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Simpor leaf extract- (*Dillenia suffruticosa* Martelli) induced apoptosis of the MCF-7 and HepG2 cell lines

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ABSTRACT

Cancer is a malignant tumor with characteristics of uncontrolled cell growth, poor metastasis, leading to the dysfunction of organs and death of patients. Many efforts have been made, including the exploration of natural products. The purpose of this study was to determine the cytotoxicity of the *Dillenia suffruticosa* leaf extract against the cell lines MCF-7 and HepG2. The method used is an experimental research design with a completely randomized design. The treatment group consisted of the leaf extract of 500, 1,000, 2,000, 5,000, and 9,000 ppm for the brine shrimp lethality test (BSLT). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test used 10, 15, 20, 25, and 50 ppm concentrations and control (without extract). The data on the BSLT test was determined by analyzing the lethal concentration 50 (LC_{50}) value using the probit analysis, while the MTT test was determined by analyzing the IC_{50} and analysis of variance test. The BSLT test results showed an LC_{50} value of 5,221 ppm, which means that the leaf extract is not toxic. Simpor leaf extract at 25 ppm with IC50 of 88.52 was seen as the best concentration for liver cancer treatment. It can be concluded that *D. suffruticosa* shows cytotoxicity against the cell lines MCF-7 and HepG2 and can be used as a candidate for breast and liver cancer treatment.

INTRODUCTION

Cancer is a group of diseases characterized by abnormal cell growth. This disease has been the leading cause of death worldwide; breast cancer is one of the most common causes of death in the world (Cao *et al*., 2021). This disease is caused by environmental and genetic factors. Both factors can spur cancer cells to proliferate or multiply cells and also prevent programmed cell death or apoptosis. Proliferation will generally spur the development of cancer cells to be massive (Greten and Grivennikov, 2019). To stop the proliferation of cancer cells, chemotherapy drugs such as cisplatin, tamoxifen, and doxorubicin are commonly used (Syafriana *et al*., 2021). Some chemotherapeutic agents have high toxicity so that they can interfere with or damage normal tissues and cause resistance. Therefore, it is necessary to develop safer therapeutic agents (Guo *et al*., 2020).

Phytochemical compounds in plant extracts have a role as antioxidants that can be used in cancer therapy with minimal side effects (Hano and Tungmunnithum, 2020). *Dillenia suffruticosa* with the local name simpor plant is a herbaceous plant that belongs to the Dilleniaceae family. Simpor plants grow in Southeast Asia, such as in West Malaysia, the Philippines, Brunei Darussalam, and Indonesia. In Indonesia, this plant grows in several areas, one of which is Bangka Belitung, and is known to have antimicrobial (Syafriana *et al*., 2021) and antiparasitic properties (Shah *et al*., 2020). Previous studies on *D. suffruticosa* also show its antioxidant effect against the 1,1-Diphenyl-2picryl Hidrazil radical (Rahayu *et al*., 2019).

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The active compounds that have cytotoxic activity against cell lines are saponins, triterpenoids, sterols, and polyphenols. Saponins can prevent antitumor effects by expanding anticancer pathways. *Dioscin* is a type of saponin that can prevent cancer through the induction of oxidative stress (Tao *et al*., 2018). Triterpenoids have anticytotoxic ability in the HT-29 cell line of colon cancer (Ren *et al*., 2018).

Simpor plants in the Bangka Belitung area are generally used by the villagers to wrap food, and so far, there has been no research found on the potential of the simpor leaf extract from Bangka Belitung to treat cancer. Therefore, this study aimed to test *in vitro* the cytotoxicity of the simpor leaf extract from Belitung for the treatment of breast cancer and liver cancer.

MATERIALS AND METHODS

Tools and materials

The tools used in this research are a rotary vacuum evaporator type IKA RV 06-ML 1-B, analytical balance, enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek, USA), incubator, micropipette, dropper pipette, separating funnel, Whatman filter paper no. 42, 96-well plate, Erlenmeyer, Baker Glass, measuring cup, oven, scissors, jar, tube glass, measuring tube, basin, 40-mesh sieve, Bunsen, tripod, microscope, mortar, and pestle.

The research raw material is the simpor leaf obtained from South Bangka, Bangka Belitung. The chemicals used were ethyl acetate, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), penicillin, streptomycin, magnesium (Mg), concentrated HCl, alcohol, $CHCl_3$, NH_4OH, H_2SO_4 2M, Mayer's reagent, Wagner's reagent, Dragendorff's reagent, and 1% $FeCl_3$.

