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Original Research Article

The Effects of Phenylpropanoid and Aurone Compounds on Cell Cycle Modulation and Apoptosis Induction in 4T1 Breast Cancer Cells

Rollando Rollando[1,2]*, Eva Monica[1,2], F.X. Haryanto Susanto[1,2], Ellyvina Setya Dhini[1]

[1]*Pharmacy Department, Faculty of Health Sciences, Ma Chung University, Malang 65151, Indonesia*
[2]*Drug Discovery and Design Research Group, Faculty of Health Sciences, Ma Chung University, Malang 65151, Indonesia*

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ABSTRACT

Breast cancer remains a leading cause of mortality among women worldwide, necessitating the continuous search for effective treatments. Natural compounds, such as phenylpropanoids and aurones, have gained attention for their potential anticancer properties. This study investigated the cytotoxic effects of these compounds on 4T1 breast cancer cells, with the aim of exploring their capacity to induce cell death and disrupt the cell cycle. 4T1 breast cancer cells were treated with Cisplatin (3.56 µg/mL), phenylpropanoid (5.12 µg/mL), and aurone (4.05 µg/mL). Cell death and cell cycle distribution was assessed by flow cytometry. Results revealed that phenylpropanoid induced the highest cell death (11.81%), followed by aurone (7.51%), and cisplatin (6.61%). Both phenylpropanoid and aurone significantly triggered apoptosis and disrupted the cell cycle. Phenylpropanoid treatment led to an accumulation of cells in the S and G2/M phases, while aurone primarily caused arrest in the S phase. These findings suggest that these compounds may have more potent anticancer effects than cisplatin, mainly through cell cycle arrest and apoptosis induction. Phenylpropanoid and aurone compounds exhibit promising anticancer potential by promoting apoptosis and disrupting the cell cycle in 4T1 breast cancer cells. Further studies are necessary to explore their therapeutic potential and underlying mechanisms.

***Keywords**:* Phenylpropanoid, Aurone, Cell cycle, Apoptosis, Cancer.

Introduction

Breast cancer remains a leading cause of morbidity and mortality among women worldwide. As the most prevalent malignancy in women, it poses a substantial public health burden, with the World Health Organization (WHO) reporting over two million new cases annually.[1] In Indonesia, breast cancer has the highest incidence rate among female cancers, as documented by the Ministry of Health, with a consistent upward trend in reported cases. This rising prevalence highlights the urgent need for more effective and less toxic therapeutic interventions.[2] Natural compounds have garnered increasing attention as potential candidates for cancer therapy due to their diverse biological activities and lower likelihood of adverse effects than conventional treatments. Among these, phenylpropanoids and aurones have demonstrated notable anticancer potential. Phenylpropanoids, a class of organic compounds commonly found in plants, exhibit a range of biological effects, including antioxidant, anti-inflammatory, and anticancer properties.[3] Aurones, a subset of flavonoids, have shown significant promise in inhibiting cancer cell proliferation and inducing apoptosis (Figure 1).[4] These natural products represent a potential alternative to conventional chemotherapy, which is often associated with severe side effects and drug resistance.

*Corresponding author. E mail: ro.llando@machung.ac.id
Tel: + 6282220379864

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The therapeutic potential of phenylpropanoid and aurone compounds in breast cancer treatment is particularly significant. Breast cancer cells often develop resistance to apoptosis, a programmed cell death mechanism critical for eliminating damaged or abnormal cells.[5] This resistance enables malignant cells to evade death and continue proliferating. Previous studies have demonstrated that phenylpropanoids induce apoptosis in various cancer cell lines. At the same time, aurones have exhibited similar effects, particularly in breast cancer models.[6] These findings suggest that both classes of compounds could play a crucial role in overcoming apoptosis resistance in breast cancer cells.

In addition to promoting apoptosis, an essential aspect of effective cancer therapy is regulating the cell cycle, a tightly controlled process governing cell growth and division. Dysregulation of the cell cycle is the hallmark of cancer, often resulting in unchecked cell proliferation.[5] Phenylpropanoids and aurones have been implicated in modulating key cell cycle checkpoints, potentially arresting cancer cell progression at specific phases. This cell cycle modulation offers a promising therapeutic strategy for controlling cancer growth and preventing metastasis.

