# Isolation of Active Compound from Zingiber Purpureum Roxb. Using Bioassay Guided Fractination Method for WIDR Colon Adenocarcinoma Cell Line 

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#### Abstract

Zingiber purpureum Roxb. was a plant that has the potential to be developed as a chemotherapy agent. Bioassay guided fractination aims to examine the anticancer activity of $n$-hexane, ethyl acetate, and methanol fractions from ethanolic extract of bangle plant rhizome to colon cancer cell type WiDr and Vero cells. The apoptotic test and modulating cell cycle were observed by flow cytometry method. Isolation process of the most active fraction. The pure isolates obtained were identified by structures with structural elucidation using ultraviolet, 1D-NMR, 2D-NMR and liquid chromatography-mass spectrometer. Obtained 2,3 -dihydro- 5 -hydroxy-7-methoxy-2-phenylchromen-4-one compound of ethyl acetate fraction number 21. With a value of $\mathrm{CC}_{50}$ to a WiDr cell of $11.24 \pm 3.44 \mu \mathrm{~g} / \mathrm{mL}$ and $342.22 \pm 4.98$ $\mu \mathrm{g} / \mathrm{mL}$ to Vero cells.


Keywords: Zingiber purpureum Roxb., WiDr, Vero, 3-dihydro-5-hydroxy-7-methoxy-2 phenylchromen-4one

## Introduction

Colorectal cancer ranked fourth in all men and women worldwide, with an incidence of $1,400,000$ new cases diagnosed in 2012 [1]. IARC states that cancers of the colon and rectum are the most common incidence of cancer with the number 3 ( 34,000 per year, $15.9 \%$ ) and the third cause of death ( 18,000 per year, $10.8 \%$ ). Chemotherapy was one of the therapies in the treatment of cancer. Chemotherapy agents generally have low selectivity properties because they are antiproliferative to normal selkanker and cell [2]. In addition, some chemotherapy has a narrow therapeutic index, and may lead to multidrug resistance (MDR) and adverse side effects [3].

One of the problems that often arise in the treatment of cancer is the resistance of chemotherapy drugs (drug resistance). Doxorubicin is a chemotherapy agent of various types of cancer of the anthracycline class that has provoked resistance. In addition to causing resistance, doxorubicin may also cause cardiotoxicity in long-term
use [4]. Increased doses of doxorubicin are not an appropriate way to overcome resistance problems because they can cause normal cells to be exposed by cytotoxic drugs that will trigger toxicity and transform normal cells into cancer cells [5].

One of the medicinal plants in Indonesia that potentially developed as a chemopreventive agent in colon cancer is bangle. The bangle plant has been used by society traditionally to cope with several diseases. Empirically, boiled water rhizome bangle used the community as a headache medicine, constipation drugs, abdominal pain, jaundice, as body warmers, slimming, helps the gas out of the digestive tract, asthma, rheumatism, and antipyretics.

The research on scientific proof of the efficacy of the rhizome of bangle plant is still minimal publication and the efficacy of rhizome of bangle plant still many that have not been explored. So in this study, conducted an active fraction search on the ethanolic extract
of rhizome plant bangle that can kill and suppress the growth of cancer cells, especially in colon cancer cell type WiDr.

## Material and Instrument

Methanol, Hexane and ethyl acetate fraction of faloak bark, DMSO 0,1\%, cisplatin (Wako), High glucose DMEM (Dulbecco's Modified Eagle Media) (Gibco), (FBS) 10\% (v/v) (Qualified FBS, Gibco, Invitrogen USA), penicillin-streptomycin 1,5\% (v/v) (Gibco, Invitrogen USA and Fungizone 0,5\% v/v Gibco). Tripsin-EDTA 0,25\% (Gibco, Invitrogen Canada), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA). MTT was prepared with a concentration of $5 \mathrm{mg} / \mathrm{mL}$ dissolved in phosphate buffer saline (PBS) $1 \times \mathrm{pH} 7,4.0$, 01 N HCl (Merck, Darmstadt, Germany). PBS containing $1 \mathrm{mg} / \mathrm{ml}$ (minimum $95 \%$ (HPLC), Sigma-Aldrich Co., St. Louise, MO, 63178 , USA) $10 \mathrm{mg} / \mathrm{ml}$ RNAse (obtained from Laboratory of Animal Sciences, NAIST, Japan) and Triton X-100 for GC, E. Merck, 64271, Darmstadt, Germany).