Cell culture

The human breast cancer cell line MCF-7 (ATCC HTB 22) and hepatocellular carcinoma HepG2 (ATCC CCL 23) were purchased from ATCC and obtained from Pusat Studi Satwa Primata (PSSP) IPB University. The morphology of both cell lines was epithelial-like. As many as 5,000 cells from each cell line were maintained in Dulbecco's modified Eagle's medium and supplemented with 5% FBS and added by penicillin 100 U/ml and streptomycin 100 µg/ml. The cell line was stored in a 5% CO_2 incubator at a temperature of 37°C.

Experimental procedures

Preparation of D. suffruticosa leaf extract

The simpor leaf of *D. suffruticosa* was cut into small pieces and then placed in an oven at 50°C for 24 hours. The dried leaf samples were ground until smooth and then sieved with a size 40 mesh. The result of sifting is simpor leaf powder.

200 g of the simpor leaf powder was macerated with ethyl acetate in a ratio of 1:9 (w/v) for 72 hours and then put into the rotary evaporator to be concentrated for 24 hours at a temperature of 25°C ± 2°C. The residual solvent was filtered using Whatman no. 42 filter paper. Then, the residue was put into the oven at 40°C for 24 hours. The extraction results were stored at −20°C until used.

Phytochemical determination test

Phytochemical determination of the leaf of Belitung simpor (*D. suffruticosa*) was done based on Shaikh and Patil (2020). A flavonoid test was done with Mg powder and HCl. The alkaloid test was done based on Mayer's, Wagner's, and Dragendorff's. The saponin was detected with the foam formed and tannin with Braymer's test. Terpenoid and steroid analysis was conducted with chloroform and a few drops of H_2SO_4.

Brine shrimp lethality test (BSLT)

The purpose of this test was to determine the LC_{50} using 2-day-old *Artemia salina* shrimp. This method is carried out by incubating *A. salina* eggs in a container filled with water with 3.8% NaCl added. After hatching day, *A. salina* aged 48 hours was ready to be treated. Next, 1 mg of the ethyl acetate extract of simpor leaf (EAESL) was dissolved in 100 ml of seawater to obtain a concentration of 10,000 ppm. The stock solution was then diluted with concentrations of 500, 1,000, 2,000, 5,000, and 9,000 ppm and put into five different tubes by adding seawater up to 5 ml.

Ten Nauplii shrimp were put into each tube. As a control, one tube was filled with 5 ml of seawater without adding the extract. After 48 hours of incubation, the tubes were observed using a magnifying glass, and the number of live shrimp in each tube was counted and recorded. The results of the data were transformed into the probit analysis to determine the lethal concentration 50 (LC_{50}) value of the extract. Calculation reference to Rasyid *et al*. (2020) was determined by looking at the percent of individual deaths in each tube, as follows:

$$\% \text{ mortality} = \frac{\text{number of death larvae}}{\text{number of total larvae}} \times 100\%.$$

Anticancer activity test with MTT method

The cell line used in this study was obtained from PSSP IPB University. First, cells (1×10^5 cells/ml) were placed into 96-well plates (100 µl/well). After 24 hours of incubation, cells were given the simpor leaf extract at concentrations of 5, 10, 20, 30, and 50 ppm, while control cells were not given the simpor leaf extract and then incubated in a CO_2 incubator at 37°C for 72 hours. After incubation with the extract, 20 µl of MTT was added to each well and incubated again for 4 hours at 37°C (Nosrati *et al*., 2020).

Mitochondria that are active in living cells will reduce MTT to purple-blue formazan crystals. The number of surviving cells is assumed to be according to the number of purple-blue formazan crystals formed. After incubation, the supernatant was taken, and 100 µl DMSO was added to dissolve the formazan purple-blue crystals. The absorbance was calculated using an ELISA plate reader (BioTek, USA) at a wavelength of 595 nm. The graph of the presentation of cell viability was compared to the concentration of the simpor leaf extract plotted, and the inhibitory concentration (IC_{50}) was calculated. The percentage of inhibition was calculated using the following formula with reference to Mardina *et al*. (2020):

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100\%.$$

Cell line morphological changes after giving simpor leaf extract

The cell lines MCF-7 and HepG2 were put into 6-well plates (3 ml, 1×10^5 cells/ml) and incubated for 24 hours. The cells were treated with the simpor leaf extract at concentrations of 5, 10, 20, 30, and 50 ppm, and controls were not given the simpor leaf extract. After treatment, the cell morphology was observed using a light microscope with a 32× magnification.