Despite these encouraging preliminary findings, the precise molecular mechanisms by which phenylpropanoid and aurone compounds exert their anticancer effects still need to be understood. A deeper understanding of these mechanisms is critical to fully elucidating their therapeutic potential and enhancing their efficacy.[7] Current evidence suggests that these compounds may interact with key signalling pathways involved in cell cycle regulation and apoptosis. However, further investigation is required to confirm these interactions and identify additional molecular targets.

Given the potential of phenylpropanoid and aurone compounds to modulate apoptosis and regulate the cell cycle, this study aims to investigate their effects in greater detail. The research seeks to elucidate the molecular mechanisms through which these compounds influence cell cycle regulation and apoptosis induction in 4T1 breast cancer cells, providing a foundation for developing novel therapeutic strategies for breast cancer.

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Materials and Methods

Equipment

The cytotoxicity assay was conducted using a range of equipment, including a Labconco purifier class II biosafety cabinet (Delta Series, USA), carbondioxide (CO_2) incubator (Heraeus), inverted microscope (Nikon, Eclipse, TE 2000-U), cell counter, micropipettes (Pipetman®neo, Gilson, France), digital camera (Sony), centrifuge (Sigma 203, B. Braun Biotech International), digital balance (Mettler Toledo, AG204 Delta Range®, USA), stirrer (Nuova, USA), mixer (Maxi Mix II, Thermolyne type 37600 mixer, Iowa, USA), oven (Memmert), and flow cytometer (FACSCalibur, USA).

Chemicals and Reagents

The materials utilized in this study included phenylpropanoid and aurone compounds previously isolated in earlier research. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Aldrich, USA), RNAse (obtained from the Laboratory of Animal Sciences, NAIST, Japan), 0.1% (v/v) Triton-X 100 (Triton X-100 for GC, E. Merck, 64271, Darmstadt, Germany), Sodium Dodecyl Sulphate (SDS) (Merck, Darmstadt, Germany), HCl (Merck, Darmstadt, Germany), Propidium Iodide (PI) (minimum 95% HPLC, Merck, Darmstadt, Germany), and Annexin V-FLOUS Apoptosis Detection Kit (Roche, USA).

Preparation of Reagents

MTT was prepared at a concentration of 5 mg/mL, dissolved in 1x phosphate-buffered saline (PBS) at pH 7.4, with the working reagent prepared by diluting the stock solution tenfold with the culture medium. The PBS (pH 7.4) was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 1.15 g Na_2HPO_4 in 1 L of distilled water. The stopper reagent contained 10% (w/v) Sodium Dodecyl Sulphate (SDS) in 0.01 N HCl. Propidium Iodide (PI) solution was prepared in PBS containing 1 mg/mL PI.

Cell line and culture medium

The cytotoxicity assays were performed using 4T1 cell line. The 4T1 cells were cultured in DMEM High Glucose medium (Gibco), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco, Invitrogen, USA), 1.5% (v/v) penicillin-streptomycin (Gibco, Invitrogen, USA), and 0.5% (v/v) Fungizone (Gibco). Cell harvesting from the Tissue Culture Dish (IWAKI) was conducted using 0.25% trypsin-EDTA (Gibco, Invitrogen, Canada).

Preparation of 4T1 Cancer Cells

The 4T1 cancer cells was thawed from cryovials in a sterile Laminar Air Flow (LAF) hood disinfected with 70% alcohol. The thawed cells were transferred to a sterile conical tube containing complete DMEM high glucose medium, then centrifuged at 600 rpm for 5 minutes to separate the cells from the supernatant. The supernatant was discarded, and the cell pellets were resuspended in fresh medium. The resuspended cells were transferred to a tissue culture dish (TCD) and incubate at 37°C in a 5% CO_2 atmosphere. After the cells have reached approximately 80% confluence, they were harvested. The culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). The cells were detached by the addition of 0.25% trypsin-EDTA, allowed to stand for 30 seconds, after which the cells were incubated for an additional minute. The trypsin was neutralized by the addition of fresh medium to resuspend the cells, and prevent clumping. The cell suspension was transferred to a new sterile conical tube, and the cell count was determined using a hemocytometer and cell counter. The cells were prepared at a density of 8×10^4 cells per well for the cytotoxicity assay. All equipment were kept sterile by regular disinfection of the LAF hood with 70% alcohol.[8]

