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Original Research Article

Synergistic Cytotoxic and Cell Cycle-Regulating Effects of *Garcinia cowa* Leaf Extract and Trastuzumab in HER2-Positive Breast Cancer Cells

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ABSTRACT

HER2-positive breast cancer is highly aggressive, with poor prognosis and resistance to standard therapies. Although trastuzumab is the standard therapy, its effectiveness is often limited by resistance and cardiotoxic side effects. Therefore, adjuvant therapy strategies are needed to improve the response to trastuzumab. This study evaluated the synergistic effect of *Garcinia cowa* leaf extract and trastuzumab on MCF-7/HER2 breast cancer cells. Cells were treated with various concentrations of *G. cowa* leaf extract and trastuzumab, either singly or in combination. Cytotoxicity was assessed using a tetrazolium reduction assay, while synergism was evaluated with the Combination Index (CI). Flow cytometry was employed for cell cycle analysis, apoptosis detection, and the assessment of protein expression (p53, Cyclin D1, and Cyclin E). The results indicated that *G. cowa* extract had an IC_{50} of 119.21 µg/mL, whereas trastuzumab had an IC_{50} of 954.52 µg/mL. The combination of 25 µg/mL extract and 125 µg/mL trastuzumab produced an optimal synergistic effect (Fa = 0.897, CI = 0.266), inducing G_2/M arrest with the cell population increasing to 54.6%, reducing cell viability to 73.4%, and elevating the apoptotic fraction by 17.2%. Protein expression analysis revealed an increase in p53 and decreases in Cyclin D1 and Cyclin E, contributing to the inhibition of cell proliferation. Overall, *G. cowa* leaf extract enhances the effectiveness of trastuzumab through cell cycle modulation and apoptosis induction, suggesting its potential as an adjuvant therapy for HER2-positive breast cancer. Further studies using *in vivo* models and clinical validation are needed to confirm these findings.

Keywords: *Garcinia cowa*, Trastuzumab, Breast cancer, Cell cycle arrest, Apoptosis, Combination therapy.

Introduction

Breast cancer remains the most prevalent malignancy among women and a leading contributor to cancer-related mortality worldwide. According to GLOBOCAN 2020, Indonesia recorded 65,858 new breast cancer cases, accounting for a significant proportion of the 396,914 total cancer cases, with 22,430 deaths attributed to the disease.[1] One of the most aggressive subtypes of breast cancer is cancer with HER2 overexpression, which is found in 15-20% of cases.[2] This subtype is characterised by rapid growth, high metastatic ability, and resistance to conventional therapies, contributing to a poor prognosis. HER2 drives cancer progression through abnormal cell proliferation, making it a key therapeutic target.[3] Given the aggressive nature of HER2-positive breast cancer, trastuzumab, a monoclonal antibody targeting HER2, has become the gold standard in its treatment. Although this therapy has shown promising effectiveness, its use still has limitations due to the emergence of resistance and side effects, such as cardiotoxicity.[4,5]

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The cytotoxicity assay showed that increasing trastuzumab concentration enhanced growth inhibition, with trastuzumab IC_{50} values of 1660 µg/mL in MCF-7 and 1780 µg/mL in AMJ13.[6] Therefore, alternative strategies are needed to increase therapeutic effectiveness while reducing toxic effects, one of which is through the combination of trastuzumab with natural-based agents.[7,8] Given these limitations, alternative strategies, including natural compounds with anticancer properties, have gained interest. One promising candidate for natural-based agents is *Garcinia cowa* Roxb. (*G. cowa*), a plant native to West Sumatra that contains various bioactive compounds with anticancer activity.[9] Compounds such as xanthones, flavonoids, cowanin, and garcinisidone-A found in *G. cowa* leaves have shown cytotoxic potential against various cancer cell lines, including T47D, MCF-7, and H-460.[10,11] The ethanol extract of *G. cowa* fruit bark showed cytotoxic effects against T47D breast cancer cells with an IC_{50} value of 19.33 µg/mL. In contrast, the ethanol extract of its stem bark had a more potent activity with an IC_{50} of 5.10 µg/mL.[1] The ethanol extract of *G. cowa* leaves also showed cytotoxic activity against T47D cells with an IC_{50} of 6.13 ± 3.51 µg/mL.[13] Cowanin acts as an anticancer agent by inhibiting the cell cycle in the G_0/G_1 phase and suppressing T47D cell migration.[14] Also, molecular docking analysis showed that garcinisidone-A can interact with HER2, indicating its potential to inhibit HER2-mediated proliferation pathways.[15] However, research on the mechanism behind the anticancer activity of the combination of *G. cowa* leaf extract and trastuzumab, especially against MCF-7/HER2 breast cancer cells, is still minimal. The mechanism of cell cycle inhibition as the main target of cancer therapy has not been explored in depth. The cell cycle is controlled by various regulatory proteins, including cyclin D1 and cyclin E, which play a role in the G_1 to S phase transition.[16] In addition, the p53 protein functions as a master regulator of the cell cycle and apoptosis, maintaining genetic stability by stopping the cell cycle or inducing DNA repair when damage

occurs.[17] Deregulation of p53, cyclin D1, and cyclin E is often found in various cancers, including breast cancer with HER2 overexpression, so regulating the expression of these proteins is a potential strategy in cancer therapy.[16,18]

