

Cowanin, a Cytotoxic Xanthone from Asam Kandis (*Garcinia cowa*, Roxb.) Reduced Cell Migration and Induced Cell Cycle Arrest on T47D Human Cancer Cell

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Abstract—This study aims to investigate and evaluate the mechanism action of cowanin, a cytotoxic xanthone isolated from ethanolic extract of the stem bark of asam kandis (*Garcinia cowa* Roxb). This compound was isolated after successive column and radial chromatography to give a yellow needless crystal, m.p. 121-124 °C. Based on ultraviolet, infrared, mass and nuclear magnetic resonance spectroscopic data and comparison with those of the literature, this compound was elucidated as cowanin. Since it had activity against T47D human breast cancer cell lines, further investigation of its mechanism activity was performed to explore the effects of cowanin on cell viability, migration of cells, and the cell cycle activities against T47D breast cancer cells. Viability of cell was carried out by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, migration of cells by scratch migration assay, and the cell cycle analysis by flow cytometry method. As a result of this investigation, it can be seen that cowanin could inhibited T47D cells' growth at concentration 0.1, 1, 10, and 100 µg/ml at 48 hours with the IC₅₀ value of 11.11 µg/ml. Cowanin exhibited inhibiting the cell migration T47D cells at concentration 11.1 µg/mL treated cells at 48 h was 0.32 fold compared to control, suggesting the potent inhibitory effect of it. Cowanin is caused in significant detention of T47D cells at the G₀-G₁ phase of the cell cycle. Based on these data, it can be concluded that cowanin is a potential candidate to be developed as an anticancer drug.

Keywords— cell cycle; cowanin; *Garcinia cowa* Roxb; T47D; breast cancer; flow cytometry.

I. INTRODUCTION

Cancer remains the leading cause of death worldwide [1]. Majority cancers are related to lifestyle. There were around 9.6 million deaths from 18.1 million cancer cases worldwide in 2018. This case is predicted to increase in 2030, with around 13 million deaths in 21.7 million cases. The most significant cause of cancer deaths each year is lung cancer, liver, stomach, colorectal, and breast cancer [2]. Cancer is a disease in which cell cycle irregularities occur in the normal processes of cell division. The cell cycle is a vital process in the life of any organism [3]. Cell proliferation can be controlled in healthy cells, whereas cell proliferation is a disorder in cancer cells. Several plant-derived compounds could inhibit at different stages of the cell cycle. Doxorubicin blocks the phase of the G₂; topotecan blocks

the phase of the S and G₂, methotrexate at the S-phase [4], [5].

Cell migration is an important process that plays a crucial role in many physiological and pathological mechanisms. The increasing capability of cancer cell migration plays an indispensable part in tumor invasiveness and metastasis [6]. In continuation of our study on Sumatran Plants, several plants have been investigated for their chemical and biological activity properties [7]–[14]. Improvements within the efficiency and toxicity profile of anticancer drugs are necessary to develop more straightforward treatments, which could be achieved through natural products [15]. Cowanin [Fig.1] was isolated from the stem bark of *Garcinia cowa*. Since this compound showed inhibited breast cancer cell lines [16], this compound's ability to inhibit cell migration and cell cycle arrest by scratch and flow cytometry method was further investigated.

II. MATERIALS AND METHOD

A. General

Ultraviolet and Infra-Red (KBr) spectra were obtained using UV spectrophotometer (Shimadzu 1900) and FTIR spectrophotometer (Thermo Nicolet iS10), respectively. Mass spectra were recorded using MS spectrometer (JEOL J HX-110A). ^1H and ^{13}C NMR spectra (CDCl_3) were obtained using NMR Spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C), respectively. The NMR spectrum was interpreted included the ^1H - ^1H COSY, HMBC, and HMQC techniques. Melting points were observed using Stuart SMP 30 melting point apparatus. Microplate reader (BioRad xMark) was used to measure the formazan solution. Cell cycle analysis was determined using FACScan flow cytometry (Becton Dickinson, Sunnyvale, CA). Column chromatography used Silica Gel 60 (70–230 mesh), and TLC analysis was executed on Silica Gel plates (Merck Kieselgel 60 GF254, 0.25 mm, 20 x 20 cm).