Determination of Cytotoxic Activity

Cytotoxic activity was evaluated using the MTT assay. Harvested cells were adjusted to a cell density of 8×10^3 per well. The cell suspension (100 µL) were seeded into each well of a 96-well microplate and incubate for 24 h in a 5% CO_2 incubator. Prior to treatment, the culture medium was removed, and the cells were washed with with 100 µL of phosphate-buffered saline (PBS). After washing, 100 µL of test solutions at 1, 10, 25, 50, 75, 100, and 200 µg/mL were added to the respective wells. The cells were then incubated for an additional 24 h, afterwhich they were washed with PBS, followed by the addition of 100 µL of MTT reagent to each well. The cells were incubated at 37°C for 3-4 h, after which 100 µL of stopping solution (10% sodium dodecyl sulfate (SDS) in 0.01 N HCl) was added to each well, and then incubated overnight at room temperature in the dark. The absorbance of the formazan product formed was measured using an ELISA reader at 595 nm. The absorbance data was converted to a percentage of cell viability using the following equation:

$$\% \text{ Cell viability} = \frac{(OD\ treatment\ cells - OD\ media\ control)}{(OD\ cell\ control - OD\ media\ control)} x\ 100\%$$

The IC_{50} value was calculated from the curve of the relationship between log concentration and percentage of cell viability.[9]

Determination of Apoptosis Effect

Apoptosis detection was performed using the Annexin V-FLOUS Apoptosis Detection Kit (Roche, USA). A total of 5×10^5 cells per well were seeded into a 6-well plate by adding 1000 µL of cell suspension to each well. The cells were incubated overnight to allow for cell adhesion in preparation for treatment. For cell treatment, 900 µL of the designated concentration of the test solution was added to each well, while 900 µL of culture medium was added to the control wells. After treatment, the cells were incubated for an additional 24 h. After incubation, the culture media were removed, and the cells were washed with 500 µL of phosphate-buffered saline (PBS), and the washing was collected in a conical flask. The cells were harvested by the addition of 200 µL of 0.25% trypsin-EDTA to each well and incubated for 3 min to detach the cells. After detachment, 1000 µL of culture medium was added to neutralize the trypsin and resuspend the cells to achieve a single-cell suspension. The cell suspension was transferred to the conical tube containing the previously collected media. The cell suspension was centrifuged at 2000 rpm for 5 min, the supernatant was discarded, and the cell pellets was resuspend in annexin V-FLOUS buffer and stained with 2 µL of propidium iodide (PI) and 2 µL of annexin V. The suspension was homogenized and incubated for 10 min at room temperature in the dark. The cell suspension was transferred to a flow cytometer tube for analysis. Flow cytometric analysis was performed to determine the percentage of cells in each of the four quadrants: LL (lower left), LR (lower right), UL (upper left), and UR (upper right). The LL quadrant represents viable cells, the LR quadrant denotes early apoptotic cells, the UL quadrant indicates necrotic cells, and the UR quadrant shows late apoptotic cells. Apoptosis induction was assed by comparing the cells in the treatment group with those of the control group.

Cell Cycle Modulation Assay

A total of 5×10^5 cells per well was seeded in a 6-well plate, 1000 µL of cell suspension was added to each well. The cells were incubated until they reached a normal state. Once normalized, 900 µL of the test solution was added to each well, while 900 µL of culture medium was added to the control wells. After treatment, the cells were incubated for an additional 24 h, after which the medium was collected into a conical tube, up to a total volume of 1.5 mL per treatment. The wells were then washed with 500 µL of phosphate-buffered saline (PBS), and the washing was collected into the same conical tube. The cells were harvested by addition of 200 µL of 0.25% trypsin-EDTA, and incubated for 3 min. After incubation, 1 mL of culture medium was added to neutralize the trypsin, and the cells were resuspended to ensure a single-cell suspension. The cell suspension was transferred to the conical tube. Any remaining cells in the wells were rinsed with PBS and added to the conical tube. The cell suspension was centrifuged at 2000 rpm for 5 min, the supernatant was discarded, and the cell pellets were washed with 500 µL of cold PBS and centrifuged again at 2000 rpm for 5 min. The PBS was discarded, and the cells were stained with a flow cytometry reagent consisting of 25 µL of propidium iodide (PI), 1 µL of RNase, 0.5 µL of Triton-X, and 500 µL of PBS per sample. The cell suspension was thoroughly mixed and transfered to flow cytometry

