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Apoptotic Effect of Simpor Leaf Extract (*Dillenia suffruticosa*) from Belitung on Colorectal Cancer Cells WiDr

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Abstract

Colorectal cancer is the second deadliest cancer worldwide. The side effects and resistance of existing chemotherapy drugs are the background for various studies looking for potential plant extracts as new anticancer agents. This study aims to determine the cytotoxic activity of simpor leaf extract from Belitung and its effect on apoptosis of WiDr colorectal cancer cells. The compound content in simpor leaf extract was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The cytotoxic activity of simpor leaf extract at different concentrations (25, 50 and 100 ppm) was determined by the MTT test and apoptosis induction by Annexin V/PI flow cytometry analysis. Based on the GC-MS chromatogram results, 13 different compounds were detected. Analysis of the relationship between extract concentration and the percentage of WiDr cell inhibition showed that an extract concentration of 100 ppm was the concentration with the highest percentage of inhibition (20.12 % ± 4.99) compared to concentrations of 50 ppm (13.95 % ± 2.32) and 25 ppm (15.90 % ± 1.38) with an inhibitory concentration value (IC_{50}) of simpor leaf extract of 10.45 ppm. Induction of WiDr cell apoptosis by the extract was influenced by the concentration of simpor leaf extract, which was increase in the percentage of apoptotic cells from 16 % at a concentration of 25 ppm to 70.5 % at a concentration of 100 ppm. The results of this study show that simpor leaf extract from Belitung is able to induce apoptosis in WiDr colorectal cancer cells.

Keywords: Simpor, GC-MS, MTT, IC_{50}, Annexin V/PI, Flow cytometry, WiDr

Introduction

Colorectal cancer is one of the most common types of cancer in the world, with the third-highest incidence and second place in terms of mortality [1]. In 2020, more than 1.9 million new cases of colorectal cancer and 93,000 deaths were recorded worldwide [1]. GLOBOCAN data for 2020 shows that in

Indonesia, 34,189 new cases of colorectal cancer were recorded, making colorectal cancer rank fourth as the cancer with the most new cases. Colorectal cancer refers to cancer that occurs in the colon and rectum of sufferers, usually starting as polyps in the wall of the large intestine and developing into cancer cells that grow abnormally to form malignant tumors when left untreated. Various treatments such as surgery, chemotherapy and radiotherapy are procedural actions that are often the first choice in the medical world for cancer sufferers [2]. However, its effectiveness is limited due to the resistance of colorectal cancer cells to these therapeutic drugs [3], and the side effects caused are considered a risk in treating cancer.

To reduce the incidence and mortality rates due to colorectal cancer, much research has been carried out exploring the potential of plant ingredients as anticancer agents. Various types of plants in Indonesia are natural resources that have great potential to be explored and developed for their compounds to become useful anticancer agents. Simpor (*Dillenia suffruticosa*) is a plant that grows in Indonesia and has the potential to be used as a traditional medicinal plant because of its pharmacological effects. People in Bangka-Belitung use simpor leaves as a traditional medicine for diarrhea [4], while people in Sabah, Malaysia use simpor to treat cancer growth [5]. Previous research has reported that simpor extract from Malaysia has high antioxidant and cytotoxic activity against several cancer cells such as HeLa, MCF-7, MDA-MB-231, A549 and HT-29 [6]. In MCF-7 cells, simpor extract from Malaysia was able to induce apoptosis by modulating many genes involved in the oxidative stress pathway, this shows that simpor extract has the potential to be further developed as an anticancer agent [7].

The anticancer effectiveness of plants is supported by the presence of phytochemicals such as saponins, triterpenes, sterols and polyphenolic compounds which are associated with cytotoxic activity and are recorded to have anticancer effects [8,9]. Some studies have shown that simpor leaf extract can induce apoptosis. Though, there have been no studies investigating the cytotoxic activity of simpor leaf extract from Belitung in inducing apoptosis in colorectal cancer cells. Therefore, in this study, an evaluation was conducted to determine the apoptotic effect of extract of simpor leaves from Belitung on WiDr colorectal cancer cells.

Materials and methods

Plant material and extraction

The simpor leaves were washed with running water until clean and air-dried for several hours. Then the leaves are oven-dried at 50 °C for 10 h. Dried leaves were ground to a fine powder using a blender and sieved to obtain simpor leaf simplicia. Plant powder about 100 g was extracted with ethanol solvent (1:9, w/v) using the maceration method for 72 h. The residue was filtered using Whatman number 1 filter paper. The resulting filtrate was then collected and concentrated until it became a thick extract in a rotary evaporator.

