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Development of Co-Chemotherapeutic Agents and Analysis of the Active Substance Content of Red Ginger (*Zingiber officinale Var Rubrum rhizoma*) in Metastatic Breast Cancer Cells

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Abstract

Metastatic breast cancer is also known as stage IV breast cancer. Current chemotherapy agents have several limitations, such as resistance events, side effects, and inadequate efficacy in advanced cancer. It is necessary to develop a more effective chemotherapy model, namely the development of co-chemotherapy agents derived from natural ingredients that have activity in counteracting oxidative stress and preventing metastatic breast cancer, namely red ginger. GC-MS analysis of red ginger oleoresin showed the composition of chemical compounds influenced by variants of soxletation time of 60, 120, and 180 minutes. GC-MS analysis showed that this essential oil consists of volatile compounds, mainly from the sesquiterpene group and namely zingiberene, βsesquiphellandrene, β-bisabolene and ar-curcumene. The results of the cytotoxic test analysis of red ginger extract with socletation time for 60, 120 and 180 minutes respectively obtained IC50 data as follows with 45.16 µg/ml, 37.23 µg/ml and 20.2 µg/ml. The compounds contained in red ginger, such as zingiberene, gingerol, shogaol, flavonoids, phenolics, alkaloids, saponins, and tannins, play an important role in its cytotoxic effect against cancer cells. The mechanism by which α-Zingiberene induces cell death in cancer cells is mainly through the apoptotic process. The compound causes the release of mitochondrial cytochrome c into the cytoplasm, which then activates caspase-3, an apoptotic enzyme.

Keywords: Red ginger; metastatic; cytotoxic; soxhlet, time of extraction.

INTRODUCTION

Cancer is one of the major health problems in various countries with the number of global sufferers continuing to increase. Based on IARC (International Agencies for Research on Cancer) data, through the GLOBOCAN project, throughout 2018 there were 18.1 million cancer cases and 9.6 million cancer deaths. Cancer deaths will continue to increase to 13.1 million by 2030 (Mutia et al., 2019) . Of these data, 56% of cases and 64% of deaths occur in developing countries. Breast cancer is the most common type of cancer among women in Indonesia, comprising 30.8% of all female cancer cases in 2020 and causing 20.4% of female cancer-related deaths in the same year. By 2040, it is predicted that there will be a 47.1% increase in the incidence and mortality, respectively, of women with breast cancer in Indonesia (Bryant et al., 2023). Based on a national health survey, DIY Province, one of 38 provinces in Indonesia, has more than twice the national cancer prevalence with breast cancer incidence ranking first in the region based on population-based cancer registry data (Nindrea et al., 2021). Many individual risk factors have been associated with breast cancer incidence including diet, lifestyle, body mass index (BMI), reproductive history, comorbidities, and genetic factors (Wakai et al., 2000).

Metastatic breast cancer, also known as stage IV breast cancer, is a type of breast cancer that has spread beyond the breast and nearby lymph nodes to other parts of the body, such as the bones, liver, lungs, or brain (Hairunisa et al., 2023). Metastatic breast cancer is considered incurable, but it can be treated to help manage symptoms, improve quality of life, and prolong survival. Common symptoms may include bone pain, shortness of breath, fatigue, weight loss, and neurological symptoms such as headaches, seizures, or confusion (Ashariati, 2019). Treatment for metastatic breast cancer usually involves a combination of systemic therapies, such as chemotherapy, hormone therapy, targeted therapy, and immunotherapy, as well as local treatments such as radiation therapy or surgery to manage specific symptoms or complications. Radiotherapy treatment

methods induce ROS formation in the process of cell death (Feng et al., 2018). Reactive Oxygen Species (ROS) are by-products of aerobic metabolism, mainly produced by mitochondria. Most types of cancer contain high amounts of ROS. An increase in endogenous ROS triggers adaptive changes, induces oxidative stress and is cytotoxic. Oxidative stress can trigger the onset of cancer. Some chemotherapeutic agents target ROS metabolism, such as doxorubicin (Dox) and cisplatin (Hikmah et al., 2021). Curcumin, a natural compound from Curcuma sp., is also a target of ROS metabolism. Curcumin increases cellular ROS levels by inhibiting ROS metabolic enzymes which results in the inhibition of cancer cell growth. Therefore, it is possible that high ROS levels in cancer cells are a critical point to target anticancer development without having significant effects on normal cells (Angraini et al., 2019).

