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Unravelling the apoptosis induction potential of *Amomum cardamomum* seed: A combination *in silico* and *in vitro* approach

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ABSTRACT

Introduction: Breast cancer remains a prevalent global malignancy and necessitates a treatment regimen, which is often accompanied by substantial side effects. To address this challenge, alternative therapies with fewer adverse effects are urgently needed. *Amomum cardamomum* has displayed promising anticancer potential. This study aimed to investigate the impact of *A. cardamomum* seed on T47D breast cancer cell viability and the ability to induce apoptosis, utilizing *in silico* and *in vitro* approaches.

Methods: The samples were extracted utilizing the maceration method using ethanol 96% solvent. In addition, the bioactive constituents were identified through phytochemicals and GC/MS analysis. Cell viability was assessed through MTT assay at various concentrations with 24 and 48-hour incubation periods and compared with the control cells. Apoptosis patterns were visualized by Immunofluorescence assay and analyzed utilizing ImageJ software. *In silico* analyses included three distinct tests, namely pharmacokinetics analysis (ADMET), bioactivity prediction (PASS), and molecular docking.

Results: The *A. cardamomum* seed extract inhibited the growth of the cells with an IC50 value of 97.28 µg/mL in 48 hours of the incubation period. Immunofluorescence assay exhibited that the extract induced apoptosis in over 50% of T47D cells. *In silico* approaches identified bicyclogermacrene, Germacrene-D, and δ-cadinene as potential JAK3, BRAF v600e, and MMP9 protein inhibitors. These compounds exhibited stronger binding affinities to critical amino acids than control ligands.

Conclusion: This research presents compelling evidence that the *A. cardamomum* extract has anticancer activity against breast cancer by preventing growth and inducing apoptosis.

Implication for health policy/practice/research/medical education:
The ethanol extract of *Amomum cardamomum* seed inhibits cancer cell growth and induces apoptosis due to its cytotoxic effects. Therefore, this extract could be valuable in the development of chemotherapeutic agents for treating breast cancer.

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Introduction

Breast cancer stands as the most prevalent global malignancy, with a reported incidence of 19 292 789 cases worldwide in 2020 (1). It represents a critical public health concern, particularly among the female population. Numerous risk factors for breast cancer are, increasing age, gender, reproductive factors, family history, genetic factors, lifestyle, and environmental factors (2). The development and progression of breast cancer are related to 14 hallmarks of cancer, including the evasion of growth suppressors, activation of invasion and metastasis, genome instability and mutation, and resistance to cell death (apoptosis) (3). Under physiological conditions, damaged cells undergo apoptosis. However, in the context of cancer,

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cells persist in abnormal growth and division (4).

Chemotherapy is a treatment commonly used for cancer. However, chemotherapy has various side effects due to low bioavailability, heavy cytotoxicity to normal cells or tissues, and chemoresistance of the drug. Chemoresistance, also known as multidrug resistance (MDR), stands as the primary cause of treatment failure in cancer and takes responsibility for 90% of cancer-related deaths. An ideal treatment should exactly kill cancer cells without breaking or destroying normal cells within the human body (5). Traditional herbal medicine emerges as a promising avenue for cancer treatment due to its minimal side effects, biocompatibility, reduced drug-related adverse effects, lower toxicity to normal cells, and its ability to promote apoptosis (4,6). Natural products have strong chemopreventive effects because of antioxidants, antimutagenics, and anti-inflammatory activities; they effectively inhibit proliferation and induce apoptosis (7). Apoptosis, a programmed cell death mechanism, plays a vital role in maintaining the delicate balance between cell growth and death in the body (8).

Cardamom, a member of the *Zingiberaceae* family, is known as the "*Queen of Spices*". Studies have revealed that its 96% ethanol extract contains terpenoids around 60-80%, alkaloids, flavonoids, terpenes, and various other bioactive components (9). These components, either independently or synergistically, exhibit promising anticancer properties by inhibiting crucial enzymes involved in cancer progression, angiogenesis, antiproliferation, and inducing apoptosis (8,10). With the high mortality rate associated with breast cancer and the emergence of MDR leading to patient death, there is a pressing need for the development of more effective medications. Inducing apoptosis represents a promising strategy in breast cancer treatment, as it selectively targets cancer cells without disrupting the body's homeostasis. The bioactive compounds present in *Amomum cardamomum* seed have shown chemopreventive effects capable of inducing apoptosis. Nowadays, there are no studies conducted to verify the apoptosis induction of *A. cardamomum* seed on breast cancer. This study utilizes both *in silico* and *in vitro* approaches to investigate anticancer efficacy and apoptosis induction of *A. cardamomum* seed extract on breast cancer cell lines.