This study aims to evaluate the synergistic effect of a combination of *G. cowa* leaf extract and trastuzumab on the MCF-7/HER2 breast cancer cell model. Specifically, this study will investigate whether this combination can regulate key cell cycle proteins (p53, cyclin D1, and cyclin E) to inhibit cancer cell proliferation. The mechanistic insights gained from this study could provide a more substantial scientific basis for integrating natural compounds into targeted breast cancer therapy. The flow cytometry technique will be used to obtain quantitative data on changes in the expression of these key proteins, thus revealing the mechanism of action of this combination therapy in more depth. Furthermore, the cytotoxic effect of the combination will be assessed through IC_{50} determination, and the synergy between these agents will be evaluated using Combination Index (CI) analysis. No studies have comprehensively investigated the molecular mechanisms underlying the combination of *G. cowa* leaf extract and trastuzumab in HER2-positive breast cancer, particularly concerning cell cycle regulation via p53, cyclin D1, and cyclin E. This study uniquely integrates natural-based and antibody-based therapies, offering a novel strategy to enhance trastuzumab efficacy while reducing its associated toxicity. The findings of this study could serve as a foundation for developing safer and more effective targeted therapies for HER2-positive breast cancer.

Materials and Methods

Materials

The materials used in this study include *G. cowa* leaf extract, trastuzumab (Hexpharm), and various chemicals and reagents that support cellular analysis. The chemicals used include 70% ethanol, sodium dodecyl sulfate (SDS, Sigma), dimethyl sulfoxide (DMSO, Merck), and Triton X-114 (Sigma). Reagents for cellular analysis included [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, Sigma), Propidium Iodide (PI, Sigma), RNase (Roche), PI RNase dye solution (BD Biosciences), and Annexin V apoptosis detection kit (BD Biosciences), including Binding Buffer. Cell culture materials included Dulbecco's Modified Eagle Medium (DMEM, Gibco), fetal bovine serum (FBS, Gibco), penicillin-streptomycin (Gibco), trypsin-EDTA (Gibco), and phosphate-buffered saline (PBS, Invitrogen). Additional reagents for protein expression analysis included Protease and Phosphatase Inhibitor (Roche), Permeabilization Buffer (BD Biosciences), and fluorophore-conjugated antibodies against Cyclin D1, Cyclin E, and p53 (BD Biosciences).

Sample preparation

The leaves of *G. cowa* were collected on February 20, 2023, from Kudu Gantiang, Padang Pariaman, West Sumatra (latitude: -0.5107, longitude: 100.1640). The plant material was identified and authenticated at the Herbarium of Andalas University (ANDA), Padang, Indonesia, under voucher number 137/K-ID/ANDA/II/2023. The leaves were air-dried in a greenhouse at 25-30°C for 72 hours, followed by additional drying in an oven at 40°C for 24 hours to ensure complete moisture removal. The dried leaves were then finely ground using a laboratory grinder. For extraction, the powdered leaves were macerated in 70% ethanol for 24 hours, and repeated three times using a fresh solvent to enhance the yield of bioactive compounds. The obtained extract was then concentrated under reduced pressure at 40°C using a rotary evaporator, resulting in a concentrated extract.[19]

Cell culturing procedure

MCF-7/HER2 breast cancer cells were obtained from the Tissue Culture Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with high glucose, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Incubation was carried out under standard conditions at 37°C with 5% CO_2 and 95% humidity. When cell confluency reached 70–80%, cells were harvested using trypsin-EDTA, centrifuged, and resuspended in a fresh medium for further experiments. All cell culture procedures adhered to Good Cell Culture Practice (GCCP) guidelines.[20,21]

Combination Design and Synergism Analysis

The combination effect of *G. cowa* leaf extract and trastuzumab was evaluated using the checkerboard assay. The tested concentrations included the IC_{50} values of each compound and a range of concentrations above and below the IC_{50}. The Combination Index (CI) was calculated using the Chou-Talalay method, classifying interactions as synergistic (CI < 1), additive (CI = 1), or antagonistic (CI > 1).[22–24]

Cytotoxicity Assessment and Combination Index Using MTT Assay

The cytotoxic effects of *G. cowa* extract, trastuzumab, and their combination were assessed using the MTT assay. MCF-7/HER2 cells were seeded in 96-well plates (1×10^4 cells/well) and incubated overnight at 37°C with 5% CO_2. After 48 hours of treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the plate was incubated for 4 hours (Model 3429, Thermo Fisher Scientific, USA) to allow formazan crystal formation. MCF-7/HER2 cells were treated with *G. cowa* extract at 12.5–100 µg/mL concentrations and trastuzumab at 250–2000 µg/mL. The medium was removed, and 100 µL of DMSO was added to dissolve the crystals. Absorbance was measured at 570 nm using a microplate reader (xMark, Bio-Rad Laboratories, Japan). Cell viability was calculated relative to the untreated control, and IC_{50} and CI values were determined to evaluate the cytotoxicity and interaction effects of *G. cowa* extract and trastuzumab.[8,25,26]