Current chemotherapeutic agents have several limitations, such as resistance events, side effects, and inadequate efficacy in advanced cancer. As a result, therapeutic inefficiency occurs, so it is necessary to develop a more effective chemotherapy model (Muna & Maulidina, 2022). One of the developments in cancer therapy is directed at the development of Co-chemotherapy Agents derived from natural ingredients that have activity in counteracting oxidative stress and preventing metastatic breast cancer compared to normal fibroblast cells (Fachreza Erdi Pratama & Rina Fajri Nuwarda, 2018). Co-chemotherapy can increase the efficacy of chemotherapeutic drugs and allow the use of low doses of chemotherapeutic agents that will decrease toxicity to normal tissues. One approach to finding chemotherapeutic compounds is through the exploration of natural materials, especially plants. Some plants have been known to have chemopreventive activity with specific targets (Muna & Maulidina, 2022). Red ginger is one of the plants that has the potential to be developed as a co-chemotherapy and anti-aging agent in treating metastatic breast cancer cases (S. Zhang et al., 2022).

Ginger is a plant containing about 169 chemical constituents, including monoterpenes, sesquiterpenes, diterpenes, vanilloids, flavonoids, etc. The biological activity of red ginger is likely due to synergistic or additive effects of these compounds (S. Zhang et al., 2022). The biological activity of red ginger is likely due to synergistic or additive effects of these compounds (S. Zhang et al., 2022). Research conducted by (Ghasemzadeh et al., 2015) reported that the total amount of phenolics and flavonoids in red ginger was higher than that of regular ginger. Research conducted by (Supu et al., 2019) used GC-MS to identify the chemical composition of essential oils contained in Red Ginger containing three dominant monoterpenes (camphene, geranial, and geranyl acetate) and 47 sesquiterpenes and flavonoid compounds. The active substance dehydrozingerone and its analog in red ginger have been found to exhibit significant cell proliferation inhibitory activity against certain cancer cells (Tjendraputra, E.; Tran, V.H.; Liu-Brennan, D.; Roufogalis, B.D.; Duke, 2001). In addition, 6-gingerol which is a component of Red Ginger has been reported to inhibit the proliferation of transgenic mouse ovarian cancer cell lines. Red Ginger inhibits cancer progression, angiogenesis, and metastasis and MMP production to block malignant tumor migration (Nishidono et al., 2018). However, it is still unclear whether these phenomena are correlated with increased intracellular and cellular ROS. Curcumin increases cellular ROS levels by inhibiting ROS metabolic enzymes which results in the inhibition of cancer cell growth. Therefore, it is possible that high ROS levels in cancer cells are a critical point to target anticancer development without having significant effects on normal cells (Angraini et al., 2019).

MATERIALS AND METHODS

Tools and Materials

Materials: Red ginger, 96% Ethanol, 4T1 cell culture, FBS, penicillin streptomycin, DMEM, MTT, SDS, PBS, Doxorubicin

Tools: 96 Well plate, 6 Well Plate, Elisa Reader, Rotary evaporator, set of Distillation, GCMS, propiper, Blue tip, Yellow Tip, set of glassware

Preparation of Red Ginger Extract

Red ginger was obtained from the Research on Medicinal Plants and Traditional Medicines and Development Center, Tawangmangu, Ministry of Health, Republic of Indonesia. Dried red ginger was cleaned from dirt, peeled and cut into pieces after which it was dried in the oven, after which it was pulverized using a blender until it became a fine powder to increase the surface area (Erlita et al., 2022). Next, 25 grams of red ginger powder was weighed and oxletated using 90% ethanol solvent with time variants of 60, 120, and 180 minutes at 70-80°C (Sidauruk et al., 2021). The extraction process was completed, the mixture was filtered to separate the pulp and filtrate containing active compounds. The extract obtained can be evaporated with a rotary evaporator at 70-80oC until a concentrated extract is formed (Ida et al., 2024)