Material and Methods

Extract preparation

Amomum cardamomum was purchased from a market in Pasa Ibuah, Payakumbuh, West Sumatera, Indonesia. Sample collection was identified at Herbarium Laboratory, Biology Department, Andalas University with voucher specimen 3/K-ID/ANDA/I/2024. The seeds were separated from the exocarp, totalling 1 kg, and were dried in an oven at 40 °C for 24 hours. Following drying, the seeds were ground using a grinder. The powder was macerated with 10 L of 96% ethanol, being soaked for 3 × 24 hours while occasionally stirred. Subsequently, the macerate was filtered and this maceration process was repeated twice. After obtaining 96% ethanol macerate of *A. cardamomum* seeds, solvent evaporation was conducted in-vacuo to yield a thick 96% ethanol of the seeds (11).

Phytochemical test

The phytochemical test was conducted employing standard protocols that involved several procedures; Alkaloids test (Mayer's test), flavonoids test (Shinod's test), phenolic test (Ferric chloride test), triterpenoids test (Horizon test), saponins test, and steroids test (Salkowski test) (12).

GC-MS analysis

GC/MS analysis was conducted at the Laboratory of the Provincial Health Department in West Sumatra. The instrument used was TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., Waltham, MA, USA), combined with a thermo spectrometer mass detector (ISQ Single Quadrupole Mass Spectrometer). Other specifications of the analysis instrument included the use of TG-5MS column (30 m × 0.25 mm id, with a film thickness of 0.25 μm), aided by helium gas as the carrier with a separation ratio of 1:10 at 80 °C for 2 minutes, gradually increased at a rate of 5 °C per minute until reaching 300 °C, and then waited for 5 minutes. The injector and detector were maintained at a temperature of 280 °C while injecting a diluted sample (1:10 hexane, v/v) at a rate of 0.2 μL. Mass spectra were obtained using electron ionization (EI) at 70 eV, covering a spectral range of m/z 35-500.

In vitro anticancer activity

Cell culture

The T47D breast cancer cell line was obtained from the Biomedical Laboratory, Faculty of Medicine, Andalas University. The chemical utilized included dimethyl sulfoxide (DMSO) (Invitrogen), Dulbecco's modified eagle medium (DMEM) (Gibco), Trypsin-EDTA (Sigma), phosphate-buffered saline (PBS) (Sigma), fetal bovine serum (FBS) (Sigma), penicillin-streptomycin (Gibco), trypan blue (Sigma), and MTT reagent 3-(4,5-dimethylthiazol-2-il) -2,5-diphenyltetrazolium bromide. The T47D cell line was cultured in a complete growth medium consisting of DMEM with 10% FBS and penicillin-streptomycin. Cultures were maintained at 37°C in a 5% CO_2 incubator. The *A. cardamomum* seed extract was dissolved in DMSO before administration to the cells for treatment (13).

MTT assay

Cells at a density of 2×10^4 cells per well were cultured across two 96-well plates in complete growth medium

until they were 70% confluent and at 37 °C in a 5% CO_2 incubator (asrec®). Various concentrations of the extract (15.625, 31.25, 62.5, 125, 250, 500 µL/mL) were added with six replicates and incubated for 24 and 48 hours at 37 °C in a 5% CO_2 incubator. Post-treatment, the MTT reagent was added to the cells and allowed to incubate for 4 hours at 37 °C in a 5% CO_2 incubator to produce formazan crystals within viable cells. DMSO was added to stop the MTT reagent reaction, followed by a 30-minute incubation period. Subsequently, the cell viability was assessed by measuring absorbance at 595 nm using an ELISA reader (BioRad®) (13).

Statistical analyses for cell viability

Data obtained from the MTT assay were involved in the determination of cell viability percentage using the following formula:

$$\%\text{Cell Viability} = \frac{\text{Absorbance treatment} - \text{Absorbance control}}{\text{Absorbance solvent control} - \text{Absorbance control}} \text{ X } 100\%$$

The absorbent data underwent statistical analysis to derive viability percentage and construct a correlation graph illustrating the relationship between treatment concentration and cell viability. Utilizing the graph, the regression equation was derived to ascertain the extract's IC_{50}. Statistical significance of the cell viability data was evaluated using the IBM SPSS Statistics software via one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, where $P < 0.05$ indicated statistical significance (13).

Immunofluorescence (Double staining AO/PI)

Morphological alteration of T47D cells was visualized using AO/PI assay. A volume of 200 µL of cancer cells was dispensed onto cover slides in 12 well plates. Subsequently, 800 µL of complete medium was added to each well. The cell distribution was observed using a fluorescent microscope, followed by a 24-hour incubation period to allow cell adherence to the well bottom. The cells were divided into two treatment groups: the control group received a complete medium, while the treatment group was exposed to 1 ml of the extract at its IC_{50} concentration. Incubation was continued in an incubator at 37 °C/5% CO_2 for 24 hours and 48 hours. Afterward, the cover slides were carefully removed and placed onto subject glasses. A solution containing 10 µg/mL of acridine orange and 10 µg/mL of propidium iodide, totaling 10 µL AO/PI solution was applied to the cells. The stained cells were examined under a fluorescent microscope (Zeizz®) and images were analyzed using ImageJ software (14).