T47D breast cancer cells obtained from the laboratory of Parasitology Faculty of Medicine Gadjah Mada University. RPMI 1640 medium and Penicillin and Streptomycin were purchased from Life Technologies (Paisley, UK), Trypsin-EDTA was obtained from GIBCO (Auckland, New Zealand), Fetal bovine serum from PAA Laboratories (Linz, Austria). Propidium iodide, Phosphate buffered saline (PBS) tablets, ribonuclease A (RNase A) were purchased from Sigma Chemicals (St. Louis, USA). Tween-20 and Dimethylsulfoxide (DMSO) were purchased from Merck (Hohenbrunn, Germany) and 3-(4,5dimethylthiazol-2yl)-diphenyltetrazolium bromide (MTT) from Phytotechnology Laboratories (Kansas, USA).

B. Plant Material

The stem bark of *G. cowa* was collected from the Biological Education and Research Forest Andalas University, Padang, West Sumatra, Indonesia, in April 2019. Dr. Nurainas identified the plant from Herbarium Universitas Andalas, Indonesia.

C. Isolation of Cowanin

Powdered stem bark stem bark (1.1 Kg) of *G. cowa* was successively extracted with ethanol yielded a dark mass (56.5 g). The ethanolic extract was poured into 500 ml water and then defatted with hexane (4 x 250 ml) followed with ethyl acetate (8x100 ml), evaporated to give hexane (5,7 g) and ethyl acetate fraction (43 g).

D. Cell Culture

T47D cells were maintained in RPMI medium supplemented with ten %v/v FBS, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 IU/mL penicillin. Cells were grown in a humidified 5% CO_2 incubator (37°C). After the cells reached 80% confluency, a 1 ml solution of trypsin-EDTA was put for 5-10 min to remove cells. The cells were observed under the inverted microscope, and 3 ml of complete growth medium was added to the flask. $0.5\text{-}1 \times 10^6$ cells were subcultured into a new flask containing fresh medium.

E. Cell Viability Assay

MTT assay was used to investigate cell viability. T47D (2×10^3 cells/well) were sown into 96-well culture plates and

incubated in 5% CO_2 at 37°C overnight. The cells were incubated with extract, fraction, and cowanin for 48 hours. Then, 100 μL MTT (0.5 mg/mL) was put to each well and were incubated for four h at 37°C. MTT was removed from the plate. 100 μL of DMSO was added to each well. The absorbance was measured with the Microplate Reader at 550 nm.

F. Scratch Assay

The migration of cells was determined by measuring cells to migrate to lesions produced on confluent cells' surface. T47D cells were collected by trypsinization, were sown in 24-well plates, and grown in complete medium for 24 h. The cells were scraped using a 100 μL tip to create a straight-line "scratch." After washing with PBS, a medium containing FBS and cowanin were added then incubated for 48 h. Two different concentrations of cowanin (11.1 and 22.2 $\mu\text{g}/\text{ml}$) and images were collected at 24 h and 48 h. The ImageJ software was used to analyzed quantitatively and determine the percentage of wound closure.

G. Flow cytometry

Amount of 5×10^5 cells in RPMI 1640 medium were sown in 6-well culture dishes. After overnight, two concentrations, 11.1 and 22.2 $\mu\text{g}/\text{ml}$ of cowanin, were added to cells for 24 – 72 hours. Trypsin was added to cells then collected in cold 1% BSA-PBS buffer. Each determination, approximately 5×10^5 – 5×10^6 cells, were selected. The cells were centrifuged for 10 min at 1000 rpm, washed twice using 1% BSA-PBS buffer, resuspended in 1 ml PBS, and moved to a polystyrene circular bottom tube. 40 μl of 2.5 mg/ml propidium iodide and 20 μl of 10 mg/ml RNase A were added and were incubated at 37°C for 30 min.

