

EFFECT OF COWANIN ON CYCLIN D1 EXPRESSION IN MCF-7/HER2 BREAST CANCER CELLS

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ABSTRACT

Objective: Cowanin, isolated from the stem bark of Asam kandis (*Garcinia cowa* Roxb.) has been known to have cytotoxic activity in MCF-7/HER2 breast cancer cells. Recent studies have reported that cowanin compounds can inhibit MCF-7/HER2 cell migration and the T47D cell cycle in the G0-G1 phase. This research aims to determine the effect of cowanin on the cyclin D1 protein expression in MCF-7/HER2 breast cancer cells.

Methods: The treatment consisted of a negative control group and a group given cowanin at a concentration of IC₅₀ value (10,51 µM). The expression of cyclin D1 protein was detected using the western blot method. Observations of protein area and density were carried out using ImageJ software. Data were analyzed using the independent T-test.

Results: The research showed that cowanin compounds induced cell cycle arrest of MCF-7/HER2 breast cancer cells by reducing the expression of cyclin D1 protein (p<0,05).

Conclusion: The findings show that cowanin can significantly decrease the area and density of cyclin D1 protein

Keywords: Cowanin, *Garcinia cowa* Roxb, Cyclin D1, Cell cycle, Western blot, MCF-7/HER2 breast cancer cells

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INTRODUCTION

Cancer is the uncontrolled growth of abnormal cells that become the leading cause of death worldwide [1]. Each year, over 19.3 million patients are diagnosed with cancer and 10 million death cases in the world [2]. The most common cancer in the world is breast cancer, which mainly occurs in women. In Indonesia, breast cancer is the first leading cause of death among women. It is shown through the data by The Global Burden Cancer, with 16.6% of total breast cancer cases [3].

Cancer cells can maintain and induce proliferation signals for their survival. High proliferative signal production supports cancer cell development [4]. Cell proliferation depends on the cell cycle. The process of cell growth and division to reproduce itself occurs in the cell cycle [5]. In the cell cycle, the DNA is only replicated once during the synthesis phase, and identical chromosomal copies are evenly distributed to two daughter cells during the mitosis phase [1]. Each stage is affected by Cyclin-Dependent Kinase (Cdk) activation, which requires binding to a cyclin to become catalytic active. Before entering the synthesis phase, Cdk 4/6 binds to D-type cyclins (D1, D2, and D3) to initiate DNA synthesis and stimulate the cycle's passage through the G1 phase [2, 5].

Cyclin D1 is a D-type cyclin protein family member, the primary molecule in cell cycle regulation. The CCND1 gene on chromosome 11q13 encodes the cyclin D1 protein. A starter protein in the cell cycle will bind directly to Cdk-4 and Cdk-6 to form a cyclin D1-Cdk4/6 complex in the G1 phase. This complex will phosphorylate the retinoblastoma protein (pRB), which codes for proteins in the cell cycle. Phosphorylation of pRB loses its ability to bind the E2F family of transcription factors, so it activates E2F and leads the transcription of several genes required in the G1 to S phase transition [1, 6].

Identification failure of extracellular signals occurs in a wide variety of human cancers. Defect regulation of cyclin D1-Cdk4/6 complex discovered the overexpression of cyclin D1 in cancer, mainly breast cancer [7]. Overexpression of cyclin D1 has been reported in more than 50% of breast cancer cases. Besides the defect of cyclin D1-Cdk4/6, this overexpression can occur due to gene amplification, increased transcription, or disruption of proteolysis of cyclin D1 and even mutations of the pRB [8].

Human Epidermal Growth Factor Receptor-2 (HER-2) is a receptor in breast cells. Excessive activity of the HER-2 gene will encourage the proliferation of breast cancer cells [9]. HER2 overexpression in breast epithelial cells deregulates control of the G1/S phase cycle through increased cyclin D1, E, and Cdk4/6 expression. HER2 will increase cyclin D1 protein, thereby activating Cdk4/6, promoting the development of cancer cells towards the S phase, which can cause uncontrolled proliferation [5].

Cowanin compounds can be isolated from *Garcinia cowa* roots, as previously studied in the research of Wahyuni *et al.* (2016), which succeeded in isolating cowanin from *Garcinia cowa* roots through various stages, ranging from extraction, column chromatology, and radial chromatography. Cowanin compounds have a molecular formula C₂₉H₃₄O₆ and a molecular weight 478 [10]. Previous research showed that cowanin has cytotoxic activity on T47D breast cancer cells with an IC₅₀ 11.11 µg/ml. Cowanin can also significantly inhibit the migration of T47D cells to 0.32 times and can inhibit the cancer cell cycle in the G0-G1 phase [11]. Further research related to the molecular mechanism of action needs to be carried out.

A breast cancer cell line named MCF-7/HER2, which characterized overexpression of HER2, is predicted to have the ability to increase the cyclin D1 protein. MCF-7/HER2 was obtained from the results of cDNA HER2 transfection through pRK5 plasmids into MCF-7 cells and selected using neomycin phosphotransferase, a marker gene [12].