Liquid nitrogen, labconco purifier class II biosafety cabinets (Delta Series, Labconco Corporation, Missouri, USA), cell counter, micropipet (Pipetman® la Gilson, France), digital camera (Sony), centrifuge (Sigma 203, B.Braun Biotech International), digital balance sheets (Mettler Toledo, AG204 Delta Rang®), mixer (Maxi Mix II, Thermolyne type 37600 mixer, Iowa, USA), oven (Memmert), ELISA reader (Bio-Rad microplate reader Bencmark serial No. 11565, Japan), FACTS calibur flow cytometer. FTIR (FTIR-100 Perkin Elmer), MS (Mariner Biospectrometry System HRESIMS) and NMR spectrometer (Delta 2 400 MHz for ${ }^{1} \mathrm{H}-\mathrm{NMR}$ and 100 MHz for ${ }^{13} \mathrm{C}-$ NMR). Autoclave (Hirayama HV 25 020585175, Hirayama Manufacturing Co., Japan), inverted microscope (Nikon, Eclipse, TE 2000-U), hemocytometer (Nebauer improved 0.100 mm Tiefe Depth Profondeur 0.0025 mm 2 , Germany), stirrer (Nuova, Thermolyne).

## Procedure

## Plant Material

Collected plant sample was washed with running tap water and shade dried at room temperature for three weeks and grounded. The plant material ( 250 g ) was successively extracted ethanol with maceration method.

All extractives were filtere through Whatman filter paper (No: 1) and concentrated.

## Fractionation with Column Chromatography

Separation of the active compound by column chromatography using silica gel $\mathrm{GF}_{254}$ as stationary phase and the 200 mL n-hexane, ethyl acetate and methanol as motion phase was carried out gradiently with the addition of 1 drop of $\mathrm{CH}_{3} \mathrm{COOH}$. Each fraction obtained was evaporated at $\pm 40^{\circ} \mathrm{C}$ to obtain a concentrated fraction and then dried in an oven at $\pm 50^{\circ} \mathrm{C}$. Each fraction tested to the cytotoxic activity to obtain the value of $\mathrm{CC}_{50}$.

## Cytotoxicity Assay

Cells were washed twice with PBS and inoculated with $200 \mu \mathrm{~L}$ of each fraction diluted in DMEM supplemented with $2 \%$ FCS (two-fold dilutions, ranging from 2000 $\mu \mathrm{g}$ to $0.975 \mu \mathrm{~g}$ ). Cells were allowed to grow for 72 h at $37{ }^{\circ} \mathrm{C} 5 \% \mathrm{CO}_{2}$. Then, medium was removed, cells were washed in PBS and 20 $\mu \mathrm{L}$ of MTT solution at $5 \mathrm{mg} / \mathrm{ml}$ (MTT, Sigma, Saint Louis, MO, USA) were added to each well. Plates were incubated for 4 h at $37{ }^{\circ} \mathrm{C}$ $5 \% \mathrm{CO}_{2}$ and the formed formazan crystals were solubilized in DMSO (Sigma, USA). The absorbance of formazan-generated dye was determined using a microplate reader (Thermo Fisher Scientific, USA) at 570 nm . The \% of viability was calculated using the following formula:

## $\% \mathrm{~V}=$ Absorbance of treated cells Absorbance of control cells

The viability of control cells was set to $100 \%$, the $\mathrm{CC}_{50}$ value was derived from the corresponding dose-response curves as the concentration of the oil that reduced cell viability by $50 \%$.

## Isolation and Purification under Bioassay-Guided Screening

According to cytotoxicity test the compounds were identified by comparing their UV, infrared, LC-MS and NMR data with those of published data test, only one fractions were the most toxic (ethyl acetate fraction 21). The fraction were separated by Sephadex LH-20 using $\quad \mathrm{CH}_{2} \mathrm{Cl}_{2}$ : MeOH (4:7). Combined fractions, according to TLC analysis, were purified by preparative TLC using a mixture of solvents.

