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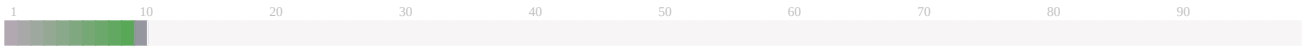

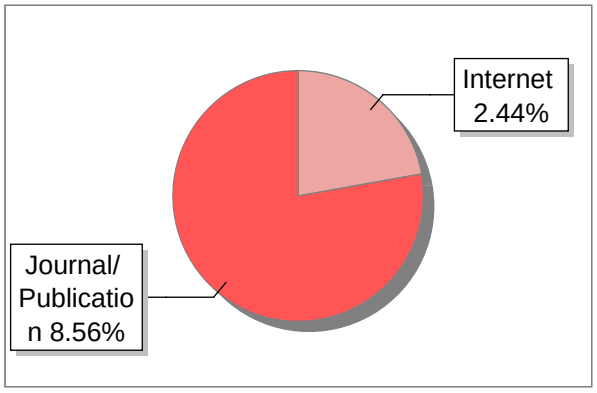

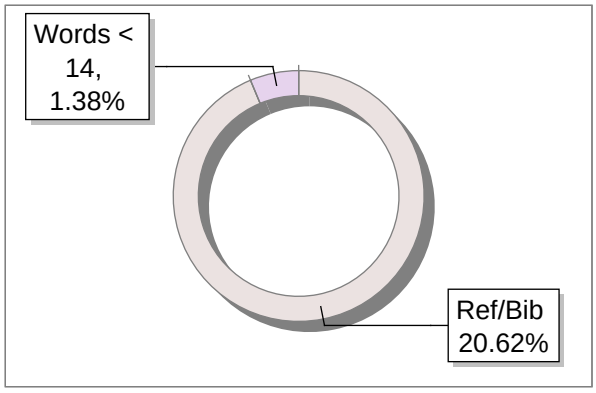

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THE APOPTOSIS INDUCTION OF *Zingiber oficinale* ETHANOLIC EXTRACT-Treated HeLa (HUMAN CERVICAL CANCER) CELLS AND ACTIVE COMPOUND PROFILING USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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ABSTRACT

Cervical cancer accounts for the highest percentage of cancer-related deaths in Indonesia, accounting for nearly 60% of all cancer cases. Therefore, research into the anticancer mechanisms needs to be conducted. The ethanolic extract of Zingiber officinale (EEZO) contains zingiberene, a chemical known for its anticancer activity. Understanding the mechanism underlying the apoptosis-inducing effects is crucial. This study aimed to elucidate the apoptotic pathway and analyze the gas chromatography/mass spectrometry (GC/MS) profile of EEZO cells. The research commenced with the maceration of *Zingiber officinale* rhizomes using 75% ethanol to obtain EEZO. Apoptosis assays were conducted on both a negative control group and an EEZO-treated group of HeLa cells (cervical cancer cells). The apoptotic mechanism was evaluated using forward scattered light-side scattered light (FSC-SSC), fluorescein isothiocyanate (FTIC), and phycoerythrin (PE) flow cytometry. Apoptotic results were analyzed by comparing the control and EEZO samples, which revealed the number of viable cells, apoptotic cells, and cells in the sub-G1 phase. The major constituent of EEZO, which is expected to be a potent apoptosis inducer, was detected using GC/MS. The FSC-SSC results indicated a lower number of viable cells in the EEZO-exposed group than in the control group. FTIC results demonstrated that EEZO significantly increased apoptotic cell death, increasing from 68 to 1537 cells. PE flow cytometry revealed an elevated sub-G1 cell population, indicating the induction of apoptosis by EEZO. GC/MS analysis revealed five dominant components in EEZO, which had the potential to induce apoptosis: L-borneol, zingiberene, farnesol, beta-sesquiphellandrene, and alpha-curcumene. In conclusion, EEZO, with its dominant compound, zingeberene, induced apoptosis in HeLa cells, indicating anticancer potential.

Keywords: HeLa cell, anticancer, *Zingiber officinale* (ginger), flow cytometry, zingiberene

INTRODUCTION

Zingiber officinale (ZO) or ginger is a plant with high levels of secondary metabolites and cytotoxic activity (Nurjannah et al., 2022). ZO has been studied *in vitro* in HCT116, B16, MCF-7 (Liao et al., 2020), colon cancer cells (Mohd Yusof et al., 2022), HuH 7 (González-Osuna et al., 2023), and HeLa cells (Hasan Mujahid et al., 2023). The mechanism of action explored in tests on HeLa cells was anti-proliferation using a cytotoxic test with MTT and an antioxidant mechanism with DPPH (Ghazemzadeh et al., 2015). The antiapoptotic mechanism was determined by Hoechst staining (Ansari et al., 2016) and propidium iodide staining (Hasan Mujahid et al., 2023).