FACScan flow cytometry in which an argon ion laser (488 nm) was used to excite PI, and it analyzed PI bound to DNA. Emission above 550 nm was recorded. Data of flow analyzed by Cell Quest program to see the distribution of cells. Each histogram represented the DNA content of the G0-G1, S, and G2-M phases of the cell cycle. The inhibition of the cell cycle that occurs can be found by comparing the test's effect with the control treatment [17].

H. Statistical Analysis

The result was analyzed using GraphPad Prism software (Intuitive Software for Science) version 7.0. Each experiment was carried out three times, and the difference is said to be significant if p-value <0,05.

III. RESULTS AND DISCUSSION

A. Isolation of Cowanin

A portion of ethyl acetate fraction was subjected to column followed by radial chromatography to give a single compound that was identified as cowanin (Fig. 1) as a yellow needles, mp 121-124 °C. The EI mass m/z 478, consistent with a molecular formula $\text{C}_{29}\text{H}_{34}\text{O}_6$ (Fig. 2). UV Absorption of UV at 243.60 nm (4.52) and 315.80 nm (4.37) (Fig. 3). The IR spectrum (Fig. 4) showed conjugated carbonyl and hydroxyl absorption at 1648 cm^{-1} and 3391 cm^{-1} , respectively. ^1H NMR spectrum displayed signals at δ 13.81 (s, 1-OH) for chelated hydroxyl group. Signal δ 6.84

(1H, s), and δ 6.30 (1H, s) were assigned as two aromatic protons, and signal at δ 3.81 (3H, s) was assigned as one methoxy group. Prenyl moiety showed by the signals at δ 3.46 (2H, d, $J=7.0$), δ 5.31 (1H, t), δ 1.61 (3H, s), and 1.83 (3H, s). The rest of the signals was assigned as a geranyl unit. The signal at δ 4.10 (2H,d) was assigned as methylene protons H1'' at two broad triplets at δ 5.31, and 5.07 of the olefinic protons were assigned as H2,'' and H6'', two multiplets at δ 2.10 and δ 2.01 was assigned as the methylene protons H4'' and H5'', respectively, and three singlets at δ 1.56, 1.85 and 1.61 of were assigned as methyl groups H8'', H9'' and H10'', respectively (Fig. 5). The ^{13}C NMR spectrum of cowanin showed several signals, one carbonyl signal at δ 182.2 (Fig. 6).

