RESEARCH ARTICLE

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Brazilin Isolated from Caesalpina Sappan Wood Induces Intrinsic Apoptosis on A549 Cancer Cell Line by Increasing p53, caspase-9, and caspase-3

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Abstract

Objective: Lung cancer is the leading cause of death among cancer patients. The majority of lung cancer is the Non-Small Lung Carcinoma (NSLC). This study evaluated the potency of brazilin isolated from Caesalpinia sappan wood to induce apoptosis on non-small lung carcinoma cell line, A549, by examining the expression of p53, caspase-9, and caspase-3. **Methods:** Brazilin was isolated from Caesalpinia sappan wood following a guided assay and it was determined by using Brazilin®SIGMA as standard. The activity of brazilin on the growth of A549 cell line was analysed by MTT assay and the apoptosis was evaluated by flowcytometer following Annexin V (FITC) and PI staining. The expression of p53, caspase-9, and caspase-3 was examined by immunocytochemistry. **Result:** The IC50 of brazilin on A549 cell line was 43µg/mL. Cell treatment with 20 µg/mL and 40 µg/mL of brazilin significantly increased early apoptosis ($p<0.001$). Cell treatment with 40 µg/mL of Brazilin significantly increased late apoptosis ($p<0.001$). Brazilin significantly increased the expression of p53, Caspase-9, and caspase-3 ($p<0.001$). **Conclusion:** This study showed evidence of the activity of brazilin to induce intrinsic apoptosis on a NSLC cell line A549.

Keywords: Brazilin- Caesalpinia sappan-A549- lung cancer- apoptosis

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Introduction

The case of lung cancer and its high mortality rate raise worldwide concern. The decrease of environmental quality and smoking habit become detrimental factors contributing to the increase of lung cancer prevalence. Air pollution is one of the environmental factors related to the increase of lung cancer cases in Southeast Asian countries (Taghizadeh-Hesary and Taghizadeh-Hesary, 2020). Although smoking consumption has a strong correlation with lung cancer incidence (Bade and Dela Cruz, 2020), the prevalence of lung cancer on nonsmokers was also contributes to a significant number of cases in East Asian countries (Zhou and Zhou, 2018).

The majority of lung cancer are Non-Small Lung Carcinoma (NSLC), which account for 80-85% of the cases. Considered as the most aggressive cancer cell type, NSLC results in high mortality cases among other types of lung cancer (Li et al., 2020). Despite many treatment methods available for NSLC, the outcome is considered substandard (Karuppasamy et al., 2021). The adverse effect of radiotherapy and pharmacotherapeutic regimen on vital organ becomes another problem. Kidney injury related to the use of pembrolizumab, a current anti PD1 checkpoint, to treat NSLC has been reported (Oki et al., 2020). Suggestion to look back at the traditional practice of consuming medicinal plant to complement cancer therapeutic strategy is worth to be explored (Lin et al., 2020). Flavonoid is an important secondary metabolite in the majority of medicinal plants. The potential anti-cancer activity of flavonoids includes both extracellular and intracellular signaling pathways (Lotha and Sivasubramanian, 2018).

This study aimed to explore the potential anti-cancer activity of Caesalpinia sappan, one of Indonesian medicinal plant with local name secang, on lung cancer cell line, A549. The wood of Caesalpinia sappan is commonly consumed as the ingredient of traditional beverage in Indonesia (Meiyanto and Larasati, 2019). Caesalpinia sappan is one of medicinal plants with

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high flavonoid content (Nguyen et al., 2020). The high flavonoid content of Caesalpinia sappan wood encourages research on anti-cancer activities. Brazilin is one of the phenolic compounds extracted from Caesalpinia sappan wood. Brazilin belong to homoisoflavonoid group, it is a heterocyclic compound with the molecular formula $C_{16}H_{14}O_5$ (Mottaghipisheh and Stuppner, 2021). The in-vitro cancer cell growth inhibition activitiy of Brazilin have been reported for several cancer cells line including breast cancer MCF7/Her-2, bladder cancer T24, colon cancer WiDr, and other type of cancer cell line HepG2, H522, COLO205, S180, Tca8113 (Qi and Jin, 2020). Brazilin was also reported to enhance the cytotoxic effect of chemotherapeutic agent. Combining brazilin with Adriamycin or cisplatin potentiates the inhibition of MCF7/Her-2 cells migration and the growth inhibition of WiDr cell lines (Utami et al., 2020).

