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Original Article

Molecular docking, synthesis, and *in vitro* activity testing of chalcone derivatives from *Boesenbergia rotunda* (L.) Mansf. against MCF-7 breast cancer cells

[Acoplamiento molecular, síntesis y actividad *in vitro* de derivados de chalcona de *Boesenbergia rotunda* (L.) Mansf. contra células MCF-7 de cáncer de mama]

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Abstract

Context: The chalcone derivative compounds (bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone) synthesized from pinostrobin, which is found in *Boesenbergia rotunda* (L.) Mansf., which has cytotoxic activity relative to the estrogen receptor alpha.

Aims: To determine the anticancer activity of chalcone derivative compounds synthesized from *B. rotunda* as an *in silico* and *in vitro* breast cancer candidate.

Methods: An *in silico* test was performed to predict the cytotoxic activity using AutoDockVina and the synthesis of chalcone and derivatives from pinostrobin compounds contained in *B. rotunda* using the green synthesis method through Williamson etherification reaction and phase-transfer catalyst (PTC)-assisted microwave irradiation. This activity was also tested *in vitro* using the microculture tetrazolium technique (MTT) assay of breast cancer cells (MCF-7) and normal cells (Vero cells).

Results: *In silico* tests predicted that chalcone derivatives were more cytotoxic at estrogen receptor alpha than lead compounds (pinostrobin and chalcone compound). *In vitro* tests showed that the bis-2-chlorobenzyloxychalcone compound showed an IC_{50} greater than 100 µg/mL, which indicated it was not active, and the bis-3-chlorobenzyloxychalcone compound exhibited an IC_{50} less than 100 µg/mL, which indicated it was moderately active. Thus, *in vitro* tests showed that two compounds were less potent against MCF-7 cells than the reference compound (tamoxifen). However, it was selective against MCF-7 breast cancer cells while not harming Vero cells.

Conclusions: The bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone compounds identified in *B. rotunda* exhibited a favorable selectivity index, suggesting moderate potential as anticancer candidates for breast cancer. However, further research and development are required to validate their efficacy and safety.

Keywords: breast cancer; chalcone; cytotoxic activity; estrogen receptor alpha; green synthesis.

Resumen

Contexto: Los compuestos derivados de chalcona (bis-2-clorobenciloxichalcona y bis-3-clorobenciloxichalcona) sintetizados a partir de pinostrobina, presente en *Boesenbergia rotunda* (L.) Mansf., muestran actividad citotóxica en relación con el receptor de estrógeno alfa.

Objetivos: Determinar la actividad anticancerígena de compuestos derivados de chalcona sintetizados a partir de *B. rotunda* como candidato para el cáncer de mama *in silico* e *in vitro*.

Métodos: Se realizó una prueba *in silico* para predecir la actividad citotóxica utilizando AutoDockVina y la síntesis de chalcona y derivados de compuestos de pinostrobina presentes en *B. rotunda* mediante el método de síntesis verde mediante la reacción de eterificación de Williamson e irradiación de microondas asistida por catalizador de transferencia de fase (PTC). Esta actividad también se evaluó *in vitro* mediante ensayo de microcultivo con tetrazolio (MTT) en células de cáncer de mama (MCF-7) y células normales (células Vero).

Resultados: Las pruebas *in silico* predijeron que los derivados de chalcona fueron más citotóxicos para el receptor de estrógeno alfa que los compuestos principales (pinostrobina y chalcona). Las pruebas *in vitro* mostraron que el compuesto bis-2-clorobenciloxichalcona presentó una CI_{50} superior a 100 µg/mL, lo que indicó su inactividad, y el compuesto bis-3-clorobenciloxichalcona exhibió una CI_{50} inferior a 100 µg/mL, lo que indicó su actividad moderada. Por lo tanto, las pruebas *in vitro* mostraron que dos compuestos fueron menos potentes contra las células MCF-7 que el compuesto de referencia (tamoxifeno). Sin embargo, fue selectivo contra las células de cáncer de mama MCF-7, sin dañar las células Vero.

Conclusiones: Los compuestos bis-2-clorobenciloxichalcona y bis-3-clorobenciloxichalcona identificados en *B. rotunda* exhibieron un índice de selectividad favorable, lo que sugiere un potencial moderado como candidatos anticancerígenos para el cáncer de mama. Sin embargo, se requiere mayor investigación y desarrollo para validar su eficacia y seguridad.

Palabras Clave: actividad citotóxica; cáncer de mama; chalcone; receptor de estrógeno alfa; síntesis verde.

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INTRODUCTION

In recent decades, cancer has been a deadly disease that is of great concern in the field of research and therapeutic development, both in Indonesia and around the world. The Global Burden of Cancer (2021) data estimate the incidence and mortality rate of cancer worldwide at 19.3 million new cases. In the case of breast cancer, it is estimated that there are 2.3 million new cases (11.7%) (Sung et al., 2021). According to (The Global Cancer Observatory, 2021), Indonesia reports 396,914 new cancer cases, with breast cancer ranking first at 65,858 (16.6%), and a reported death rate of 22,430 (9.6%) from breast cancer.