Flow cytometry Assay

MCF-7/HER2 cells (5×10^5 cells per well) were seeded into 6-well plates and incubated at 37°C with 5% CO_2 for 24 hours. The cells were then subjected to different treatment conditions: a control group, *G. cowa* extract at $1\times$ IC_{50}, trastuzumab at $1\times$ IC_{50}, and a combination of *G. cowa* extract and trastuzumab at ¼ × IC_{50} and 1/8 × IC_{50}, respectively. After 24 hours of treatment, adherent and floating cells were collected, washed three times with cold PBS, and fixed in 70% ethanol at -20°C for 2 hours. The fixed cells were then rewashed with PBS before being analysed using flow cytometry.[27,28]

For cell cycle analysis, fixed cells were stained with propidium iodide (PI) solution (40 µg/mL) containing RNase (100 µg/mL) and incubated at 37°C for 30 minutes. Samples were analysed using a flow cytometer (FACScan, BD Biosciences, USA), and the distribution of G_1, S, and G_2/M phases was determined using ModFit LT 3.0 software.[29]

Apoptosis was assessed using the Annexin V assay, where cells were stained with Annexin V and PI, incubated at 37°C for 30 minutes, and analysed by flow cytometry to determine the proportion of cells undergoing early apoptosis, late apoptosis, and necrosis.[30,31]

Protein Expression Analysis

Intracellular staining was conducted using fluorophore-conjugated antibodies against Cyclin D1, Cyclin E, and p53 to assess protein expression levels. Fixed and permeabilised cells were incubated with primary antibodies at 4°C for 30 minutes. After washing, fluorescence intensity was quantified using a flow cytometer (FACScan, BD Biosciences, USA), and the results were analysed relative to the control group.[32]

Data Analysis

Cytotoxic activity was evaluated based on IC_{50} values using GraphPad Prism 9 (GraphPad Software, 2020, San Diego, CA, USA). Combination effects were assessed using CI values calculated in CompuSyn (v1.0). Statistical comparisons among groups were performed using one-way ANOVA followed by Tukey's post-hoc test.

Results and Discussion

The MTT assay was used to evaluate the cytotoxicity of *G. cowa* leaf extract and trastuzumab against MCF-7/HER2 breast cancer cells.

Figure 1 illustrates the viability of breast cancer cells at various concentrations of *G. cowa* leaf extract and trastuzumab. At extract concentrations ≥100 µg/mL, MCF-7/HER2 cells experienced a significant decrease in viability, similar to the effect observed with trastuzumab at concentrations approaching 1000 µg/mL. These results suggest that the extract has a more selective cytotoxic effect against cancer cells than trastuzumab under these test conditions. The superior cytotoxicity of *G. cowa* extract could be attributed to its diverse phytochemical constituents, including xanthones, flavonoids, and benzophenones, which have been reported to exhibit anticancer properties.[11,33,34]

The cytotoxicity of a compound is generally assessed based on the IC_{50} value, which is the concentration required to inhibit 50% of cell growth. The National Cancer Institute (NCI) classifies compounds with lower IC_{50} as more potent inhibitors of cancer cell proliferation.[19] In this study, *G. cowa* leaf extract showed an IC_{50} of 119.21 µg/mL, while trastuzumab had an IC_{50} of 954.52 µg/mL in MCF-7/HER2 cells (Figure 1). The higher IC_{50} value of trastuzumab indicates that MCF-7/HER2 cells are less sensitive to this drug or have a particular resistance mechanism.