Zingiber officinale contains several bioactive compounds such as gingerols, shogaols, and zingiberene, which have been shown to induce apoptosis in cancer cells (Lee, 2016). The

proapoptotic effect of ginger is mediated through various signaling pathways, with the main pathway being mitochondrial apoptosis. This pathway begins by increasing the expression of the proapoptotic proteins Bax and Bad, while decreasing the expression of antiapoptotic proteins, such as Bcl-2 and Bcl-xL (Mao et al., 2019). This causes the release of proapoptotic proteins, namely cytochrome c, from the mitochondria into the cytosol. Cytochrome c binds to apoptotic protease activating factor 1 (APAF1) to form an apoptosome complex, which activates caspase-9. Caspase-9 then activates effector caspases, namely caspase-3 and caspase-7, resulting in apoptosis (Hasan Mujahid et al., 2023).

Gingerol, shogaol, and zingiberene are also known to promote apoptosis through inhibition of NF-κB activation by blocking the degradation of IκBα (NF-κBα inhibitor), which prevents NF-κB translocation to the nucleus and subsequent gene expression (Shanmugam et al., 2022). In addition, ZO stimulates apoptosis by inhibiting the PI3K/Akt/mTOR and MAPK/ERK pathways. This results in reduced cell viability and increased apoptosis of cancer cells (Manna et al., 2020).

The method used to research anticancer mechanisms through apoptosis can be performed by observing cells stained using immunostaining. Observations can be made with an electron microscope, fluorescence microscope, time-lapse microscope, and atomic force microscope (Banfalvi, 2017). Before observation using a microscope, relatively complicated preparations must be made. Moreover, the research results cannot directly obtain the cell cycles and their numbers quantitatively and accurately. Another method for observing apoptosis, complete with the cell cycle and quantitative cell counts, is flow cytometer (McKinnon, 2018). However, the effect of this method on the cancer cell cycle to the level of the number of cells is unknown. Therefore, it is necessary to assess this mechanism using flow cytometry method is required. The flow cytometry results can provide information on the G1 cycle (the cell cycle enters the inactive phase). Thus, it is important to use a flow cytometer to determine the mechanisms of apoptosis orcell death. Research on the apoptosis of ginger extract with ethanol solvent in HeLa cells using a flow cytometer has never been carried out.

The flow cytometer method is used to analyze and sort cells based on their physical and chemical properties, one of which is to observe proapoptotic mechanisms. This method can measure the number of cells undergoing apoptosis during the apoptotic phase. To measure the number of cells undergoing apoptosis, the cells were stained with a fluorescent dye that selectively labels cells undergoing apoptosis. Staining with Annexin V resulted in protein binding to phosphatidylserine, which was externalized on the surface of apoptotic cells. By staining cells with Annexin V and secondary antibodies labeled with fluorescent dyes, the size of cells undergoing apoptosis can be identified. The advantage of flow cytometry is that it is a valid, easy, and rapid tool for determining the effect of a sample as an anticancer agent using an apoptotic mechanism. Flow cytometry allows for the simultaneous analysis of multiple cellular parameters, such as cell size, shape, and fluorescence intensity, making it an ideal technique for studying apoptosis (Crowley, 2016).

In addition to these markers, flow cytometry can be used to analyze other cellular characteristics, such as cell cycle status, surface marker expression, and intracellular signaling pathways involved in apoptosis (Adan et al., 2016). By combining several parameters, flow cytometry can explain the apoptotic phase in cancer cells in this study using ZO extract.

An extract is expected to possess anticancer activity when it can stimulate apoptosis, as indicated by the number of dead and viable cells, as well as the number of cells experiencing apoptosis and necrosis, compared with the negative control. In addition, flow cytometry results showed that the sub-G1 phase by sample intervention showed accelerated apoptosis compared to the negative control (McKinnon, 2018). Apoptosis testing with a flow cytometer can be done by staining with anexin V. The mechanism of apoptosis can be observed from the test results using forward scattered light-Side scattered light (FSC-SSC), fluorescein isothiocyanate (FTIC), and phycoerythrin (PE) flow cytometry methods (Gadalla et al., 2019).