Data analysis

Phytochemical content test analysis was carried out qualitatively. The BSLT test was analyzed using the probit analysis (probability unit) to get the LC_{50} value and then tested using the one-way analysis of variance (ANOVA) to determine the effect of giving the simpor leaf extract concentration on the mortality of *A. salina* shrimp larvae. The MTT test was analyzed using regression analysis to get the value of IC_{50} and then analyzed using ANOVA. Results on *p* values of less than 0.05 were considered significant. *Post hoc* was done with Duncan's multiple range test. Observation of cell line morphology was analyzed by a descriptive test.

RESULTS

Phytochemical characterization of simpor leaf extract (*D. suffruticosa*)

Semipolar ethyl acetate was used as a solvent to resolve the phytochemical contents of the simpor leaf extract (*D. suffruticosa*) in this study. Due to its semipolar nature with a polarity index of 4.4, the secondary metabolites of simpor leaf, including both polar and nonpolar compounds, will be preserved in the solvent. The results revealed that several phytochemical contents, including flavonoids, saponins, alkaloids, and terpenoids, were identified from the EAESL (Table 1).

EAESL-mediated toxicity on *A. salina* shrimp larvae

The BSLT provides a relatively simple and high-throughput methodology to screen and determine the cytotoxicity of bioactive compounds. To evaluate the toxicity of EAESL, the BSLT was first performed and the LC_{50} value of EAESL was determined (Janakiraman and Johnson, 2016). The LC_{50} value is a concentration that can cause the death of 50% of the test animals (*A. salina* shrimp larvae) up to a certain time.

As the concentration of toxic biological compounds increases, the mortality rate increases proportionally. This is in accordance with research conducted by Suzuki *et al*. (2021), which states that there is a relationship between shrimp larval mortality rate and concentration. Brine shrimp larvae were exposed to a series of concentrations of 500, 1,000, 2,000, 5,000, and 9.000 ppm, and then their mortality rates were measured ranging from 74% to 88%. As shown in Table 2, the differences in mortality rates among groups showed statistical significance (*p* value less than 0.05), but after observation using Duncan's test ($n = 4$) there was no significant difference between the group mortality rate of shrimp and EAESL BSLT concentration, suggesting that the extract was not harmful to shrimp larvae. Furthermore, LC_{50} values were measured at a concentration of 5,221 ppm. According to Rasyid *et al*. (2020), the value of the toxicity of secondary metabolites of plants if $LC_{50} \leq 30$ ppm is very toxic, extracts with a value of $31 \leq LC_{50} \leq 1{,}000$ ppm are toxic, whereas if the $LC_{50} >$ 1,000 ppm this means nontoxic. The LC_{50} obtained in this study is nontoxic.

Table 1. Phytochemical contents of simpor leaf extract (*D. suffruticosa*).

Phytochemicals	Test result
Flavonoids	+
Saponins	+
Tannins	−
Alkaloids	+
Steroids	−
Terpenoids	+

Remarks: plus sign (+) means detected; minus sign (−) means not detected.

Table 2. EAESL-mediated mortality rate on *A. salina* shrimp larvae.

Concentration (ppm)	*N*	Mortality (%)	LC_{50} (ppm)
9,000	5	88.00 ± 5.83	
5,000	5	74.00 ± 7.48	
2,000	5	88.00 ± 2.0	5,221
1,000	5	82.00 ± 7.35	
500	5	74.00 ± 5.09	
Sig.		0.000	

Mortality value is the mean ± std. error using ANOVA at < 0.05.
N is the number of repetitions.
Sig. value got from the ANOVA test.

Morphology changes in cells exposed to simpor leaf extract (*D. suffruticosa*)

To evaluate the anticancer activity of the extract, two human cancer cell lines MCF-7 and HepG2 were exposed to a culture medium without or with the extract at 10, 15, 20, 25, and 50 ppm for 72 hours at 37°C. EAESL-mediated changes in the morphology of cancer cells were monitored and analyzed using microscopy, and their morphological differences were demonstrated as shown in Figures 1 and 2. Apoptosis and necrosis are two major events in leading to cell death (Nosrati *et al*., 2021). A previous study conducted by Armania *et al*. (2013) showed that the simpor root extract from Malaysia induced breast cancer cell death undergoing apoptotic signaling. Apoptotic cells can be distinguished by their unique morphological changes from other cells. These signatures of apoptotic cells in appearance include shrinkage of cells, chromatin condensation, plasma membrane blebbing, and formation of apoptotic bodies, which was previously described by Smith *et al*. (2017).

