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Original Research Article

Evaluation of *Garcinia cowa* Leaf Extract as A Potential Anticancer Agent: Cytotoxicity, Selectivity, and Apoptotic Effects on MCF-7/HER-2 Cells

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ABSTRACT

The global burden of cancer highlights the urgent need for therapeutic agents that are both effective and selective, with natural plant-derived compounds offering promising medicinal potential.This study aims to evaluate the anticancer potential of *Garcinia cowa* Roxb. leaf extract, a plant known for its medicinal properties, especially the cytotoxic effect, cell cycle inhibition, apoptosis in MCF-7/HER-2 breast cancer cell lines, and selectivity against normal Vero cells. The cytotoxic activity of *G. cowa* leaf extract against MCF-7/HER-2 cell lines was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry was conducted at the extract's IC_{50} concentration for cell cycle modulation and apoptosis analysis, and the results were analysed using a FACSCalibur flow cytometer. *G. cowa* leaf extract demonstrated strong cytotoxic activity with an IC_{50} of 15.68 µg/mL against MCF-7/HER-2 cell lines and 303.6 µg/mL against normal Vero cells, yielding a selectivity index above 3. The extract significantly altered the MCF-7/HER-2 cell cycle, reducing G_0-G_1 phase cells from 38.1% to 29.9% and increasing the S phase (7.5% to 9.7%) and G_2-M phase (49.5% to 55.3%), indicating cell cycle arrest. Additionally, the extract reduced early apoptosis from 5.5% to 2.7% and increased late apoptosis from 5.4% to 12.2%, while necrosis rose from 3.8% to 14.6%, reflecting enhanced apoptosis, necrosis, and cell accumulation in the S and G_2-M phases. *G. cowa* leaf extract showed strong anticancer potential against MCF-7/HER-2 cells by disrupting the cell cycle and promoting apoptosis and necrosis, with high selectivity for cancer cells.

Keywords: Garcinia cowa, Selectivity Index, Breast cancer, Cytotoxicity, Apoptosis, Cell cycle

Introduction

Breast cancer is the leading cause of cancer-related deaths among women worldwide, with 65,858 new cases reported in Indonesia in 2020, accounting for 16.6% of all new cancer cases.[1] Breast cancer cells grow and differentiate rapidly, driven by the expression of abnormal proteins or genes.[2] HER-2 plays a crucial role in cancer development by producing abnormal proteins that drive cancer cell proliferation. HER-2 overexpression is often associated with aggressive tumour growth, poor prognosis, and resistance to conventional therapies, underscoring the need for targeted and selective anticancer agents.[3]

In the search for effective anticancer treatments, researchers are exploring natural sources like *Garcinia cowa* Roxb. (*G. cowa*), a plant native to West Sumatra, which shows promise against breast cancer.[4] Phytochemical studies of *G. cowa* leaves have identified the presence of xanthones, phloroglucinol, flavonoids, triterpenoids, steroids, tannins, and saponins.[5]

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Various studies have investigated the cytotoxic activity of *G. cowa* plant extracts against cancer cells. The ethanol extract of *G. cowa* fruit rind showed cytotoxic effects against T47D breast cancer cells, with an IC_{50} value of 19.33 µg/mL, while the ethanol extract of its stem bark exhibited more potent activity, with an IC_{50} of 5.10 µg/mL.[6] Additionally, the dichloromethane fraction of the fruit bark induced apoptosis in cervical cancer cells (HeLa).[7] Compounds such as rubraxanthones, *α*-mangosteen, and cowanin, isolated from the stem bark, demonstrated potent cytotoxicity against MCF-7 breast cancer and H-460 lung cancer cells.[8] Cowanin acts as an anticancer agent by arresting the cell cycle at the G_0-G_1 phase and inhibiting the migration of T47D cells.[9] Meanwhile, *β*-mangosteen, isolated from the leaves, showed significant antiproliferative and apoptotic effects against non-small cell lung cancer (NSCLC).[10] The ethanol extract of *G. cowa* leaves was also cytotoxic to T47D cells, with an IC_{50} of 6.13 ± 3.51 µg/mL.[11] Furthermore, computational analyses using molecular docking and dynamics indicated that Garcinisidone-A, isolated from the leaves, can interact with HER-2 in breast cancer cells via hydrogen, carbon-hydrogen, and alkyl bonds, suggesting its potential as an effective anticancer agent.[12] Despite this, the anticancer potential of *G. cowa* leaf extract on HER-2 overexpressing breast cancer cells remains unexplored.