Although the antiproliferative activity of compounds extracted from Caesalpinia sappan has been reported, the previous researches have not figured out the anti-cancer mechanism of brazilin. This study focused on investigating the activity of brazilin to induce apoptosis on A549 lung cancer cell lines.

Materials and Methods

The heartwood of Caesalpinia sappan was purchased from the Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT) in Karanganyar Region, Central Java Province, Indonesia and by which it was authenticated. Common chemical reagents used in this study were purchased from Merck and Sigma -Aldrich, USA, unless otherwise stated.

Isolation of Brazilin from Caesalpinia sappan heartwood

Brazilin was isolated from the hearthwood of Caesalpinia sappan adapted from the guided assay published by Jung et al., (2015). In brief, simplicia of Caesalpinia sappan heartwood was macerated using 100% (v/v) methanol for 24 hours and then filtered using Whatman Quantitative Filter Papers, Ashless, Grade 42 (GE healthcare Lifescience). The procedure was repeated three times. The filtrate was concentrated using a rotary evaporator at 40^{o}C to obtain the solid extract. Further serial separation was conducted using n-hexane, ethyl acetate, and water saturated butanol solvents. The ethyl acetate fraction was then subjected in to a glass column chromatography (Iwaki). Silica gel bead was used as solid phase, while the mobile phase was chloroform: ethyl acetate (50:50 v/v) with 1 ml/min flow rate. Four fractions from 18 total fractions were further analyzed using a High-Performance Liquid Chromatography (HPLC) (Shimadzu Corp., Tokyo, Japan) to identify the brazilin compound. Brazilin®SIGMA was used as standard. Finally, the fraction 10 (F10) had the same peak with the standard brazilin and was used for further experiment, and therefore it was confirmed as brazilin. The chromatograph of F10 and Brazilin®SIGMA was showed in figure 1. In the rest of this paper, Fraction 10 was then mentioned as brazilin.

Antiproliferative Assay

The A549 cell line was from ATCC® provided by Prof. Chiou-Feng Lin from department of Immunology and Microbiology, Taipei Medical University.

The antiproliferative activity of F10 was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Brazilin was dissolved in dimethyl sulfoxide (DMSO), the concentration of DMSO was adjusted not to exceed 0.1% final concentration in each experiment. A total number of $1x10^4$ A549 cell line was cultured in a 96 well plate using DMEM supplemented with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, and 1% fungizone (Gibco, USA), and incubated in a 5% CO2 incubator at 37°C for 24 hours. Following 24 hours incubation, a serial concentration of brazilin (100, 80, 60, 40, 20, 10, 5, and 0µg/mL) was added and the cells were re-incubated for 24h. After that, the MTT reagent was added into each well to make 5µg/ml final concentration and the plate was incubated at 37oC for further 4 h. The stop solution (10% SDS in 0.01N HCL) was added after 4 hours incubation and then the plate was gently shaken for 15 minutes. Formazan formation in each well was measured using an ELISA reader (Biorad, USA) at λ595. All experiments were done in triplicate and the percentage of cell viability was calculated using the following formula:

%cell viability = (OD treated cell)/(OD control cell) x 100

Finally, the IC_{50} value of brazilin against lung cancer A549 cell line was calculated using the following formula(Mathieu et al., 2008):

$$IC_{50} = ((X2-X1) \times (50-Y1))/((Y2-Y1))+X1$$

X1 and X2 are the higher and the lower drug concentrations that reduce 50% cell viability, while Y1 and Y2 are percentage of cell viability at X1 and X2.