In silico studies

In silico methodologies comprised absorbtion, distribution, metabolism excretion, and toxicity (ADMET) prediction, prediction of activity spectra for substances (PASS) Analysis, and molecular docking. Canonical SMILES strings representing the eight identified compounds derived from the *Amomum cardamomum* seed extract were sourced from PubChem and subsequently inputted into the SwissADME tool. SwissADME was utilized to estimate key ADME parameters, such as lipophilicity (LogP), solubility, and gastrointestinal absorption (GIA) (15). Lipinski's rule of five was applied to evaluate the drug-likeness of these compounds. For PASS analysis, the interaction between the target protein and the predicted compound was assessed by comparing the probability of activity (Pa) and probability of inhibition (Pi) values. A Pa value >0.3 (medium confidence) indicated computationally supported outcomes, necessitating further analysis. The three-dimensional structures of the previously screened *A. cardamomum* seed compounds were acquired in SDF format from the PubChem website. Target proteins pivotal in inducing apoptosis within various breast cancer pathways were identified using data retrieved from the KEGG pathway website (https://www.kegg.jp/) (16).

BRAF V600E, JAK3, and MMP9 proteins, identified as crucial to apoptosis induction in breast cancer, were selected based on these pathways. The three-dimensional structures of the chosen target proteins were downloaded in .pdb format from http://www/rcsb.org. Preparation of each *A. cardamomum* seed compound and target protein was conducted using Biovia Discovery Studio v19. Subsequently, AutoDock Tools v1.5.7 repaired all target proteins for molecular docking purposes.

Molecular docking procedures were executed via PyRx application, customizing the grid box according to the specific binding site of each target protein obtained (Table 1). Visualization of the molecular docking outcomes was performed using the Biovia Discovery Studio v19 application (16).

Results

Phytochemical test

Table 2 presents a detailed breakdown of the compounds identified in the ethanol extract derived from *A. cardamomum* seeds. The extract contained bioactive constituents, alkaloids, steroids, triterpenoids, and phenolic compounds.

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GC-MS analysis

GCMS analysis was conducted for the identification and quantification of major components within the *A. cardamomum* seed extract (Table 3, Figure 1). The analysis revealed the presence of several significant compounds.

Table 1. Grid setting for specific molecular docking

Proteins	Binding site	Grid	
		Center	Dimension
BRAF V600E (16)	Asp594, Trp531, Gly596, Ala481, Phe595, Leu514, Gln530, Lys483, Cys532, Thr529, Gly593, Leu505, Phe583	X: 18.3651 Y: 11.3884 Z: -11.9067	X: 11.0885 Y: 19.9546 Z: 12.5355
JAK3 (16)	Leu828, Val836, Ala853, Lys855, Met902, Glu903, Leu905, Leu956, Ala966	X: 7.9469 Y: -4.0153 Z: 8.1208	X: 16.6071 Y: 18.1068 Z: 16.2023
MMP9 (17)	Leu187, Leu188, Ala189, His226, His236, Tyr248	X: 18.5126 Y: -16.5618 Z: 18.9554	X: 18.9854 Y: 15.8434 Z: 14.2079

Relative abundance assessment involved the calculation of individual peak areas, expressed as a percentage of the total peak area, thereby providing the concentration percentage of the compound.

Table 3 presents the concentration of the key compound identified within the sample. Oleic acid exhibited the highest concentration at 83.52%. Following this, norsabinane demonstrated a concentration of 9.73%, while the trans-caryophyllene compound was observed at the concentration of 2.29%. In addition, several other compounds were detected at lower concentrations.

In vitro anticancer activity

MTT assay was conducted to evaluate cell viability following two treatment duration of 24 hours and 48 hours. The acquired dataset provides the cell viability percentage under each treatment condition, facilitating a comprehensive analysis. Cell viability, representing the number of viable cells within a sample, serves as an indicator reflecting survival or mortality of cells subsequent to exposure to a potentially cytotoxic agent.

An increase in the concentration of *A. cardamomum* seed extract corresponded to a significant reduction in cell viability (Figure 2). This indicates that the higher concentration administered resulted in increased cancer cell death. The one-way ANOVA test confirmed that the extract significantly inhibited the growth of the cells. Based on the findings, the extract exhibited an IC_{50} value of 220.82 µg/mL for the 24-hour incubation period and 97.28 µg/mL for the 48-hour incubation period. These results underscored the potency of *A. cardamomum* seed extract in impeding the proliferation of breast cancer cells, suggesting its potential as a promising candidate for further investigation in anticancer therapeutic strategies.

Table 2. Phytochemical analysis of *Amomum cardamomum* seed extract

Component	Test result
Alkaloid	+
Flavonoid	-
Saponin	-
Steroid	+
Triterpenoid	+
Phenolic	+

(+) Detected, (-) Not detected.