ZO is a widely used traditional medicine with various health benefits including anti-inflammatory, antioxidant, and anticancer properties. The active compounds responsible for these effects occur in varying types and amounts in different ginger extracts, and their identification is essential for understanding the mechanism of action and developing new therapeutic applications. The type and amount of volatile compounds in ZO are suitable for analysis by Gas Chromatography/Mass Spectrometry (GC/MS) (Nur et al., 2020).

GC/MS analysis allows the separation, identification, and quantification of individual compounds in ginger extracts based on their chemical properties such as molecular weight, structure, and volatility. GC/MS results can be used to identify chromatograms and spectra that provide information based on retention time and m/z of the compounds contained in them (Nur et al., 2020). This study aimed to determine the mechanism by which EEZO induces apoptosis using flow cytometry, and to identify the dominant compound responsible for this effect.

RESEARCH METHODS

Equipment and Materials

The material used is EEZO which is extracted with 75% technical ethanol, methanol, aquadest (Bratachem), NaOH (Merck), methanol pa (Merck), HeLa cell culture collection from the Parasitology Laboratory Faculty of Medicine Public Health and Nursing UGM, aquabidest, sodium bicarbonate (Sigma), hepes (Sigma), Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco), fetal bovine serum (FBS) 10% v/v (Gibco), penicillin-streptomycin 1% v/v (Gibco), fungizone 0.5% (Gibco), phosphate buffer saline (PBS) 20% (Sigma), dimethyl sulfoxide (DMSO), MTT Reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg in 1 mL PBS (Sigma), stopper, sodium dodecyl sulphate (SDS) 10% in 0.01 N HCl (Merck), 96 well-plate, trytan blue stain 0.25% (Gibco). The equipment used is a CO_2 incubator (New Bronswick, Galaxy 170R), centrifuge (Hermle Siemensstr-25 D-78564), laminar air flow cabinet (Mascotte LH-S), micropipette (Soccorex), autoclave, hemocytometer, ELISA microplate reader (Robonik), microscope inverted (Olympus CKX41-2), GC-MS (QP2010 SE Shimadzu with type of column: Rtx-5MS). EEZO was extracted using the maceration method and 75% ethanol solvent on Z. officinale rhizomes, which were obtained from the UPT Batu Herbal Materia Medica Laboratory, East Java Provincial Health Service (number 067/1410/102.20/2023).

Research Procedures

1. Flow cytometry test

The flow cytometer test was carried out on HeLa cells that had been cultured in 96-well plates with a cell density of 5 × 104 cells/well and were then incubated at 37°C in a CO_2 incubator for 24 hours. The test sample solution (EEZO) was then added to the cell culture and incubated in a CO_2 incubator for 24 hours. Next, readings were taken on the flow cytometer with the output settings of FSC-SSC, FTIC, and PE in the Pharmacology Laboratory at the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Yogyakarta.

2. GC/MS analysis

Qualitative analysis using GC-MS was performed by dissolving EEZO in a NaOH solution in methanol. A sample of 1 µL was injected under the following GC conditions: column oven temperature, 70.0 °C; injection temperature, 280.00 °C; injection mode splitless; sampling time, 1 minute, pressure 53.5 kPa, total flow 21.1 mL/min, column flow 0.91 mL/min, linear velocity 34.9 cm/sec, purge flow, 2. mL/min; split ratio 20.0, equilibrium time 3.0 min, MS, ion source temp 250.00 °C, interface temp 160.00 °C, detector gain 1.10 kV+0.00 kV.

Data Analysis

The flow cytometry data were analyzed by comparing the flow cytometer results between the control and sample (EEZO). The identification process using GC-MS produced several bioactive compounds that could be identified from the chromatogram peaks. The chromatography results were followed by mass spectrometry (MS) testing of the mass spectrum for each molecular weight of the bioactive compound. The chromatogram obtained was analyzed by comparing the retention times, similar to the standard library provided by the instrument database. The spectra from the GC/MS samples were analyzed by comparing the similarity index (SI) in the instrument database.