However, some research gaps remain. No studies have specifically tested *G. cowa* leaf extract on HER-2 overexpressing breast cancer cells, such as MCF-7/HER-2. Additionally, information on the safety selectivity of this extract against normal cells, such as Vero cells, is lacking. Furthermore, no studies have used flow cytometry to observe cancer cell cycle inhibition and apoptosis induced by *G. cowa* leaf extract in MCF-7/HER-2 cells.

This study addresses these gaps by evaluating the cytotoxicity, selectivity, and mechanisms of action of *G. cowa* leaf extract on HER-2 overexpressing breast cancer cells. It provides detailed insights into cell cycle modulation and apoptosis by employing flow cytometry. Implementing flow cytometry will also provide detailed data on the extract's mechanism of action on cancer cells. This study aims to develop *G. cowa* extract as a scientifically validated traditional medicinal product. It is expected to contribute significantly to the evaluation of *G. cowa* leaf extract's effects on breast cancer, particularly concerning HER-2 overexpression, extract cytotoxicity, selectivity, and its impact on cell cycle and apoptosis in MCF7/HER-2 cells.

Materials and Methods

Materials

G. cowa leaves, ethanol 70% (Brataco), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), Dimethyl Sulfoxide (Merck), Propidium Iodide (Sigma), PI RNAse staining buffer (BD Biosciences), fungizone 0.5% (Gibco), Sodium Dodecyl Sulfate (Sigma), HCl (Merck), Annexin PI apoptosis kit (BD Biosciences), Dulbecco's Modified Eagle Medium (Gibco), M199 (Gibco), Phosphate Buffered Saline (Invitrogen), Trypsin-EDTA (Gibco), Fetal Bovine Serum (Gibco), Penicillin-Streptomycin (Gibco), RNAse (Roche), and TritonX-114 (Sigma).

Sample preparation

The leaves of *G. cowa* were collected from Kudu Gantiang, Padang Pariaman, West Sumatra (the coordinates, with a latitude of about -0.5107 and a longitude of 100.1640). The plant material was identified and authenticated at the Herbarium of Andalas University (ANDA), Padang, Indonesia, with voucher no 137/K-ID/ANDA/II/2023. After collection, the plant materials were air-dried in a greenhouse at ambient temperature (approximately 25–30°C) for 72 hours, followed by oven-drying at 40°C for 24 hours to ensure complete dehydration. The dried leaves were then ground into a fine powder using a laboratory mixer. Approximately 750 g of the powdered material was macerated in 7.5 L of 70% ethanol for 24 hours. The maceration process was repeated three times with fresh solvent to maximise extraction efficiency. The combined ethanol extracts were concentrated using a rotary evaporator at 40°C under reduced pressure, yielding 190 g of a dark, concentrated extract.[13]

Cell culturing procedure

The MCF-7/HER-2 and Vero cell lines were obtained from the Tissue Culture Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. MCF-7/HER-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose supplementation, while Vero cells were maintained in M199 medium; both media were enriched with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin to prevent contamination. Cells were incubated in a CO_2 incubator at 37°C with 95% humidity to maintain optimal growth conditions. Upon reaching 70-80% confluency, cells were harvested using trypsin-EDTA, centrifuged, and transferred to a fresh medium for further analysis. Daily observations were conducted using an inverted microscope to monitor cell health and growth. All procedures were performed by Good Cell Culture Practice (GCCP) guidelines to ensure quality and safety.[14,15]

Cytotoxicity and Selectivity Assessment Using MTT Assay

The cytotoxic effects of the extract on cell lines were evaluated using the MTT assay, a colourimetric method established for assessing cell viability by measuring mitochondrial dehydrogenase activity in viable cells.[16] In this assay, the MTT reagent, initially pale yellow, was converted by mitochondrial enzymes into an insoluble purple formazan product within viable cells, serving as an indicator of cell viability. The absorbance of the resulting formazan was measured at 570 nm with a microplate reader (Model xMark, Bio-Rad Laboratories, Japan) to quantify cell survival.[17] Cells in each treatment group were exposed to various concentrations of the extract for 48 hours. MCF-7/HER-2 cells were treated with *G. cowa* extract at concentrations of 0.1, 1, 10, and 100 µg/mL, while Vero cells were treated with concentrations of 10, 100, 500, and 1000 µg/mL. After treatment, MTT solution was added to each well, and cells were further incubated for 4 hours to promote formazan crystal formation. Following this incubation period, the medium was carefully removed, and the crystals were dissolved in Dimethyl Sulfoxide to release the purple colour, which was subsequently measured spectrophotometrically (Model xMark Microplate Absorbance, Bio-Rad, Japan).[13] The assay was performed in triplicate to ensure the reliability of the results. Cell survival was determined by calculating the percentage of cell survival by comparing the average absorbance of treatment wells with control wells, which indicates the proportion of viable cells.[14,18]

