

***In vitro* study of pinostrobin propionate and pinostrobin butyrate: Cytotoxic activity against breast cancer cell T47D and its selectivity index**

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Abstract

Backgrounds: Pinostrobin has the potential activity as an anti-cancer. However, its activity is still lower than the anticancer drugs on the market. To increase its activity, pinostrobin derivatives have been synthesized, namely pinostrobin propionate and pinostrobin butyrate, which are predicted to have better activity and lower toxicity than pinostrobin after being tested by *in silico* approach. So the compound deserves to be tested for its anticancer activity and selectivity on normal cells.

Objective: This study aims to determine the anticancer activity of pinostrobin propionate and pinostrobin butyrate against the T47D breast cancer cell line and its selectivity against the Vero cell line.

Methods: The cytotoxicity test which is anticancer activity test and its selectivity on normal cell were carried out using the MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The cells used were breast cancer cell line T47D and normal Vero cells. The test results were analyzed using a microplate reader with a wavelength of 570 nm.

Results: From the analysis of anticancer activity on T47D cells, the IC₅₀ values of pinostrobin, pinostrobin propionate, and pinostrobin butyrate were 2.93, 0.57, and 0.40 mM, respectively. While the results of the cytotoxicity test on Vero cells obtained the CC₅₀ value of pinostrobin, pinostrobin propionate, pinostrobin butyrate was 1.27, 0.94, and 0.89 mM, respectively. So the SI value of pinostrobin (SI=0.4) is smaller than its derivatives (SI=1.7 and 2.2). Meanwhile, pinostrobin butyrate is more selective than pinostrobin propionate.

Conclusions: It can be concluded that pinostrobin propionate and pinostrobin butyrate compounds have greater activity and selectivity than pinostrobin so these compounds are promising to be further developed as anticancer candidates.

Introduction

Based on data from the Global Cancer Observatory (Globocan) 2020 in Indonesia, a new case of breast cancer in women occurred in as many as 65,858 (30.8%) cases. New cases of breast cancer ranked first with a percentage of 16.6% beating cervical cancer (9.2%) and deaths caused by breast cancer ranked second with a percentage of 9.6%. So the breast cancer is cancer with the most sufferers.¹

Breast cancer is one of the most common malignant diseases and is the leading cause of death in women. As the time being, breast cancer treatment has not been effective as it affects normal cells.^{2,3} It is necessary to develop a new cancer drug that selective-

ly kills cancer cells.

In the development of new drugs, herbal plants are widely used as an option, especially for the development of cancer treatment. In addition to having lower side effects, the price is also more affordable. One of the plants that have the potential to be anticancer is fingerroot.⁴

Fingerroot (*Boesenbergia pandurata*) is part of the family *Zingiberaceae*, which is widely found in the Southeast Asian region.⁵ In fingerroot, there are phytochemical components that can act as antibacterial, anticancer, anti-inflammatory, and antioxidant. One of the flavanones found is pinostrobin (Figure 1A), which is known to have anticancer activity, namely the induction of apoptosis in breast cancer cells.⁶ On T47D cells, pinostrobin causes DNA fragmentation by inhibiting the work of the DNA topoisomerase enzyme which plays a role in the process of replication, transcription, and DNA recombination as well as the proliferation of cancer cells. The inhibition of the work of these enzymes can form protein-linked DNA breaks (PLDB) so that the DNA of cancer cells will be damaged and lead to the death of cancer cells.⁷

In the rational design of a drug, the pharmacological activity of a compound can be improved for better activity by modifying the structure based on chemical and physical properties, namely lipophilic parameters (LogP), electronic parameters (Etot), and steric parameters (MR). Previously, studies related to pinostrobin showed that its cytotoxic activity against cancer cells was still low compared to anticancer drugs on the market.^{2,8,9} Then supported by the research of Jones & Gehler (2020), pinostrobin selectively decreases the spread of but has less effect on cancer cell death. Although a lot of research has been conducted related to pinostrobin, research related to the modification of its structure is still very rare, especially in the compound pinostrobin propionate and pinostrobin butyrate (Figure 1B,C).¹⁰ Structural modifications by adding acyl compounds can improve anticancer activity in the breasts where the selection of substituents is based on lipophilic, electronic, and steric parameters.¹¹