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tubes for cell cycle analysis. Cell cycle distribution in the G1, S, and G2/M phases was analyzed with Flowing Software. Cell cycle inhibition was evaluated by comparing the effects of the test solutions with the control cells.[11]

Data analysis
Cell viability was analyzed using Microsoft Excel 2007 to obtain the linear correlation coefficient (r) from the curve depicting the relationship between log concentration and percentage of cell viability. The significance of linear correlation was tested at 5% alpha level. The effectiveness of the test compounds was determined based on the percentage reduction in cell viability.

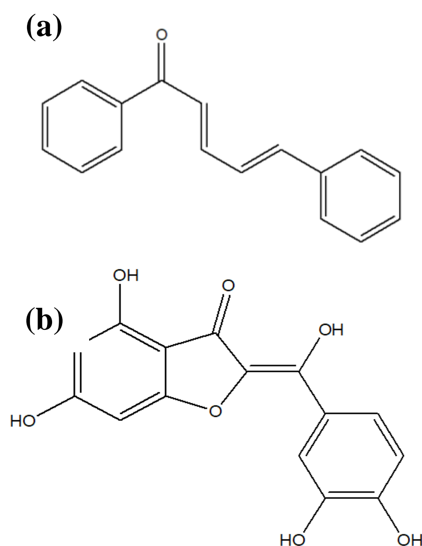

Figure 1: Structure of phenylpropanoid (a) and aurone (b) compounds

Results and Discussion

Cytotoxic Activity
The IC_{50} value, defined as the concentration of an inhibitor required to reduce cell viability by 50%, is a critical parameter for evaluating the efficacy of compounds in inhibiting cancer cell growth relative to normal cells. This study noted significant differences in IC_{50} values and selectivity indices among Cisplatin, phenylpropanoid, and aurone, providing insights into their therapeutic potential and associated risks.
Cisplatin exhibited notably low IC_{50} value of 3.56 µg/mL in 4T1 cells, reflecting its potent anti-cancer activity. Conversely, its IC_{50} value in Vero cells was considerably higher at 261.88 µg/mL, highlighting its high selectivity for cancer cells. With a selectivity index of 73.56, Cisplatin demonstrates a favourable balance between efficacy and reduced toxicity to normal cells, positioning it as a promising therapeutic agent. Phenylpropanoid also displayed significant cytotoxic activity, with an IC_{50} value of 5.12 µg/mL in 4T1 cells, indicating effective suppression of cancer cell proliferation. The IC_{50} value in Vero cells was 334.31 µg/mL, suggesting adequate selectivity towards cancer cells. The selectivity index of phenylpropanoid was 65.30, indicating substantial therapeutic potential with relatively low adverse effects on normal cells. In contrast, aurone presented an IC_{50} value of 4.05 µg/mL in 4T1 cells, denoting its capacity to inhibit cancer cell growth. However, its IC_{50} value in Vero cells was significantly lower at 43.27 µg/mL, indicating reduced selectivity compared to cisplatin and phenylpropanoid. The selectivity index of aurone was 10.68, suggesting a higher likelihood of side effects on normal cells, which necessitates careful consideration in its therapeutic application (Table 1).
Cisplatin and phenylpropanoid exhibited superior selectivity towards cancer cells compared to aurone. This differential selectivity underscores the need for a comprehensive evaluation of the balance between anti-cancer efficacy and potential side effects, which is crucial for determining the clinical utility of these compounds.

Post-treatment, morphological assessments of 4T1 cells were performed to elucidate cytotoxic effects (Figure 2). As a positive control, cisplatin significantly reduced viable cell numbers relative to the untreated control group. Morphological changes observed in 4T1 cells treated with Cisplatin (Figure 2b) included cell rounding and coagulation, indicating cell death. Similarly, treatment with phenylpropanoid and aurone led to substantial reductions in cancer cell viability (Figure 2c-d). At IC_{50} concentration, the test compounds induced notable morphological changes such as cell shrinkage, rounding, nuclear condensation, and membrane blebbing, although distinct cell junctions were not observed. These findings demonstrate that the test compounds effectively induce cytotoxicity in cancer cells, with marked visual differences between treated and normal cancer cells.[12]