## Determination of Cell Cycle and Apoptosis

WiDr cells were grown on glass coverslips in tissue culture dishes (Falcon) and were allowed to attach for 24 hours prior to the addition of any drug. After the cells were incubated with a drug for 24 hours, the coverslips were washed once in PBS and fixed in object glass. Treated cells were stained with acridine orange and ethidium bromide $5 \mu \mathrm{~L}$ and visualized by fluoresence microscopy.

## Result and Discussion

## Results of Cytotoxic Test

The cytotoxic activity of the fraction was expressed as $\mathrm{CC}_{50}$. The test was performed by dissolving 44 fractions of fractionation of ethanolic extract with gradient system (n-
hexane, ethyl acetate and methanol), then into it added DMSO solution until obtained the desired concentration. A single cytotoxic effect showed decreased cell viability and morphological changes in colon cancer cells of WiDr. The test results (Fig.1) show that Ethyl Acetate Faction 21 treatment (Fig. 1bc) shows the wrinkled cell. This indicates a decrease in living cells when compared to controls (Fig. 1a).

The cells appear round and fragmented to indicate a change in cell morphology, but unknown changes were due to cell deaths due to necrotic or apoptotic processes and proliferative inhibition processes. Meanwhile, the $\mathrm{CC}_{50}$ value of the largest was ethyl acetate faction 21 with concentrations of $11.24 \pm 3.44 \mathrm{\mu g} / \mathrm{mL}$ and doxorubicin was $13.88 \pm 0.34 \mu \mathrm{~g} / \mathrm{mL}$.


Fig.1: Ethyl Acetate Faction 21 testing was performed using cell controls (a) and Vero cells (d) Effect of Ethyl Acetate Faction 21 on WiDr colon cancer cells (b-c). Figure (b) at concentrations of $200 \mu \mathrm{~g} / \mathrm{mL}$ and image (c) at a concentration of $300 \mu \mathrm{~g} / \mathrm{mL}$. The Vero cells were treated with a fraction of concentration of $200 \mu \mathrm{~g} / \mathrm{mL}$.