Based on *in silico* approach using pkCSM online and ChemOffice Professional software ver 19.0. Pinostrobin, pinostrobin propionate, and pinostrobin butyrate have an LD₅₀ value of 2.359 mol/kg (637.6 g/kg), 2.543 mol/kg (829.9 g/kg), and 2.537 mol/kg (863.5 g/kg). Then, pinostrobin has a LogP value of 2.28, Etot of 46.2543 kcal/mol, and MR of 75.29 cm³/mol. Pinostrobin propionate has a LogP value of 2.91, an Etot of 66.9378 kcal/mol, and MR of 89.42 cm³/mol. Pinostrobin butyrate has a LogP value

of 3.33, Etot of 66.6182 kcal/mol, and an MR of 94.02 cm³/mol. After that, the docking process is carried out using Molegro Virtual Docker (MVD) ver 6.0 against estrogen receptors taken from Protein Data Bank (PDB) with PDB code: 5W9C. Based on the docking results, rerank score (RS) value was obtained. Pinostrobin, pinostrobin propionate, and pinostrobin butyrate have RS values of -76.3237 kcal/mol, -89.0797 kcal/mol, and -93.6329 kcal/mol. It can be concluded that based on these predictions by *in silico* approach, pinostrobin propionate and pinostrobin butyrate have better anticancer activity and lower toxicity than pinostrobin.¹² So that the anticancer activity of pinostrobin propionate and pinostrobin butyrate deserve more research continued by *in vitro* approach.

Materials and Methods

Compounds

Pinostrobin, pinostrobin propionate, and pinostrobin butyrate were obtained from Research Center, *In Vitro*-1 Laboratory, Faculty of Pharmacy, Airlangga University. They are the results of a synthesis of previous studies that have confirmed their purities.

Cell culture

T47D breast cancer cell lines and Vero normal cell lines were obtained from Research Center, *In Vitro*-1 Laboratory, Faculty of Pharmacy, Airlangga University. T47D cells were grown in RPMI 1640 (Sigma Aldrich) medium supplemented with 10% fetal bovine serum (FBS, Gibco), 1% amphotericin B (Gibco), and 1% Penicillin-Streptomycin (Sigma Aldrich) at 37°C with 5% CO₂.

Cytotoxic assay

The cytotoxicity was determined by MTT assay. T47D and Vero cells at a density of 5x10⁴ cells/mL were distributed into 96 wells of plates, treated with various concentrations of pinostrobin, pinostrobin propionate, and pinostrobin butyrate dissolved in dimethyl sulfoxide (DMSO), followed by incubation for 24 hours. Each well was added with 100 µl 0.05% MTT in PBS, and after 3 hours the formation of purple formazan crystal was identified, and the reaction was stopped by 10% SDS 0.01N HCl. The test results were analyzed using a microplate reader with a wavelength of 570 nm. The IC₅₀ was determined, as the concentration required to

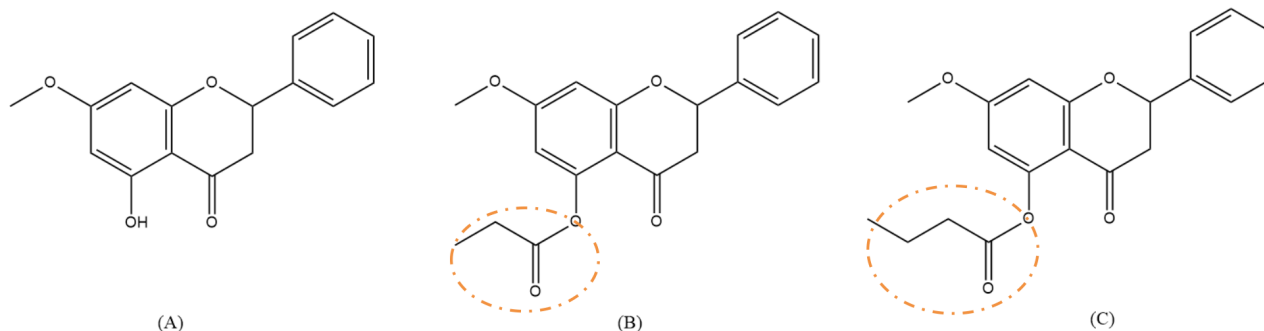


Figure 1. Structure of (A) pinostrobin, (B) pinostrobin propionate and (C) pinostrobin butyrate (chemdraw professional).