Flowcytometry assay

A total number of $3x10^5$ A549 cells /well were seeded in 6 well plate. Following 24 hours incubation, the cells were treated with 0, 20, or 40µg/mL brazilin and re-incubated for 24 h. The analysis of apoptotic cells was conducted by using the Fluorescently Labeled Annexin V and Propidium Iodide (Annexin V(FITC)-PI) assay method (Annexin V-FITC Apoptosis Detection Kit Roche) with the BD FACS Diva 8.0.2 flowcytometer.

Immunocytochemistry

Protein expressions of p53, caspase-9, and caspase-3 in A549 cells treated with F10 were evaluated using the immunocytochemistry. A number of $1x\ 10^5$ of A549 cells were cultured on coverslip® (Nunc™ Thermanox™) for 24 well plate. Following 24 hours incubation the cells were treated with 0, 10, 20, or 30µg/mL of brazilin and re-incubated for further 24 hours. At the end of 24 hours of cells incubation the culture media was removed and the cells was washed with cold Phosphate Buffered Saline and then fixed with methanol. The primary antibodies against p53 (ABclonal; 1:200 dilution), caspase-9 (ABclonal;

1:200 dilution), caspase-3 (ABclonal; 1:200 dilution) and the secondary antibody StarTrek Anti-Polyvalent (Biocare) were used for immunocytochemistry. The enzymatic reaction of antigen-antibody complexes was performed by adding the diaminobenzidine substrate and the nuclear counterstaining used the hematoxylin. Interpretation of ICC staining was conducted by counting the percentage of A549 cells with positive expression of p53, caspase-9, and caspase-3. Positive expression of p53 was defined as positive staining of p53 immunocytochemistry (ABclonal; 1:200 dilution) in the nuclei of A549 cells. Positive expression of caspase-9 anda caspase-9 was defined as positive staining in the cytoplasm of A549 cells.

Statistical analysis

The IBM SPSS Statistic software version 28.0.0.0 (190) was used to analyze all collected data. Numerical data were presented as mean ± standard deviation and the different mean among groups was determined using the one-way analysis of variance (ANOVA) with p-values < 0.05 considered as statistically significance.

Results

Antiproliferative Assay

The MTT assay showed a dose dependent antiproliferative effect of brazilin on A549 cancer cell line. The concentration of brazilin required to inhibit 50% of A549 cell growth (IC_{50}) was 43µg/mL. Figure 2. showed that brazilin isolated from Caesalpinia sappan wood inhibit the proliferation of A549 cancer cell line.

Flowcytometry analysis

The potency of brazilin to induce apoptosis on A549 cancer cell line was evaluated based on the Annexin V-FITC-PI staining. Figure 3. showed the cell population according to Annexin V-FITC-PI staining. The healthy