Table 1: Cytotoxic activity of cisplatin and test compounds

Sample	IC_{50} (µg/mL)		Selectivity Index
	4T1	Vero	
Cisplatin	3.56	261.88	73.56
Phenylpropanoid	5.12	334.31	65.30
Aurone	4.05	43.27	10.68

Effect of phenylpropanoid and aurone on apoptosis
To evaluate the apoptotic effects of cisplatin, phenylpropanoid, and aurone on 4T1 breast cancer cells, cells were treated for 24 hours with compounds at concentrations corresponding to their respective IC_{50} values. Apoptosis induction was assessed using the Annexin V assay and detected via flow cytometry. Annexin V, a phospholipid-binding protein, binds specifically to negatively charged phosphatidylserine on the inner leaflet of the cell membrane, which is exposed during early apoptosis. Propidium Iodide (PI) staining, which intercalates with DNA, was used to differentiate between apoptosis and necrosis (Figure 3).[13]

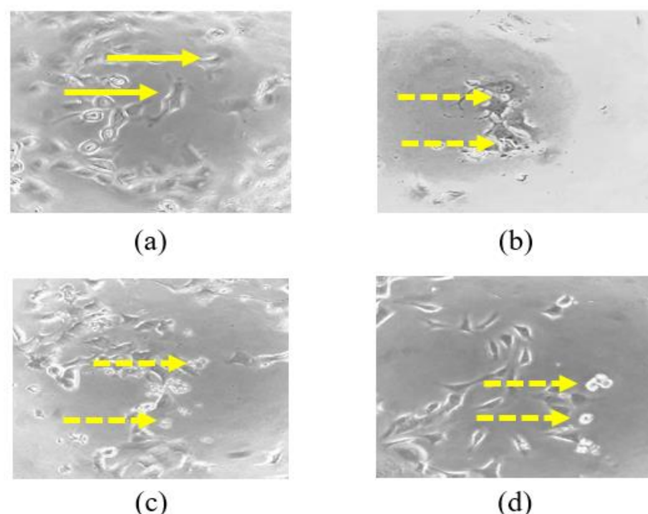

Figure 2: Effect of cisplatin and compound treatment on 4T1 cells with IC_{50} concentration. 6000 cells/well in 96 well plates, incubated for 24 hours DMEM Hi-glucose. Observations were carried out under an inverted microscope with 300x magnification. Image caption: (a) Cell control; (b) Cisplatin (3.56 µg/mL); (c) Phenylpropanoid (5.12 µg/mL); (d) aurone (4.05 µg/mL); The morphology of viable 4T1 cells is indicated by arrows and cells undergoing morphological changes are indicated by dotted arrows.

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The analysis revealed that untreated cells had a cell viability of 96.50% with 3.50% cell death. Cisplatin treatment resulted in a cell death rate of 6.61%. Phenylpropanoid treatment led to 11.81% cell death, while aurone treatment caused a cell death rate of 7.51%. These results indicated that cisplatin increased cell death by 3.11% compared to the control, phenylpropanoid compounds increased cell death by 8.31%, and aurone increased cell death by 11.81%. This study demonstrated that both phenylpropanoid compounds and aurone effectively induced apoptosis (Table 2).

Phenylpropanoid compounds, synthesized through the shikimate pathway from precursors L-phenylalanine or L-tyrosine, have shown promising anticancer activity through apoptosis induction. Hematpoor *et al*.[14] reported that asaricin and isoasarone, phenylpropanoids from *Piper sarmentosum*, exhibit cytotoxic effects on MDA-MB-231 breast cancer cells by inhibiting Bcl-2 and promoting Bax expression. Similarly, Qi *et al*.[15] identified novel phenylpropanoid compounds from *Leptopus lolonum* that induce apoptosis in MCF-7 breast cancer cells via MAPK and Akt pathways. Additionally, 1′S-1′-acetoxy eugenol acetate from *Alpinia conchigera* Griff suppresses MCF-7 cell growth by inducing apoptosis through Bcl-2 inhibition and Bax induction.[16]