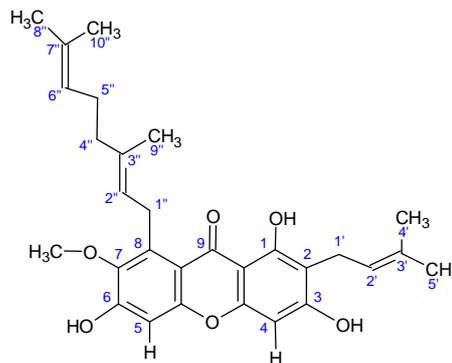


Fig. 1 The chemical structure of Cowanin

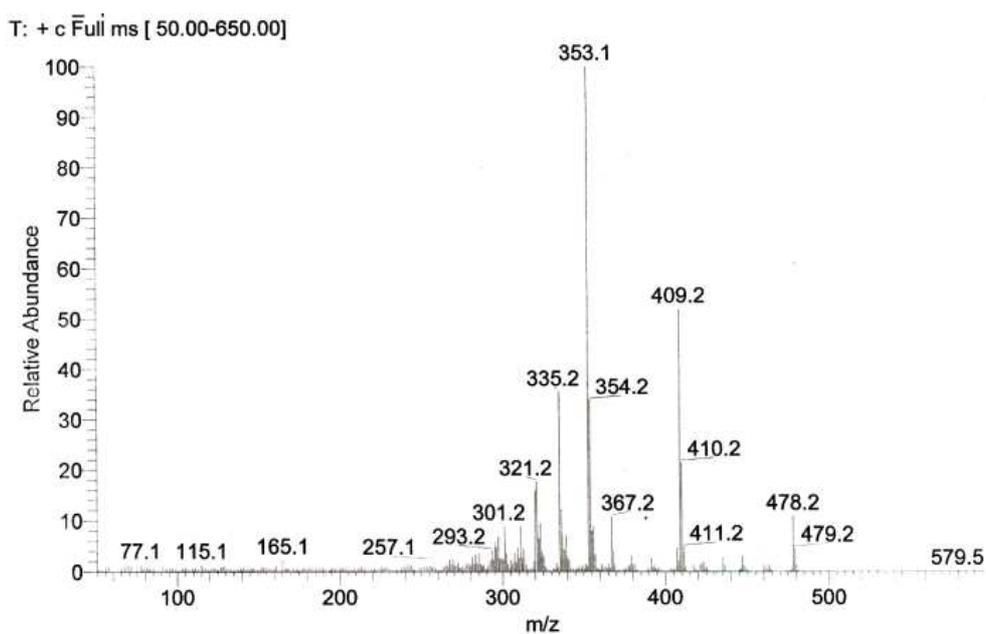


Fig. 2 EI-MS spectrum of cowanin

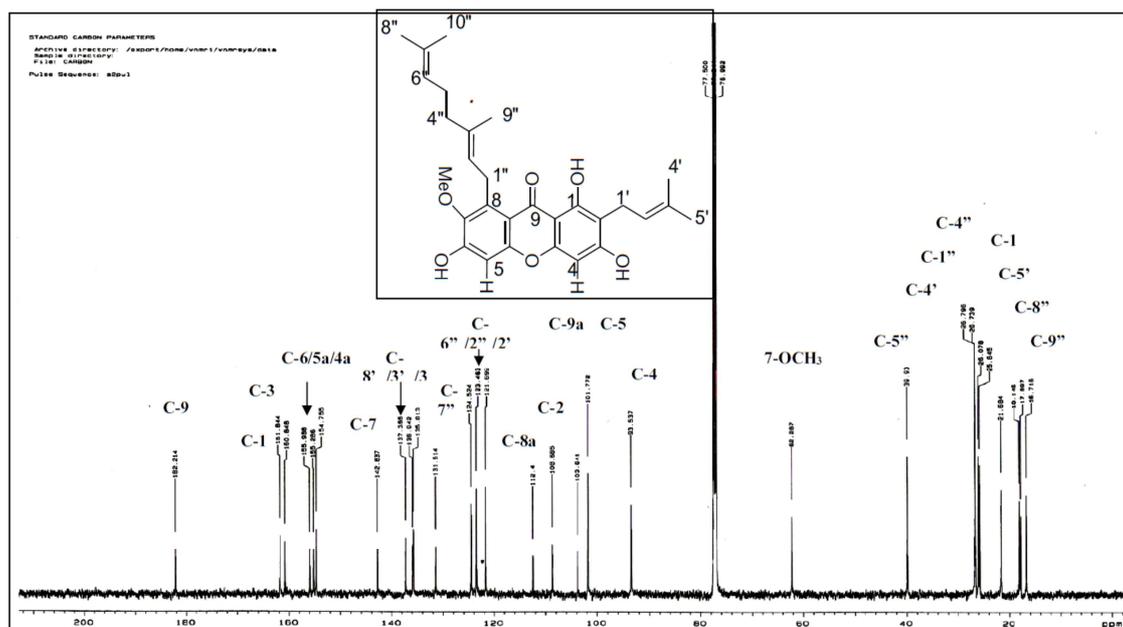


Fig. 3 The ultraviolet spectrum of cowanin

B. Inhibition of T47D Cancer Cell Viability by Cowanin

Cowanin inhibited the T47D cells. The 50% inhibitory concentration were $11.11 \pm 0.13 \mu\text{g/ml}$ after incubation 48 h (Fig. 7).

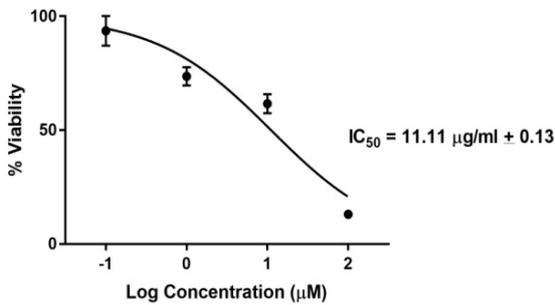


Fig. 7 Inhibition of T47D cancer cell viability by cowanin. The data represent the means \pm SEM of three independent experiments.