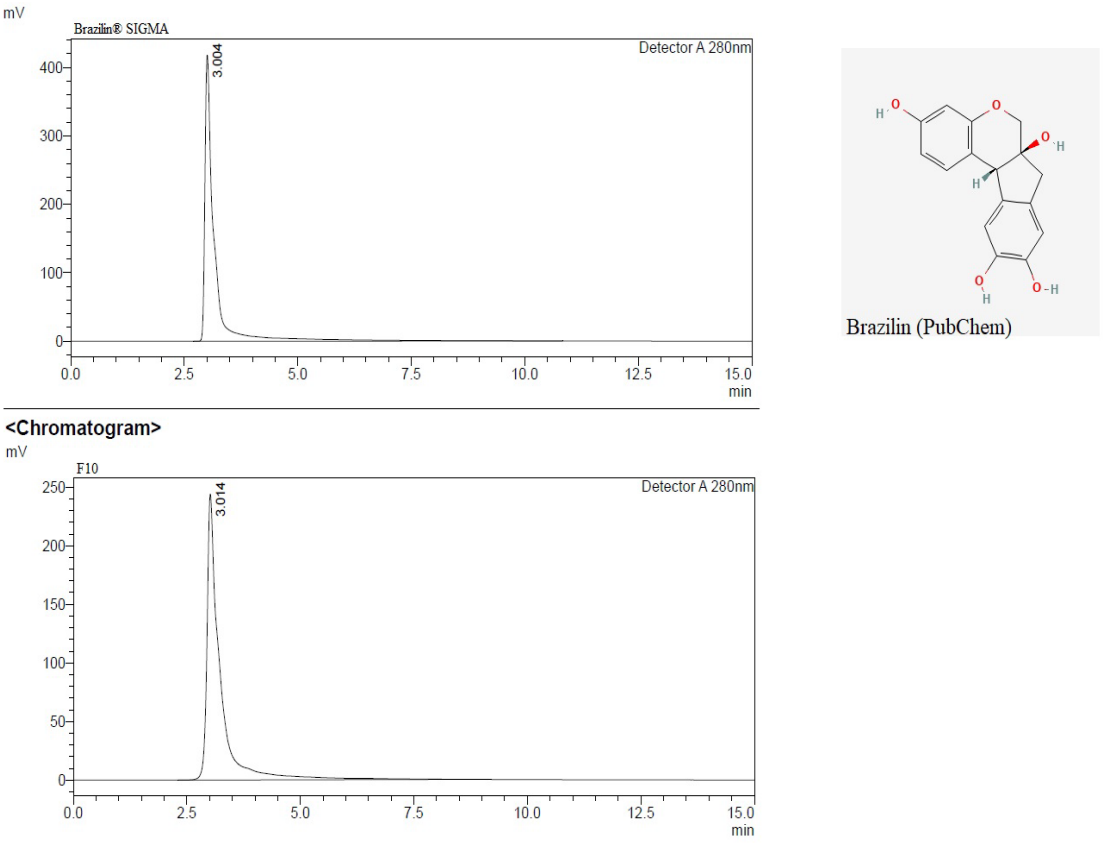

Figure 1. The Comparative HPLC analysis of F10 with Brazilin® SIGMA as the Standard Reference. The chromatograph showed similar retention time of the peak for F10 and Brazilin® SIGMA. The chromatograph confirm that F10 is Brazilin

Table 1. The Effect of Brazilin on Apoptosis. Treatment with brazilin 20µg/mL and 40µg/mL on A549 cells during 24 hours significantly increased the event of apoptosis.

	The percentage of cell population			
Brazilin dose	Healthy cell	Early apoptosis	Late apoptosis	Necrosis
0µg/mL	94,25 ± 0.35	1,85 ± 0.35	2,75 ± 0.35	1,1 ± 0.00
20µg/mL	81,3 ± 0.57†	11.4% ± 0.14% †	3.2% ± 0.84% †	3.7% ± 0.35% †
40µg/mL	80,55†	8.6% ± 0.28% †	6.7% ± 0.42% †	3.6%± 0.46%†