GC-MS analysis

Determination of extract contents was performed using GC-MS system consisting of an Agilent 7890A GC device combined with an Agilent 5975C MS. Briefly, 1 g of simpor leaf extract was diluted with 5 mL of HPLC methanol and filtered with a 0.45 nm syringe filter into a GC vial. Samples were injected into a GC-MS system. The GC-MS conditions were adjusted as in previous research [10], which used the capillary column used was HP-5MS 30 m × 0.25 mm with a film thickness of 0.25 µm. The injector temperature is set at 250 °C, while the temperature setting is as follows: The temperature starts at 40 °C and is held for 3 min, then the temperature rises from 40 to 200 °C at a rate of 3 °C/min, then remains constant for 3 min. The carrier gas used is helium gas (He) with a flow rate of 1 mL/min. Gas chromatograms were carried out in splitless mode. Identification of compound components of simpor leaf

samples was carried out by referring to the National Institute of Standards and Technology (NIST) library database.

Cell lines and treatments

The WiDr cells culture were obtained from The Primate Animal Study Center, Institute for Research and Service to The Community-Bogor Agricultural Institute (PSSP, LPPM-IPB). The WiDr cells is grown on D-MEM medium containing Fetal Bovine Serum 10 %, Glutamine, 1,000 units/mL Penicillin antibiotics and 10,000 µg/mL Streptomycin. WiDr cells routinely maintained as monolayers in a humidified, 5 % CO_2 atmosphere at 37 °C on a flask until 50 % confluence.

Morphological changes of cells treated with simpor leaf extract

The 5×10^3 WiDr cells were seeded in 96-well plates and treated with simpor leaf extract at 25, 50 and 100 ppm concentrations for 48 h. The untreated cell as negative control and doxorubicin 1 ppm as positive control were also included. Then morphological changes were observed under an inverted light microscope.

MTT assay

The 5,000 WiDr cells were placed in 96-well plates (100 µL/well) and incubated for 24 h. Then each cell was treated with 100 µL of simpor leaf extract with different concentrations (25, 50 and 100 ppm). For the positive control cells were given 1 ppm doxorubicin while the negative control cells were not given simpor leaf extract. Then incubation was carried out in a CO_2 incubator for 72 h (37 °C). Next, each well was added with 10 µL of MTT solution (5 mg/mL in PBS) and incubated for 4 h (37 °C) in dark conditions. The MTT solution added to the well is reduced by mitochondria within living cells, resulting in the formation of purple-blue formazan crystals, which can be utilized to indicate the number of viable cells. After the incubation was complete, the supernatant formed was taken out and 100 µL of absolute ethanol was added so that the formazan crystals could be dissolved. Absorbance was measured at 595 nm using an ELISA plate reader. The results of the OD (optical density) value obtained from the absorbance reading are then used to calculate the %inhibition value. Linear regression curve of inhibition percentage and IC_{50} concentration (inhibits 50 % cell growth) compared with control using the following equation [6].

$$\text{Inhibition (\%)} = \left(\frac{\text{Control absorbance} - \text{Treatment absorbance}}{\text{Control absorbance}}\right) \times 100\ \% \quad (1)$$

Flow cytometry apoptosis assay

The steps for apoptosis testing were carried out according to the procedures in the Annexin V-FITC Kit from Miltenyi Biotec. The 5,000 WiDr cells were counted in 100 µL of growth medium each placed into wells (12-well plates) and incubated for 24 h. The cells were then treated with negative control, positive control and simpor leaf extract with different concentrations (25, 50 and 100 ppm) and incubated for 24 h at 37 °C (5 % CO_2). Cells were harvested using the trypsinization method and cell suspensions in each treatment were placed in propylene tubes for centrifugation (1,500 rpm, 5 min). After that, the supernatant was aspirated while the pellet was added with 1 mL of PBS. The pellet was then centrifuged (1,500 rpm, 10 min), and the supernatant was aspirated. The pellet was resuspended with 1 mL PBS and centrifuged again. The pellet was added with 10 µL of annexin V dye, then homogenized. Incubate cells for 15 min in dark conditions at 4 °C. Then 1 mL of PBS was added, followed by centrifugation for 10 min. The supernatant was then discarded and the pellet was added with 5 µL of PI dye before being analyzed by flow

cytometry. When analyzing cells using flow cytometry (BD Accuri C6 Plus), a cell population of 10,000 cells was used to obtain representative data in accordance with the instructions [11].