Analysis of oleoresin with GCMS

Gas Chromatography-Mass Spectrometry (GC-MS) analysis first prepared the sample and solvent, then the sample was extracted and rinsed twice with presolvent, for the third time the main solvent, only one rinse on the sample. Then, the sample was injected into the device using a suction speed needle and high injection at normal split injection to avoid overloading the column. After that, the sample is vaporized at 175°C, then the vapor was carried by helium gas with a pressure of 45.9 kPa and a flow rate of 83.8 mL/min through the

chromatographic column at temperatures ranging from 75°C to 300°C to separate compounds that have volatiles. Components that enter and exit the ion source at 200 ° C will pass through a surface of 225 ° C, broken down into ions of a certain mass and detected with a gain of 1.10 kV. The recorded data with the chromatogram has the peak of the analyzed compound at the 2nd to 40th minute with a mass ion range of about m/z 10-500 for a speed in scanning 2000 seconds per scan. Identification of compounds carried out by matching the standard spectrum data. The analysis of red ginger extract showed that major compounds such as zingiberene at a retention time of about 15 minutes, as well as beta-bisabolene and βeta-sesquiphellandrene, contributed to the extract's pharmacological activity and characteristic aroma (Situmorang & Ricky, 2022)

Cell Culture Preparation

4T1 breast cancer cells (ATCC® CRL-2359) were obtained from Faculty of Medicine UGM. Cells were cultured in Dulbecco's modified Eagle's Medium with 10% FBS and 1% penicillin-streptomycin under standard conditions (37°C, 5% CO2).

MTT Assay

Cells were cultured into 96 well plates at a concentration of 3 x 104 cells/mL and treated with various concentrations of Red Ginger Extract in combination with Doxorubicin (Sigma) for 24 hours. After incubation, the medium was discarded, and the cells were washed 1X with phosphate buffered saline (PBS). Then, MTT reagent (Sigma) was added and incubated for 4 hours. SDS was added, incubated overnight, and then the absorbance was measured with λ = 595 nm (Ahlina et al., 2020).

Cytotoxic Analysis

In the cytotoxicity test method using the MTT method, the calculation of the % of living cells due to treatment is by comparing the difference in absorbance of the solution from the cell control with the absorbance of the solution from the treated cells to the absorbance of the solution from the cell control itself multiplied by 100%. The results of absorbance readings on live cells were converted in units of % live cells by means of:

$$\% \text{ living cells} = \frac{Abs\ Cells\ with\ treatment - Abs\ Media\ control}{Abs\ Cell\ control - Abs\ Media\ control}\ x\ 100\%$$

To determine whether or not there is a cytotoxic effect, a linear regression analysis was performed between the concentration of the test compound and the % viability with Microsoft excel to obtain the IC_{50} value. The smaller the IC_{50} concentration of the test compound, the more potent the compound is for its cytotoxic effect.

RESULTS AND DISCUSSION

Results Extraction Results

One kg of fresh red ginger rhizome was dried at 50°C using an oven until it became simplisia and mashed using a blender. Red ginger that had been mashed each weighed as much as 70 grams and then inoculated at a temperature of 70-80 ° C using 96% ethanol solvent as much as 250 ml. This is because the oleoresin content in red ginger is effectively extracted using the sokletation method with a solvent and time that is more efficient than the maceration method (Prasetiyo, 2015). Oleoresin extracted using ethanol has the highest yield. This may be because ethanol is a universal polar solvent capable of dissolving many compounds both polar, semipolar and nonpolar. The properties of ethanol result in other phytochemicals and extracted oleoresin (Q. W. Zhang et al., 2018).The socletation process was carried out three times with time variations of 60, 120, and 180 minutes in order to obtain different compound results. The results of red ginger extraction were evaporated at 50ºC to obtain a thick red ginger extract.

Figure 1. Process of making thick red ginger extract. Description: A. Fresh red ginger, B. Red ginger symplisia, C. Red ginger powder, D. Succulent extract of red ginger.

The results of the analysis of the content of Red Ginger compounds with GC MS

The results of red ginger extract were analyzed for oleoresin content using GCMS analysis to see differences based on the length of time of the soxhletation process. Oleoresin obtained from the extraction of each organic solvent was then tested with GC-MS instrument. Helium gas is used as the mobile phase, which does not react

with the chemical compounds present in the oleoresin. As the temperature increases, the materials will separate and be carried away in the mobile phase according to their respective volatility levels. The retention time and structure fragmentation pattern of each substance are not the same. The chromatogram pattern of substance separation, retention time (RT), and peak area percent are information obtained from the GC instrument. Mass spectroscopy produces information about the fragmentation spectrum of each compound equipped with its molecular weight (BM) value. Based on the fragmentation pol and molecular weight can determine the identity of each compound present in oleoresin (Yuni Subhi Isnaini, Bahrah Bahrah, 2024). The results of GC-MS analysis showed that red ginger oleoresin from ethanol solvent with 60 minutes of socletation time contained 15 chemical compounds, 120 minutes of 13 chemical compounds, and 180 minutes of 22 chemical compounds. The chemical composition of each resin is shown in Table 1.