C. Cowanin Inhibits Cell Migration in T47D Cancer Cell

The ability of tumor cells to migrate is closely associated with their metastatic potentiality [18]. Inhibition T47D cell migration through cowanin was examined by a scratch assay and shown in Fig. 9. Photographs in Fig. 8 presented the migration with two concentrations of cowanin, and the quantification analysis was shown in Fig. 9. The untreated group cells moved into the scraped area over time, while the cowanin treatment group significantly inhibited cell migration. The quantitative assay demonstrated that the migratory potential of cowanin at concentration $11.1 \mu\text{g/mL}$ treated cells at 48 h was 0.32-fold compared to control, suggesting the potent inhibitory effect of cowanin. It might be concluded that cowanin effective as anti-metastasis, and it was thus confirmed to be dose-dependent and accompanied by cell growth inhibition.

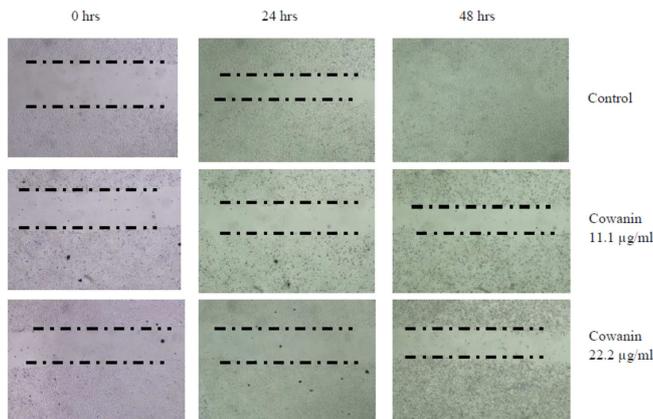


Fig. 8 Cowanin inhibits the migration of T47D cells.

D. Cell cycle Analysis Shows G₀-G₁ Arrest by Cowanin

The population in the G₀-G₁ phase after 24 h treated T47D cells with cowanin increased, and cells will be colored with propidium iodide (PI) for DNA labeling. As can be seen in Fig. 10-11 abnormal cell cycle progression pattern by the control cells, whereas treatment ($11.1 \mu\text{g/mL}$) and ($22.2 \mu\text{g/mL}$) of cowanin increased in the G₀-G₁ phase and a decrease in the S and G₂-M phase. Flow cytometry

analysis showed that cowanin could inhibit T47D breast cancer cells at the G₀-G₁ phase.

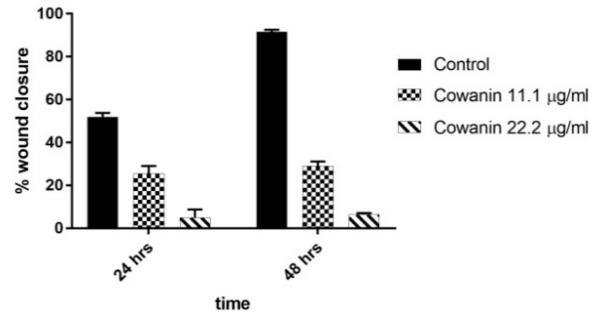


Fig. 9 Quantification of the migration of T47D treated cowanin. The data represent the means \pm SEM of three independent experiments.

Cowanin treatment group caused in significant detention of T47D cells at the G₀-G₁ phase of the cell cycle. The G₀-G₁ phase of the control cells was 52.19%, and the ratio of cells in G₀-G₁ phase was increased (54.12% and 67.72%) at with 1x concentration IC₅₀ ($11.1 \mu\text{g/ml}$) and after treatment with cowanin with 2x concentration IC₅₀ ($22.2 \mu\text{g/ml}$) respectively. An increase G₀-G₁ population of cells was related by a decrease in cell numbers in the S and G₂-M phases.