Table 1: Cytotoxic activity on WiDr and Vero

| Number | Experiment | $\mathrm{CC}_{50}$ on WiDr Cell | $\mathrm{CC}_{50}$ on Vero Cell |
| :---: | :---: | :---: | :---: |
| 1 | Doxorubicin | $13,88 \pm 0,34 \mu \mathrm{~g} / \mathrm{mL}$ | 160,67 $\pm 0,76 \mu \mathrm{~g} / \mathrm{mL}$ |
| 2 | Methanol fraction 1 | $56,54 \pm 1,67 \mu \mathrm{~g} / \mathrm{mL}$ | $240,89 \pm 0,96 \mu \mathrm{~g} / \mathrm{mL}$ |
| 3 | Methanol fraction 2 | $70,88 \pm 0,92 \mu \mathrm{~g} / \mathrm{mL}$ | $140,92 \pm 1,22 \mu \mathrm{~g} / \mathrm{mL}$ |
| 4 | Methanol fraction 3 | $31,22 \pm 2,22 \mu \mathrm{~g} / \mathrm{mL}$ | $341,67 \pm 2,39 \mu \mathrm{~g} / \mathrm{mL}$ |
| 5 | Methanol fraction 4 | $48,88 \pm 0,88 \mu \mathrm{~g} / \mathrm{mL}$ | $131,33 \pm 1,96 \mu \mathrm{~g} / \mathrm{mL}$ |
| 6 | Methanol fraction 5 | $74,38 \pm 0,55 \mu \mathrm{~g} / \mathrm{mL}$ | $402,43 \pm 1,07 \mu \mathrm{~g} / \mathrm{mL}$ |
| 7 | Methanol fraction 6 | 99,68 $\pm 0,87 \mu \mathrm{~g} / \mathrm{mL}$ | $148,56 \pm 3,84 \mu \mathrm{~g} / \mathrm{mL}$ |
| 8 | Methanol fraction 7 | $58,33 \pm 1,99 \mu \mathrm{~g} / \mathrm{mL}$ | $340,44 \pm 1,45 \mu \mathrm{~g} / \mathrm{mL}$ |
| 9 | Ethyl Acetate Faction 1 | $41,11 \pm 2,68 \mu \mathrm{~g} / \mathrm{mL}$ | $441,44 \pm 0,34 \mu \mathrm{~g} / \mathrm{mL}$ |
| 10 | Ethyl Acetate Faction 2 | $66,78 \pm 1,03 \mu \mathrm{~g} / \mathrm{mL}$ | $561,90 \pm 1,52 \mu \mathrm{~g} / \mathrm{mL}$ |
| 11 | Ethyl Acetate Faction 3 | $36,99 \pm 0,86 \mu \mathrm{~g} / \mathrm{mL}$ | $445,95 \pm 1,14 \mu \mathrm{~g} / \mathrm{mL}$ |
| 12 | Ethyl Acetate Faction 4 | $98,82 \pm 3,35 \mu \mathrm{~g} / \mathrm{mL}$ | $123,98 \pm 3,22 \mu \mathrm{~g} / \mathrm{mL}$ |
| 13 | Ethyl Acetate Faction 5 | $95,85 \pm 3,08 \mu \mathrm{~g} / \mathrm{mL}$ | $248,07 \pm 2,58 \mu \mathrm{~g} / \mathrm{mL}$ |
| 14 | Ethyl Acetate Faction 6 | $88,55 \pm 2,88 \mu \mathrm{~g} / \mathrm{mL}$ | $338,77 \pm 4,95 \mu \mathrm{~g} / \mathrm{mL}$ |
| 15 | Ethyl Acetate Faction 7 | $13,11 \pm 2,08 \mu \mathrm{~g} / \mathrm{mL}$ | $565,44 \pm 1,08 \mu \mathrm{~g} / \mathrm{mL}$ |
| 16 | Ethyl Acetate Faction 8 | $36,88 \pm 1,07 \mu \mathrm{~g} / \mathrm{mL}$ | $341,69 \pm 0,11 \mu \mathrm{~g} / \mathrm{mL}$ |
| 17 | Ethyl Acetate Faction 9 | $32,54 \pm 1,91 \mu \mathrm{~g} / \mathrm{mL}$ | 249,22 $\pm 0,65 \mu \mathrm{~g} / \mathrm{mL}$ |
| 18 | Ethyl Acetate Faction 10 | $16,22 \pm 0,79 \mu \mathrm{~g} / \mathrm{mL}$ | $241,53 \pm 0,96 \mu \mathrm{~g} / \mathrm{mL}$ |
| 19 | Ethyl Acetate Faction 11 | $99,11 \pm 2,07 \mu \mathrm{~g} / \mathrm{mL}$ | $299,19 \pm 4,98 \mu \mathrm{~g} / \mathrm{mL}$ |
| 20 | Ethyl Acetate Faction 12 | $88,19 \pm 5,88 \mu \mathrm{~g} / \mathrm{mL}$ | $349,93 \pm 0,96 \mu \mathrm{~g} / \mathrm{mL}$ |
| 21 | Ethyl Acetate Faction 13 | $14,33 \pm 4,33 \mu \mathrm{~g} / \mathrm{mL}$ | $240,11 \pm 1,93 \mu \mathrm{~g} / \mathrm{mL}$ |
| 22 | Ethyl Acetate Faction 14 | $88,11 \pm 3,34 \mu \mathrm{~g} / \mathrm{mL}$ | $360,24 \pm 5,11 \mu \mathrm{~g} / \mathrm{mL}$ |