Table 1. Chemical composition of red ginger oleoresin from 3 variations of Time 60, 120, and 180 minutes.

60 minutes			120 minutes			180 minutes		
No	**Component**	**% Area**	**No**	**Component**	**% Area**	**No**	**Component**	**% Area**
1	trans-Farnesol	0.38	1	endo-Borneol	0.95	1	endo-Borneol	0.30
2	beta.-Elemene	0.51	2	Acetid Aacid 1,7,7-Trimethyl-Bicyclo[2.2.1]Hept-2-YL Ester	0.27	2	beta.-Elemene	0.19
3	Benzene, 1-(1,5-Dimehtyl-4-Hexenil)-4-Methyl-	7.87	3	.alpha.-Copaene	0.25	3	Germacrene D	0.30
4	Zingiberene	32.55	4	6,10,11,11-Tetramehtyl-Tricyclo[5.3.0.1(2,3)]Undec-1(7)Ene	0.39	4	Farnesol	0.25
5	beta.-Bisabolene (CAS)	18.56	5	trans-Caryophyllene	0.85	5	KW3 AUS Epiglobulol	0.24
6	beta.-Sesquiphellandrene (CAS)	21.34	6	Benzene, 1-(1,5-Dimethyl-4-Hexenyl)-4-Mehtyl-	9.53	6	farnesol	0.18
7	2,5-Furandione, 3-(dodecenyl)dihydro-	1.91	7	Zingiberene	37.21	7	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (CAS)	3.19
8	Pyridinium, 1-hexadecyl-, chloride, monohydrate (CAS)	0.48	8	beta.-Bisabolene (CAS)	24.28	8	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- (CAS) ar-Curcumene	5.46
9	4-Methyl-5-(3'-methyl-2'-butenyl)-6-methyl-1-formyl-6-(4"-methyl-3"-pentenyl)-1,3-cyclohexadiene	0.62	9	beta.-Sesquiphellandrene (CAS)	24.25	9	Zingiberene (CAS)	35.52
10	Ergost-5-en-3-ol, (3.beta.)-(CAS) .delta.5-Ergostenol	1.05	10	-)-.beta.-Elemene	0.08	10	beta.-Bisabolene	21.83
11	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.,22Z)-(CAS)	1.06	11	Alpha-Bisabolol	0.4	11	beta.-Sesquiphellandrene (CAS) 2-Methyl-6-(4-Methylenecyclohex-2-Enyl)-2-Heptene	27.83
12	Pregn-4-ene-1,20-dione, 12-hydroxy-16,17-dimethyl-	2.75	12	Veridiflorol	1.03	12	Elemol	0.24
13	Methyl arachidonate	0.24	13	Bicyclo[4.1.0]Hept-3-En, 2-Isoprenyl-5-Isopropyl-7,7-Dimehtyl	0.52	13	Citronellyl acetate	0.18
14	3.alpha.,4.alpha.,9.beta.,11-Diepoxymuurolan-10-ol	1.29				14	3-(1-Acetoxy-ethyl)-2,4,4-trimethyl-2-cyclohexen-1-one	0.23
15	Stigmast-5-En-3-Ol, Oleat	9.37				15	trans-Farnesol	0.51
						16	V eridiflorol	1.03
						17	4-Methyl-5-(3'-methyl-2'-butenyl)-6-methyl-1-formyl-6-(4"-methyl-3"-pentenyl)-1,3-cyclohexadiene	0.78
						18	3,6-Dimethyl-2,3,3a,4,5,7a-	0.23

60 minutes			120 minutes			180 minutes		
No	Component	% Area	No	Component	% Area	No	Component	% Area
							hexahydrobenzofuran	
						19	Benzeneacetic acid, 4-hydroxy-3-methoxy-, methyl ester (CAS)	0.32
						20	Geranyl propionate	0.29
						21	2-(2'-Nitro-2'-propenyl)-1-cyclohexanone	0.49
						22	Secoisolariciresinol	0.32