Analysis

The compound content in the samples were identified by mass spectrometry on GC-MS using spectral comparison with database values and analyzed qualitatively. The MTT results were analyzed using linear regression calculations using 1-way ANOVA and followed by DMRT (Duncan's Multiple Range Test). Apoptosis assay results were analyzed using flow cytometry and represented in a dot plot of annexin V (x-axis) against PI (y-axis).

Results and discussion

Analysis of simpor leaf extract compounds using GC-MS

Based on the chromatogram results of GC-MS of simpor leaf extract from Belitung, shows the presence of several bioactive compounds that were detected with different retention times. The GC-MS chromatogram of simpor leaf extract can be seen in **Figure 1**. The mass spectrum pattern produced by the MS detector was compared with the NIST database to identify compounds contained in simpor leaf extract. Compounds that have been identified in simpor leaf extract along with retention time, peak area percentage, and biological activity can be seen in **Table 1**.

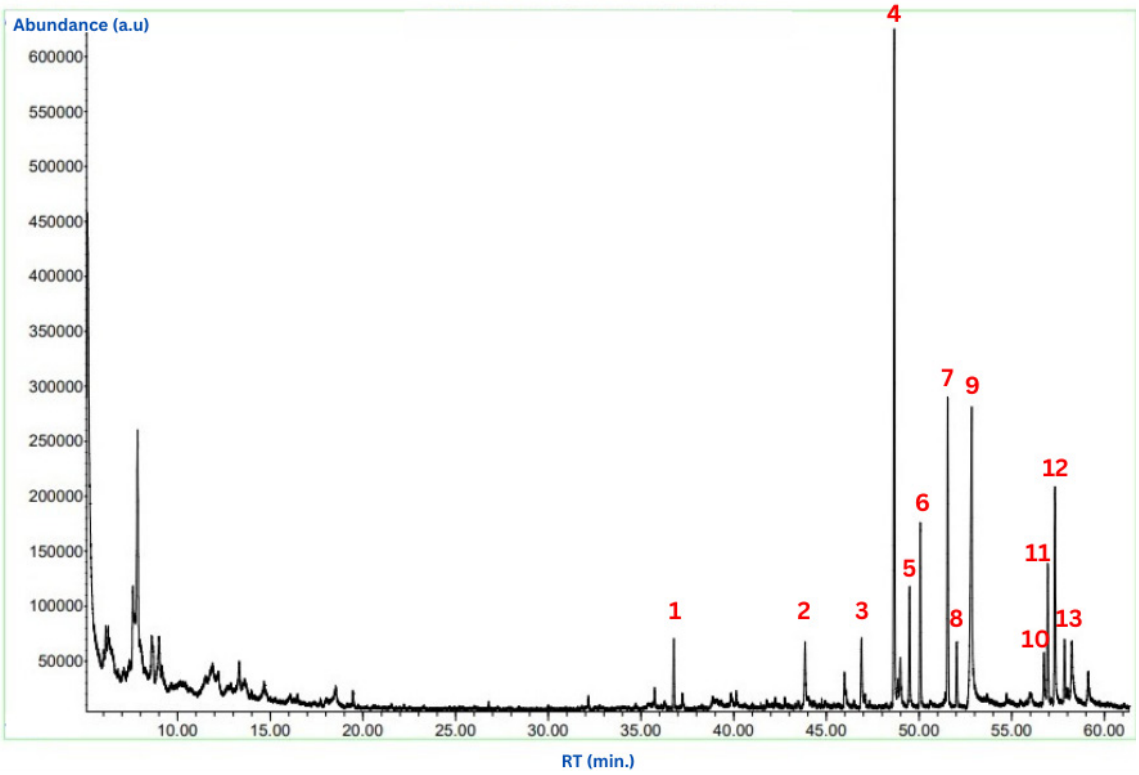

Figure 1 Chromatogram of GC-MS results of simpor leaf extract.

Table 1 Phytochemical compounds from simpor leaf extract were detected by GC-MS.