Currently, several cancer therapies, including chemotherapy, radiotherapy, and immunotherapy, have demonstrated benefits for cancer patients. However, these therapies often come with side effects or multidrug resistance (MDR) mechanisms, which remain the primary cause of clinical treatment failure. These mechanisms can lead to reduced efficacy of chemotherapy drugs (Kesuma et al., 2022; Pratama et al., 2022), gradually diminishing their effectiveness, and causing cancer cells to develop resistance to anticancer drugs (Goldman, 2003; Kar, 2007; Tartarone et al., 2013). In the case of tamoxifen administration to patients with breast cancer, resistance developed after 3–5 years of use. Moreover, tamoxifen functions as an agonist in the endometrium, leading to the development of endometrial cancer over time. There are 30% of breast cancer patients in the early stages who develop endometrial cancer after Tamoxifen administration at a certain time (Ali et al., 2016).

ER-α is considered to be a receptor that is primarily involved in breast cancer development, making it an important target in breast cancer therapy. One adherent cell line that expresses estrogen receptor alpha (ER-α) is MCF-7 breast cancer cells (Ogba et al., 2014). However, MCF-7 cells develop resistance to selective estrogen receptor modulator (SERM) chemotherapeutic agents, namely tamoxifen (TAM). Breast cancer patients with estrogen receptor-positive (ER+) status primarily receive TAM (Clusan et al., 2023; Moerkens et al., 2014).

Therefore, a systematic elaboration process is necessary to further develop existing drugs, with the aim of obtaining new cancer drugs. According to Sun et al. (2023), this process involves using natural materials that can act as chemotherapeutic agents, making cancer cells more sensitive so that chemotherapeutic agents work better, and lessening the side effects of chemotherapeutic agents.

Fingerroot (*Boesenbergia rotunda* (L.) Mansf., family *Zingiberaceae*), also known as temu kunci in Indonesia, has the ability to increase the number of lymphocytes and specific antibodies, as well as kill cancer cells. Flavanones, flavones, and chalcones are the main flavonoids found in temu kunci extract (Atun and Handayani, 2017). Chalcone compounds play a significant role in the synthesis of various heterocyclic compounds, including flavones, isoxazoles, benzodiazepines, pyrazolines and their derivatives, and flavonols. This leads to the widespread use of chalcone analog compounds and their derivatives as target molecules in the search for active compounds, particularly as potential anticancer drugs (Dona et al., 2019). Strong biological activity has shown chalcone's potential as a chemotherapeutic agent, but its limited availability in nature compared to other flavonoid compounds necessitates an effective synthesis method (Villa et al., 2024). The process of creating chalcone compounds involves isolating pinostrobin from *B. rotunda*. The next step involves structurally modifying the lead compound to produce compounds that exhibit the desired activity. Therefore, structural modifications are anticipated to enhance selectivity, improve pharmacological activity, and minimize toxicity (Siswandono, 2016).

Therefore, in an effort to conserve the environment and utilize biodiversity, we must develop the latest cancer treatment methods based on natural resources. One such method involves synthesizing chalcone derivative compounds from the isolated *B. rotunda*. We used estrogen-a receptors with the PDB ID 6CHZ to do *in silico* tests on chalcone compounds and their derivatives for this study. The next stage is the isolation of compounds from the *B. rotunda*, the synthesis of chalcone and its derivatives from pinostrobin compounds isolated from the *B. rotunda* using the green synthesis method through the Williamson ether reaction and using microwave irradiation with the help of a phase-transfer catalyst (PTC). Purity and structure were tested using an IR spectrometer and a ^{1}H-NMR spectrometer. We further tested its cytotoxic activity *in vitro* using the MTT assay on MCF-7 cancer cells and Vero normal cells. This research aims to obtain and develop breast cancer chemotherapy drug candidates.

MATERIAL AND METHODS

Materials

Materials for molecular docking: marker compounds from *B. rotunda,* specifically pinostrobin and its derivatives (chalcones and chalcone derivatives),

which were made structural drawings using the MarvinSketch application, and estrogen-receptor with PDB ID 6CHZ, as well as the tamoxifen reference compound downloaded from the protein data bank (PDB).

Materials for physicochemical isolation, synthesis and analysis: *B. rotunda* powder (*B. rotunda*) (determined by UPT Laboratorium Herbal Materia Medica Batu, Indonesia), N-hexane p.a. (E.Merck, Germany), ethyl acetate p.a. (E.Merck, Germany), methanol, pinostrobin (compound isolated from *B. rotunda* powder), chalcone (2,6-dihydroxy-4-metoxychalcone) (compound synthesized from pinostrobin), 2-chlorobenzyl chloride (TCI, America), 3-chlorobenzyl chloride (TCI, America); NaOH p.a (E.Merck, Germany), tetrabutylammonium chloride p.a (E.Merck, Germany), chloroform (E.Merck, Germany), and $MgSO_4$ anhydrous (University of Surabaya, Indonesia).

Materials for *in vitro* test: Bis-2-chlorobenzyloxychalcone and bis-3- chlorobenzyloxychalcone synthesized compounds; tamoxifen (Indonesian FDA Laboratory Services (INFALABS, BPOM Indonesia); MCF-7 cell culture and Vero cells; DMEM culture medium; MI99 culture medium; fetal bovine serum (FBS); phosphate buffer saline (PBS); dimethylsulfoxide (DMSO); tripsin; penicillin-streptomycin, fungizon; 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) 0.5 mg/mL, SDS 10% in HCl 0.1 N.