inhibit 50% T47D cell growth and CC_{50} was determined, as the concentration required to inhibit 50% Vero cells growth.^{9,13}

Statistical analysis

Absorbance value from cytotoxic assay converse to viability cell (%).

$$\% \text{ viable cells} = \frac{\text{Abs of cells} - \text{Abs of medium control}}{\text{Abs of cells medium control} - \text{abs of medium control}} \times 100\%$$

Numerical data for the treatment of T47D cells was analyzed with One-Way ANOVA. The significance of differences among treatments on the cells was determined by Dunnett's C as the Post Hoc Test used IBM SPSS Statistics (version 26.0). The Sig. <0.05 were considered statistically significant differences.

Numerical data for the treatment of Vero cells was analyzed with Independent-Samples T Test using IBM SPSS Statistics (version 26.0). The Sig. <0.05 were considered statistically significant differences.

Selectivity index

Based on IC_{50} and CC_{50} values that were obtained, the selectivity index (SI) can be obtained by calculating the ratio of CC_{50} over IC_{50} . A compound is declared to be selective against cancer cells if $SI \geq 2$.¹⁴⁻¹⁶

Results

Cytotoxic activity

There are visible differences in cell conditions before and after being treated with various concentrations. Treatment of T47D with increasing concentrations of pinostrobin and its derivatives results in reduced cell viability (Figure 2).

From the data (Table 1) and graphs (Figure 3) that have been presented, pinostrobin butyrate has the lowest IC_{50} value compared to pinostrobin and pinostrobin propionate and has a slightly different than 5-fluorouracil's IC_{50} value which is a positive control on this research which is 399 μ M.

Furthermore, the results of the percentage of cell viability of Vero against the treatment of Pinostrobin, Pinostrobin propionate, and Pinostrobin butyrate were obtained which are shown in Table 2. Then the cell viability curve was made to calculate the CC_{50} value (Figures 4 and 5).

Selectivity index

Based on the IC_{50} and CC_{50} values, the selectivity index value can be calculated as in Table 3.

Table 1. Percentage of T47D cell viability against the treatment of pinostrobin, pinostrobin propionate, pinostrobin butyrate and 5-Fu.

Concentration (μ M)	P (%)	SD	PP (%)	SD	PB (%)	SD	5FU (%)	SD
10	-	-	-	-	-	-	63.877	7.307
50	-	-	62.997	6.738	69.240	2.731	-	-
100	98.264	1.251	64.245	7.002	74.404	2.044	67.100	2.185
250	96.311	5.358	62.514	3.637	62.429	2.167	45.738	9.434
500	94.286	4.286	52.015	3.272	41.544	3.637	38.877	3.849
750	91.356	0.381	47.503	2.432	21.283	0.354	23.701	7.848
1000	90.127	2.465	37.188	1.746	4.540	1.595	20.374	3.107
1500	86.944	4.300	17.509	3.960	1.022	1.461	-	-

C= Concentrations; P= Pinostrobin; PP= Pinostrobin Propionate; PB= Pinostrobin Butyrate; 5-FU= 5-Fluorouracil.

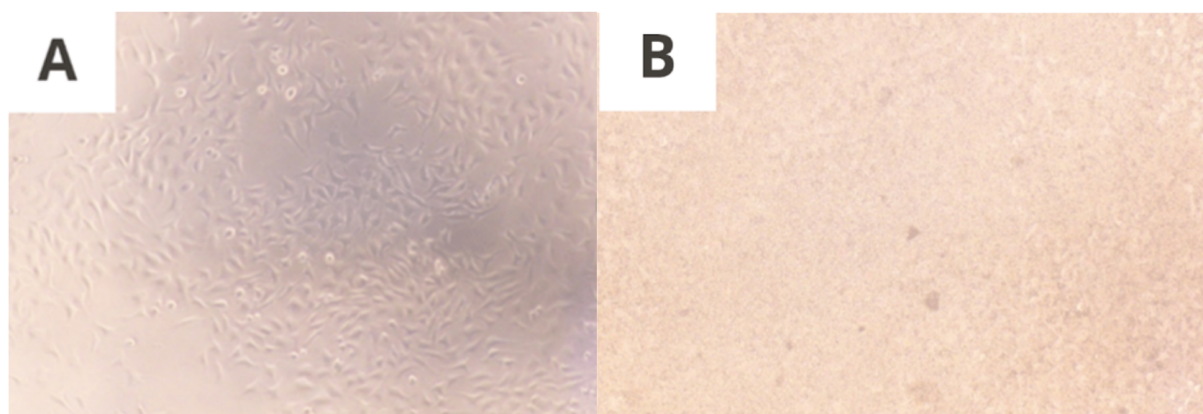


Figure 2. The condition of T47D cells after being treated with pinostrobin derivatives. [A] T47D before treatment, [B] T47D after treatment.