No	Compounds	RT (min.)	Peak Area (%)	Biological activity
1	Phenol, 2,4-bis(1,1-dimethylethyl)-	36.765	2.47	Anti-cancer, antioxidant, antifungal, antibacteria [12] and antiparasitic [13]
2	2-Cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl	43.849	2.40	-
3	2-Cyclohexen-1-one, 4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-	46.896	2.97	Inhibitory effect [14]
4	Phytol acetate	48.661	27.31	Antioxidant [15] and antimicrobial [16]
5	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	49.482	4.43	Anti-inflammatory and antimicrobial [17]
6	1-Hexadecyne	50.068	7.46	Antibacterial [18]
7	Hexadecanoic acid methyl ester	51.542	12.47	Anti-cancer, anti-inflammatory, antimicrobial [15] and antioxidant [19]
8	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	52.027	2.43	Antioxidant and antifungal [20]
9	n-Hexadecanoic acid	52.835	18.27	Anti-cancer [21], antioxidant [18] and anti-inflammatory [22]
10	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	56.732	1.05	Cancer preventive and anti-inflammatory [23]
11	9-Octadecenoic acid, methyl ester, (E)-	56.942	5.78	Cancer preventive, anti-inflammatory [24] and antiandrogenic [25]
12	Phytol	57.332	10.27	Anti-cancer, anti-inflammatory, antimicrobial, Diuretic [25] antioxidant, anti-convulsant and antinociceptive [15]
13	Heptadecanoic acid, 16-methyl-, methyl ester	57.850	2.70	Potent skin cancer activity [15]

Based on the chromatogram in **Figure 1**, it can be seen that there were 13 compounds detected by GC-MS. Among the 13 compounds detected, the compound that had the largest peak area percentage was phytol acetate (27.31 %). The peak area percentage has a linear relationship with the compound concentration as indicated by the height of the peak on the y-axis which shows the abundance in arbitrary units (a.u.). The larger the peak area, the greater the concentration of compounds contained in the extract [26]. Thus, the phytol acetate compound which has the largest peak area percentage is the compound with the largest concentration contained in simpor leaf extract.

The compound that has the fastest retention time is the Phenol compound, 2,4-bis(1,1-dimethylethyl)- with a retention time of 36.765. The retention time of each compound is different from each other. The interaction of each compound with the type of column is the cause of the difference in retention time. A non-polar column, which holds non-polar compounds in the column longer, while polar compounds exit

the column more quickly so that the retention time is faster [26]. The GC column used in this research was the HP-5MS UI from Agilent Technologies. The HP-5MS column is known to contain (5 %-phenyl)-methylpolysiloxane which renders the column non-polar [27].

In this study, specific compounds have been identified from simpor leaf extract originating from Belitung, including phytol acetate, Hexadecanoic acid methyl ester, n-Hexadecanoic acid, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, Phytol, and Heptadecanoic acid, 16-methyl-, methyl ester. Interestingly, previous research has successfully identified similar compounds from simpor leaf extract originating from Malaysia using solvents other than ethanol. This indicates consistency in the presence of certain compounds in simpor leaf extracts from various geographical locations, highlighting the potential applications and chemical composition variations of this plant species.

The compounds detected in simpor leaf extract have various potentials with a biological activity which has been reported in several studies as can be seen in **Table 1**. Among the 13 compounds, 7 have been identified as having anticancer and cancer preventive activities. These compounds include Phenol, 2,4-bis(1,1-dimethylethyl), Hexadecanoic acid methyl ester, n-Hexadecanoic acid, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, 9-Octadecenoic acid, methyl ester, (E)-, phytol and Heptadecanoic acid, 16-methyl-, methyl ester. Several compounds that have been reported as potential anti-cancer and cancer-preventing compounds can support this research to inhibit the growth of cancer cells and induce the death of WiDr colorectal cancer cells by apoptotic death.

Morphological changes of WiDr treated with simpor leaf extract

WiDr cells grow together and form a thin layer (monolayer) which is attached to the culture container. In evaluating the ability of simpor leaf extract on WiDr cells, an analysis of morphological changes was carried out in WiDr cells after being treated with simpor leaf extract with various concentrations. The results show morphological changes in the cells as shown in **Figure 2**.