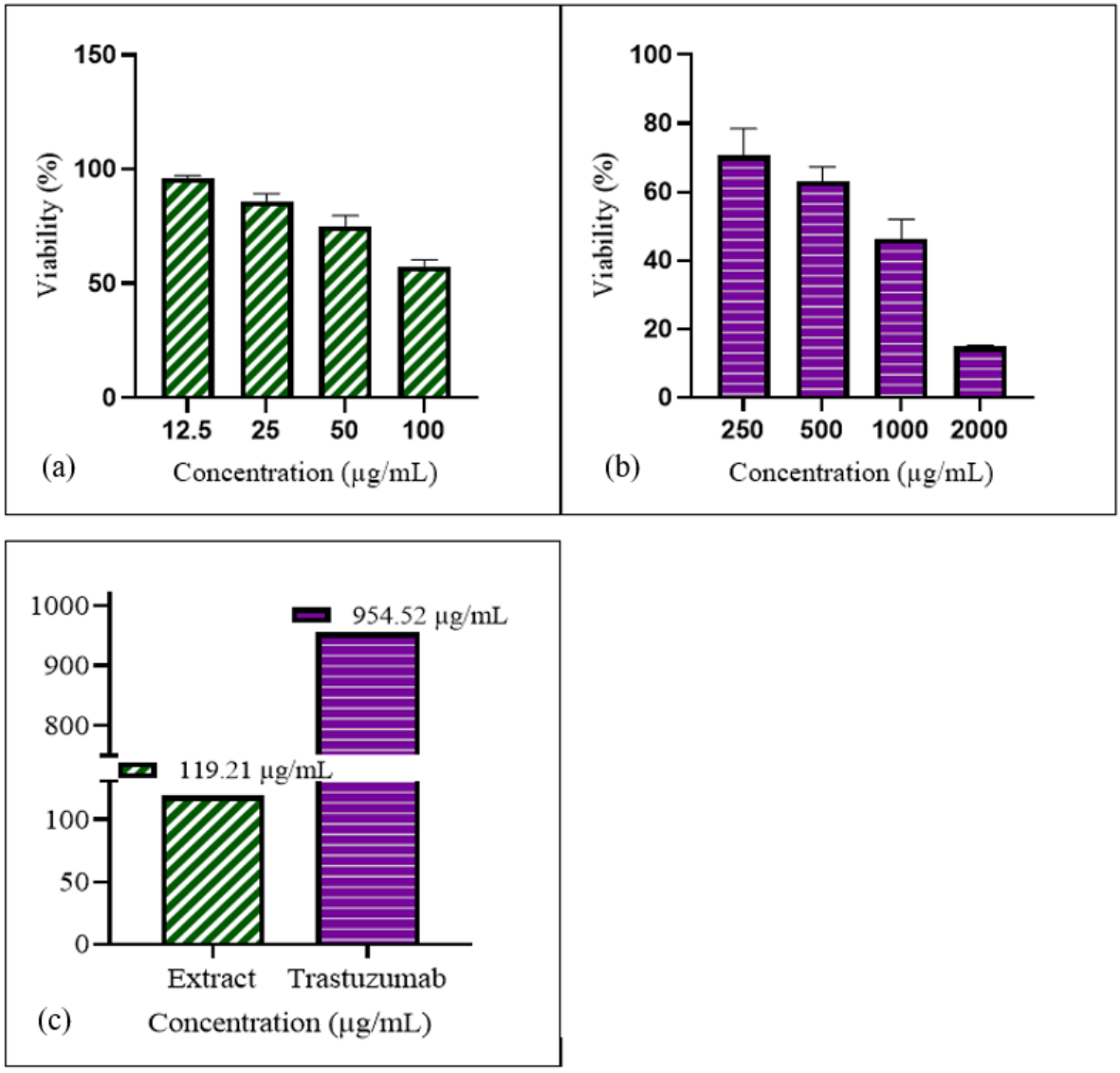

Figure 1: Percentage of Cell Viability and IC_{50} Values After Treatment (a). *G. cowa* extract, and (b). trastuzumab (c). IC_{50} values

Several factors can cause resistance of MCF-7/HER2 cells to trastuzumab.[5] Trastuzumab is designed to target cancer cells with HER2 overexpression, but MCF-7 cells are known to have lower HER2 expression than HER2-positive breast cancer cells such as SK-BR-3 or BT-474.[35–37] This lower HER2 expression reduces the effectiveness of trastuzumab due to an insufficient number of molecular targets for optimal binding.[38] In addition, resistance mechanisms may involve alternative signalling pathways, such as PI3K/AKT and MAPK activation, increased IGF-1R expression, or increased expression of anti-apoptotic proteins.[39,40] These resistance mechanisms may cause trastuzumab to be less effective in inhibiting cancer cell proliferation, requiring higher concentrations to achieve significant cytotoxic effects.[38,41]

The cytotoxic mechanisms of trastuzumab include inhibition of HER2 growth signalling, HER2 receptor internalisation, and mediation of Antibody-Dependent Cellular Cytotoxicity (ADCC) by the immune system.[42–44]

However, in this MTT assay, the ADCC mechanism cannot be fully realised due to the absence of immune system components. This may explain why the IC_{50} values obtained are higher than the observations in more complex biological systems. Therefore, combining trastuzumab with other agents, such as *G. cowa* leaf extract, may offer a more effective strategy to enhance cancer cell sensitivity. In modern

medicine, combination therapies have become essential for managing complex and chronic diseases by improving treatment efficacy, reducing adverse effects, and preventing drug resistance. [22] This approach is particularly beneficial in conditions such as cancer, infectious diseases, and autoimmune disorders, where single-agent therapies may be insufficient.