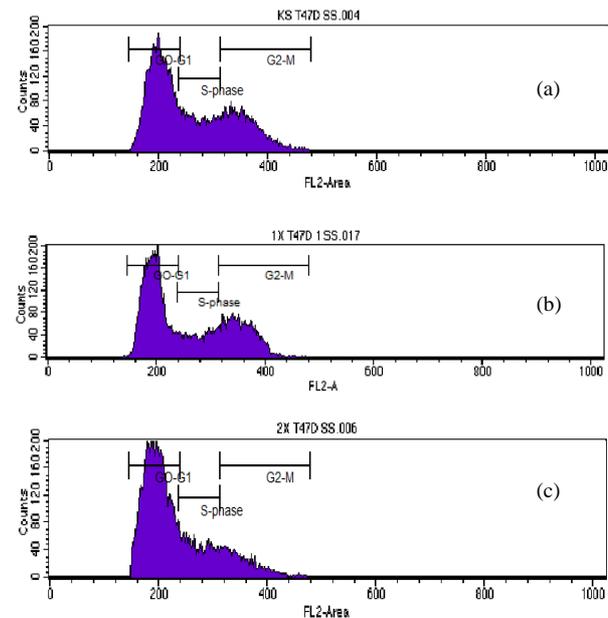


Fig. 10 Histogram T47D breast cancer cells were analysed by flow cytometer, control (a), cowanin $11.1 \mu\text{g/ml}$ (b), cowanin $22.2 \mu\text{g/ml}$ (c).

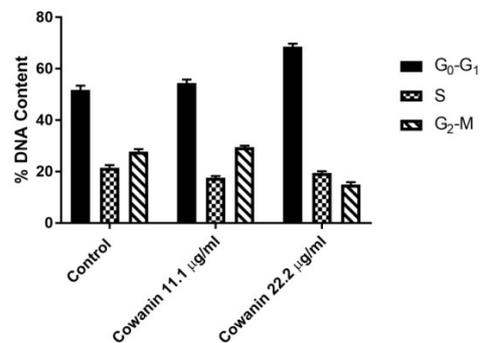


Fig. 11 Percentages of G₀-G₁, S, and M phase after treatment by cowanin. The data represent the means \pm SEM of three independent experiments.

The proliferation and growth of the cells are mediated through the cell cycle. Numerous studies have been carried out to see the relationship between cell cycle parameters with cancer, and the arrest of the cell cycle has become potentially object on cancer treatment [19].

One of the targets of cytotoxic compounds work on the G0-G1 phase is as an inhibitor of Cdk (Cyclin-Dependent Kinase) to bind the Cdk Cyclin-Cdk complexes. Cyclin and Cdk is a cell cycle regulator protein. After doing cell division, the cells will enter the G0 phase. With growth factor stimulation, then the cell will enter a phase of G1. In the early phases of G1, Cdk4 and Cdk6 enabled by Cyclin D. The complex between Cdk 4/6 with Cyclin D will initiate the phosphorylation of the retinoblastoma protein so can release transcription factors required in cell cycle progression to S phase [20]. By inhibiting the bond of Cyclin-Cdk, it will require no phosphorylation of protein retinoblastoma, and the cell cycle will be interrupted.

Other cytotoxic compounds working mechanism in phase G1 is on the restriction point (R) that appears at the end of the G1 phase [3]. The restriction point is a G1 phase checkpoint mechanism to detect whether there is damage to the cells' DNA. Since cowanin prevents the development of breast cancer cells in the G0-G1 phase T47D, it causes damage to the dominant cell so that the cell's DNA cannot perform the repair of damaged DNA and restriction point was unable to breakthrough. When R is not exceeded, the cell will return to the G0 phase, and the cell cycle does not continue. This mechanism so that the expected T47D breast cancer cells do not experience progression gives the more malignant.

Cowanin is a compound derived from stem bark Asam Kandis (*Garcinia cowa* Roxb.). From its structure, seen that cowanin has a cluster of hydroxy, prenyl, and methoxy group. Some studies mention that the existence of the cluster, prenil, hydroxy, and methoxy group can affect the compound's activity in inhibiting cancer [21].

IV. CONCLUSION

The mechanism of anticancer activity of cowanin on T47D cells was determined as stimulation of G₀-G₁ phase cell cycle arrest and reduced migration T47D breast cancer cell.

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