Aurone compounds, biosynthesized from the shikimate and acetate pathways, have been studied for their anticancer properties. Huang *et al*.[17] isolated hamiltrone from *Uvaria hamiltonii*, demonstrating its ability to inhibit MCF-7 and T47D breast cancer cell growth by upregulating p53. A novel aurone derivative, isoaurostatin, produced by *Thermomonospora alba*, inhibited the proliferation of MCF-7 and 4T1 cells by inhibiting topoisomerase enzymes.[18] Additionally, Kumar *et al*.[19] synthesized aurone derivatives with cytotoxicity against MCF-7 cells, and Hassan *et al*. developed furoaurones that exhibited activity against T47D cells by inhibiting ATP-dependent enzymes.[20]

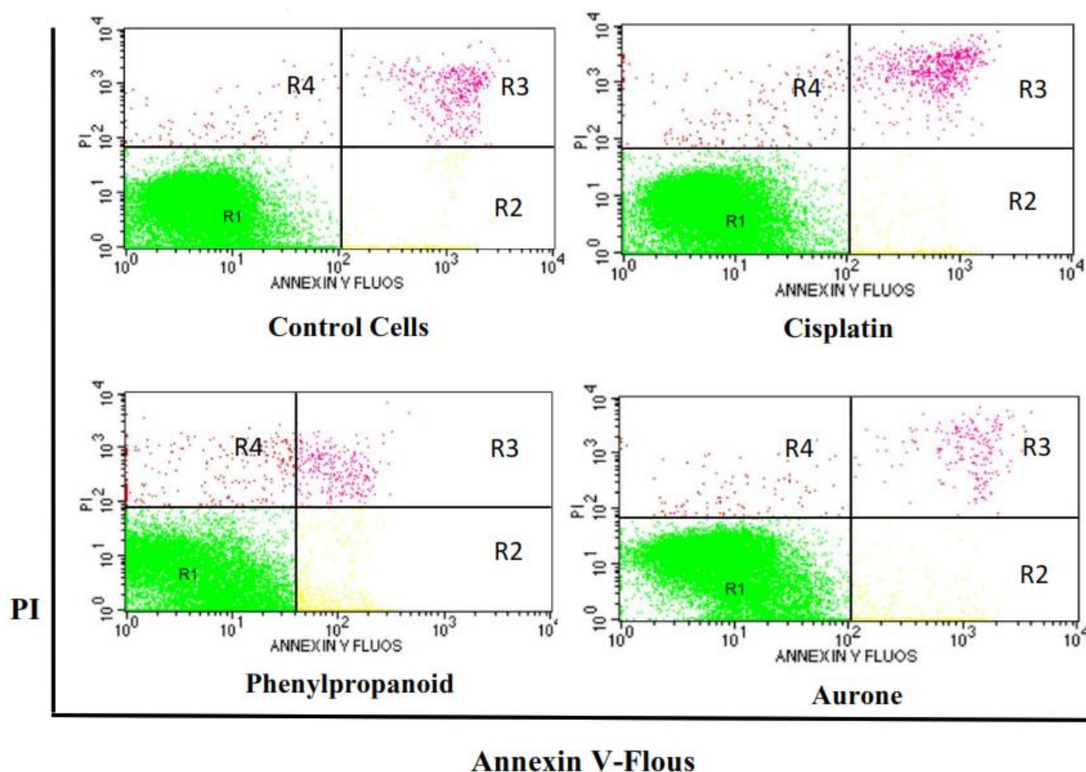

Figure 3: The effects of apoptosis induction after treatment with Cisplatin and test compounds. Cells were seeded at a density of 5x10^5 in a 6-well plate and incubated for 24 hours in DMEM High Glucose medium, either without treatment or with treatment. Flow cytometry detection of cell death using Annexin V FLUOS was conducted on 4T1 breast cancer cells after treatment with 3.56 µg/mL Cisplatin, 5.12 µg/mL phenylpropanoid, and 4.05 µg/mL aurone. Quadrant R1 indicates live cells, R2: early apoptosis, R3: late apoptosis, R4: necrosis

Effect of phenylpropanoid and aurone on cell cycle progression of cancer cell

Cancer cell proliferation involves continuous DNA synthesis through the cell cycle, typically characterized by distinct phases: G1, S, and G2/M. The ability to inhibit cancer cell proliferation by modulating the cell cycle has become a strategic approach in cancer therapy. Flow cytometry can assess this modulation, a technique that allows the detection of each cell cycle phase based on chromosomal content.[21]