CompuSyn software combined analysis showed that the interaction between trastuzumab and *G. cowa* leaf extract was dose-dependent, as shown in Table 1. This evaluation was based on the Fraction Affected (Fa), which reflects the degree of cancer cell inhibition, and the Combination Index (CI), which determines the nature of the drug interaction. CI values < 1 indicate synergism, CI = 1 indicates an additive effect, while CI > 1 indicates antagonism.[45,46]

Table 1: CI values of combination *G. cowa* extract and trastuzumab on MCF-7/HER2 cells

Extract (µg/mL)	Trastuzumab (µg/mL)	Total Dose (µg/mL)	Fraction affected (Fa)	Combination Index (CI) Value
100	1000	1100	0.999	26.199
50	1000	1050	0.982	3.142
25	1000	1025	0.916	1.941
12.5	1000	1012.5	0.952	2.271
100	500	600	0.999	7.537
50	500	550	0.982	1.646
25	500	525	0.964	1.281
12.5	500	512.5	0.966	1.271
100	250	350	0.999	4.397
50	250	300	0.999	3.768
25	250	275	0.941	0.577
12.5	250	262.5	0.981	0.767
100	125	225	0.960	0.529
50	125	175	0.905	0.318
25	125	150	0.897	0.266
12.5	125	137.5	0.935	0.281

The analysis showed a variety of interactions, from strong synergism to antagonism. Optimal synergism was found in certain dose combinations, especially in 25 µg/mL Extract + 125 µg/mL Trastuzumab (Fa = 0.897, CI = 0.266) and 50 µg/mL Extract + 125 µg/mL Trastuzumab (Fa = 0.905, CI = 0.318). The combination of 12.5 µg/mL extract + 125 µg/mL trastuzumab (Fa = 0.935, CI = 0.281) also showed strong synergy. However, this dose was not prioritised due to the slight differences in Fa and CI values and the potential risk of toxicity from excessive synergism. Conversely, at high concentrations of trastuzumab (≥1000 µg/mL) combined with *G. cowa* leaf extract (≥100 µg/mL), CI values exceeded 3, indicating an antagonistic interaction. This suggests that excessively high doses do not enhance therapeutic benefits and may instead contribute to drug resistance or increased side effects. [22] The synergism observed is likely due to the different pharmacological mechanisms of these two agents. Trastuzumab targets the HER2 receptor, inhibiting cancer cell proliferation through growth signal suppression.[36,39] Meanwhile, *G. cowa* leaf extract has antioxidant and pro-apoptotic activities, which may increase the sensitivity of cancer cells to trastuzumab.[47–50] Based on these results, the combination of *G. cowa* leaf extract 25-50 µg/mL with trastuzumab 125 µg/mL was identified as the optimal dose, providing significant synergistic effects without increasing the risk of excessive toxicity. This suggests that *G. cowa* extract enhances the efficacy of trastuzumab, potentially by sensitising cancer cells to HER2-targeted therapy. At low to moderate doses (125-275 µg/mL), drug combinations tend to exhibit synergism, where their combined effect exceeds the sum of individual effects. However, at high doses (above 300 µg/mL), they often become antagonistic, likely due to toxicity or target saturation. [45]

This interaction can be explained by models such as Loewe's additivity, which predicts synergy when the combined effect surpasses the expected additive outcome. Similarly, the Bliss independence model, used for drugs with different mechanisms, defines synergy or antagonism based on deviations from independently expected effects.[24] Previous studies have indicated that certain natural compounds can modulate signalling pathways associated with HER2 resistance, thereby improving the response to trastuzumab.[51–53] Further mechanistic investigations are needed to elucidate the precise molecular interactions involved in this synergy.

Flow cytometry analysis demonstrated that the combination of *G. cowa* leaf extract and trastuzumab altered cell cycle distribution in MCF-7/HER2 cancer cells (Table 2, Figures 2 and 3). Flow cytometry analysis of cell cycle distribution revealed distinct treatment effects. In untreated control cells, 38.1% were in the G_0/G_1 phase, 7.5% in the S

Table 2: Cell Cycle Distribution of MCF-7/HER2 Cells Following Treatment with *G. cowa* Leaf Extract and Trastuzumab

Treatment	Concentration	Cell Phase (%)		
		G_0/G_1	S	G_2/M
Control	0	38.1	7.5	49.5
Extract	1x IC_{50}	29.9	9.7	55.3
Trastuzumab	1x IC_{50}	40.9	9.2	44.2
Extract-Trastuzumab	¼ IC_{50}- $^1/_8$ IC_{50}	32.0	8.6	54.6

G_0/G_1: G_0 (Resting) phase and G_1 (Growth 1) phase
S: Synthesis phase
G_2/M: G_2 (Growth 2) and Mitosis

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phase, and 49.5% in the G_2/M phase. Treatment with *G. cowa* extract at 1x IC_{50} reduced the G_0/G_1 population to 29.9% while increasing the S phase to 9.7% and the G_2/M phase to 55.3%, indicating induction of G_2/M arrest. Conversely, trastuzumab at 1x IC_{50} increased the G_0/G_1 phase to 40.9% and decreased the G_2/M phase to 44.2%, with the S phase remaining relatively unchanged at 9.2%, suggesting inhibition of the G_1-S transition.