Blebbing is one of the stages during an apoptotic process in which the plasma membrane of the cell is formed into bulges, round or bulge in shape (Caruso *et al*., 2019). Blebbing results from the disarrangements or damage of the cytoskeleton in cells, causing the membrane to protrude, followed by separation of the bulge by carrying the cytoplasm and then forming apoptotic bodies (Figure 3 a and b) (Morris, 2018). These apoptotic bodies will eventually be eaten and cleared by phagocytic cells. In addition to morphology, it was seen that there were fewer differences in the number of cells given the extract compared to the control without the simpor leaf extract.

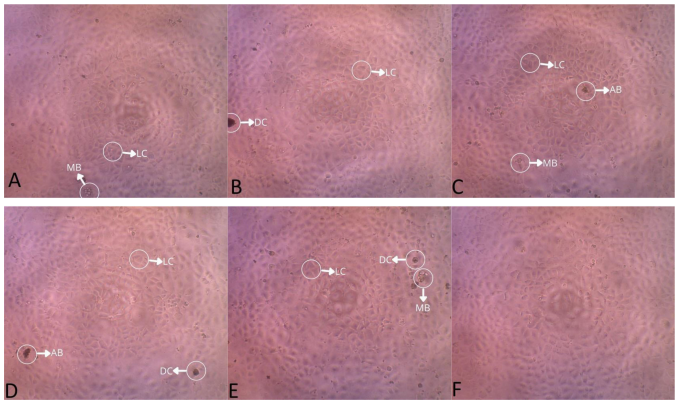

Figure 1. The morphological changes of MCF-7 induced by EAESL. MCF-7 cells were exposed to simpor extract at concentrations of (a) 10 ppm, (b) 15 ppm, (c) 20 ppm, (d) 25 ppm, and (e) 50 ppm and (f) control without extract (32× magnification). Death cell (CD), live cell (LC), membrane blebbing (MB), and apoptotic bodies (AB).

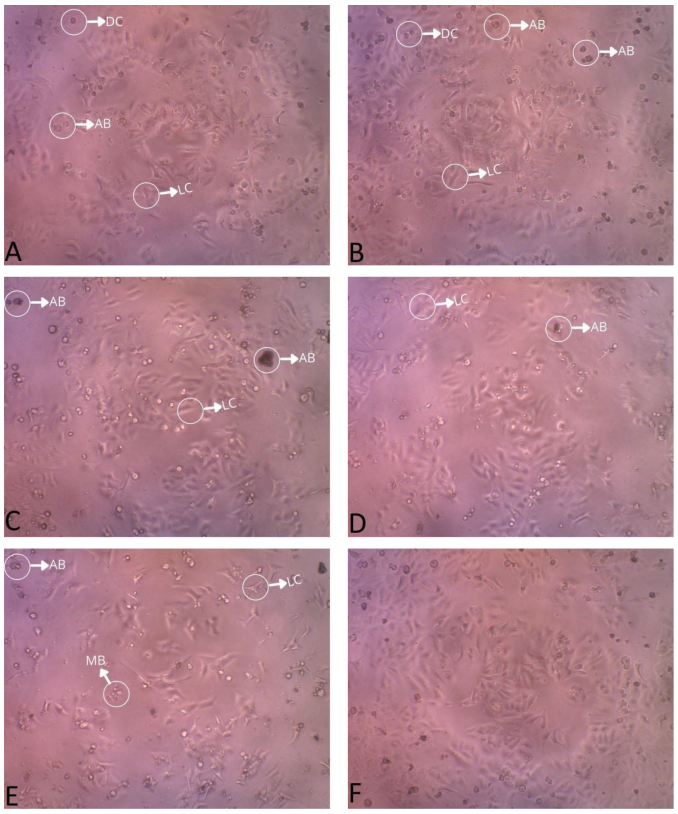

Figure 2. The morphological changes of HepG2 induced by EAESL. HepG2 cells were exposed to extraction at concentrations of (a) 10 ppm, (b) 15 ppm, (c) 20 ppm, (d) 25 ppm, and (e) 50 ppm and (f) control without extract (32× magnification). Death cell (CD), live cell (LC), membrane blebbing (MB), apoptotic bodies (AB).