The Selectivity Index (SI) was calculated by dividing the IC_{50} value of a compound against normal cells by its IC_{50} value against cancer cells. A compound was considered highly selective if the SI value was greater than 3 and less selective if the SI value was less than 3.[19–21]

Flow cytometry Assay

MCF-7/HER-2 cells were seeded at a density of 5 x 10^5 cells per well in a 6-well plate and incubated at 37°C in a 5% CO_2 environment for 24 hours to ensure proper cell adherence. Following the incubation period, the cells were treated with *G. cowa* extract at a concentration corresponding to its IC_{50} value, as well as with MCF-7/HER-2 control cells. The treated cells were then incubated (Model 3429, Thermo Fisher Scientific, USA) for an additional 24 hours. After treatment, both floating and adherent cells were collected by trypsinisation using 0.025% trypsin, and the cell suspension was transferred into a conical tube. The cells were washed three times with cold PBS, with centrifugation (Biofuge Primo R, Thermo Fisher Scientific, Germany) at 2500 rpm for 5 minutes between each wash. The supernatant was discarded, and the cell pellet was carefully retained. To fix the cells, the pellet was resuspended in cold 70% ethanol prepared in PBS and incubated at -20°C for 2 hours. After fixation, the cells were washed three more times with cold PBS and centrifuged at 3000 rpm for 3 minutes each time to remove residual ethanol, followed by examination using a flow cytometer (FACScan, BD Biosciences, USA) to view cell cycle and apoptosis.[22–25]

Cell Cycle Inhibition Analysis

After the initial process was carried out, the cell pellet was then stained with a Propidium Iodide (PI) kit, which included 40 µg/mL of PI and 100 µg/mL of RNAse. The staining mixture was incubated at 37°C for 30 minutes to ensure adequate staining of the cellular DNA. Finally, the stained cells were analysed using a FACScan flow cytometer, and data regarding the distribution of cells across different phases of the cell cycle (G_1, S, and G_2/M) were collected. The resulting data were analysed using ModFit LT 3.0 software to determine the percentage of cells in each phase, providing insights into the impact of the treatments on the cell cycle progression.[26,27]

Apoptosis assay

Subsequently, the cell pellet was stained using an Annexin V kit to detect apoptotic cells. The staining was performed by resuspending the cells in the staining solution and incubating at 37°C for 30 minutes. Finally, the stained cells were analysed using a FACScan flow cytometer to quantify the extent of apoptosis.[28,29]

Statistical analysis

Statistical analysis and data visualisation, including the determination of IC_{50} values for cytotoxic activity and Tukey's posthoc test for group comparisons, were performed using GraphPad Prism version 9 (GraphPad Software, 2020, San Diego, CA, USA)[13,30,31]

Results and Discussion

The MTT assay was used to evaluate the cytotoxicity of *G. cowa* leaf extract on MCF-7/HER-2 (breast cancer) and Vero (normal) cell lines.

Figure 1 illustrates significant differences in cell viability between the two cell lines across various extract concentrations. At concentrations of 10 µg/mL and above, MCF-7/HER-2 cells show a pronounced decrease in viability compared to Vero cells, indicating the extract's selective cytotoxic effect on cancer cells. The Tukey statistical test results show a p-value of less than 0.001 ($p < 0.0001$), suggesting that this difference is highly statistically significant. The significant viability differences at these concentrations are also marked by red asterisk symbols, supporting the hypothesis that *G. cowa* extract has a selective cytotoxic effect on MCF-7/HER-2 cancer cells while being relatively safer for normal Vero cells. These findings reinforce the potential of *G. cowa* extract as a promising anticancer candidate for further investigation, particularly due to its selectivity in targeting cancer cells while minimising effects on healthy cells.