In the G1 phase, cells possess 2n (diploid) chromosomes; during the S phase, cells replicate their DNA, resulting in a chromosome count between 2n and 4n. By the G2 phase, cells have 4n (two diploid sets) chromosomes. Propidium iodide (PI) is used to stain DNA, as it interacts with nucleic acids and allows for the measurement of DNA content through fluorescence intensity. Higher DNA content results in greater fluorescence intensity. Flow cytometric analysis was conducted at the 24-hour mark (Figure 4).[22]

Control cells exhibited typical distribution across the G1, S, and G2/M phases. Cisplatin was used as a positive control, which led to an accumulation of cells in the S and G2/M phases. Phenylpropanoid compounds similarly caused accumulation in the S and G2/M phases, whereas aurone compounds primarily induced accumulation in the S phase. Specifically, phenylpropanoid compounds increased S phase accumulation to 12.18% and aurone compounds increased S phase accumulation to 19.17%, compared to 10.32% in the control cells. Additionally, phenylpropanoid compounds caused a distribution percentage of 27.86% in the G2/M phase, which is higher than the 22.91% observed in control cells. These findings suggest cell cycle arrest at these phases (Table 3).

Research has demonstrated that phenylpropanoid compounds possess significant cytotoxic activity against breast cancer cells. Hematpoor *et al*.[14] reported that asaricin and isoasarone, derived from *Piper*

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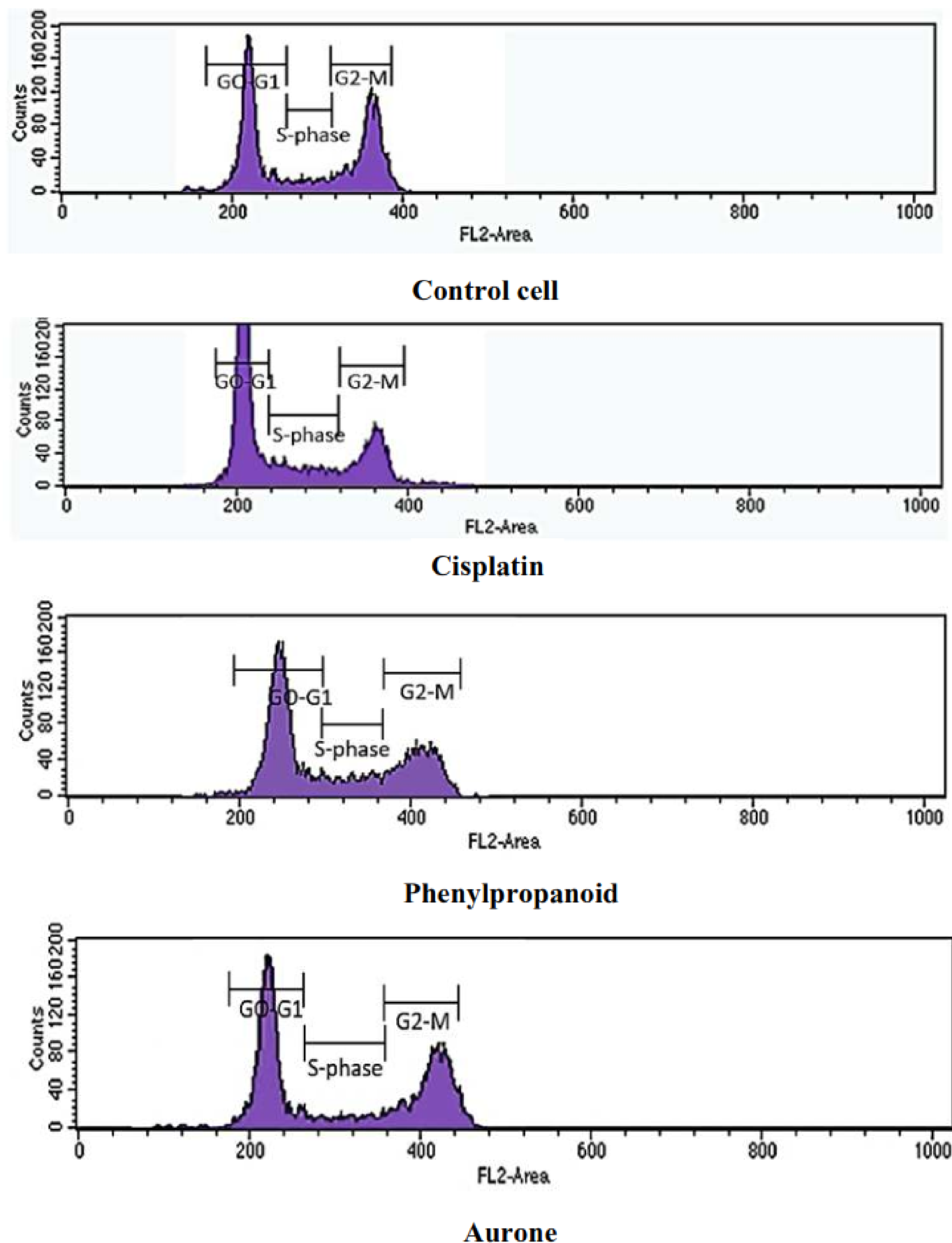