Anticancer activity of simpor leaf extract with MTT assay

Currently, the MTT assay is used to measure cellular metabolic activity and is useful for screening cytotoxicity of drug candidates, agents, or compounds in various types of cancer cell lines. This colorimetric method is very useful for assessing cell viability, activity, and proliferation of cells. The cell viability in the MTT assay is determined by the activity of dehydrogenase in the mitochondria of cells to convert micro tetrazolium (MTT) as yellow color to formazan as purple color followed by resolving in the DMSO solution (Aziz *et al*., 2019). The changes in color can be quantitatively assessed as UV absorbance value at 570 nm (Abs 570) using a multiwell spectrophotometer. Based on the results of the MTT assay, it demonstrated that EAESL showed cytotoxic activity against the MCF-7 and HepG2 cancer cells, while extract-mediated inhibition on MCF-7 appeared in a dose-dependent manner (Tables 3 and 4).

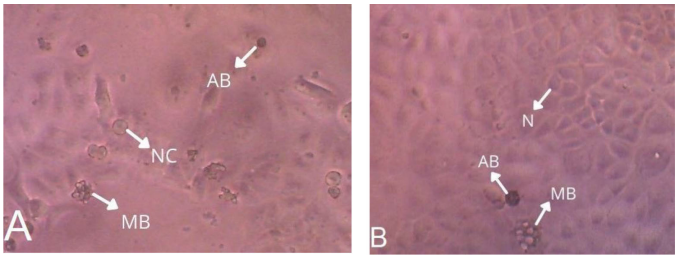

Figure 3. Cell morphology after treatment of 10 ppm leaf extract to cell lines HepG2 ppm (a) and MCF-7 (b). Cells undergo apoptosis with characteristics such as nuclear compaction (NC), apoptotic bodies (AB), and membrane blebbing (MB).

50 ppm EAESL caused the highest inhibition of the MCF-7 cancer cells by 48%, while 25 ppm reached the best inhibition of HepG2 cancer cells by 28.47%. Compared to inhibition data between the MCF-7 and HepG2 cell lines, 10 ppm extract-mediated inhibition in the proliferation of MCF-7 and HepG2 was 11.40% ± 1.87% and 24.90% ± 2.25%, respectively, suggesting HepG2 is more sensitive to the extract than MCF-7 cells at concentrations ranging from 10 to 25 ppm. This result is consistent with a previous study reported by Crespo *et al*. (2020), which states that the HepG2 cell line is sensitive when measured using MTT. In the ANOVA test for the MCF-7 cancer cells, a significance value of 0.000 was obtained, which means that there is an effect of concentration on MCF-7 cell inhibition with the best effect on 50 ppm concentration (Table 3). The same result occurred in the HepG2 cancer cells. The concentration given had an effect on the inhibition of the cell growth, with 25 ppm as the best concentration (Table 4). The value of IC_{50} is expressed from the relationship between the extract concentration curve (*x*-axis) and the inhibition (%) curve (*y*-axis) with the linear regression analysis. According to the National Cancer Institute, the IC_{50} value is the concentration of the extract used to inhibit the growth of 50% of cancer cells (Isrul *et al*., 2019). Categorization of IC_{50} value if the IC_{50} value < 4 ppm is highly cytotoxic, 4 ppm ≤ IC_{50} value < 20 ppm is moderately cytotoxic, 20 ppm ≤ IC_{50} value < 100 ppm is weakly cytotoxic, and IC_{50} ≥ 100 ppm is not cytotoxic (Mutiah, 2020). The IC_{50} value of the extract in the MCF-7 cancer cells was 52.39 ppm, while in the HepG2 cancer cells the IC_{50} value was 88.52 ppm so that the simpor extract was weakly cytotoxic against both types of cell lines (Tables 3 and 4).

DISCUSSION

Phytochemical content of simpor leaf extract (*D. suffruticosa*)

The results of the research showed that simpor leaves contain secondary metabolites in the form of tannins, polyphenols,

triterpenoids, steroids, and saponins. The composition of secondary metabolites possessed by plants can be influenced by environmental factors, one of which is due to soil conditions (Prinsloo and Nogemane, 2018).