Decreased expression of cyclin D1 is a new target for cancer therapy. As previously found, reduced expression of cyclin D1 protein can stop the development of the cancer cell cycle. Therefore, to understand the contribution of cyclin D1 to cancer progression, a detailed analysis of its expression will be conducted in this study.

MATERIALS AND METHODS

• General

MCF-7/HER2 breast cancer cell line obtained from CCRC (Cancer Chemoprevention Research Center) Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. RPMI 1640 medium, Fetal bovine serum, Trypsin-EDTA, and Penicillin-Streptomycin were

obtained from GIBCO (Auckland, New Zealand), Phosphate buffered saline (PBS) was obtained from Thermo Fischer Scientific (Massachusetts, US), and trypan blue were purchased from Merck (Hohenbrunn, Germany).

• **Plant material**

The pure cowanin compound isolated from the stem of Asam kandis (*Garcinia cowa* Roxb.) was obtained from the extraction carried out by Dira hefni at the Research Laboratory of the Faculty of Pharmacy of Andalas University [11].

• **Cell culture**

The MCF-7/HER2 cancer cell line was cultured on the RPMI complete medium, which was added with 10% FBS (Fetal Bovine Serum) as a nutrient for cells and 1% Penicillin-Streptomycin antibiotic. Cell lines are incubated in the cell culture process using a CO₂ incubator at 37 °C and 5% CO₂. After the cell reaches 80% confluent, the cells in the flask are given 3 ml of trypsin-EDTA, which is then incubated for 5-10 min so that the cell detaches from the base of the flask. Observe the cells under an inverted microscope, and add trypsin-EDTA containing the cells into a centrifugation tube to obtain the cells to be suspended with 3 ml of medium. Put the cell suspense into a new flask to subculture. Change the growth medium every two days.

• **Western blotting**

The cells were washed twice with ice-cold PBS and lysed in lysis buffer (25 mmol Tris-HCl pH 7.6, 150 mmol NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) that also added 10 µl *phosphatase* dan *protease inhibitor* for 5 min on ice. Lysates were cleared by centrifugation. Protein concentration was measured with

the Invitrogen Qubit 4 Starter Package. Cell lysates containing 40 µl were resolved on SDS-PAGE and transferred to nitrocellulose at a constant current of 20 volts for 7 min. Nitrocellulose membranes were blocked by starting block T20 (PBS) blocking buffer for one hour at room temperature. Incubate with primary (cyclin D1 recombinant rabbit monoclonal antibody) and secondary antibody (goat anti-rabbit IgG) separately and block using PBS T20 buffer. The nitrocellulose was washed with a TMB Blotting solution to detect the protein band.

• **Statistical analysis**

The study's results were analyzed statistically with IBM SPSS software version 25. The experiment was carried out with as many as three repetitions, and the results were significant if the p-value was <0.05.

RESULTS AND DISCUSSION

The supernatant from MCF-7/HER2 breast cancer cells was used to detect the presence of the target antigen using the western blotting methods. The protein bands obtained after the blotting procedure were observed using ImageJ software (fig. 1).

The cyclin D1 protein has a molecular weight of 36 kDa, so the band at 30-40 kDa indicates the cyclin D1 protein band [13]. The observations show that the cyclin D1 protein band in the negative control group is thicker and broader than in the cowanin compound treatment group, indicating decreased cyclin D1 protein expression. Beta-actin is used as "housekeeping," which is used as an internal standard because of its high and uniform expression in cells, stability under various conditions, stability throughout the cell's life, and resistance to environmental changes that occur [14].

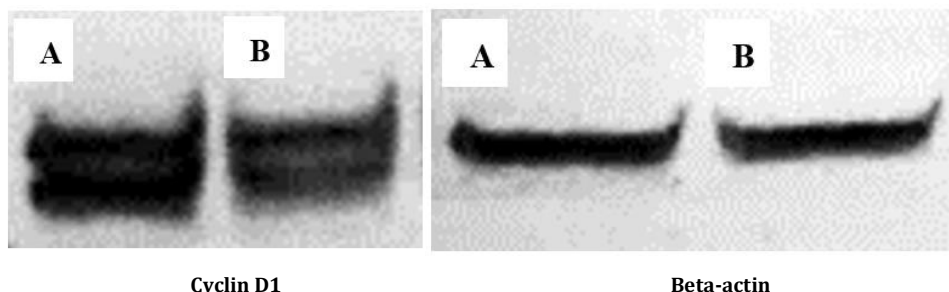


Fig. 1: Cyclin D1 protein and beta-actin band in MCF-7/HER2 cells. Negative control (A) and cowanin 10.51 µM (B)

The bands were analyzed for the area and density values of the protein bands formed using ImageJ software. The results of the analysis of protein area values can be seen in fig. 2. It was found that the area values of the cowanin treatment groups were smaller than the area values of the negative control group.