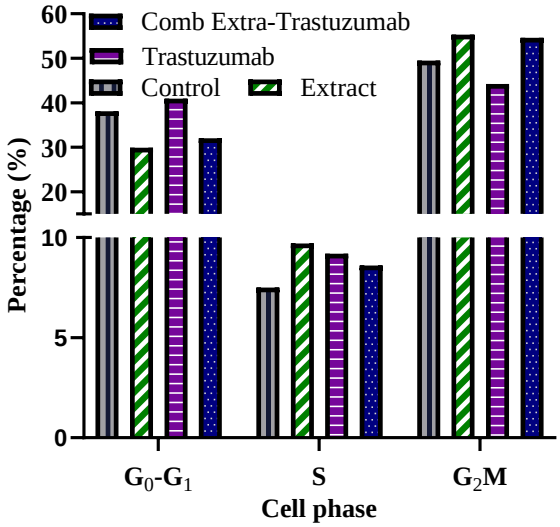

Figure 2: The combined effect of *G. cowa* Leaf Extract and trastuzumab on Cycle Phase Distribution of MCF-7/HER2 Cells.

The combination treatment (¼ IC_{50}–1/8 IC_{50}) resulted in an intermediate distribution, with 32.0% in the G_0/G_1 phase, 8.6% in the S phase, and 54.6% in the G_2/M phase, supporting the predominance of G_2/M arrest and indicating a complementary mechanism that may enhance cytotoxic effects. *G. cowa* extract specifically induced G_2/M phase arrest, while trastuzumab inhibited the G_1-S transition. This combination maintained the G_2/M arrest effect, indicating a synergistic role in cell cycle regulation. The shift in cell cycle distribution was characterised by a decrease in the G_0/G_1 phase population and an increase in the S and G_2/M phases. The rise in S phase cells indicates an accelerated transition from G_1 to S. In contrast, the accumulation in G_2/M suggests impaired mitotic progression due to DNA damage or disruption of mitotic spindle formation.[18,54,55] This effect likely involves regulating key cell cycle proteins, including cyclins and cyclin-dependent kinases (CDKs), which govern cell cycle progression.[56,57]

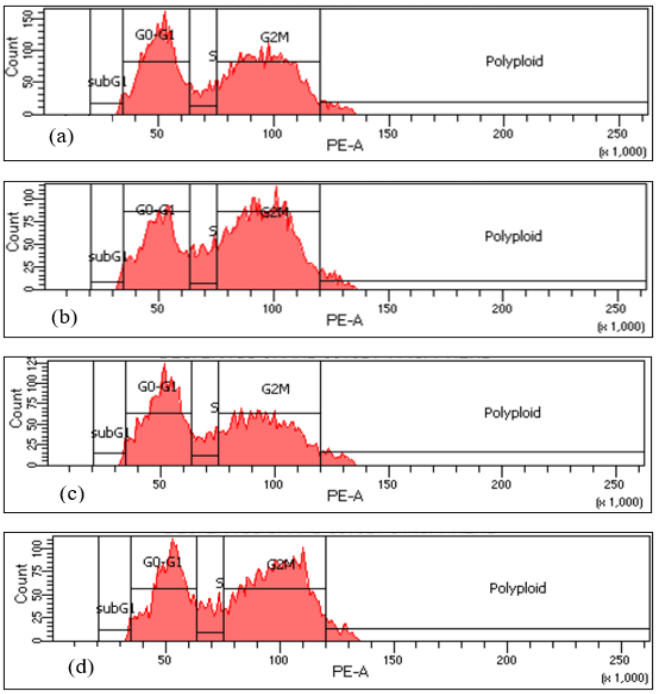

Figure 3: MCF-7/HER2 cell cycle analysis using flow cytometry. (a) Control cells (b) Treatment with *G. cowa* leaf extract (c) Trastuzumab (d) Combination Extract and Trastuzumab

Arresting cancer cells at critical checkpoints is a well-established strategy for inhibiting tumour growth, aligning with the mechanisms of many anticancer agents.[58] Specifically, targeting the G_2/M phase during the DNA repair checkpoint can enhance cytotoxic chemotherapy effects while preventing further genomic instability.[59] These findings support previous reports demonstrating that phytochemicals in *G. cowa* modulate cell cycle regulators, leading to cell growth inhibition.[60,61] The ability of this combination therapy to induce cell cycle arrest at multiple checkpoints underscores its potential as a more effective treatment strategy for HER2-positive breast cancer. In addition to cell cycle inhibition, the combination of *G. cowa* extract and trastuzumab significantly enhanced apoptosis, as evidenced by increased Annexin V expression in flow cytometry analysis (Table 3, Figures 4 and 5). In the apoptosis analysis using flow cytometry, significant differences were observed among the control, monotherapy, and combination treatment groups. In the control group, 85.7% of cells were viable, with 10.9% undergoing apoptosis and 3.8% necrotic. Treatment with the extract at 1× IC_{50} reduced viability to 72.0%, increased apoptosis to 14.9%,

Table 3: Apoptosis Cells Distribution of MCF-7/HER2 Cells Following Treatment with *G. cowa* Leaf Extract and Trastuzumab