Toxicity with BSLT method

Shrimp *A. salina* L., a low-level shrimp that belongs to the family Artemiidae, is zooplankton commonly used to determine the toxicity of a compound that is considered toxic (Cong *et al*., 2021). Toxicity testing using the BSLT test showed a correlation with cytotoxicity activity against tumors in humans. The mechanism of secondary metabolites in the BSLT method is one of them by inhibiting the metabolic process of *A. salina* shrimp. Alkaloids and flavonoids can poison the stomach or what is known as stomach poisoning so that if these two compounds enter the digestive system of the *A. salina* larvae they will interfere with their metabolism (Weny *et al*., 2018). However, in this study, the LC_{50} value was above 1,000 ppm, which is nontoxic. However, the potential of a plant extract as an anticancer agent can continue to be sought with other toxicity methods other than common toxicity methods such as BSLT.

Table 3. Anticancer activity of simpor leaf extract using the MTT assay against the MCF-7 cell line.

Concentration (ppm)	*N*	Inhibition (%)	IC_{50} (ppm)
50	4	48 ± 1.05^{e}	55.39
25	4	24.97 ± 2.53^{d}	
20	4	17.25 ± 1.19^{c}	
15	4	11.40 ± 1.87^{b}	
10	4	10.70 ± 1.70^{b}	
Control	4	0	
Sig.		0.000	

Inhibition value is the mean ± std. error using ANOVA at < 0.05.
N is the number of repetitions.
The superscript letter after the number is Duncan's test result value at a 95% confidence interval.

Table 4. Anticancer activity of simpor leaf extract using the MTT assay against the HepG2 cell line.

Concentration (ppm)	*N*	Inhibition (%)	IC_{50} (ppm)
50	4	27.67 ± 2.26^{a}	88.52
25	4	28.47 ± 3.29^{a}	
20	4	23,02 ± 4.79^{a}	
15	4	22.40 ± 0.38^{a}	
10	4	24.90 ± 2.25^{a}	
Control	4	0	
Sig.		0.000	

Inhibition value is the mean ± std. error using one-way ANOVA at < 0.05.
N is the number of repetitions.
The superscript letter after the number is Duncan's test result value at a 95% confidence interval.

Anticancer activity of simpor leaf extract with MTT method

The simpor leaf extract that has been tested contains flavonoids, saponins, alkaloids, and terpenoids. Secondary metabolite compounds such as flavonoids, saponins, alkaloids, and terpenoids have been shown to have antioxidant activity, anticancer activity, and toxicological activity (Eswaraiah *et al*., 2020). In this study, the simpor leaf extract from Belitung has the ability to be a cancer preventive agent. It is difficult for cancer cells to control cell cycle regulation, so cancer cells continue to divide or proliferate. Normal cells have control of cell cycle regulation so that cell division does not continue to occur. The p53 protein has antiproliferative properties that play a role in cell cycle regulation. In addition, the cell cycle can be delayed by suppressing the activity of cyclin-dependent kinases (cyclin-CDK) and other protein kinases. About 50% of breast cancer cases are caused by having hormone receptor proteins such as CDK2. CDK2 is a protein that plays a role in cell cycle progression driven by estrogen (Tadesse *et al*., 2020) so that it can activate other oncoproteins such as Myc and CycD1. Both of these oncoproteins can stimulate the process of breast cancer cell development and are transcription factor proteins (Xiao *et al*., 2018). Flavonoids have an influence in stopping the G_1 phase (Tavsan and Kayali, 2019), and the phenolic compounds contained in the simpor root extract can stop the HeLa cancer cell cycle in the G_2/M phase so that cells are prevented from entering the G_0 phase and cells are stimulated to perform apoptosis.

Programmed cell death or apoptosis can be triggered by certain stimuli, for example, through the B-cell lymphoma protein