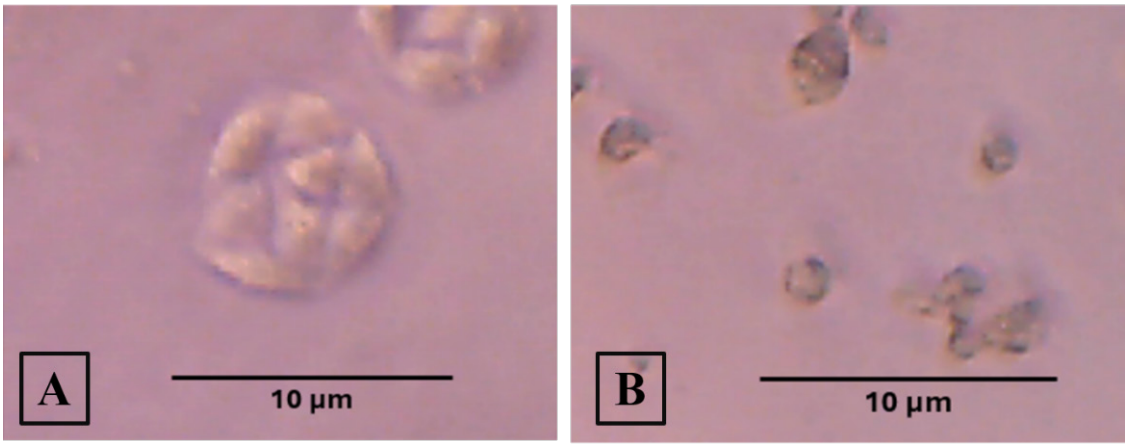

Figure 2 Changes in WiDr cell morphology; (A) normal cells and (B) rounded cells, 32× magnification.

Cell morphology was observed under an inverted light microscope at 32× magnification. The results indicated that untreated WiDr cells exhibited normal cell morphology with an epithelial-like structure, as shown in **Figure 2(A)**. In contrast, WiDr cells treated with doxorubicin as a positive control showed a complete transformation into round cells, as depicted in **Figure 2(B)**. Cells treated with simpor leaf extract

also exhibited morphological changes, becoming round, similar to those treated with the positive control, although the number of round cells was lower than in the doxorubicin-treated group. The extent of morphological changes correlated with the increasing concentration of the extract used. These morphological changes could be attributed to the activation of caspase-3, which is associated with apoptosis [7]. However, caspase-3 activity was not assessed in this study, so the mechanism underlying these morphological changes remains unclear.

Based on observations of WiDr cells, distinct differences were noted between cells treated with simpor leaf extract and the negative control. Untreated WiDr cells grew in colonies, whereas WiDr cells treated with doxorubicin, a chemotherapy drug, as a positive control, did not form colonies due to its cytotoxic effects (**Figure 2(B)**). A relative reduction in WiDr cell colonies was observed at extract concentrations ranging from 25 to 100 ppm, with a significant reduction at 100 ppm. Cancer cells typically grow in colonies when in contact with neighboring cells [28]. If cancer cells lose contact with neighboring cells, they undergo cell death [28]. Therefore, it can be concluded that simpor leaf extract has potential as an anticancer agent in WiDr cells.

Cancer cells generally have irregular size and shape morphology compared to normal cells [29]. Apart from that, the nuclear morphology of cancer cells is different from normal cells, which have a larger nucleus size, irregular nuclear contours, and abnormally distributed chromatin. On the other hand, normal cells are able to maintain a constant ratio of nucleus volume to cell volume [30]. The differences in nuclear morphology can be influenced by several factors such as mutations, dysregulation of signal transduction pathways, abnormal gene expression patterns, altered nuclear envelope proteins and chromatin, as well as aneuploidy factors [30].

Cytotoxicity of simpor leaf extract on WiDr cells

In this study, the cytotoxicity of simpor leaf extract against WiDr cells was tested using the MTT method. The MTT method is a test method that can be used to measure cell proliferation colorimetrically which is based on changing the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into crystals purple formazan that is converted by living cells in mitochondria [31]. The hydrogenase enzyme is an enzyme in mitochondria that plays a role in the process of converting MTT salt into formazan crystals. The formazan crystals formed will be dissolved in absolute ethanol and then the absorbance reading will be carried out using a spectrophotometer at a wavelength of 595 nm to obtain the optical density value. The OD value from absorbance measurements indicates cell metabolic activity. The more living cells there are, the more formazan crystals will be formed, so the OD value will be higher.

The results obtained show that simpor leaf extract has the ability to inhibit the growth of WiDr colorectal cancer cells. The inhibition percentage and IC_{50} value of simpor leaf extract are shown in **Table 2** and **Figure 3**. The inhibitor concentration (IC_{50}) value obtained is the value of the relationship between the extract concentration (x-axis) and the percentage inhibition curve (y-axis) using linear regression analysis. The IC_{50} value category is based on the cytotoxic category of a compound issued by the National Cancer Institute of the US (NCI), the IC_{50} value $\leq$ 20 ppm is a category for compounds that have a high cytotoxic effect, moderate cytotoxic if 20 < IC_{50} $\leq$ 200 ppm, weak cytotoxic if 200 < IC_{50} $\leq$ 500 ppm and has no cytotoxic effect if the IC_{50} value is > 500 ppm [32].