Tools and equipment

Tools used for *in silico* tests: Computer hardware with CPU specifications Intel(R) Core (TM) i7-9700F CPU @ 3.00GHz, 16384MB RAM equipped with Windows 10 Education 64-bit operating system (Dell, Dell Technologies Inc., China), AutoDock Vina (The Scripps Research Institute, USA) and AutoDockTools 1.5.6 programs (The Scripps Research Institute, USA), PyRx program (PyRx LTD., UK), MarvinSketch program (ChemAxon, Hungaria), Lipinski Rule of Five website (URL), and BIOVIA Discovery Studio Visualizer (BIOVIA Foundation, USA).

Tools used for isolation, synthesis, and structure confirmation were rotary evaporator (Buchi, Swiss); water bath (Memmert GmbH, Germany); Corning Hot Plate P351 (Thermo Fisher Scientific Inc., USA); Fisher-John Electrothermal Mel-Temp (Biobase Biodusty, China); IR Spectrophotometer (Agilent, Agilents Tehnologies, USA) and ^{1}H-NMR Spectrometer 400 MHz (Agilent, Agilents Tehnologies, USA).

Tools used for *in vitro* tests were CO_2 incubator (Thermo Fisher Scientific Inc., USA); laminar air flow (Gelman Sciences); test tubes (Agilents Tehnologies, USA); vortex (Thermo Fisher Scientific Inc., USA); 96 well microplate (Falcon, Corning, China); inverted microscope (Zeiss 451235, Zeiss Axiovert, Zeiss, USA); haemocytometer (Thermo Scientific, Thermo Fisher Scientific Inc., USA); cell counter (Thermo Scientific, Thermo Fisher Scientific Inc., USA); ELISA-reader (Bio-Rad, Agilent, Agilents Tehnologies, USA).

Docking method validation

When performing docking protocols on chalcone derivative compounds using the Autodock 4.2.6 and Autogrid (AutoDock, The Scripps Research Institute, USA) programs, it is necessary to validate the method by redocking the native ligand (tamoxifen) to the protein whose native ligand has been removed. The parameter for validation of this method is the root mean square deviation (RMSD) value, with a tolerable value of ≤3.0 (Ramírez and Caballero, 2018).

***In silico* prediction of cytotoxic activity**

Autodock software (Version 4.2.6) was used to predict the cytotoxic activity of these compounds. Molecular tethering simulations were performed between the test compound and estrogen receptor alpha PDB ID 6CHZ, containing the tamoxifen ligand, which was downloaded from the protein data bank (PDB) server.

To prepare the protein and ligand molecules, Mgl tools (version 1.5.6) were used, and to get a perfect/good grid parameter assignment of each ligand, the grid box was created by trial and error. Grid box centres X: -30.000, Y: -3.957, Z: -22.374 were used for docking calculation (Amelia et al., 2024). Subsequently, the docking process was performed by running the Lamarckian Genetic Algorithm. Here, default parameters and one hundred independent docking runs were performed for each chemical structure. Structure modification to obtain derivative compounds was performed using the Topliss approach method. The structure modification was carried out with the aim of obtaining new drugs that are more effective and reducing the side effects that have been known before.

Plant material

The *B. rotunda* rhizome (GPS coordinates: -7.867687882049943, 112.51928107745778) used in this study has been identified by the UPT Laboratorium Herbal Materia Medica Batu, Indonesia, with identification number 000.9.3/516/102.20/2024. The botanical classification of this plant is as follows:

Kingdom: *Plantae*

Division: *Magnoliophyta*

Class: *Monocotyledonae*

Order: *Zingiberales*

Family: *Zingiberaceae*

Genus: *Boesebergia*

Species: *Boesebergia rotunda* (L.) Mansf.

Synonym: *Boesebergia pandurata* (Roxb.) Schlecht. = *Kaempferia pandurata* Roxb.

The reference for this determination was derived from the textbook (Backer and Bakhuizen Van Den Brink, 1968; Van Steenis, 2008), which served as the authoritative source for the plant's taxonomic classification.

Isolation of pinostrobin from *B. rotunda*

The determined *B. rotunda* powder was weighed as much as 2 kg of fine powder, then extracted using 20 L of n-hexane solvent by maceration and then remacerated three times. The remaining solvent was evaporated at 40°C under low pressure until the volume reached 1/3 of the initial volume using a rotary evaporator and evaporated in a fume hood. Then recrystallization was carried out by dissolving the crude crystals with hot methanol solvent and cooled in a refrigerator for 24 hours until crystals formed. The crystals formed were washed with hot methanol four times and allowed to stand at room temperature. Chemical structure identification was carried out using an IR spectrophotometer and a ^{1}H-NMR spectrophotometer at 400 MHz.