Immunofluorescence (Double staining AO/PI)

The observation of both viable cells and apoptosis was conducted using the Carl Zeiss fluorescent microscope.

Table 3. Analysis results of peak area and molecular formula of GC/MS compounds of *Amomum cardamomum* seed extract

Compounds	Retention time (Rt)	Area (%)	Molecular weight (%)	Formula
β-pinene	2.660	0.03	0.72	$C_{10}H_{16}$
Trans-Sabinene Hydrate	3.477	0.18	1.98	$C_{10}H_{18}O$
Sabinene Hydrate	3.847	0.26	3.33	$C_{10}H_{18}O$
α-Terpineol	4.205	0.06	1.09	$C_{10}H_{18}O$
γ-Terpineol	4.933	0.25	2.90	$C_{10}H_{20}O$
Linalylacetate	5.401	0.41	2.97	$C_{12}H_{20}O_2$
Norsabinane	6.774	9.73	25.43	C_9H_{14}
Trans-Caryophyllene	7.391	2.29	6.63	$C_{15}H_{24}$
Germacrene-D	8.302	0.52	3.35	$C_{15}H_{24}$
Bicyclogermacrene	8.482	1.10	4.65	$C_{15}H_{24}$
δ-Cadinene	8.808	1.08	3.35	$C_{15}H_{24}$
Oleic acid	13.060	83.52	41.64	$C_{18}H_{34}O_2$
Olealdehyde	17.232	0.28	0.77	$C_{18}H_{34}O$

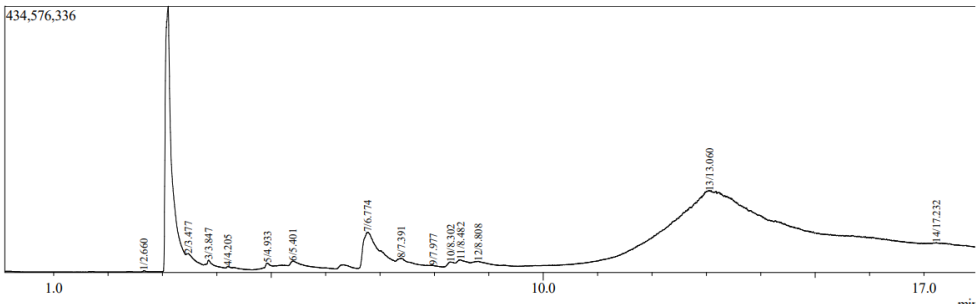


Figure 1. GC/MS chromatogram of *Amomum cardamomum* seed extract.

Subsequently, quantitative analysis was performed utilizing ImageJ 1.53v Software. The qualitative assessment revealed a marked escalation in apoptosis within the T47D cell line after treatment with the extract. Notably, the cell viability in the treated group exhibited a significant increase, denoted by green fluorescence colour, in contrast to the untreated control group. Only a limited number of cells exhibited signs of apoptosis, indicated by orange-red fluorescence colour (Figure 3).

The quantitative analysis demonstrated an increase in apoptosis within the T47D cell line. In the 24-hour incubation period, the apoptosis percentage was 3.23% for the control group and notably elevated to 60,61% for the treatment group. Similarly, during the 48-hour incubation period, the apoptosis percentage stood at 2.18% for the control group and substantially higher at 65.39% for the treatment group. These findings strongly indicate the potential of *A. cardamomum* seed extract to effectively induce apoptosis.

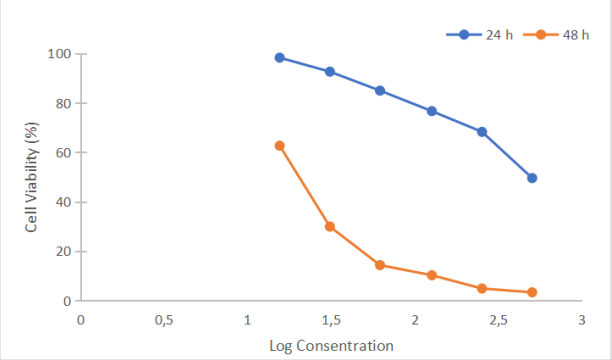


Figure 2. Cell viability of T47D breast cancer cell line.

In silico

ADMET prediction

Analysis of the pharmacokinetic properties (Table 4) revealed that all the selected compounds conformed to Lipinski's rule, with none of the compounds violating more than one rule. Table 4 outlines the oral bioavailability and other physicochemical properties of the selected compounds.

Based on the pharmacokinetic properties analysis, all the selected compounds were in an acceptable range as stated in Lipinski's rule and no compound violates more than one rule. The oral bioavailability and other physicochemical properties of the selected compounds are shown in Table 4.