RESULTS AND DISCUSSION

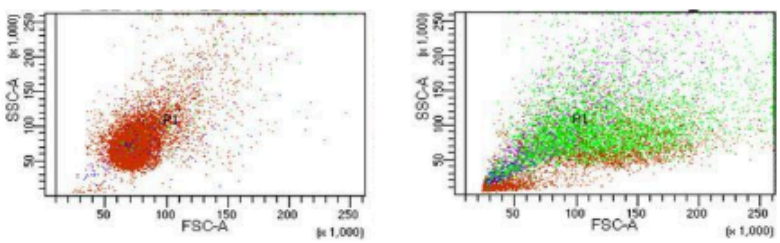
This research was conducted with ethical clearance from the UAD Ethics Committee number 0123071177. Apoptosis induction testing was carried out on HeLa cells under the influence of EEZO with a flow cytometer by looking at the FSC-SSC, FTIC, and PE images.

Testing apoptosis with a flow cytometer

1. *Apoptosis and the flowcytometer FSC-SSC*

Apoptosis can be detected with a flow cytometer based on the number of cells observed as a result of the excitation scattering of cells. This is because scattering produces colors that can be detected by an instrument (Adan et al., 2016). The FSC-SSC flow cytometer images of HeLa cells exposed to EEZO are shown in Figure 1. The cells in the negative control did not appear to be spread out, but were homogeneous in the same place at a position that showed the number of viable cells. In contrast, the color produced in cells exposed to EEZO showed visible scattering at heterogeneous cell image positions (Figure 1). Apoptotic or necrotic cells may influence these differences in appearance.

The differences among cell images in the FSC-SSC flow cytometer results were caused by photons when they hit the cell and were deflected around the cell, causing light scattering, based on which the FSC and SSC images were formed. FSC is proportional to the cell surface area, cell size, nucleus, cell granularity, cell shape, and cell membrane. However, SSC light is mostly refracted, reflected, and collected, which is proportional to the cell granularity or internal complexity, which is proportional to the cell type in a heterogeneous population (Adan et al., 2016). The test was carried out at IC_{50} EEZO on HeLa cells so that the cells could be clearly observed in sufficient numbers and was carried out in one test. Cells affected by EEZO appear heterogeneous, possibly because of the presence of live cells, apoptotic cells, and necrosis cells. Meanwhile, the control images showed cells in a homogeneous position as the dominant living cells (Figure 1).



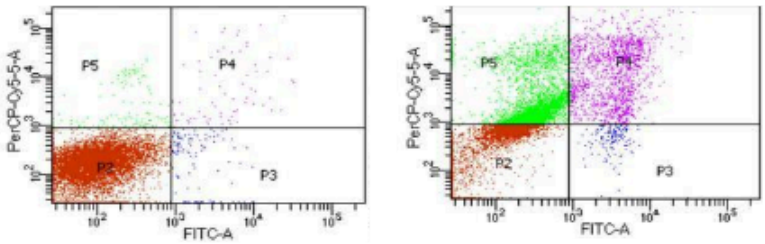
(a) **(b)**

Figure 1. Image of Cells From The FSC-SSC Flow Cytometer of HeLa cells (a) Control; (b) Treated With *Zingiber Officinale* Ethanolic Extract (●: Viable Cells; ●: Early Apopotosis; ●: Final Apoptosis; ●: Necrosis)

2. *Apoptosis and the flow cytometer results FTIC*

The results of the FTIC flow cytometer provided an overview of cells in all four quadrants. The lower right quadrant shows the percentage of early apoptotic cells, the lower left quadrant shows the percentage of viable cells, the upper right quadrant shows the percentage of late apoptotic cells, and the upper left quadrant shows the percentage of

necrotic cells (Mamat et al. 2021). The FTIC results of HeLa cells in controls compared to those exposed to EEZO were different, especially in the upper-right and lower-right quadrants (Figure 2). The right quadrant shows cells undergoing apoptosis. In addition, EEZO also increased cell death via apoptosis, as indicated by the increase in the number of cells in the upper left quadrant compared to the control. EEZO also increased cell death, as shown in the lower left quadrant, and the number of living cells decreased from 9718 cells to 4159 cells (Table I). The cell death process is by apoptosis and necrosis mechanisms.