Discussion

The IC_{50} value is the amount of concentration that can inhibit cancer cell activity by 50% of the initial activity as a cytotoxic parameter in breast cancer cells in this study. The United State National Cancer Institute classifies cytotoxic where the IC_{50} value <20 g/ml belongs to high cytotoxic activity, IC_{50} 21-200 g/ml belongs to moderate cytotoxic, IC_{50} 201-500 g/ml belong to weak cytotoxic activity and $IC_{50} >500$ g/ml did not have cytotoxic activity. Pinostrobin propionate and pinostrobin butyrate belong in the category of a moderate cytotoxic compound because the IC_{50} is 185 g/mL (0.57 mM) and 136 g/mL (0.40 mM) so pinostrobin propionate and pinostrobin butyrate is considered to have promising potential anticancer activity, while pinostrobin belong to the non-cytotoxic category (2.93 mM = 793 g/ml). There is not a statistically significant difference between pinostrobin derivatives and 5-fluorouracil (positive control) on T47D Cells using One-Way ANOVA with Dunnett's C as Posthoc Test.¹⁷

The increased activity of pinostrobin propionate and pinos-

trobin butyrate compared to pinostrobin is caused by an increase in their lipophilicity because of propionate and butyrate substitution which increases the ability of the compound to penetrate biological membranes and can help orient the compound correctly to interact optimally with receptors so that there is an increase in biological activity.¹⁸ Increased anticancer activity was also associated with the results of the *in silico* study of pinostrobin propionate and pinostrobin butyrate, which has a lower rerank score compared to pinostrobin (Pinostrobin propionate = -89.0797 kcal/mol; pinostrobin butyrate = -93.6329 kcal/mol, pinostrobin = -72.5487 kcal/mol). This is because of a higher affinity for pinostrobin propionate and pinostrobin butyrate as an estrogen receptor antagonist that prevents the proliferation of breast cancer cells.^{12,19}

The CC_{50} values of pinostrobin propionate, butyrate, and pinostrobin are quite high. This shows that pinostrobin propionate, butyrate, and pinostrobin have weak cytotoxic activity against normal cells. Pinostrobin's CC_{50} value which is greater than pinostrobin propionate and butyrate is due to the greater lipophilicity of pinostrobin propionate and pinostrobin butyrate than pinostrobin so that the Vero cell membrane is more easily penetrated by pinos-

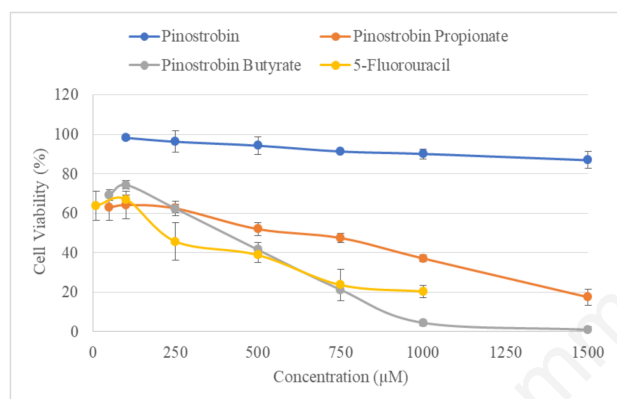


Figure 3. T47D cells viability after being treated by pinostrobin, pinostrobin propionate, pinostrobin butyrate, 5-fluorouracil.