Synthesis of bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone

A 12.5 N NaOH solution was made in a beaker. The manufacturing step can be done by weighing 50 grams of NaOH (1.250 mmol). After that, add 100 mL of distilled water solution and stir until homogeneous. Pipette 2 mL of the finished 12.5 N NaOH solution using a syringe, then put it in a round-bottom flask. After that, add 0.2 g (0.74 mmol) of chalcone and 0.28 mL (2.22 mmol) of 2-chlorobenzyl chloride and 3-chlorobenzyl chloride, respectively, to each round-bottom flask. Add 0.01 g (0.025 mmol) tetrabutylammonium chloride. Heat the mixture in a microwave for 10 minutes at 200 watts. The purity test was characterized by thin layer chromatography (TLC) and melting point, while the structure identification of active bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone compounds utilized the following instruments: IR spectrophotometer and ^{1}H-NMR (McMurry, 2011; Pavia et al., 2009).

The reaction between chalcone from a synthesis of pinostrobin with 2-chlorobenzyl chloride and 3-chlorobenzyl chloride can be seen in Fig. 1.

In vitro test and selectivity index

The cell culture was incubated in DMEM and M199 media for 24 h in a 96-well plate (Kesuma et al., 2025). Cells were withdrawn individually using a micropipette, and then a sample solution consisting of synthesized compounds (chalcone derivatives) and the reference drug tamoxifen was added. The plate was incubated in a CO_2 incubator at 37°C for 24 hours. After incubation, the microplate was removed from the incubator, and the treatment media was discarded. Then, 100 µL of MTT solution was added to each well, including the cell control and media control wells. The plate was incubated in a CO_2 incubator at 37°C for 2 to 4 h and regularly observed under an inverted microscope to check for formazan formation. Once formazan formation was observed, the MTT reaction was stopped by adding 10% DMSO.

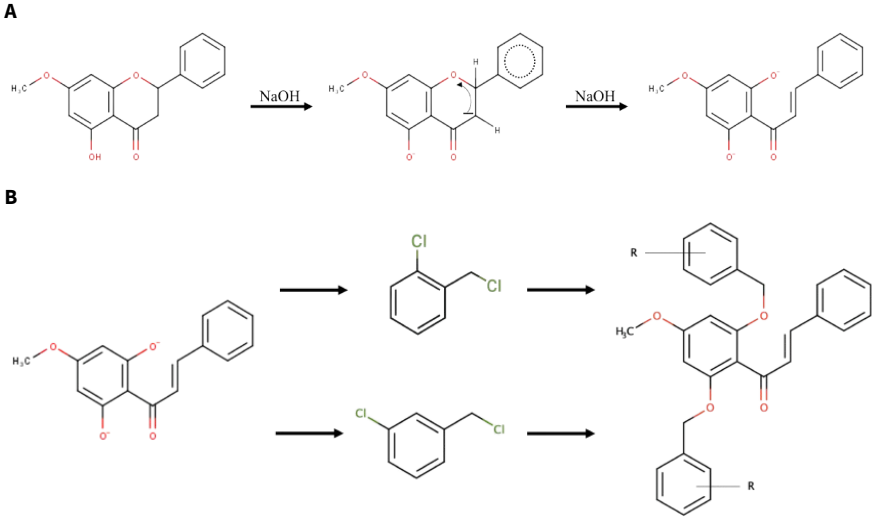


Figure 1. The synthesis reaction of chalcone derivatives.

(A) The reaction mechanism of converting pinostrobin into a chalcone compound involves the addition of a strong base, NaOH, which deprotonates the phenol group, turning the phenoxide ion into a nucleophilic group. This phenoxide ion then attacks the carbon atom in the benzyl chloride compound, which acts as the electrophilic group. The reaction results in the formation of a bond between the phenoxide ion and the benzyl chloride, followed by the cleavage of the bond between the carbon atom and the halide ion, leading to the formation of a benzyl oxychalcone derivative. **(B)** The mechanism of the synthesis reaction strengthens the bond between the phenoxide ion and the carbon atom. This chalconate ion gives the compound two nucleophilic sites at C-2 and C-6. When reacting with 2-chlorobenzyl chloride and 3-chlorobenzyl chloride, the resulting bonds form bis-2-chlorobenzyloxy-chalcone and bis-3-chlorobenzyloxychalcone compounds.

The microplate was then incubated in the dark at room temperature overnight. Absorbance of each well was then measured using a microplate reader. The selectivity index (SI) was determined by dividing the IC_{50} value for normal Vero cells by the IC_{50} value for MCF-7 cancer cells. The SI is a parameter used to measure the safety of a drug, calculated using equation [1].

$$SI = \frac{IC_{50} \text{ normal cells}}{IC_{50} \text{ cancer cells}} \quad [1]$$

A SI greater than 2 indicated that a sample exhibits cytotoxic activity against cancer cells without affecting normal cells (Rashidi et al., 2017).

Statistical analysis

The *in vitro* test presents the cytotoxic activity test data as absorbance values. These values are then used to calculate the percentage of viable cells (% cell viability) and to determine the statistical significance as well as the 50% inhibitory concentration (IC_{50}) values for the compounds bis-2-chlorobenzyloxychalcone, bis-3-chlorobenzyloxychalcone, and the reference compound tamoxifen, through probit analysis using SPSS version 27. The cell viability percentage (% cell viability) is calculated using the following equation [2].

$$\text{Cell viability (\%)} = \frac{(At \,.\, Ab)}{(Ac \,.\, Ab)} \times 100 \quad [2]$$

Where At: Absorbance of test; Ab: Absorbance of blank (media); and Ac: Absorbance of control (cells).