| 23 | Ethyl Acetate Faction 15 | $16,92 \pm 2,88 \mu \mathrm{~g} / \mathrm{mL}$ | $130,11 \pm 8,22 \mu \mathrm{~g} / \mathrm{mL}$ |
| 24 | Ethyl Acetate Faction 16 | $28,22 \pm 3,30 \mu \mathrm{~g} / \mathrm{mL}$ | $223,11 \pm 2,30 \mu \mathrm{~g} / \mathrm{mL}$ |
| 25 | Ethyl Acetate Faction 17 | 92,22 $\pm 3,93 \mu \mathrm{~g} / \mathrm{mL}$ | 292,12 $\pm 2,33 \mu \mathrm{~g} / \mathrm{mL}$ |
| 26 | Ethyl Acetate Faction 18 | $31,21 \pm 3,33 \mu \mathrm{~g} / \mathrm{mL}$ | $234,44 \pm 4,33 \mu \mathrm{~g} / \mathrm{mL}$ |
| 27 | Ethyl Acetate Faction 19 | $39,92 \pm 4,22 \mu \mathrm{~g} / \mathrm{mL}$ | $234,11 \pm 3,43 \mu \mathrm{~g} / \mathrm{mL}$ |
| 28 | Ethyl Acetate Faction 20 | $21,22 \pm 2,22 \mu \mathrm{~g} / \mathrm{mL}$ | $134,21 \pm 3,11 \mu \mathrm{~g} / \mathrm{mL}$ |
| 29 | Ethyl Acetate Faction 21 | $11,24 \pm 3,44 \mu \mathrm{~g} / \mathrm{mL}$ | $342,22 \pm 4,98 \mu \mathrm{~g} / \mathrm{mL}$ |
| 30 | Ethyl Acetate Faction 22 | $41,98 \pm 9,92 \mu \mathrm{~g} / \mathrm{mL}$ | $232,22 \pm 1,92 \mu \mathrm{~g} / \mathrm{mL}$ |
| 31 | Ethyl Acetate Faction 23 | $34,22 \pm 2,33 \mu \mathrm{~g} / \mathrm{mL}$ | $234,03 \pm 3,22 \mu \mathrm{~g} / \mathrm{mL}$ |
| 32 | Ethyl Acetate Faction 24 | 92,23 $\pm 2,22 \mu \mathrm{~g} / \mathrm{mL}$ | $445,11 \pm 2,39 \mu \mathrm{~g} / \mathrm{mL}$ |
| 33 | Ethyl Acetate Faction 25 | 82,11 $\pm 3,98 \mu \mathrm{~g} / \mathrm{mL}$ | $354,73 \pm 1,39 \mu \mathrm{~g} / \mathrm{mL}$ |
| 34 | Hexane Fraction 1 | $77,92 \pm 2,93 \mu \mathrm{~g} / \mathrm{mL}$ | $551,82 \pm 3,11 \mu \mathrm{~g} / \mathrm{mL}$ |
| 35 | Hexane Fraction 2 | 98,28 $\pm 3,34 \mu \mathrm{~g} / \mathrm{mL}$ | $458,11 \pm 1,28 \mu \mathrm{~g} / \mathrm{mL}$ |
| 36 | Hexane Fraction 3 | 90,01 $\pm 2,88 \mu \mathrm{~g} / \mathrm{mL}$ | $388,29 \pm 3,29 \mu \mathrm{~g} / \mathrm{mL}$ |
| 37 | Hexane Fraction 4 | $34,22 \pm 1,98 \mu \mathrm{~g} / \mathrm{mL}$ | $123,21 \pm 2,92 \mu \mathrm{~g} / \mathrm{mL}$ |
| 38 | Hexane Fraction 5 | $22,11 \pm 2,62 \mu \mathrm{~g} / \mathrm{mL}$ | $220,91 \pm 0,16 \mu \mathrm{~g} / \mathrm{mL}$ |
| 39 | Hexane Fraction 6 | $97,14 \pm 2,62 \mu \mathrm{~g} / \mathrm{mL}$ | $240,11 \pm 0,22 \mu \mathrm{~g} / \mathrm{mL}$ |
| 40 | Hexane Fraction 7 | $36,22 \pm 4,11 \mu \mathrm{~g} / \mathrm{mL}$ | $233,11 \pm 0,92 \mu \mathrm{~g} / \mathrm{mL}$ |
| 41 | Hexane Fraction 8 | 90,11 $\pm 2,66 \mu \mathrm{~g} / \mathrm{mL}$ | $102,11 \pm 0,81 \mu \mathrm{~g} / \mathrm{mL}$ |
| 42 | Hexane Fraction 9 | $83,29 \pm 2,98 \mu \mathrm{~g} / \mathrm{mL}$ | $358,09 \pm 0,12 \mu \mathrm{~g} / \mathrm{mL}$ |
| 43 | Hexane Fraction 10 | $73,12 \pm 2,93 \mu \mathrm{~g} / \mathrm{mL}$ | $241,72 \pm 0,02 \mu \mathrm{~g} / \mathrm{mL}$ |
| 44 | Hexane Fraction 11 | $99,54 \pm 1,67 \mu \mathrm{~g} / \mathrm{mL}$ | $229,11 \pm 0,88 \mu \mathrm{~g} / \mathrm{mL}$ |
| 45 | Hexane Fraction 12 | 96,11 $\pm 2,22 \mu \mathrm{~g} / \mathrm{mL}$ | $221,11 \pm 0,11 \mu \mathrm{~g} / \mathrm{mL}$ |