The concentration-viability relationship is detailed in Figure 2. Graph (a) shows that MCF-7/HER-2 viability remains near 100% at low concentrations but declines sharply as concentrations increase, reflecting the extract's potent cytotoxicity against cancer cells. Conversely, graph (b) shows a more gradual decline in Vero cell viability, which remains relatively high at lower concentrations but decreases significantly at higher concentrations.

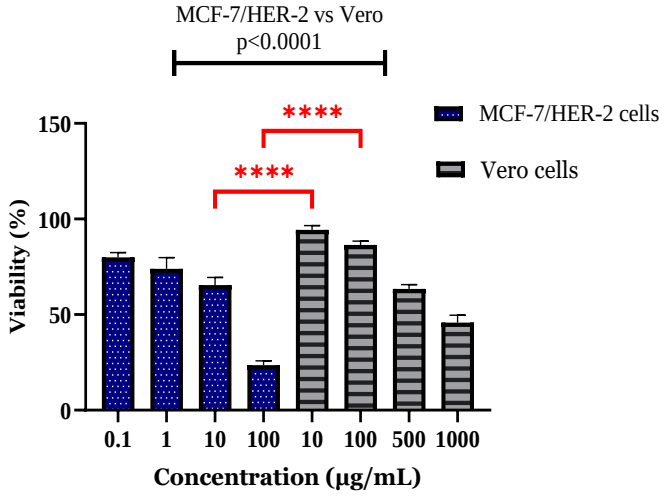

Figure 1: Percentage of test cell viability after *G. cowa* leaf extract treatment

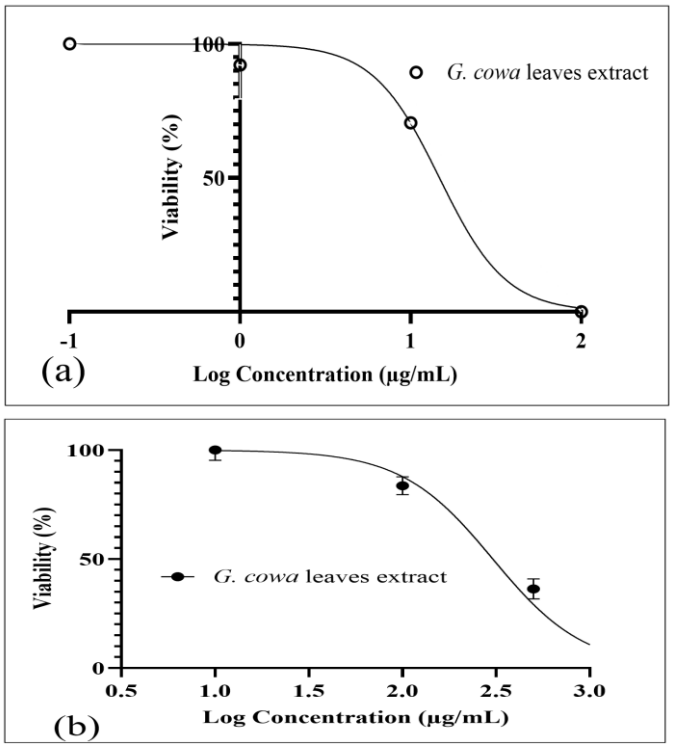

Figure 2: Concentration-viability response of extracts on (a). MCF-7/HER-2 and (b). Vero cell lines

The purpose of this study was to evaluate the anticancer potential of *G. cowa* Roxb. leaf extract, focusing on its cytotoxic, cell cycle inhibition, and apoptotic effects on MCF-7/HER-2 breast cancer cell lines, with selective safety against normal Vero cells. The MTT assay, a colourimetric technique based on cellular metabolism, was used to assess cell viability by measuring the reduction of MTT into formazan crystals in living cells.[16] MCF-7/HER-2 and Vero cells were selected for their growth properties and sensitivity to chemotherapeutic agents, making them suitable models for cytotoxicity analysis.[13,32–34] The results, shown in Figures 1 and 2, reveal dose-dependent cell viability inhibition, supported by IC_{50} values calculated via GraphPad Prism software.