RESULTS AND DISCUSSION

Docking validation score

After performing the validation method by re-docking the native ligand on the protein, whose native ligand was removed, the RMSD value was obtained: 2.42 Å. The tolerable RMSD value was ≤3.0 Å (Ramírez and Caballero, 2018).

In silico molecular docking

The molecular docking results for the reference compound tamoxifen, along with the test compounds bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone, using the Autodock 4.2.6 program, are summarized in Table 1. The interactions between the ligand molecules and receptors, as presented in Table 1, show that bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone demonstrated docking scores of -11.54 kcal/mol and -11.92 kcal/mol, respectively, which are lower than those of tamoxifen (-10.00 kcal/mol) and pinostrobin (-10.07 kcal/mol). This lower binding energy suggests that the interactions between bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone with their receptors are more stable, as less energy is required for binding (Amelia et al., 2024). The binding energy values and interactions between ligands and amino acids are detailed in Table 1. The binding energy of chalcone compounds and their derivatives can be used to predict their biological activity, with lower binding energy indicating stronger ligand-receptor interactions and greater potential activity. The more hydrogen bonds and steric interactions (Van der Waals and hydrophobic interactions) formed between the ligand and receptor, the more stable the ligand-receptor complex is expected to be bis-2-chlorobenzyloxychalcone interacts with the ER-α receptor through steric interactions (Van der Waals and hydrophobic interactions) with the amino acids Arg 394, Glu 353, Gly 521, Leu 349, Leu 428, Phe 404, Thr 347, Asp 351, Met 421, Leu 525, Met 388, Met 343, Leu 346, Trp 383, Ala 350, Leu 354, Leu 384, Leu 387, and Leu 391. Similarly, bis-3-chlorobenzyloxychalcone interacts with amino acids Arg 394, Asp 351, Gly 521, Leu 354, Leu 428, Leu 384, Met 343, Phe 404, Thr 347, Glu 353, Leu 346, His 524, Ala 350, Ile 424, Leu 349, Leu 387, Leu 391, Leu 525, Met 388, and Met 421. In comparison, tamoxifen interacts with the ER-α receptor through hydrogen bonds with the amino acid Thr 347 and steric interactions with amino acids Arg 394, Asp 351, Glu 353, Gly 521, His 524, Ile 424, Leu 354, Leu 384, Leu 391, Met 343, Met 421, Trp 383, and Thr 347. Therefore, it can be concluded that chalcone and its derivatives can interact with the ER-α receptor due to their ability to form bonds with the same residues amino acids as tamoxifen, including Arg 394, Asp 351, Glu 353, Gly 521, His 524, Leu 349, Leu 354, Leu 384, Met 343, Phe 404, Trp 383, Thr 347, Ala 350, Leu 346, Leu 387, Leu 391, Ile 424, Leu 428, Leu 525, Met 388, and Met 421.

Isolation of compounds

From powdered *B. rotunda* simplicia (2 kg), 10.50 grams of pinostrobin crystals were isolated, yielding white crystals with a yield of 0.525% and a melting point of 99.5°C-101.4°C. The IR spectrum showed ν max (cm^{-1}): 3058 (–OH phenolic); 2972-2912 (=C–H aromatic); 1638-1526 (C=C aromatic); 1284-1034 (C-O ether); and 887-698 (C-H aromatic). ^{1}H-NMR (chloroform-D, 400 MHz). δ 2.82 (d, J=2 Hz, 1H); δ 3.09 (d, J= 12 Hz, 1H); δ 3.80 (s, 3H); δ 5.42 (d, J=4 Hz, 1H); δ 6.06 (m, 2H); δ 7.41 (m, 5H); δ 12.02 (s, 1H), The IR and ^{1}H-NMR spectrum can be seen in Fig. 2A-B.

Synthesis of chalcone compounds

The synthesis procedure was based on a modified green synthesis method using the Williamson ether reaction, with microwave irradiation and a phase-transfer catalyst (PTC). Chalcone compounds were synthesized from pinostrobin, which was isolated from *B. rotunda*. Chalcones have been shown to exhibit strong biological activity and potential as chemotherapeutic agents, but their natural occurrence is limited compared to other flavonoid compounds. Therefore, an efficient synthetic method is needed, following the principles of Green Chemistry. The synthesis involved reacting pinostrobin with 2-chlorobenzyl chloride and pinostrobin with 3-chlorobenzyl chloride.

Table 1. Binding energy and interaction binding between chalcone derivatives and receptors.