Treatment	Concentration	Percentage (%)			
		Live cells	Early apoptotic	Late apoptotic	Necrosis
Control	0	85.7	5.5	5.4	3.8
Extract	1x IC_{50}	72.0	2.7	12.2	14.6
Trastuzumab	1x IC_{50}	78.1	1.1	5.3	16.6
Extract-Trastuzumab	¼ IC_{50}- $^1/_8$ IC_{50}	73.4	6.5	10.7	10.8

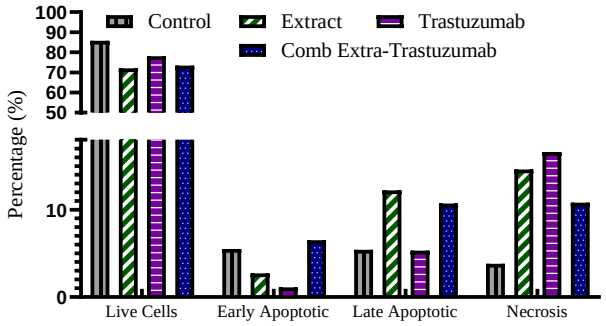

Figure 4: Apoptosis Analysis of MCF-7/HER2 Cells Following Treatment with *G. cowa* Leaf Extract and Trastuzumab

and resulted in 14.6% necrosis. Similarly, trastuzumab at 1× IC_{50} yielded 78.1% viability, 6.4% apoptosis, and 16.6% necrosis. Notably, the combination treatment (¼ IC_{50}–1/8 IC_{50}) further reduced viability to 73.4% while significantly increasing apoptosis to 17.2%, with necrosis observed in 10.8% of cells.

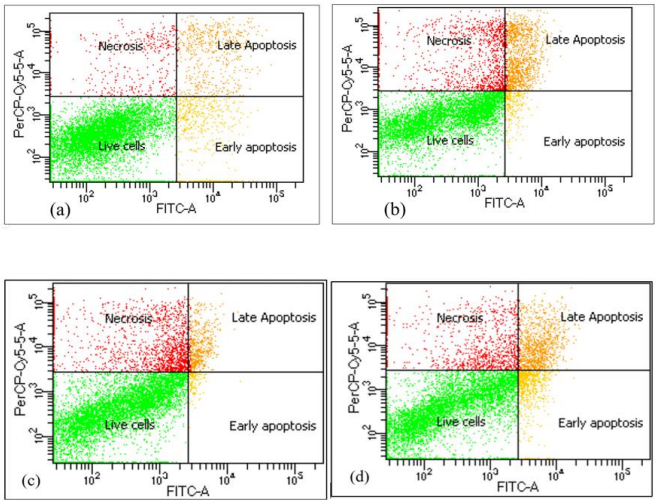

Figure 5: Apoptosis Analysis of MCF-7/HER2 Cells Using Flow Cytometry. (a) Control Cells (b) *G. cowa* leaf extract treatment (c) Trastuzumab treatment (d) Combination of *G. cowa* Leaf Extract and Trastuzumab
FITC-A: Fluorescein Isothiocyanate Area
PerCP-Cy5.5-A: Peridinin-Chlorophyll Protein Cyanin 5.5 Area

These findings suggest that the combination treatment induces a higher level of apoptosis than either monotherapy, indicating a synergistic effect that may enhance therapeutic efficacy against MCF-7/HER2 cells. Apoptosis is a programmed cell death mechanism regulated by intrinsic (mitochondrial) and extrinsic (death receptor) pathways.[62] The bioactive compounds in *G. cowa*, including flavonoids, triterpenoids, and steroids, have been reported to disrupt the cell cycle and trigger apoptosis.[10] Specifically, depsidone and its derivatives, key secondary metabolites in *G. cowa*, contribute to apoptosis induction by modulating cell cycle regulators such as Cyclin D1 and Cyclin E. Depsidone has also been shown to induce G_2/M phase arrest, further disrupting cell cycle progression and enhancing apoptosis.[63,64] The increased apoptosis following combination treatment may result from mitochondrial dysfunction, caspase activation, or modulation of apoptotic regulators such as Bcl-2 and Bax.[65–67] These findings suggest that *G. cowa* extract enhances trastuzumab-induced apoptosis through complementary mechanisms involving both apoptotic pathways. Further studies are required to identify the key molecular mediators responsible for this enhanced apoptotic response.

In the protein expression analysis using flow cytometry, significant changes were observed in several key proteins related to cell cycle regulation and apoptosis (Table 4, Figure 6). In the control group, p53, Cyclin D1, and Cyclin E expression levels were 4.3%, 7.4%, and 8.1%, respectively. Treatment with *G. cowa* extract at 1× IC_{50} significantly increased p53 expression to 9.5% ($p < 0.05$) and reduced Cyclin D1 to 6.9%, while Cyclin E expression slightly increased to 9.4% (not significant). Conversely, treatment with trastuzumab at 1× IC_{50} resulted in decreased expression of p53 (5.3%), Cyclin D1 (5.7%), and Cyclin E (7.9%). Notably, the combination treatment (¼ IC_{50}–1/8 IC_{50}) produced a moderate increase in p53 expression to 7.5% and a further reduction in Cyclin D1 to 5.2%, while Cyclin E expression remained stable at 8.1%. These findings suggest that the combination therapy promotes apoptosis by increasing p53 expression, which activates the pro-apoptotic pathway, while simultaneously suppressing cell proliferation through the downregulation of Cyclin D1, a crucial regulator of G_1 phase progression. Meanwhile, Cyclin E expression remains essentially unchanged.