Prediction of compound bioactivity

The GC-MS analysis revealed the identification of 13 compounds, among which 9 were predicted to exhibit apoptosis agonist properties. Table 5 presents the bioactivity analysis results. Utilizing the PASS web server for compound bioactivity prediction, 9 compounds derived from *Wurfbainia compacta* seed exhibited a high probability of activation (Pa) with a standard of medium confidence standard (Pa score > 0.3).

Potential of breast anticancer ligands based on molecular docking analysis

The docking results (Table 6) revealed the binding affinity of compounds to protein-ligand active sites. Based on

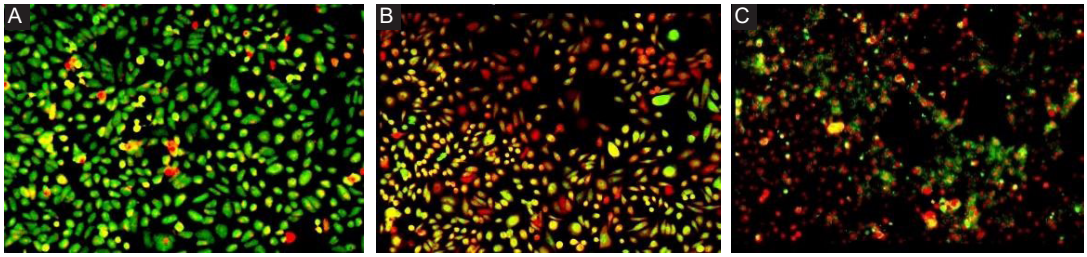


Figure 3. Immunofluorescence AO/PI staining of T47D breast cancer cell. (A) control group, (B) 24 hour treated group, (C) 48 hour treated group. Green fluorescence indicates viable cells and red fluorescence indicates apoptosis cells.

Table 4. Result of ADMET prediction from *Wurfbainia compacta* seed extract

Pharmacological Properties	Compounds (ligands)												
	β-pinene	Bicylogermacrene	Oleic acid	Olealdehyde	Trans-sabinene hydrate	Sabiene hydrate	α-Terpineol	Germacrene-D	γ-Terpineol	trans-Caryophyllene	δ-Cadinene	Norsabinane	Linalylacetate
Molecular weight (g/mol)	136.23	204.35	1.543	266.46	155.25	154.25	154.25	204.35	100.16	204.35	128.17	82.14	196.29
Lipophilicity (XLOGP3)	4.16	4.14	1.836	7.58	2.12	2.12	1.98	4.74	1.23	4.38	3.30	2.45	3.93
Water solubility (Log S)	-3.87	-3.85	1.679	-7.78	-2.18	-2.18	-2.03	-4.47	-1.25	-4.10	-2.98	-2.09	-4.18
Polarity	0.00	0.00	1.753	17.07	20.23	20.23	20.23	0.00	20.23	0.00	0.00	0.00	26.30
Fraction Csp3	0.80	0.73	2.118	0.83	1.00	1	0.80	0.60	1.00	0.73	0.00	1	0.58
GI absorption	Low	low	1.687	low	high	high	high	low	high	low	low	low	high
Lipinski	yes (1 violation)	yes (1 violation)	1.615	yes (1 violation)	yes	yes	yes	yes (1 violation)	yes	yes (1 violation)	yes (1 violation)	yes	yes
Synthetic accessibility	3.73	4.34	1.815	3.20	2.82	2.82	3.31	4.55	1.19	4.51	1.00	2.33	2.75
Bioavailability score	0.55	0.55	3.07	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55

Table 5. Result of bioactivity prediction analysis of metabolite compounds in *Wurfbainia compacta* seed extract

Compounds (ligands)	Bioactivity	
	Apoptosis agonist probability activation (Pa)	Apoptosis agonist probability inhibition (Pi)
β-Pinene	0.515	0.038
Bicyclogermacrene	0.398	0.074
Oleic acid	0.471	0.004
Olealdehyde	0.441	0.055
Trans-Sabinene hydrate	-	-
Sabinene hydrate	-	-
α-Terpineol	0.315	0.119
Germacrene-D	0.529	0.036
γ-Terpineol	0.481	0.044
Trans-Caryophyllene	0.847	0.005
δ-Cadinene	0.279	0.139
Norsabinane	-	-
Linalylacetate	0.750	0.011

docking results, Germacrene-D exhibited binding to the active site of BRAF V600E with a binding affinity close to the comparator ligand, vemurafenib (-8.1 kcal/mol). Despite forming fewer hydrophobic bonds than vemurafenib, Germacrene-D interacted with key amino acids of the BRAF V600E receptor Trp531, Phe595, Val471, and Ile463 (Table 7). These interactions suggest the potential of Germacrene-D to induce apoptosis by inhibiting the BRAF V600E receptor activity in breast cancer.