The results of GC-MS analysis of red ginger oleoresin showed the composition of chemical compounds influenced by variants of soxhletation time of 60, 120, and 180 minutes. GC-MS analysis showed that this essential oil consists of volatile compounds, mainly from the sesquiterpene and monoterpene groups (Amin et al., 2025). The main components of essential oil are zingiberene, βsesquiphellandrene, β-bisabolene and ar-curcumene (Mawadah, 2024). The essential oil component in red ginger is a terpene group that has ethanol polarity, essential oils contained in oleoresin from essential oil components are polar (Nur et al., 2020). The compound is included in the sesquiterpene hydrocarbon group, is a terpenoid derivative with a basic structure consisting of 15 carbon atoms (C_{15}), is non-polar and has a molecular mass (Kriswiyanti et al., 2021). The structures of these three compounds have aliphatic or cyclic skeletons containing double bonds and are not polar functional groups, so they are easily soluble and easily extracted at low relative temperatures.

Zingiberene, a monocyclic sesquiterpene compound which is the main component in red ginger essential oil (Verenzia et al., 2022). Then for, βeta-bisabolene and βeta-sesquiphellandrene function as sesquiterpene isomers with open chain structures containing double bonds, being the main volatile components in red ginger essential oil. These three compounds tend to be extracted optimally at solvent boiling temperatures in soxhlet of 70-80 °C (using ethanol), because these temperatures are sufficient to facilitate the release of volatile components without causing thermal degradation and at this temperature close to the boiling point of ethanol, which boiling point is about 78 C (Maspupah et al., 2022).

Cytotoxic Test Results of Red Ginger Extract

The cytotoxic test was performed using the MTT assay method. The principle of using this method is colorimetric measurement of the formation of formazan salts that are insoluble in water and purple in color derived from the reduction reaction of water-soluble tetrazolium by producing a yellow solution (Ghasemi et al., 2021). In the cytotoxic test of red ginger ethanolic extract using several concentration levels, namely 15.625; 31.25; 62.5; 125 µg/ml. In this study, the higher the concentration of red ginger ethanolic extract, the higher the concentration of the test compound affects the % cell viability. A compound if it has IC_{50} more than 1000µg/ml is less potent when developed into a cytotoxic agent (Machana et al., 2011). The observation result of cytotoxic test of ethanolic extract of red ginger can be seen in Figure 2.

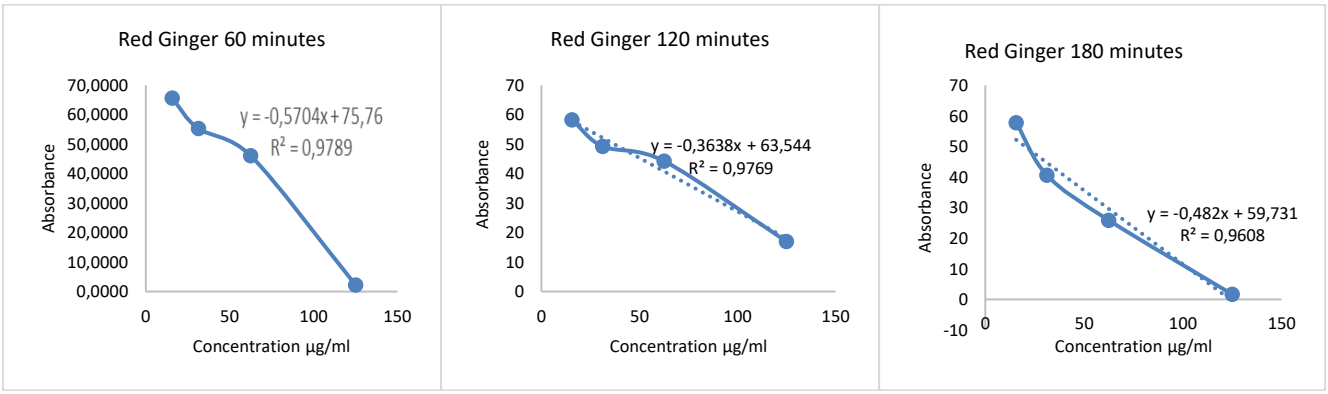

Figure 2. Linear regression of red ginger with soaking times of 60, 120, and 180 minutes.