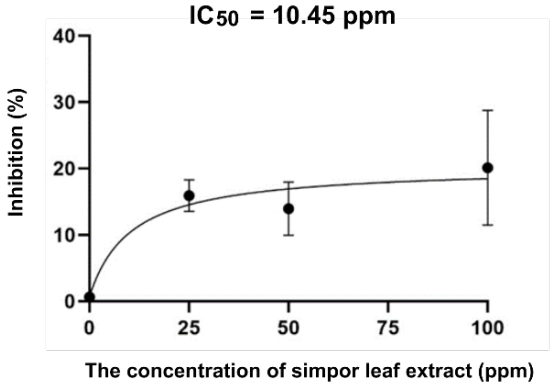

Figure 3 Linear regression curve of the effect of simpor leaf extract concentration on the percentage of WiDr colorectal cancer cell inhibition.

Table 2 Effect of simpor leaf extract with several concentrations on the percentage of WiDr colorectal cancer cell inhibition.

Simpor leaf extract (ppm)	n	Inhibition (%) (Mean ± SE)	IC_{50} (ppm)
100	3	20.12 ± 4.99^{b}	10.45
50	3	13.95 ± 2.32^{b}	
25	3	15.90 ± 1.38^{b}	
0	3	0.65 ± 0.00^{a}	
Positive control	3	91.10 ± 0.86^{c}	

Data are expressed as mean ± SE of 3 replicates. Data with different superscripts $^{(a, b, c)}$ are considered significant.

Based on the results of 1-way ANOVA analysis on the percentage of inhibition, it shows a significance value of less than 0.05 ($p < 0.05$), so it can be interpreted as a real difference between the treatment groups in the percentage of WiDr cell inhibition. After that, further analysis was carried out using the Duncan Multiple Range Test (DMRT) at the 5 % level to determine which treatment significantly increased the percentage of cell inhibition. The results of the DMRT analysis in **Table 2** show that the extract treatment groups were not significantly different, but the extract treatment was significantly different from the control. DMRT analysis also showed that the highest percentage of inhibition was found in the positive control treatment with doxorubicin administration.

Doxorubicin is a chemotherapy drug that is often used in the treatment of cancer, so of course giving 1 ppm of doxorubicin as a positive control will produce a greater percentage of inhibition compared to giving simpor leaf extract. This drug works by binding to the DNA of cancer cells and blocking the work of the topoisomerase II enzyme, causing the DNA to shrink and preventing the growth of cancer cells [33]. Doxorubicin has side effects, which include dangerous cardiotoxicity, where this drug can cause heart failure [33]. Mitochondria and sarcoplasmic reticulum are the 2 parts of the heart that are most damaged due to the side effects of doxorubicin [33]. However, the use of doxorubicin as a positive control can provide an overview of the cytotoxic effects on WiDr cells and can be used as a comparison for the simpor leaf extract treatment group.

In the simpor leaf extract treatment group, the results of the percentage of inhibition showed that the concentration of 100 ppm had the highest percentage of inhibition (20.12 % ± 4.99) compared to the concentration of 50 ppm (13.95 % ± 2.32) and 25 ppm (15.90 % ± 1.38). These results show that a dose of

simpor leaf extract of 100 ppm shows the best cytotoxic ability in inhibiting WiDr cells. This cytotoxic ability can also be seen based on the IC_{50} value of simpor leaf extract of 10.45 ppm which is included in the high cytotoxic category (**Figure 3**).

The cytotoxic ability in this study is supported by the compounds contained in simpor leaf extract. Simpor leaf extract which has been analyzed for its compound content using GC-MS has several compounds which are reported to have potential as anticancer and cancer preventive as previously mentioned. These compounds can play a role in inhibiting WiDr cell growth and inducing apoptosis. The apoptosis induction ability was further confirmed by testing using flow cytometry.