(a) (b)

Figure 2. Image of Cells From The FTIC Flow Cytometer of HeLa cells (a) Control; (b) Treated With *Zingiber Officinale* Ethanolic Extract (●: Viable Cells; ●: Early Apopotosis; ●: Final Apoptosis; ●: Necrosis)

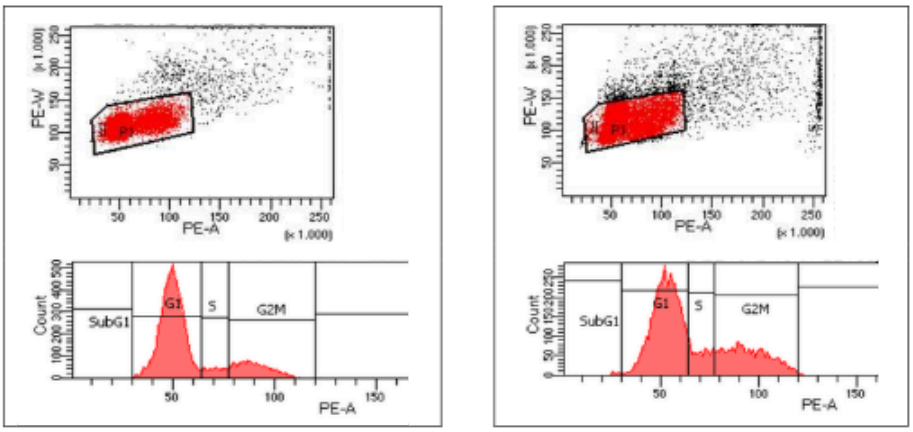
Table I. Number of Cells In Each Quadrant of FITC Flow Cytometer Results After *Zingiber Officinale* Ethanolic Extract (EEZO) Treatment

Quadrant	Remark	Number of cell	
		Control	EEZO
Bottom left (P2)	viable cells	9718	4159
Top right (P4)	early apoptosis	109	156
Bottom right (P3)	final apoptosis	68	1537
Top left (P5)	Necrosis	122	4426

3. *Apoptosis and the PE flow cytometer results*

The cell cycle observed from the flow cytometry results included the number of cells in the cell growth (G1), synthesis (S), arrest (G2/M) phases, and apoptosis in the sub cell growth (sub-G1) phase. Apoptosis could be observed from the results of the sub-G1 phase of the cell cycle from the results of cell counts on the flow cytometer between intervention samples compared to controls. The PE flow cytometer image produced an image of the number of cells entering the sub-G1 phase, where this phase showed cells in the resting phase. The sub-G1 phase also shows cells undergoing the initial apoptosis process (Al-Sheddi et al., 2015). Figure 3 shows that the sub-G1 phase (leftmost quadrant of the figure) of HeLa cells was increased by EEZO. This increase was in line with the results of FSC-SSC and FTIC, which supports the occurrence of apoptosis due to the influence of EEZO. This result was confirmed in Table II, which shows a decrease in G1 (beginning of cell division) from 6,799 to 4619 and an increase in sub-G1 from 29 to 68. The results that support the mechanism by which apoptosis occurs are an increase in the cell resting or antiproliferation phase (G2/M) from 1813 to 2318, although the S phase increases from 626 to 849 as a consequence of increasing G2/M. However, the increased synthesis due to EEZO did not cause the number of cells to increase because it then entered the G2/M phase. This is also supported by the smaller number of cells compared to the control, namely 15744 by the influence of EEZO from the number 18538 in the control. In this study, the active compound responsible for the apoptotic effect was not identified. To overcome this limitation, we identified the presence of zingibererene, shigeol, and gingerol in the ginger

extract, although they could not be confirmed as active compounds that act as apoptotic inducers.



(a) (b)

Figure 3. Forward Scattered light-Side Scattered light (FSC-SSC), Fluorescein Isothiocyanate (FITC), and Phycoerythrin (PE) Results From (a) Negative Control and (b) Ethanol Extract of *Zingiber officinale*

Table II. Number of Cells From PE Flow Cytometer Images of HeLa cells By The Influence of *Zingiber officinale* Ethanol Extract

Cell phase	Number of Cells	
	Control	EEZO
P1	9269	7872
G1	6799	4619
S	626	849
G2M	1813	2318
Sub-G1	29	68
Polyploid	2	18
Total	18538	15744

Identification of the dominant compound in EEZO as an inductor apoptosis using GC/MS

The chromatogram profile and identity of metabolite compounds in the 75% ethanolic extract of ginger rhizomes and the results of analysis using gas chromatography (GC) are presented in Figure 4 and Table III. The results of the analysis showed that there were metabolite compounds included in the group of terpenoid compounds (monoterpenes and sesquiterpenes), ketones, aldehydes, lipids, and phenols. Sample analysis using GC/MS was used to determine the compound content based on the retention time on the GC and SI value for structural similarity to standards in the database on the MS instrument (Al-Rubaye et al., 2017).