In the analysis of a single cytotoxic test of sample A, namely red ginger which was inoculated for 60 minutes, a linear regression curve equation y = 0.5704x + 75.76 was obtained so that the IC50 of red ginger extract sample A could be calculated as 45.16 µg/ml. In the single cytotoxic test analysis of sample B, the linear

regression curve equation y=-0.3638x+63.544 was obtained so that the IC_{50} of red ginger extract of sample B could be calculated as 37.23 µg/ml. In the single cytotoxic test analysis of sample C, the linear regression curve equation y=-0.482x+59.731 was obtained so that the IC_{50} of red ginger extract sample C could be calculated as 20.2 µg/ml. Based on the single cytotoxic analysis of these samples, it can be seen that the cytotoxic test against 4T1 cancer cells is best in sample C with a succulent time of 180 minutes. Temperature significantly affects the stability of red ginger bioactive compounds during extraction. High temperatures can increase extraction efficiency by increasing the diffusion rate (Handayani et al., 2024). However, high temperatures also increase the degradation of thermally labile compounds such as gingerol and shogaol. In this study, the soxhletation process of red ginger at 70-80°C, so that the extracted compounds are zingiberene (Azian et al., 2014). Zingiberene is a compound of the red ginger essential oil group that gives a spicy taste and a very strong fragrant aroma of ginger. This compound is classified as a sesquiterpene hydrocarbon, an organic compound derived from isoprene composed of 15 carbon atoms ($C_{15}H_{24}$), has volatile properties and is commonly found in essential oils (Nur et al., 2020). The structure of Zingiberene has a cyclic skeleton with one double bond on the ring, which shows stability in contributing to the distinctive aroma of red ginger. The time used in the soxhletation process can also affect the decrease in bioactive concentration over time, indicating that thermal instability causes degradation. However, in this study, the time used for soxhletation up to 180 minutes showed an increase in zingiberene compounds due to the high stability of the compounds (Maulida et al., 2023).

The compounds contained in red ginger, such as zingiberene, gingerol, shogaol, flavonoids, phenolics, alkaloids, saponins, and tannins, play an important role in its cytotoxic effect against cancer cells. Research conducted by (Dwi Oktaviani, 2020) explains that red ginger extracted using maceration method with 96% ethanol solvent has an IC_{50} of 69.86 µg/ml against 4T1 breast cancer cells. The mechanism of α-Zingiberene induces cell death in cancer cells mainly through the apoptosis process. This compound causes the release of mitochondrial cytochrome c into the cytoplasm, which then activates caspase-3, an important executing enzyme in apoptosis. This pathway leads to DNA fragmentation and an increase in the sub-diploid (sub-G1) cell population, hallmarks of apoptosis. Evidence from DNA fragmentation assays, flow cytometry analysis of cell cycle distribution, and caspase activity assays support that α-zingiberene triggers apoptosis through the mitochondrial pathway, resulting in cancer cells (Lee, 2016). In addition, Zingiberene content on cell viability and apoptotic cell death in cancer cells Zingiberene was able to modulate DMBA-stimulated physiological and hematological changes and decrease transaminases and lipid peroxidation in DMBA-stimulated animals. Zingiberene also increased antioxidant levels and suppressed inflammatory markers. Zingiberene is able to induce apoptotic cell death. (Seshadri VD, Oyouni AAA, Bawazir WM, Alsagaby SA, Alsharif KF, Albrakati A, 2022).

CONCLUSIONS

The results of GC-MS analysis of red ginger oleoresin showed the composition of chemical compounds influenced by variants of sox**h**letation time of 60, 120, and 180 minutes. GC-MS analysis showed that this essential oil consists of volatile compounds, mainly from the sesquiterpene group and namely zingiberene, βsesquiphellandrene, β-bisabolene and ar-curcumene. The results of the cytotoxic test analysis of red ginger extract with soxhletation time for 60, 120 and 180 minutes respectively obtained IC50 data as follows with 45.16 µg/ml, 37.23 µg/ml and 20.2 µg/ml. The compounds contained in red ginger, such as zingiberene, gingerol, shogaol, flavonoids, phenolics, alkaloids, saponins, and tannins, play an important role in its cytotoxic effect against cancer cells. The mechanism by which α-Zingiberene induces cell death in cancer cells is mainly through the apoptotic process. The compound causes the release of mitochondrial cytochrome c into the cytoplasm, which then activates caspase-3, an important executing enzyme in apoptosis.

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Competing Interests: The authors declare that there are no competing interests.

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