Apoptotic effect of simpor leaf extract on WiDr cells

Apoptosis is characterized and begins with the process of releasing phosphatidylserine (PS) from the inner layer to the outer layer of the cell membrane for recognition by phagocytes. Apoptosis testing utilizing flow cytometry with Annexin V and PI dyes was conducted to detect apoptosis, wherein the 2 dyes were capable of distinguishing cells undergoing the early stages of apoptosis, late stages of apoptosis, necrosis and viable cells. Annexin V has a high affinity for binding to PS but is unable to penetrate the cell membrane layer. If PS does not move to the outer layer of the membrane, then Annexin V cannot bind PS and the apoptosis test results will be negative. Meanwhile, PI dye is a dye that is only able to enter cells and bind to DNA when cell membrane damage occurs which is in line with late apoptosis and necrosis but not in cells undergoing early apoptosis because the integrity of the membrane is still intact. DNA binding to PI will produce a positive test. Therefore, combining Annexin V with PI helps in differentiating live cells (Annexin V^-, PI^-), early apoptosis (Annexin V^+, PI^-), late apoptosis (Annexin V^+, PI^+) and necrosis (Annexin V^-, PI^+). Based on the results of flow cytometry analysis, simpor leaf extract was able to trigger apoptosis in WiDr colorectal cancer cells. This apoptosis induction ability is shown in **Figure 4**.

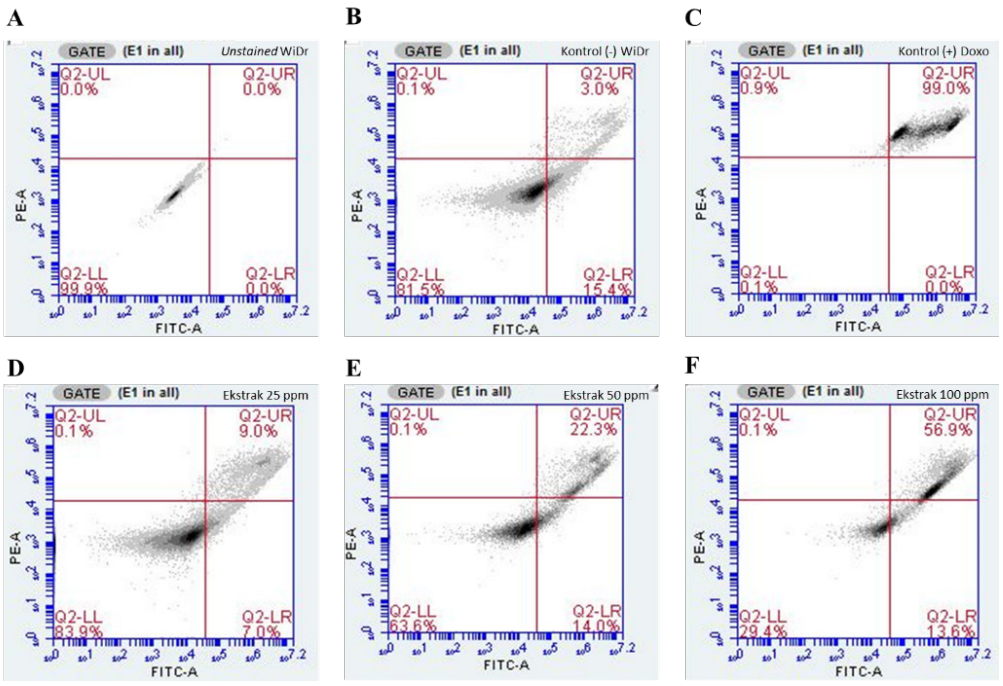

Figure 4 WiDr cell apoptosis testing without treatment and treatment with various concentrations of simpor leaf extract. (A) Control cells without staining, (B) negative control, (C) positive control (doxorubicin), (D-F) 25, 50 and 100 ppm simpor leaf extract treatment. The LL (Lower Left), LR (Lower Right), UR (Upper Right) and UL (Upper Left) quadrants indicate live cells, early apoptosis, late apoptosis and necrosis.

In this study, simpor leaf extract with concentrations of 25, 50 and 100 ppm showed the ability to trigger apoptosis in WiDr cells (**Figure 4**). The percentage of cells undergoing apoptosis increased significantly with increasing extract concentration: 16 % at 25 ppm, 36.3 % at 50 ppm and 70.5 % at 100 ppm. Specifically, in 25 ppm simpor leaf extract, annexin V/PI staining on WiDr cells showed that of the 16 % of cells that underwent apoptosis, 7 % were in the early stages and 9 % were in the late stages. At this concentration, living cells had a higher percentage (83.9 %) than apoptotic cells. Meanwhile, the results of an extract concentration of 50 ppm showed that of the 36.3 % of apoptotic cells, 14 % of them underwent the early stages of apoptosis and 22.3 % were in the final stages of apoptosis, while for living cells it was 63.6 %. Finally, the highest apoptosis results were at a concentration of simpor leaf extract of 100 ppm showing that of 70.5 % of cells undergoing apoptosis, 13.6 % of them underwent the early stages of apoptosis and 56.9 % in the final stages of apoptosis, while 29.4 % of the cells were alive. A comparison between apoptosis and live cells at a concentration of 100 ppm showed significant results, which indicated that the percentage ratio of cells undergoing apoptosis was above 50 %, exceeding the percentage of live cells.