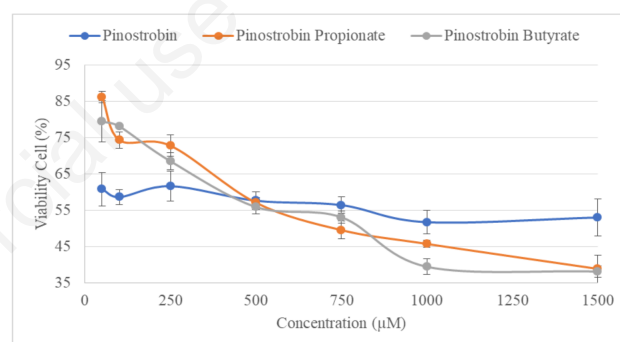


Figure 4. Vero cells viability after being treated with pinostrobin, pinostrobin propionate and pinostrobin butyrate.

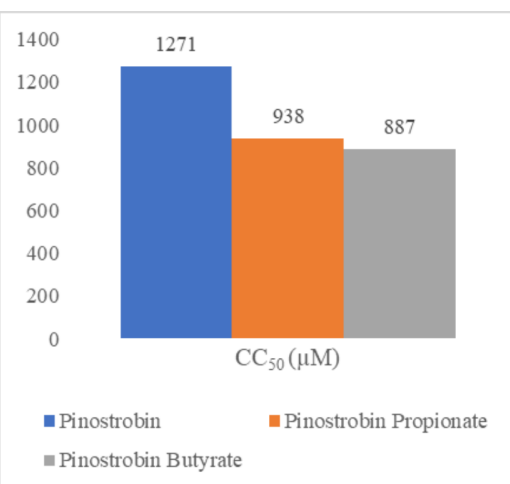
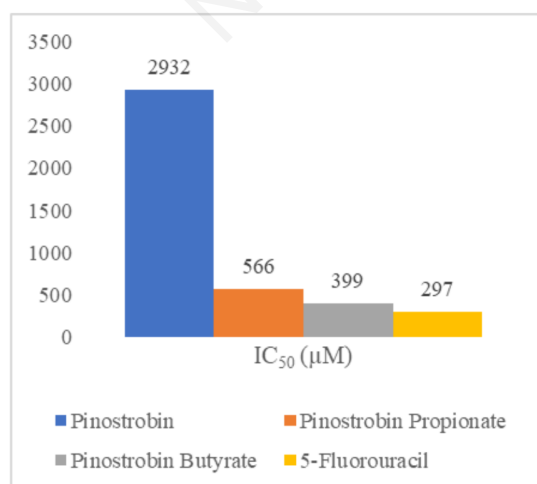


Figure 5. The IC_{50} and CC_{50} values of pinostrobin, pinostrobin propionate, and pinostrobin butyrate on T47D and Vero cells.

Table 2. Percentage of Vero cell viability against the treatment of pinostrobin, pinostrobin propionate and pinostrobin butyrate.

Concentration (μM)	P (%)	SD	PP (%)	SD	PB (%)	SD
50	60.882	4.617	86.230	1.637	79.512	5.677
100	58.677	2.003	74.418	2.265	78.178	0.438
250	61.695	4.209	72.832	2.919	68.543	2.283
500	57.690	2.464	57.104	1.516	55.949	1.980
750	56.471	2.266	49.578	2.452	53.105	1.618
1000	51.770	3.231	45.764	1.014	39.466	2.118
1500	53.047	5.067	38.880	3.848	38.131	1.618

Table 3. The selectivity index (si) of pinostrobin, pinostrobin propionate, and pinostrobin butyrate.

Compounds	IC 50	CC50	SI
Pinostrobin	2932	1271	0.4
Pinostrobin Propionate	566	938	1.7
Pinostrobin Butyrate	399	887	2.2

trobin propionate and pinostrobin butyrate and causes the cytotoxic effect of pinostrobin propionate and pinostrobin butyrate on Vero cells to be greater than pinostrobin.¹⁷ There isn't a statistically significant difference between pinostrobin and its derivatives on Vero Cells using Independent-Samples T-Test.

A compound is declared to be selective against cancer cells if the Selectivity Index (SI) ≥ 2 .¹⁴⁻¹⁶ Based on the acceptance criteria, pinostrobin butyrate is selective against T47D cells compared to normal cells (Table 3).

Conclusions

It can be concluded that both pinostrobin propionate and pinostrobin butyrate compounds have great activity and selectivity than pinostrobin, where pinostrobin butyrate showed a better selectivity index. So, these compounds are promising to be further developed as anticancer candidates.

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