Figure 4: Detection of cell cycle distribution using flow cytometry. Approximately 5 x10^5 cells were seeded in 6 well plates and incubated for 24 hours in DMEM high glucose medium without or with treatment. Flow cytometry detection using PI reagent on 4T1 cells after treatment with 3.56 µg/mL Cisplatin, 5.12 µg/mL phenylpronaoid, 4.05 µg/mL aurone. Flow cytometry analysis using flowing software

sarmentosum, induced cell cycle arrest in the S phase of MDA-MB-231 breast cancer cells. Similarly, Hasima *et al*.[23] identified 1′S-1′-acetoxy eugenol acetate from *Alpinia conchigera* Griff as inducing G0/G1 phase arrest in MCF-7 breast cancer cells. Additionally, ardicrephenin, a phenylpropanoid glycoside from *Ardisia crenata*, inhibited MCF-7 and MDA-MB-231 breast cancer cells growth by inducing S phase arrest.[24] Azavedo-Barosa *et al*.[16] synthesized eight phenylpropanoid derivatives that induced cell cycle arrest in MCF-7 cells at S and G1/G2 phases, associated with downregulation of cyclin D1 and cyclin E.

Aurone compounds have also demonstrated anticancer activity against breast cancer cells. Huang *et al*.[17] isolated isoaurone 4′,6-dihydroxy-4-methoxyisoaurone from *Trichosanthes kirilowii* seeds, which inhibited T47D and MCF-7 cell growth by blocking cyclin-dependent kinases (CDKs). Additionally, Kafle *et al*.[25] achieved the total synthesis of aurone glycoside, which was highly active against 4T1 breast cancer cells with an IC_{50} of 2.34 µM and inhibited cyclin D1 and cyclin E. These findings underscore the potential of both phenylpropanoid and aurone compounds in cancer therapy through cell cycle modulation.

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Table 2: The percentage of 4T1 cell death after treatment with cisplatin and test compounds

Type of Treatment	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)	Total (%)
Cell control	1.23	1.74	0.23	3.50
Cisplatin	2.85	2.92	0.84	6.61
Phenylpropanoid	9.21	1.27	1.33	11.81
Aurone	2.04	2.97	2.50	7.51

Table 3: Percentage of cell cycle distribution in 4T1 breast cancer cells after cisplatin and test compound treatment

Type of Treatment	G1 phase (%)	S phase (%)	G2/M phase (%)	CV (%)
Cell control	47.78	10.32	22.91	9.13
Cisplatin	37.12	14.28	24.14	8.65
Phenylpropanoid	43.87	12.18	27.86	8.88
Aurone	43.92	19.17	16.73	8.83

Conclusion

The study reveals that phenylpropanoid compounds and aurone exhibit potent cytotoxic effects against 4T1 breast cancer cells, significantly enhancing cell death compared to untreated controls. These findings strongly indicate that both compounds induce cell cycle arrest, with phenylpropanoids exerting a more extensive influence across multiple phases. Notably, their capacity to halt cell cycle progression and trigger apoptosis positions phenylpropanoid compounds and aurone as up and coming candidates for cancer therapy. Their dual actions of impeding cancer cell proliferation and promoting cell death underscore their therapeutic potential in combating aggressive breast cancers.

Conflict of interest

The author declare no conflict of interest.

Authors' declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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