The *G. cowa* leaf extract showed significant cytotoxicity against MCF-7/HER-2 cells, with an IC_{50} of 15.68 µg/mL, while its effect on Vero cells was much weaker, with an IC_{50} of 303.60 µg/mL (Figure 3). The resulting selectivity index was 19.36, indicating a high selectivity (greater than 3) of the extract towards cancer cells compared to normal cells. These findings underscore the potent cytotoxic effect of the extract, as evidenced by its IC_{50} value being below 20 µg/mL.[13] An important parameter in determining anticancer efficacy is the selectivity index, which compares toxicity to normal cells and cancer cells. An SI value exceeding 3 indicates high selectivity for cancer cells, suggesting the extract's stronger efficacy in targeting cancer cells while minimising harm to healthy cells. This characteristic enhances the extract's potential as a safer anticancer agent.[19,35,36] Nevertheless, further research is necessary to explore potential synergistic effects with standard therapies and to elucidate the underlying mechanisms responsible for inducing cell death.

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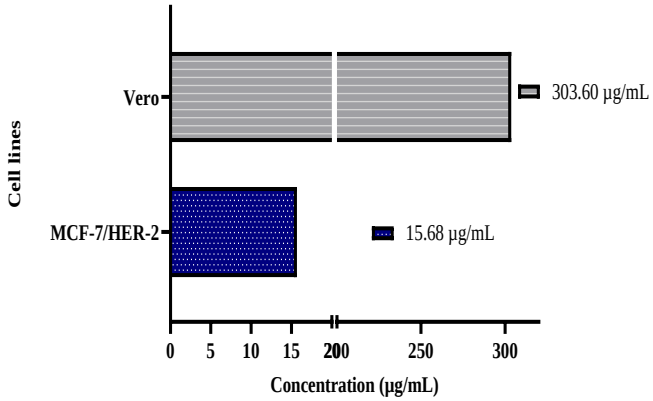

Figure 3: IC_{50} value of *G. cowa* leaves extract.

The distribution of cells in different phases of the cell cycle following treatment can be observed using cell cycle inhibition and apoptosis, allowing for an estimation of how *G. cowa* leaf extract inhibits cell cycle pathways. Cell cycle analysis of MCF-7/HER-2 cells using flow cytometry was performed under various treatment conditions, including control and extract treatment at a concentration of 1 x IC_{50} (Fig. 4 and 5). The cell cycle profile of MCF-7/HER-2 cells post-treatment is presented in Table 1. The flow cytometry method was employed to assess apoptosis, aiming to quantify the number of viable cells, necrotic cells, and apoptotic cells within a short time frame. MCF-7/HER-2 cells were treated with Annexin V, which binds to phosphatidylserine on the plasma membrane during the early stages of apoptosis, detected via fluorescence.[25] As shown in Table 2 and Figures 6 and 7, MCF-7/HER-2 cells were treated with the extract at a concentration of 1xIC_{50}. This study investigated the effects of *G. cowa* leaf extract on the cell cycle of MCF-7/HER-2 cancer cells by examining how cells were distributed across different cell cycle phases before and after treatment. The extract of *G. cowa* leaves at IC_{50} concentration causes changes in cell cycle distribution in MCF-7/HER-2 cancer cells, with a decrease in cells in the G_0-G_1 phase and an increase in the S and G_2-M phases. The extract causes an accumulation of cells in the G_2-M phase, indicating cell cycle arrest and inhibition of cancer cell proliferation while also accelerating the transition through the G_1, S, and G_2-M phases, which could enhance DNA replication and mitosis. The rise in cells in the S phase implies that the extract speeds up the transition from the G_1 to the S phase, increasing DNA replication. Similarly, the increase in the G_2-M phase suggests the extract may hasten the transition from the S phase to mitosis or speed up mitosis itself. These effects could involve inhibiting the G_1 phase, accelerating phase transitions, or regulating cell cycle proteins like cyclins and cyclin-dependent kinases (CDKs).[23–25] Inhibition in both the S phase and G_2-M phase suggests interference with DNA replication and mitosis, leading to DNA damage that ultimately triggers apoptosis when repair is not possible.[37–39]

Table 1: Distribution of MCF-7/HER-2 after treatment with *G. cowa* leaf extract

Treatment	Concentration	Cell phase (%)				
		SubG$_1$	G$_0$-G$_1$	S	G$_2$M	Polyploid
Control	0	1.1	38.1	7.5	49.5	3.3
Extract	1x IC_{50}	1.3	29.9	9.7	55.3	3.5