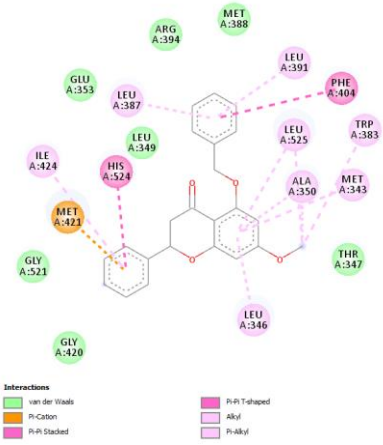
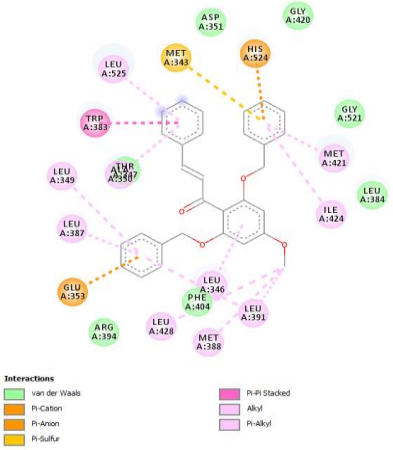
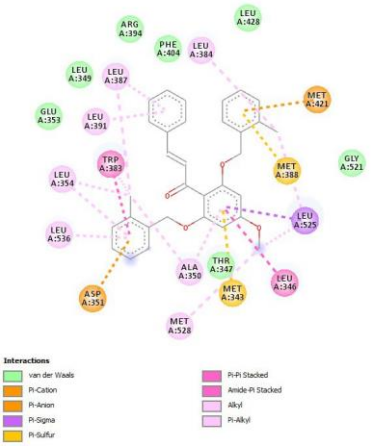
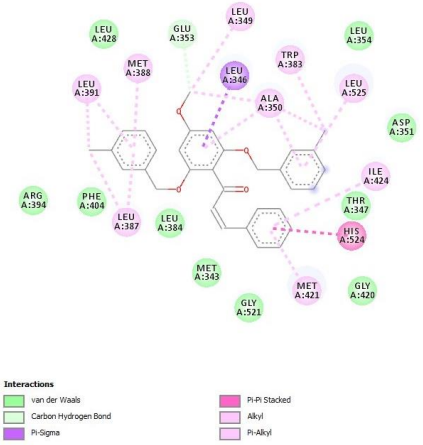
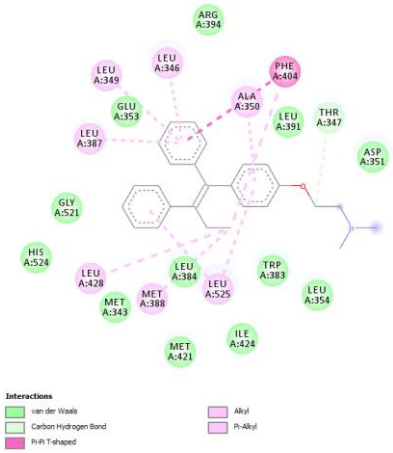
Ligand	Binding energy (kcal/mol)	Interaction binding	Amino acid residues
Pinostrobin	-10.07		Arg 394; Glu 353; Gly 420; Gly 521; Leu 349; Met 388; Thr 347; Met 421; His 524; Phe 404; Ala 350; Ile 424; Leu 387; Leu 346; Leu 525; Leu 391; Met 343; Trp 383
Chalcone	-11.35		Arg 394; Asp 351; Gly 420; Gly 521; Leu 384; Phe 404; Thr 347; His 534; Glu 353; Met 343; Trp 383; Leu 525; Ala 350; Ile 424; Leu 387; Leu 346; Leu 349; Leu 391; Leu 428; Met 388; Met 421
Bis-2-chlorobenzyloxychalcone	-11.54		Arg 394; Glu 353; Gly 521; Leu 349; Leu 428; Phe 404; Thr 347; Asp 351; Met 421; Leu 525; Met 388; Met 343; Leu 346; Trp 383; Ala 350; Leu 354; Leu 536; Leu 384; Leu 387; Leu 391; Met 528

Table 1. Binding energy and interaction binding between chalcone derivatives and receptors (continued...)

Ligand	Binding energy (kcal/mol)	Interaction binding	Amino acid residues
Bis-3-chlorobenzyloxychalcone	-11.92		Arg 394; Asp 351; Gly 420; Gly 521; Leu 354; Leu 428; Leu 384; Met 343; Phe 404; Thr 347; Glu 353; Leu 346; His 524; Ala 350; Ile 424; Leu 349; Leu 387; Leu 391; Leu 349; Leu 525; Met 388; Met 421; Trp 383
Tamoxifen	-10.00		Arg 394; Asp 351; Glu 491; Gly 420; Gly 521; His 524; Leu 349; Leu 354; Leu 384; Met 343; Phe 404; Trp 383; Thr 347; Ala 350; Leu 346; Leu 387; Leu 391; Ile 424; Leu 428; Leu 525; Met 388; Met 421

Bis-2-chlorobenzyloxychalcone

Melting points 99.6-101.6°C. The IR spectrum showed, v max (cm^{-1}): 2920-2849 (=C–H aromatic); 1675-1602 (C=C aromatic); 1293-1034 (C–O ether). ^{1}H-NMR (chloroform-D, 400 MHz). δ 3.77 (s, 2H); δ 3.77 (m, 1H); δ 3.93 (m, 1); δ 4.075 (s, 1H); δ 4.813 (s, 1H); δ 5.15 (m, 1H); δ 5.23 (s, 1H); δ 6.08 (m, 1H); δ 6.45 (s, 1H); δ 6.82 (m, 6H); δ 7.27 (m, 7H); and δ 7.77 (m, 1H), The IR and ^{1}H-NMR spectrum can be seen in Fig. 2 C-D.