Moreover, bicyclogermacrene exhibited binding to the ATP binding pocket of JAK3, exhibiting the lowest binding affinity (-8.4 kcal/mol) compared to ATP (-8.0 kcal/mol) and other compounds (Table 6). The interaction analysis showcased 8 hydrophobic interactions, including significant binding to specific residues (Leu956, Ala966, Lys855, Leu828, and Ala853), also bound by ATP (Table 6). This result suggests the potential of bicyclogermacrene as a competitive inhibitor of ATP, potentially inhibiting JAK3 phosphorylation.

Additionally, bicyclogermacrene exhibited binding to MMP9 protein active sites with the lowest affinity compared to other inhibitors, forming three hydrophobic interactions. Interestingly, two interactions occurred within the same residues as MMP9 inhibitors I, Leu188, and His226 (Table 7). Consequently, Bicyclogermacrene showed promise in effectively inhibiting MMP9 activity. The molecular docking results of *Wurfbainia compacta* seed with BRAF v600e, MMP9, and JAK 3 proteins are summarized in Table 6 and Table 7.

Table 6. Result analysis of potential bioactive compounds of *Wurfbainia compacta* seed extract based on binding affinity values

Compound name	Binding affinity (kcal/mol)		
	BRAF V600E	JAK3	MMP9
Control ligand	-11.0	-8.0	-6.4
β-Pinene	-5.9	-6.0	-5.0
Bicyclogermacrene	-7.3	-8.4	-6.6
α-Terpineol	-6.1	-6.1	-6.6
Germacrene-D	-8.1	-8.1	-5.6
γ-Terpineol	-4.9	-4.8	-5.4
Trans-Caryophyllene	-5.7	-8.3	-5.5
δ-Cadinene	-6.9	-6.6	-7.4
Norsabinane	-6.1	-5.7	-6.4
Linalyl acetate	-6.2	-6.0	-6.1

Discussion

The principle of the MTT assay is based on enzymatic reduction by intracellular dehydrogenase of a yellowish tetrazolium salt, which dissolves in water forming purple formazan crystals in living cells (18). The cells were incubated at two different times, 24 hours and 48 hours, to prove Menten's theory. The IC_{50} value of the extract against the T47D cell line was determined to be 220.82 µg/mL after a 24-hour incubation period and 97.28 µg/mL after the 48-hour incubation period. According to the National Cancer Institute, the compound exhibits a cytotoxic effect if it has an IC_{50} value <100 µg/mL, while an IC_{50} value >100 µg/mL indicates inactivity. Based on the results, *W. compacta* seed extract exhibited anticancer potential after the 48-hour incubation period. This result aligns with Menten's theory, asserting that the effects of bioactive compounds result from their interaction with cell receptors. The intensity of the effect of a material is equal to the fraction of receptors to which it is bound, as well as the fraction of receptors depending on the dose and duration of exposure (19).

Fluorescence staining was employed to observe the cell's activities. This technique differentiates viable cells from apoptosis by utilizing *acridine orange* (AO), which binds to double-stranded DNA, inducing green fluorescence in viable cells. Conversely, in apoptosis or dead cells, AO binds to single-stranded DNA, resulting in orange-red fluorescence. Additionally, *Propidium iodide* (PI) is exclusively absorbed by dead cells that experience membrane blebbing by interacting with the nucleic acid. Notably, PI absorbs AO in dead cells to ensure unbiased

results (14). The results of this study indicated the ability of *W. compacta* seed extract to induce apoptosis in T47D breast cancer cells. Following treatment with IC_{50} concentration, apoptosis induction surpassed 50% within both the 24-hour and 48-hour incubation period.

Apoptosis is the most effective method of treating cancer, as it can control and possibly stop the uncontrolled growth of cancer cells (20). *W. compacta* seed extract is proven to induce apoptosis due to the secondary metabolites having strong chemo-preventive effects which allow them to suppress proliferation and increase apoptosis (7). The *W. compacta* seeds used in this study contain alkaloids, triterpenoids, steroids, and phenolic compounds. Alkaloids can balance cell proliferation and cell cycle by blocking the G1 phase and increasing the expression of the p53 gene and p21 inhibitor gene, which are cell cycle

Table 7. Comparison between comparator ligands and *Wurfbainia compacta* seed compounds