SubG$_1$: Sub-G$_1$ phase
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

Table 2: Distribution of MCF-7/HER-2 after treatment with *G. cowa* leaf extract

Treatment	Concentration	Percentage (%)			
		Live cells	Early apoptotic	Late apoptotic	Necrosis
Control	0	85.7	5.5	5.4	3.8
Extract	1xIC_{50}	72.0	2.7	12.2	14.6

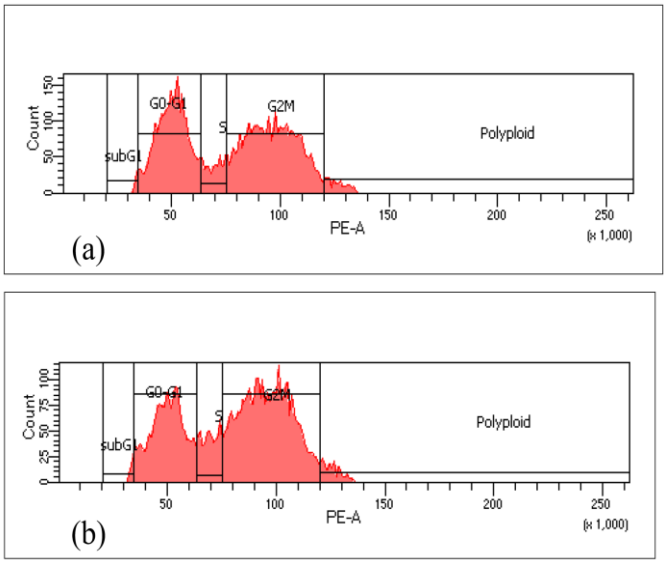

Figure 4: MCF-7/HER-2 cell cycle analysis using flow cytometry. (a) Control cells
(b) Treatment with *G. cowa* leaf extract

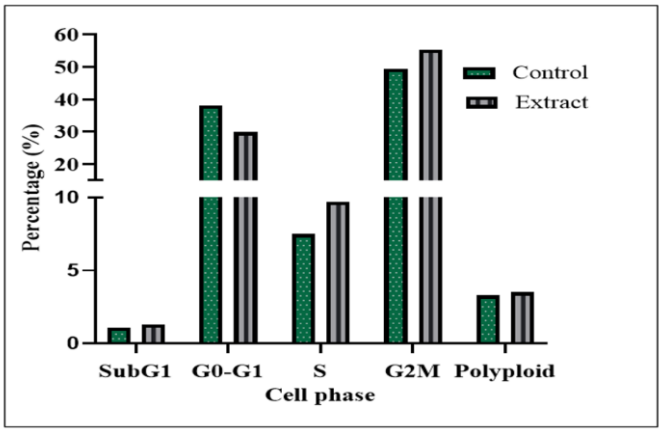

Figure 5: Effects of *G. cowa* Leaf Extract on MCF-7/HER2 Cell Cycle Phase Distribution.