The upregulation of p53 suggests enhanced transcriptional activation of pro-apoptotic genes, contributing to cancer cell death.[68] Additionally, the combination treatment led to a marked downregulation of Cyclin D1 ($p < 0.01$) and Cyclin E ($p < 0.05$), indicating effective inhibition of cell cycle progression. Cyclin D1 is essential for G_1 phase progression, and its suppression results in cell cycle arrest and reduced proliferation.[32] Interestingly, although Cyclin E expression showed a slight increase following *G. cowa* extract treatment, this change was not statistically significant ($p > 0.05$). The concurrent decrease in Cyclin E further supports the disruption of cell cycle transitions, particularly in the G_1 to S phase transition. However, while Cyclin E expression slightly increased following *G. cowa* extract treatment, it remained stable after trastuzumab and combination therapy. This suggests that the primary mechanism of cell growth inhibition depends more on Cyclin D1 regulation than Cyclin E, which may vary depending on the cancer cell type.[53,69]

Table 4: Percentage of Protein Expression in MCF-7/HER2 Cells After Treatment with *G. cowa* Leaf Extract and Trastuzumab

Treatment	Concentration	Expression Percentage (%)		
		p53	Cyclin D1	Cyclin E
Control	0	4.3	7.4	8.1
Extract	1x IC_{50}	9.5	6.9	9.4
Trastuzumab	1x IC_{50}	5.3	5.7	7.9
Extract-Trastuzumab	¼ IC_{50}- $^1/_8$ IC_{50}	7.5	5.2	8.1

Overall, the combination of *G. cowa* extract and trastuzumab produced more potent effects than monotherapy, reinforcing its potential as a more effective therapeutic strategy for HER2-positive breast cancer.

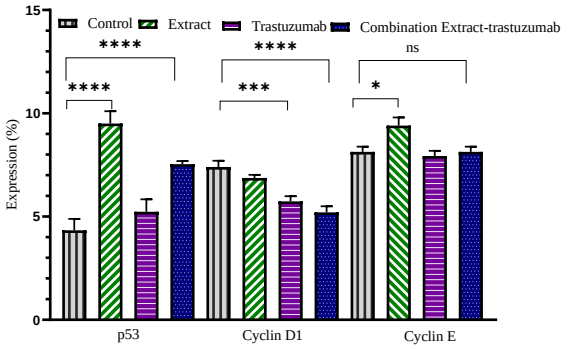

Figure 6: The Combined Effect of *G. cowa* Leaf Extract and Trastuzumab on Protein Expression in MCF-7/HER2 Cells

These findings suggest that *G. cowa* extract enhances trastuzumab efficacy through multiple mechanisms, including cell cycle modulation and apoptosis induction, highlighting its potential as an adjuvant therapy for HER2-positive breast cancer. However, while these *in vitro* results are promising, their clinical relevance remains uncertain due to the limitations of cell culture models, which do not fully replicate the physiological environment of the human body. To confirm the therapeutic benefits of this combination, further studies using *in vivo* models and clinical trials are essential. Future research should focus on identifying the molecular targets responsible for the observed effects and evaluating the pharmacokinetics and safety profile of *G. cowa* extract in preclinical models. Overall, the combination of *G. cowa* extract and trastuzumab presents a promising strategy for overcoming trastuzumab resistance and improving treatment outcomes in HER2-positive breast cancer. Further investigation is necessary to translate these findings into clinical applications and establish their feasibility as a novel therapeutic approach.

Conclusion

This study demonstrated that *G. cowa* leaf extract exhibited more potent cytotoxicity than trastuzumab against MCF-7/HER2 cells, as evidenced by lower IC_{50} values. Combination analysis revealed that a low dose of trastuzumab (125 µg/mL) and *G. cowa* extract (25 µg/mL) produced a significant synergistic effect, enhancing therapeutic efficacy while potentially reducing toxicity. Mechanistically, the combination therapy induced G_2/M phase arrest and significantly increased early and late apoptosis compared to monotherapies. These effects were accompanied by upregulation of p53, indicating activation of the tumour-suppressor pathway, and downregulation of Cyclin D1, supporting the inhibition of G_1-S phase transition. These findings suggest that the combination of *G. cowa* leaf extract and trastuzumab may be a more effective therapeutic strategy for HER2-positive breast cancer than monotherapy. However, further *in vivo* and clinical studies are required to validate these findings, optimise dosing strategy, and assess long-term safety. A deeper understanding of the molecular mechanisms underlying this synergy will be essential for translating this combination therapy into clinical applications, offering a potential alternative for HER2-positive breast cancer treatment.

Conflict of Interest

The authors declare no conflict of interest.

Author's 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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