## Structure Elucidation

Compound 1, yellow crystal, m.p. 97-99 ${ }^{\circ} \mathrm{C}$, UV ( MeOH ) $\lambda_{\text {maks }} \mathrm{nm}(\log \varepsilon): 232$ (3.92), 290 (4.10), dan 325 sh (3.66) nm. EIMS: $m / z$ (270, $\mathrm{M}^{+}, 100$, base peak), 193 (76.1), 166 (80.3), 138 (41.4), $10^{3}$ (21.2), and 77 (16.4). ${ }^{1} \mathrm{H}$ NMR ( 400 MHz in acetone $d 6$ ), $\delta_{\text {H }}$ ppm: 12.17 ( $1 \mathrm{H}, b r, s, 5-\mathrm{OH}$ ), 7.58 ( $3 \mathrm{H}, m, \mathrm{H}-3^{\prime}, 4^{\prime}, 5^{\prime}$ ), 7.41 ( $2 \mathrm{H}, m, \mathrm{H}-2^{\prime}, 6^{\prime}$ ), $6.09(1 \mathrm{H}, d, J=2.2 \mathrm{~Hz}, \mathrm{H}-8)$, $6.07(1 \mathrm{H}, d, J=2.2 \mathrm{~Hz}, \mathrm{H}-6), 5.61(1 \mathrm{H}, d d$, $J=3.8 ; 12.3 \mathrm{~Hz}, \mathrm{H}-2), 3.35\left(3 \mathrm{H}, s, 7-\mathrm{OCH}_{3}\right)$,
3.24 ( $1 \mathrm{H}, d d, J=12.8 ; 16.4 \mathrm{~Hz}, \mathrm{H}-3_{\mathrm{ax}}$ ), and $2.81(1 \mathrm{H}, d d, J=3.8 ; 16.4 \mathrm{~Hz}, \mathrm{H}-3 \mathrm{eq}) .{ }^{13} \mathrm{C}$ NMR (100 MHzin acetone d6), 8c ppm: 196.3 (C-4), 168.6 (C-7), 164.8 (C-5), 163.3 (C-8a), 140.1 (C-1'), 129.6 ( $\left.\mathrm{C}-3^{\prime}, 5^{\prime}\right), 128.5$ (C-4'), 123.9 (C$2^{\prime}, 6^{\prime}$ ), 101.5 (C-4a), 95.09 (C-8), 95.66 (C-6), $79.6(\mathrm{C}-2), 56.1\left(7-\mathrm{OCH}_{3}\right)$, and $43.9(\mathrm{C}-3)$. The elucidation results showed that the compound of the ethyl acetate fraction 21 was 2,3-dihydro-5-hydroxy-7-methoxy-2-phenylchromen-4-one.


Fig. 2: 2,3-dihydro-5-hydroxy-7-methoxy-2-phenylchromen-4-one Compound ( ${ }_{\text {HMBC }}$ )

## Observation of Apoptosis and the Cell Cycle

Furthermore, modulation test of cell cycle. The process of DNA synthesis in cancer cells through the same cell cycle as normal cells [6]. One of the main targets in inhibiting cancer cell proliferation is the modulation of cell cycle that can be observed using flowcytometry method (Rollando, 2000).