Bis-3-chlorobenzyloxychalcone

Melting points 99.3-101.2°C. IR, v max (cm^{-1}): 2920-2853 (=C–H aromatic); 1623 (C=C aromatic); 1298-1034 (C–O eter). ^{1}H-NMR (chloroform-D, 400 MHz). δ 3.77 (s, 3H); δ 5.03 (s, 4H); δ 6.16 (s, 1H); δ 7.01 (d, 1H); δ 7.18 (m, 7H); δ 7.30 (m, 2H); δ 7.37 (m, 4H); and δ 7.50 (m, 2H), The IR and ^{1}H-NMR spectrum can be seen in Fig. 2E-F.

In vitro tests

The summary of IC_{50} values for the test compounds and control against MCF-7 cancer cells and normal Vero cells can be seen in Table 2. The table shows that the IC_{50} values for the test compounds bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone against MCF-7 cells were 251.175 µg/mL (0.484 mM) and 81.050 µg/mL (0.156 mM), respectively, while the reference compound tamoxifen showed an IC_{50} of 1,46 µg/mL (0.002 mM). According to the National Cancer Institute (2021) guidelines, a compound is classified as highly cytotoxic if it exhibits an IC_{50} value below 30 µg/mL. A compound is considered moderately cytotoxic when its IC_{50} value falls between 30 µg/mL and 100 µg/mL. In contrast, a compound is regarded as inactive if its IC_{50} value exceeds 100 µg/mL. Based on these results, it was concluded that bis-2-chlorobenzyloxychalcone demonstrates no significant cytotoxic activity, whereas bis-3-chlorobenzyloxychalcone exhibits moderate cytotoxic effects. In contrast, tamoxifen displayed potent cytotoxic activity against MCF-7 cells.

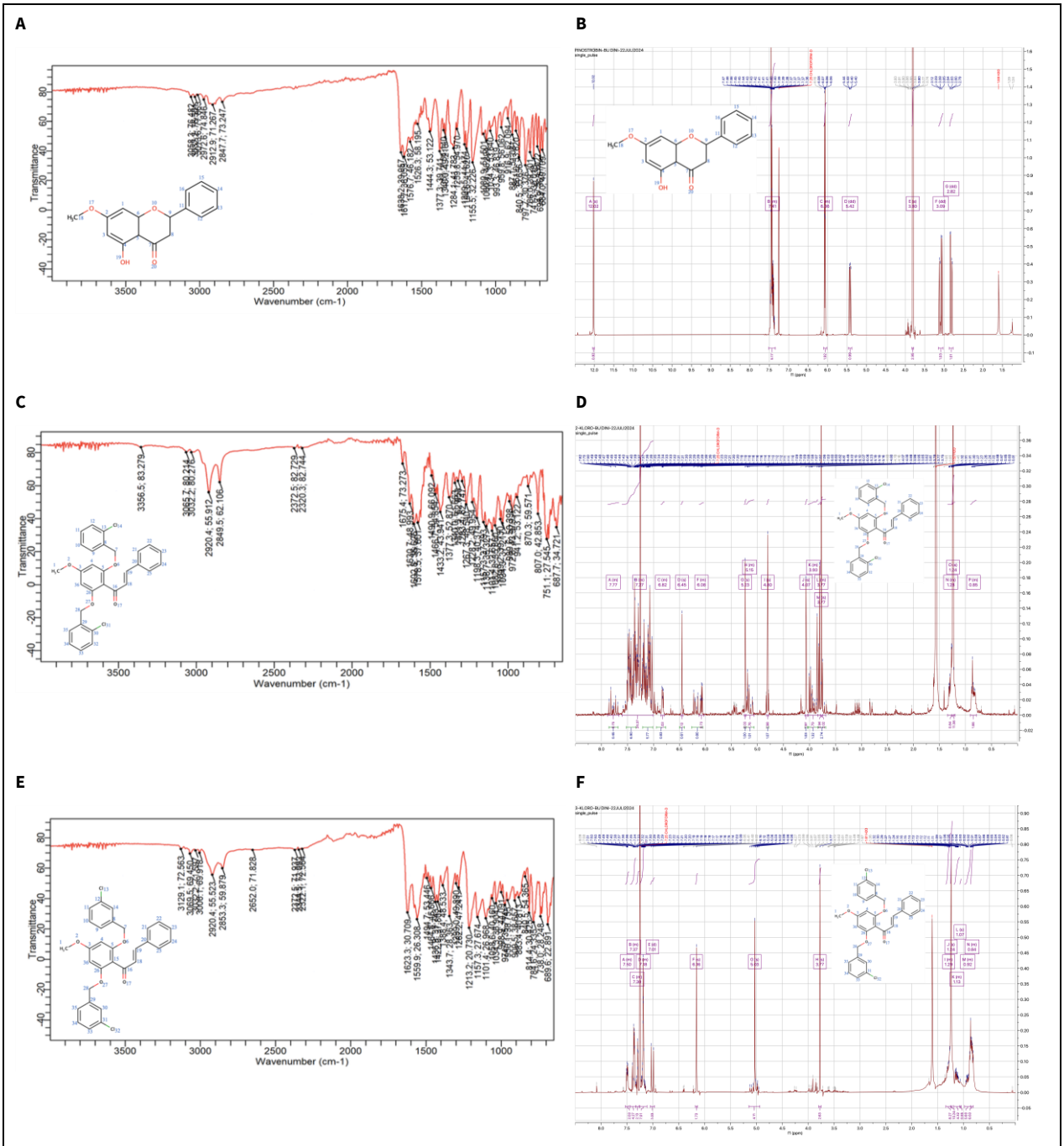


Figure 2. The structure and spectra of pinostroin, bis-2-chlorobenzyloxychalcone, and bis-3-chlorobenzyloxychalcone.