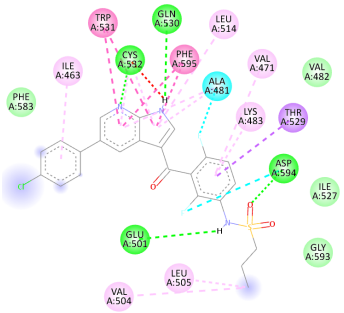
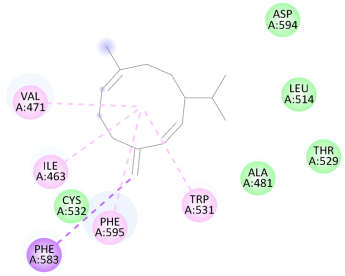
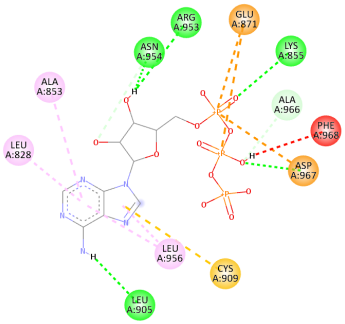
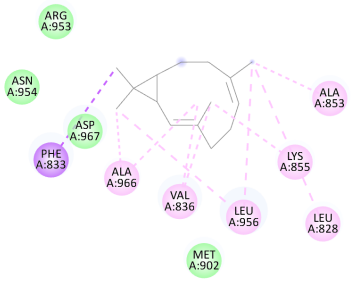
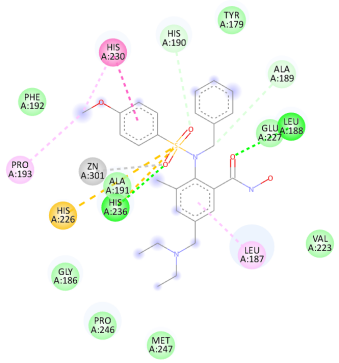
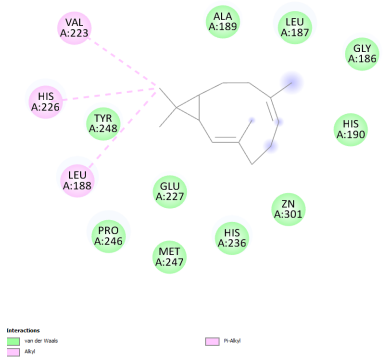
Protein	Ligand	Binding affinity (kcal/mol)	2D Protein-ligand interactions diagram	Chemical interaction	Amino acid residue
BRAF V600E	Vemurafenib	-11.0		Hydrogen bond	Cys532, Asp594, Gln530, Glu501
				Hydrophobic bond	Thr529, Trp531, Phe595, Val504, Leu505, Ala481, Leu514, Val471, Lys483, Ile463
	Germacrene-D	-8.1		Hydrogen bond	-
				Hydrophobic bond	Phe583, Ile463, Val471, Trp531, Phe595
JAK3	ATP	-8.0		Hydrogen bond	Lys855, Asp967, Arg953, Asn954, Leu905, Ala966
				Hydrophobic bond	Leu956, Leu828, Ala853
	Bicyclogermacrene	-8.4		Hydrogen bond	-
				Hydrophobic bond	Phe833, Leu956, Ala966, Val836, Lys855, Leu828, Ala853

Table 7. Continued

Protein	Ligand	Binding affinity (kcal/mol)	2D Protein-ligand interactions diagram	Chemical interaction	Amino acid residue
MMP9	MMP9 Inhibitor I	-6.4		Hydrogen bond	Leu188, His236, Ala189, His190
				Hydrophobic bond	His230, Pro193, Leu187
	Bicyclogermacrene	-6.6		Hydrogen bond	-
				Hydrophobic bond	Leu188, Val223, His226

inhibitors (21). Alkaloids can induce apoptosis through the intrinsic pathway by activating the pro-apoptosis p53 protein and producing the pro-apoptosis Bcl-2 protein Bax that leads to caspase 3 activation and induces apoptosis (22,23). Multiple studies have proven the anticancer activity of triterpenoids by suppressing cell proliferation and increasing cell apoptosis through caspase activation in MCF-7 and T47D breast cancer cell cultures (24). Steroid compounds show anticancer activity with low toxicity, low susceptibility to MDR, and high bioavailability (25).

According to *in silico* analysis, the parameters in ADMET prediction included lipophilicity (XLOGP3), water solubility (Log S), polarity, fraction Csp3, gastrointestinal (GI) absorption, and bioavailability score. Most of the selected compounds align with the recommended range for XLOGP3 (-0.7 to +5.0), with exceptions found in olealdehyde and oleic acid (26). Compounds with values at or above 0 are considered highly soluble, those within the 0 to -2 range as soluble, -2 to -3 as slightly soluble, and those less than -4 as insoluble (27), most of these compounds can dissolve in water except olealdehyde, Germacrene-D, and trans-caryophyllene. The ideal polarity value was within the 20-130 Å^2 range. The fraction of carbon Sp3 (CSP3) is anticipated to fall between 0.25 and 1 range (26). A bioavailability score of 0.55 or 0.56 indicates favorable bioavailability for the compound (28). Among the compounds examined, linealyl acetate, γ-terpineol, α-terpineol, norsabinane, and trans-sabinene hydrate exhibited desirable bioavailability profiles based on their physicochemical properties.