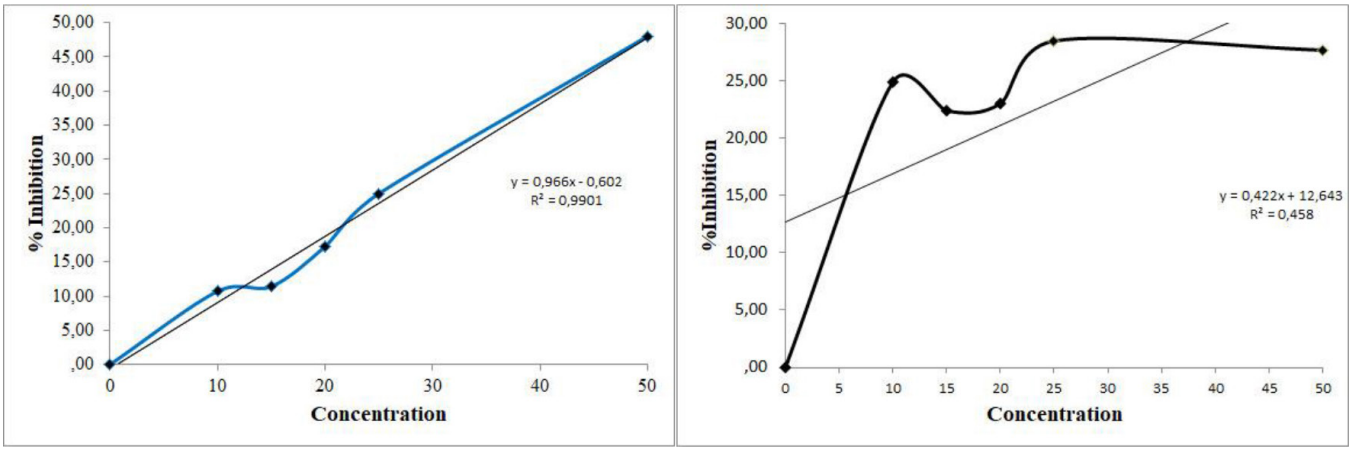

Figure 4. Linear regression curve of the relationship between the concentration of EAESL and the percentage of cell line inhibition: (a) MCF-7 and (b) HepG2.

group (Bcl-2). Bcl-2 proteins consist of two groups: these are antiapoptotic and proapoptotic Bcl-2 proteins. The antiapoptotic Bcl-2 protein group is responsible for preventing apoptosis from occurring, for example, Bcl-2, Bcl-X, metacaspase type II (McII), and Bag. On the other hand, the proapoptotic Bcl-2 protein group stimulates apoptosis, for example, Bak, Bax, and Bad. Inhibition of apoptosis of the MCF-7 cells is caused by Bcl-2 (Hernandez-Valencia *et al*., 2018). Flavonoids will work in suppressing the antiapoptotic Bcl-2 group. Flavonoids, which are chemopreventive agents, have a role in promoting apoptosis through inhibition of the expression of topoisomerase I and II enzymes that play a role in DNA replication. Flavonoids will inhibit the topoisomerase complex and cause DNA to stop replicating. Furthermore, apoptotic proteins are formed, namely, Bak and Bax, which can reduce the expression of antiapoptotic proteins (Smith *et al*., 2020), and the role of the two proteins will also stimulate the p53 gene to start apoptosis.

In liver cancer cells, the secretion of cytokines by interleukin- (IL-) 6 cells will activate the inflammatory signal protein nuclear factor-kB (NF-kB) present in hepatocyte cells. NF-kB is a protein that induces DNA transcription for cell proliferation (Tonello *et al*., 2017). Flavonoids will activate extracellular signal-regulated kinase, which is triggered by IL-6 to reduce proliferation (Lee *et al*., 2019).

Inhibition concentration (IC_{50}) is determined by the relationship between the extract concentration curve (x-axis) and inhibition (%) curve (y-axis) with linear regression analysis. The R^2 value indicates the relationship between the concentration of Simpor leaf extract used and cell line inhibition (Fig. 4). The R^2 value in the MCF-7 cell line was greater than in the HepG2 cell line, which means that concentration determined MCF-7 cell line inhibition even higher than HepG2.

CONCLUSION

The simpor leaf extract from Belitung contains phytochemicals in the form of flavonoids, saponins, alkaloids, and terpenoids. The administration of the simpor leaf extract from Belitung to the BSLT test did not have toxic properties, with an LC_{50} value of 5,221 ppm. However, it has cytotoxicity against the tested cell lines MCF-7 and HepG2. The Simpor leaf extract at 25 ppm with IC50 of 88.52 was seen as the best concentration for liver cancer treatment. It can be concluded that *D. suffruticosa* shows cytotoxicity against thet cell lines MCF-7 and HepG2 and can be used as a candidate for breast and liver cancer treatment.

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CONFLICT OF INTERESTS

The authors report no financial or any other conflicts of interest in this work.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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