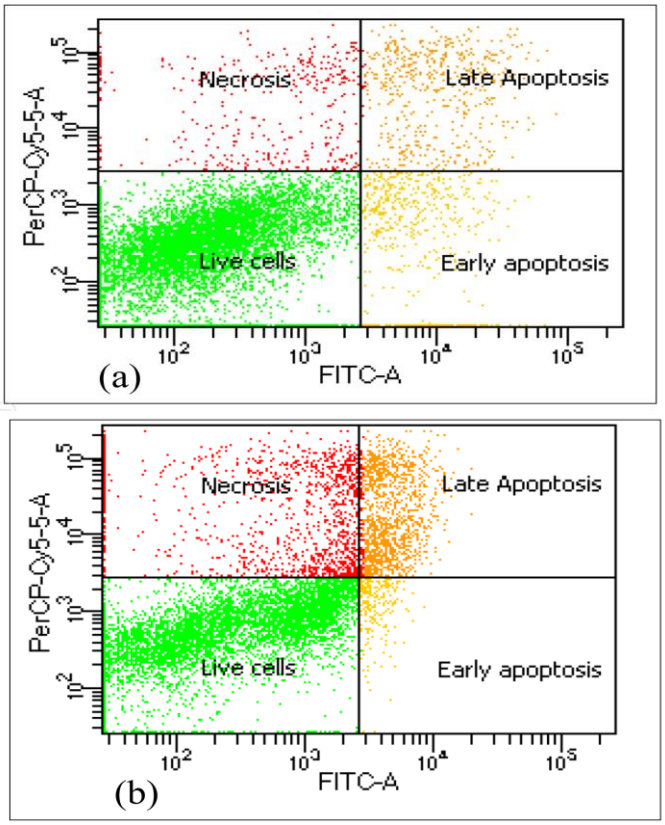

Figure 6: MCF-7/HER-2 apoptosis analysis using flow cytometry. (a) Control cells (b) Treatment with *G. cowa* leaf extract
FITC-A: Fluorescein Isothiocyanate Area
PerCP-Cy5.5-A: Peridinin-Chlorophyll Protein Cyanin 5.5 Area

Further analysis of apoptosis using flow cytometry revealed that before treatment, cells were distributed as follows, with changes after treatment suggesting that the extract may induce cell death by increasing late apoptosis and necrosis, potentially inhibiting cancer cell growth. The extract's cytotoxic effects on MCF-7/HER2 cells were characterised by increased late apoptosis and necrosis, suggesting that it accelerates cell death through both apoptotic and necrotic pathways.[40–42] The extract's secondary metabolites, including flavonoids, triterpenoids, and steroids, disrupt the cell cycle and induce apoptosis.[5,8] *G. cowa* contains active secondary metabolites such as depsidone and its derivatives, which can inhibit the cell cycle and induce apoptosis in breast cancer cells through various mechanisms. Depsidone also inhibits the cell cycle by arresting cells in the G_2/M phase, which in turn affects the regulation of cyclins D1 and E in the G_1 and S phases.[43,44] Arresting the cell cycle at critical phases is a recognised strategy to inhibit cancer cell growth, aligning with the mechanisms employed by many anticancer drugs. Specifically, targeting the G_2-M phase during the DNA repair checkpoint enhances the effects of cytotoxic chemotherapy while preventing further DNA damage.[45–47]

The limitations of this study include the use of *in vitro* models with MCF-7/HER-2 and Vero cells, which, although relevant for initial testing, do not fully represent the biological complexity of the human body, necessitating further *in vivo* models and clinical trials. The specific molecular mechanisms underlying the anticancer activity of this extract have also not been fully explored, requiring additional research to identify essential signalling pathways involved. Furthermore, the synergistic effects between the extract and conventional cancer therapies were not evaluated in this study, even though their collaborative potential could increase effectiveness or reduce the side effects of existing cancer treatments.

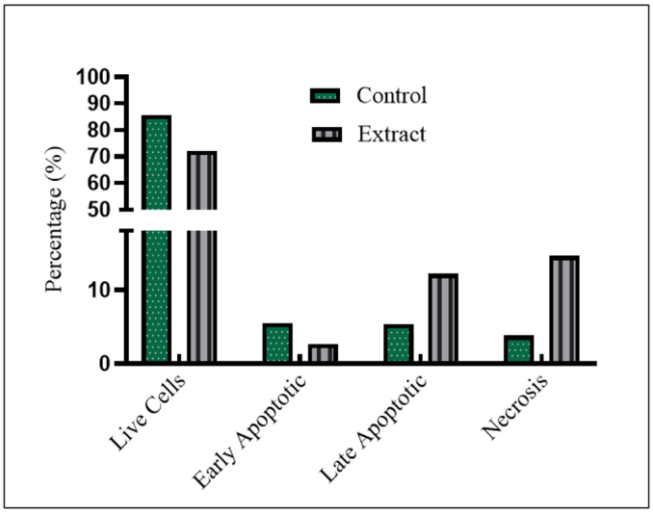

Figure 7: Distribution of MCF-7/HER-2 after treatment with *G. cowa* leaves extract

Conclusion

The *G. cowa* leaf extract demonstrated promising anticancer activity by inducing apoptosis and inhibiting the cell cycle in the S and G_2-M phases. With an IC_{50} value of 15.68 µg/mL against MCF-7/HER-2 cancer cells and a selectivity index above 3, the extract exhibits significant cytotoxic potential. However, further research is required to fully understand its mechanism of action and assess its potential for clinical application, particularly in combination with other cancer therapies. Future studies should focus on elucidating its molecular mechanisms, evaluating preclinical and clinical efficacy, investigating pharmacokinetics, and optimising formulation strategies. Addressing these aspects will provide stronger evidence for the extract's potential as a selective, safe, and effective 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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