Bioactivity prediction using PASS Online proved that the compounds contained in *W. compacta* seed extract had a high score potential to be anti-cancer. This score was chosen based on indicative computational evidence. Notably, these 9 compounds exhibited Pa scores higher than the probability score (Pi), indicating their potential to induce apoptosis within the human body (29). Computational validation confirmed the likelihood of these compounds acting as apoptosis agonists. Importantly, the activity potential inhibition (Pi) value for these compounds was lower than the Pa value. This suggests that upon introduction into the human body, these compounds are not anticipated to hinder apoptosis induction (29).

According to the molecular docking analysis results, compounds present in *W. compacta* seed exhibited promising potential as apoptosis inducers on breast cancer. bicyclogermacrene and Germacrene-D exhibited notable potential in inhibiting the activity of proteins JAK3, BRAF v600e, and MMP9 by binding to important amino acids within these proteins. The binding affinity of these three compounds surpassed that of the control ligands.

Janus kinase 3 (JAK3) known for its anti-apoptosis role, tends to be overexpressed in breast cancer. The continuous

activation of JAK/STAT signalling, especially JAK/STAT3, is recognized in various malignancies, contributing to carcinogenesis and cancer progression. The inhibition of the JAK-STAT pathway, particularly by targeting JAK3, holds promise in inhibiting STAT3/5 activity, hindering their transcription factors (30). Notably, inhibiting the JAK3/STAT3 pathway has shown efficacy in inducing apoptosis and cell cycle arrest in colon cancer cells (31). Studies on breast cancer cell metastasis to the lungs have revealed a significant correlation between MMP-9 expression and the process (32). Downregulation of MMP-9 gene expression through siRNA-mediated silencing demonstrated a notable pro-apoptosis effect in MDA-MB-231 and MCF-7 breast cancer cell lines, significantly reducing viable cancer cells through apoptosis induction (33). Mutations in BRAF have been associated with a potent inhibitor of apoptosis effects. Upregulation of BRAF inhibits apoptosis through mitochondrial regulation and inhibits cytochrome C-induced apoptosis and cytosolic caspase activation (34).

Studies in colorectal tissue with BRAF mutations highlight significantly reduced apoptosis rates in tumour cells (35). Notably, targeted inhibition of BRAF using vemurafenib has shown promising therapeutic efficacy, especially in patients with advanced triple-negative breast cancer (TNBC) carrying BRAF V600E mutations (36). However, the efficacy of vemurafenib varies, as observed in melanoma with BRAF V600E mutations, where the response range is between 50 and 80%. Recent investigations have shed light on the suppression of breast cancer by vemurafenib, targeting the PI3K/AKT pathway and inhibiting the anti-apoptotic protein BCL2A1, regulated by PI3K/AKT and linked to resistance against chemotherapeutic and targeted agents (37).

The combined utilization of computational (*in silico*) and experimental (*in vitro*) assays enhances the depth and reliability of exploring *W. compacta* seed effects on breast cancer cells. This integrated approach underscores its relevance in breast cancer treatment by targeting crucial proteins involved in cancer progression and emphasizing apoptosis induction, suggesting promising therapeutic applications. The MTT assay, evaluating cell viability and cytotoxicity, provides crucial insights into the extract's potential as an anticancer agent. Additionally, the apoptosis assay employing double staining (AO/PI) visualizes viable and apoptotic cells, augmenting the understanding of *W. compacta* seed-induced apoptosis in breast cancer cells. The inclusion of *in silico* analysis enriches the study by identifying molecular interactions between *W. compacta* seed compounds and specific cancer-related proteins. This computational modeling enhances comprehension of the extract's molecular mechanisms, reinforcing implications for breast cancer therapy.

The integration of *in silico* predictions, the MTT assay, and apoptosis assays using double staining (AO/PI) offers a comprehensive analysis of *W. compacta* seed impact on cell viability and apoptosis induction. This multidimensional approach strengthens the research findings' credibility and depth, providing valuable insights into its therapeutic potential for breast cancer treatment. However, a limitation of this study pertains to the GC-MS analysis. The research lacks a comprehensive exploration of the GC-MS analysis, potentially restricting the detailed characterization of bioactive compounds in *W. compacta* seed extract. Identified compounds might lack comprehensive representation due to potential limitations in the sensitivity or scope of the analytical method.

Conclusion

The treated cells demonstrated notable cytotoxicity to cancer cells within a 48-hour incubation period. Further studies on molecular-level investigations are essential to examine the expression of pro-apoptosis gene markers by breast cancer cells. Docking results revealed that the compounds of *W. compacta* seed had potential as apoptosis inducer agents by inhibiting the activities of the JAK3, BRAF V600E, and MMP9 proteins by binding to critical amino acids within these proteins. Based on research results, *W. compacta* seeds have substantial potential as an anticancer agent by significantly reducing cell proliferation and inducing apoptosis.

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Conflict of interests

The authors declare that they have no competing interest

Ethical considerations

This Research was approved by The Research Ethics Committee Faculty of Medicine at Universitas Andalas (Approval Reference Number: 347/UN.16.2/REP-FR/2023).

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