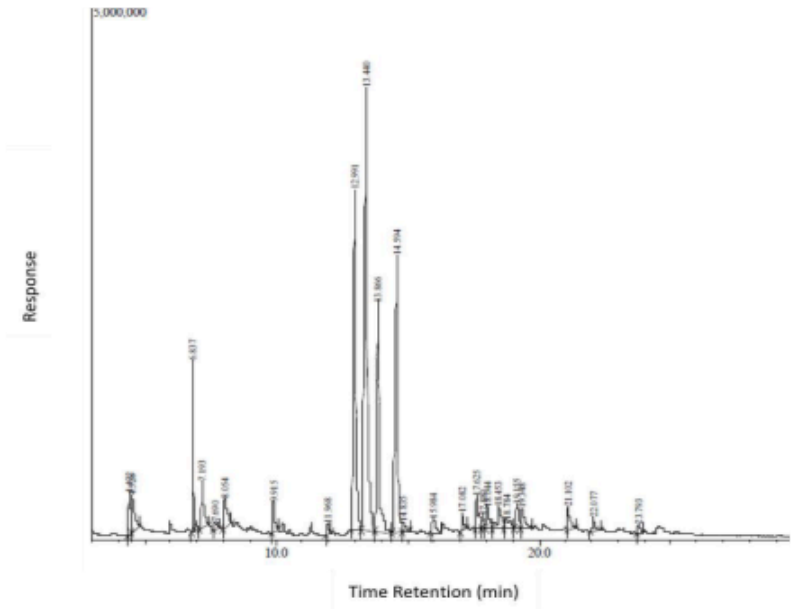


Figure 4. Chromatogram Profile of Metabolite Compounds From *Zingiber officinale* Ethanolic Extract

The chromatogram data in Figure 4 show that there was a peak at 13.440 minutes with an AUC (area under the curve) of 25.16% of the total area of all detected compounds. These data indicate the abundance of these compounds in EEZO. The compounds at the retention time were identified as zingiberene (IUPAC: (5R)-2-methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene), which belongs to the sesquiterpene group of compounds as shown in Table III. These results are in agreement with the MS results, showing the zingiberene compound in Figure 5. This was based on the results compared with the GC/MS database.

Table III. Identity of The Dominant Metabolite Compounds of *Zingiber officinale* Ethanolic Extract From Analysis Using Gas Chromatography

Retention time (minute)	Molecular Weight (m/z)	Molecular Formula	Compound Identity	Compound Classes (ChemIDplus)	Similarity Index
6.837	154	$C_{10}H_{18}O$	Borneol L	Monoterpenoids	98
12.991	202	$C_{15}H_{22}$	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Sesquiterpenes	97
13.440	204	$C_{15}H_{24}$	Zingiberene	Monocyclic Sesquiterpenes	94
13.866	222	$C_{15}H_{26}O$	Farnesol	Prenols	93
14.594	204	$C_{15}H_{24}$	beta.-Sesquiphellandrene	Bisabolane sesquiterpenoids	94

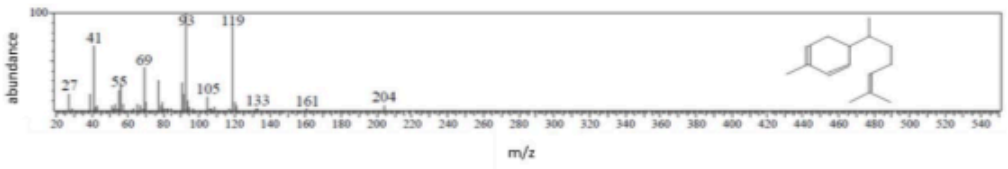


Figure 5. Mass Spectrum of The Compound Resulting From Peak Number 3. This Was Estimated To Be Zingiberene

Induction of apoptosis in HeLa cells by EEZO was made possible by the active ingredient, Zingiberen. Zingiberen can significantly increase the formation of ROS which causes cell apoptosis (Hasan Mujahid et al., 2023) and an increase in sub-G1 in SiHa cells (Lee, 2016). In *vivo* tests were performed in rats induced by dimethyl benzo anthracene (DMBA), which can act as a chemopreventive agent (Seshadri, 2022). In addition, zingiberene can inhibit the growth of colon cancer cells *in vitro* and *in vivo* by inducing autophagy (Chen et al., 2019).

CONCLUSION

Based on the GC/MS results, EEZO was predicted to contain zingiberene. The potency of EEZO as an anticancer agent was confirmed to increase apoptosis in HeLa cells, based on flow cytometry analysis.

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