Flowcytometry is able to detect each phase in the cell cycle based on the number of chromosomes on each phase (G1, S, and G2 / M) [7]. Propidium iodide is used to color each phase because it is capable of interacting with DNA [8]. Observation of cell cycle profile was done at 24 hours [9]. The percentage of cell cycle distribution in detail is shown in Table 2.

Table 2: Percentage of cell cycle distribution

| Sample | G1 (\%) | S (\%) | G2/M (\%) | \% CV |
| :--- | :--- | :--- | :--- | :--- |
| Control | 38,11 | 12,34 | 13,34 | 3,87 |
| Doxorubicin 2,5 $\mu \mathrm{M}$ | 42,88 | 13,11 | 24,55 | 2,22 |
| 2,3-dihydro-5-hydroxy-7-methoxy-2- <br> phenylchromen-4-one 2,5 $\mu \mathrm{M}$ | 34,11 | 23,11 | 11,23 | 5,98 |



Fig. 3: Distribution of cell cycle percentages. (a) Control (b) Doxorubicin (c) 2,3-dihydro-5-hydroxy-7-methoxy-2-phenylchromen-4-one Compound

The test results showed doxorubicin causing accumulation in phase S . The 2,3-dihydro-5-hydroxy-7-methoxy-2-phenylchromen-4-one
compound causes cell accumulation in phase S when compared with cell control. In the combination treatment the percentage of cell
cycle distribution in the $S$ phase amounted to $23.11 \%$ greater than the single doxorubicin of $13.11 \%$. Cell accumulation in the S-phase of combination was increased compared with
cells without treatment (cell control) of $12.34 \%$. Cell accumulation is possible because of cell cycle arrest in that phase.

Table 3: Percentage of death after treatment

|  | Control | Doxorubicin <br> $\mathbf{2 . 5 ~} \boldsymbol{\mu M}$ | 2,3-dihydro-5-hydroxy-7- <br> methoxy-2-phenylchromen-4-one <br> $\mathbf{2 . 5} \boldsymbol{\mu M}$ |
| :--- | :--- | :--- | :--- |
| Initial apoptosis (\%) | 2,11 | 2,33 | 9,87 |
| End apoptosis (\%) | 1,63 | 2,53 | 1,24 |
| Necrosis (\%) | 0,86 | 0,87 | 1,62 |
| Total | 3,94 | 6,04 | 13,78 |

Observation of apoptotic compounds 2,3-dihydro-5- hydroxy- 7- methoxy-2 phenylchromen-4-one with flow cytometer. Induction of apoptosis was used to investigate the mechanism of cell death from treatment of the 2,3 -dihydro- 5 -hydroxy-7-methoxy-2-phenylchromen-4-one compound on colon cancer cell WiDr incubated for 24 hours [10].

The method used in this study is Annexin V method that was detected using flowcytometry to see the induction of apoptosis that occurred in the treated cells. Annexin V is a member of the family of phospholipid binding proteins that are on strongly negatively charged cellular membranes. Cell cell death caused by apoptosis or necrosis can be distinguished by the staining of Propidium Iodide (PI) through intercalation with DNA [11]. The percentage of cell death after treatment of 2,3-dihydro-5-hydroxy-7-methoxy-2-phenylchromen-4-one induced by apoptosis or necrosis is shown in Table 3.

Analysis of the percentage of cell death after 2 , 3- dihydro- 5 - hydroxy-7-methoxy-2-phenylchromen-4-one (Table 3) showed that

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control cells showed a $3.94 \%$ percentage of cell death. Single-treated cells with doxorubicin showed $6.04 \%$ cell death, whereas 2 , 3-dihydro-5- hydroxy-7- methoxy2 -phenylchromen-4-one compound resulted in $13.78 \%$ cell death. It showed an increase in the percentage of cell death by $7.74 \%$ in the 2,3 -dihydro- 5 -hydroxy- 7 -methoxy- 2 -phenylchromen-4-one compound compared with doxorubicin.

## Conclusion

The results showed that isolate 2 was 3 -dihydro-5-hydroxy-7-methoxy-2-
phenylchromen-4-one having activity cytotoxic in breast cancer cell type T47D with active category with $\mathrm{IC}_{50} 11,24 \pm 3,44 \mu \mathrm{~g} / \mathrm{mL}$.

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## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.
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