The study found that in negative control, 3 % of the cells exhibited early apoptosis and 15.4 % of the cells exhibited late apoptosis. These findings could be attributed to the trypsinization process during cell harvesting, which aims to avoid wasting cells after treatment. Trypsin is a protease enzyme commonly used to release cells from culture vessels and causes cell surface damage during the trypsinization process [34]. Therefore, cells that exhibit apoptosis in the negative control are assumed to be caused by the trypsinization process.

According to the results of apoptosis, simpor leaf extract from Belitung has the potential as a candidate for an anticancer agent because it can induce apoptosis (**Figure 4**). This induction ability may be related to the presence of compounds contained in simpor leaf extract. The phenol compound, 2,4-bis(1,1-dimethylethyl) detected in simpor leaf extract is a phenolic group compound with very high cytotoxic activity against HeLa cancer cells and can induce apoptosis genes and increase the expression of p53 and caspase-7 in MCF-7 and A431 cells [35]. The compounds Hexadecanoic acid methyl ester and n-hexadecanoic acid are compounds that belong to the methyl ester fatty acid group. The previous research has proven that the hexadecanoic acid compound can inhibit HT-29 cancer cells through induction of apoptosis and cell cycle arrest [36,37]. The ability to induce apoptosis by this compound has also been reported [38]. Then the compounds 9,12-Octadecadienoic acid (Z,Z)-, methyl ester and 9-Octadecenoic acid, methyl ester, (E)- are methyl ester fatty acid compounds which respectively belong to the linoleic acid and oleic acid groups [39]. The octadecadienoic acid compound has been reported to have the ability to inhibit the development of breast cancer cells and induce apoptosis [21]. These 2 compounds are said to have the potential to prevent cancer [23,27]. The phytol compound is a diterpenoid compound that is reported to have anticancer activity [25]. Previous studies have reported that phytol compound showed cytotoxic activity on MCF-7 and PC-3 cancer cells [36]. Furthermore, the compound Heptadecanoic acid, 16-methyl-, methyl ester was mentioned can act as a potent skin cancer activity, which the activity of this compound can treat skin cancer [15]. This is based on previous research which found that this compound was able to inhibit skin cancer proteins [40]. Based on several articles that have reported the ability of these compounds as anticancer, it can be assumed that the cytotoxic and apoptosis-inducing ability of simpor leaf extract against WiDr cells is supported by the presence of these compounds.

Cell death in the form of apoptosis is the death that is expected to occur in this study because it does not trigger inflammation in normal cells. This is because apoptosis can inhibit inflammation with an anti-inflammatory effect. When phagocytes engulf apoptotic bodies, the release of cell contents that can trigger inflammation can be avoided so as not to worsen the condition of normal cells in the body [41]. Meanwhile,

cell necrosis, which is a form of passive and unprogrammed death due to environmental disturbances [41], is the other side of apoptosis. Necrosis is capable of releasing cell contents uncontrollably. In addition, necrosis causes an inflammatory response in cells due to membrane rupture [7]. In this study, simpor leaf extract did not cause necrosis in cells. Thus, simpor leaf extract can maintain homeostatic conditions by eliminating damaged cells without disturbing the normal cells around them.

Conclusions

Based on the chromatogram results obtained from GC-MS analysis of the simpor leaf extract sourced from Belitung, a total of 13 bioactive compounds were identified. Among these, 7 compounds have been previously documented for their anticancer properties, included Phenol, 2,4-bis(1,1-dimethylethyl), Hexadecanoic acid methyl ester, n-Hexadecanoic acid, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, 9-Octadecenoic acid, methyl ester, (E)-, phytol and Heptadecanoic acid, 16-methyl-, methyl ester.

The MTT assay results demonstrate the cytotoxic activity of the simpor leaf extract against WiDr cells, as evidenced by alterations in cellular morphology and an observed IC50 value of 10.45 ppm. The most effective concentration inhibiting cell growth was found to be 100 ppm (20.12 % ± 4.99), followed by 50 ppm (13.95% ± 2.32) and 25 ppm (15.90 % ± 1.38).

Furthermore, flow cytometry analysis confirmed the extract's ability to induce apoptosis in WiDr cells, thereby substantiating its cytotoxic potential.

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