(A) IR spectrum of pinostroin. **(B)** ^{1}H- NMR spectrum of pinostroin (400 MHz). **(C)** IR spectrum of bis-2-chlorobenzyloxychalcone. **(D)** ^{1}H- NMR spectrum (400 MHz) of bis-2-chlorobenzyloxychalcone. **(E)** IR spectrum of bis-3-chlorobenzyloxychalcone. **(F)** ^{1}H- NMR spectrum (400 MHz) of bis-3-chlorobenzyloxychalcone.

Table 2. Summary of IC_{50} values of the test and reference compounds against MCF-7 cancer cells and normal Vero cells.

Compound	IC_{50} MCF-7 cancer cell		IC_{50} Vero cell		Selectivity index (SI)
	(µg/mL)	(mM)	(µg/mL)	(mM)	
Bis-2-chlorobenzyloxychalcone	251.175	0.484	1068.36	2.057	4.253
Bis-3-chlorobenzyloxychalcone	81.040	0.156	304.32	0.586	3.755
Tamoxifen	1.460	0.002	1.472	-	1.008

For normal Vero cells, the IC_{50} values of bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone were 1068.36 µg/mL (2.057 mM) and 304.32 µg/mL (0.586 mM), respectively, while tamoxifen had an IC_{50} value of 1.472 µg/mL. From these results, it was concluded that both bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone have no cytotoxic effect on normal Vero cells, whereas tamoxifen is highly cytotoxic to normal cells.

Neither bis-2-chlorobenzyloxychalcone nor bis-3-chlorobenzyloxychalcone is more potent against MCF-7 cancer cells compared to the reference compound, tamoxifen. As the concentration increases, tamoxifen becomes more potent, while bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone require much higher concentrations to achieve comparable potency in killing MCF-7 cancer cells.

The IC_{50} value of bis-2-chlorobenzyloxychalcone indicates inactive cytotoxic activity as its value exceeds the required threshold and is significantly higher than that of bis-3-chlorobenzyloxychalcone. This may be due to the steric hindrance at the ortho position of the benzene ring. In contrast, bis-3-chlorobenzyloxychalcone has its substituent at the meta position on the benzene ring. This observation is supported by previous studies conducted by Karki et al. (2010) and Guo et al. (2021), which state that compounds with substituents in the ortho position tend to exhibit weaker activity compared to those with substituents in the meta or para positions. The position of substituents on the benzene ring can significantly influence a compound's biological activity, including its cytotoxicity. The terms ortho, meta, and para refer to the relative positions of substituents on an aromatic ring. If the substituent is in the ortho position (adjacent to each other on the ring), steric hindrance can occur, leading to unfavorable interactions and reduced cytotoxic activity. In the meta position (separated by one carbon on the ring), the steric and electronic properties may enhance interactions, thereby improving cytotoxicity. Meanwhile, in the para position (opposite on the ring), interactions with the biological target are often more optimal due to reduced steric hindrance and more favorable electronic effects.

Based on the SI data, tamoxifen has an SI value of 1.008, while bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone have SI values of 4.25 and 3.76, respectively. These results suggest that both compounds have SI values above 2, which indicates greater selectivity in targeting MCF-7 cancer cells over normal Vero cells. In other words, the synthesized compounds (bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxychalcone) could be potential alternatives for breast cancer therapy. However, in terms of their activity against MCF-7 cancer cells, bis-2-chlorobenzyloxychalcone is categorized as not very active, while bis-3-chlorobenzyloxychalcone is considered moderately active. Despite this, both compounds can still be viable options due to their favorable SI values, which could help reduce the side effects typically associated with cancer treatments.

CONCLUSION

The compound bis-2-chlorobenzyloxychalcone has an IC_{50} value greater than 100 µg/mL, indicating that it exhibits inactive activity. In contrast, bis-3-chlorobenzyloxychalcone has an IC_{50} value of less than 100 µg/mL, suggesting that it possesses moderately active cytotoxic activity. The reference compound, tamoxifen, shows highly active cytotoxicity against MCF-7 cells. Chalcone derivatives require higher concentrations to increase their potency in killing MCF-7 breast cancer cells. However, they exhibit selectivity in targeting these cancer cells. The compounds bis-2-chlorobenzyloxychalcone and bis-3-chlorobenzyloxy-chalcone demonstrate cytotoxic activity against breast cancer cells, making them candidates for anticancer medications.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Amelia MA	Kesuma D	Kirtishanti A	Sumartha IGA	Claudya M
Concepts or ideas	x	x	x	x	x
Design		x			
Definition of intellectual content	x	x	x	x	x
Literature search	x	x	x	x	x
Experimental studies	x			x	x
Data acquisition		x	x	x	
Data analysis		x	x	x	
Statistical analysis		x	x		
Manuscript preparation	x	x	x		
Manuscript editing	x	x	x		
Manuscript review	x	x	x	x	x

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