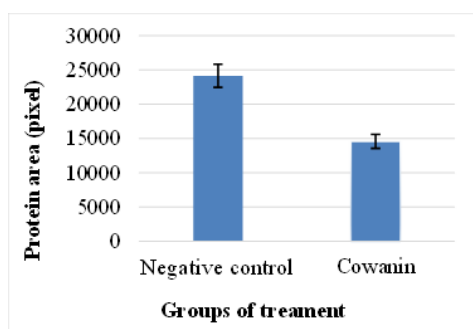


Fig. 2: The average area of cyclin D1 protein in MCF-7/HER2 cells

The results of the analysis of the protein density values are shown in fig. 3. It was found that the protein density values of the cowanin

treatment group were lower when compared to the density values of the negative control group. This corroborates the results obtained on the area value. The expression level of cyclin D1 protein in MCF-7/HER2 breast cancer cells treated with cowanin was significantly different (p<0.05) compared to the negative control. This indicates that the administration of cowanin compounds derived from the *Garcinia cowa* Roxb plant on breast cancer cells MCF-7/HER2 was able to suppress the overexpression of the cyclin D1 protein.

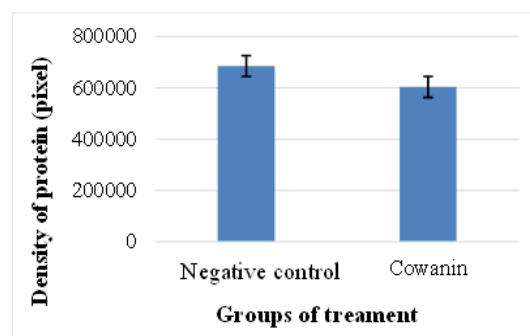


Fig. 3: The average density of cyclin D1 protein in MCF-7/HER2 cells

Cancer cells grow indefinitely, characterized by an increase in cyclin D1 protein in the cell, which can be observed through the formation of protein bands on the blotting membrane. The greater the area and density of the protein band indicates the greater the amount of protein on the blotting membrane.

Cowanin is a xanthone derivative compound of the oxygenated xanthones, which is found in the form of yellow crystals [15]. Cowanin has a xanthone ring with several functional groups, isoprenyl groups at positions C-2 and C-8, and hydroxyl groups at positions C-3 and C-7. Based on its structure, cowanin has the same position and type of functional group as other xanthone members, α -mangostin, β -mangostin, and γ -mangostin. Mangostin has been shown to have antitoxic activity through its involvement in cell cycle progression [16]. Thomas *et al.* (2013) reported that α -mangostin can induce cell cycle arrest in the G1 phase by reducing the expression of cyclin D1 in prostate cancer cells [17]. Zhu *et al.* (2021) also reported the activity of α -mangostin in inhibiting the cell cycle in the G0/G1 phase, as evidenced by the decreased expression of the cyclin D1 protein in MDA-MB-231 breast cancer cells [18]. This ensures that cowanin can have the same activity as mangostin in inhibiting and even stopping cell cycle regulation by reducing the expression of cyclin D1 protein.

Research conducted by Hefni *et al.* (2020) proved that cowanin can prevent cancer cell progression by inhibiting the T47D breast cancer cell cycle in the G1 phase, as indicated by the decreased presence of cancer cells in the S phase [11]. This proves that cowanin can inhibit the cell cycle by reducing the cyclin D1 protein. As the role of the cell cycle regulator, the fundamental task is to promote cell cycle progression through the phosphorylation of substrates. The cyclin D1 protein, which activates the early phase of G1 through the cyclin D1-Cdk4/6 complex, will initiate phosphorylation of retinoblastoma protein, which will release E2F (transcription factor) so that the cell cycle can continue into the S phase [1]. Cowanin will reduce the expression of excess cyclin D1 protein in cancer cells so that the cyclin D1-Cdk4/6 complex is not formed, the cell cycle will not continue, and the proliferation of cancer cells will stop. The decrease in cyclin D1 protein expression, which is expected to inhibit the development of cancer cells, was proven in this study. The Cowanin's ability to induce MCF-7/HER2 breast cancer cell cycle arrest indicates its potential as a new chemotherapeutic agent that needs further development [19].

CONCLUSION

The cowanin compound reduced the expression of cyclin D1 protein in MCF-7/HER2 breast cancer cells, as seen in the decrease in the area and density of cyclin D1 protein.

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AUTHORS CONTRIBUTIONS

Dira Hefni: Writing-Original Draft, Writing-Review and Editing, Methodology; Angelica Maysya Nahda: Writing-Original Draft, Methodology; Dachriyanus: Supervision, Resources, Review and Editing; Fatma Sri Wahyuni: Conceptualization, Supervision, Resources and Funding acquisition.

CONFLICT OF INTERESTS

Authors state no conflict of interest.

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