P.value†<0.001; ANOVA P.value<0.05†

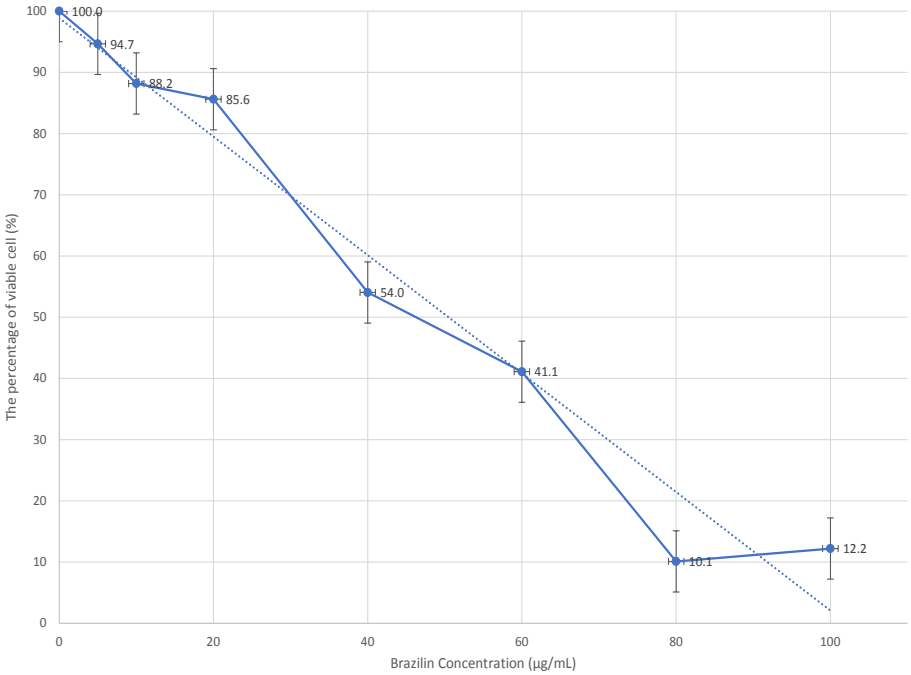

Figure 2. The Effect of Brazilin of A549 Cells Proliferation for 24 hours. The graph showed a dose dependent growth inhibitory activity on A549 cancer cell line. The concentration of F10 required to inhibit 50% growth of the cells (IC50) was 43µg/mL

cells were stained negatively for both Annexin and PI. The binding of Annexin with phosphatidylserine on cells underwent apoptosis give rise to positive staining for annexin, while the dead cells with membrane cell rupture showed positive staining for PI. The cells showed double positive staining for both Annexin and PI were considered as late apoptotic cells. Table 1. and figure 4. summarized the effect of brazilin on apoptotic event. The data showed that treatment of A549 cells with 20 µg/mL during 24 hours induced A549 cells apoptosis. The proportion of the

Table 2. Percentage of A549 Cells Expressing p53, Caspase-9, and Caspase-3

	Expression of p53, caspase-9, caspase-3		
Brazilin dose	P53	Casapse-9	Caspase-3
0µg/mL	0	0	0
10µg/mL	17.6% ± 4.3%†	24.8% ± 3.8%†	6.4%± 1.3%
20µg/mL	51.6% ± 6.2%†	50.2% ± 5.5% †	50.6%± 3.1%
30µg/mL	63.4%±1.7%†	90%±2.7%	94.4%% ± 2.3% †

P.value†<0.001; ANOVA P.value<0.05†

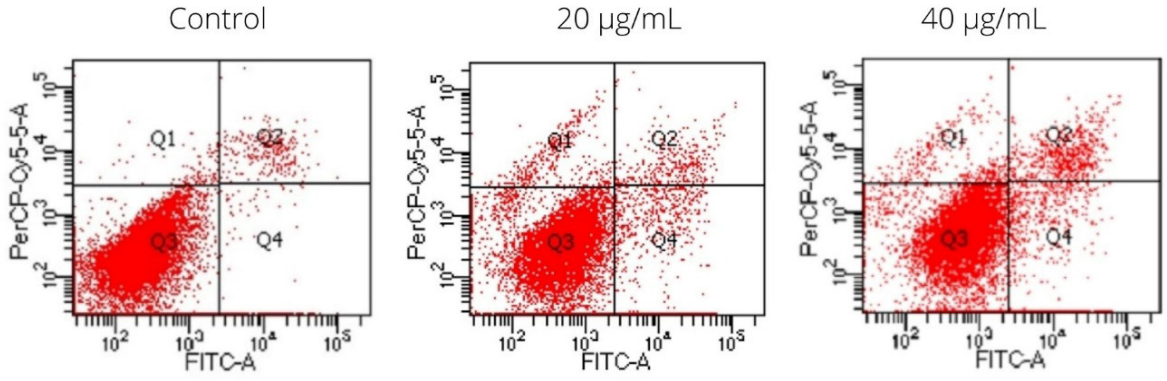

Figure 3. The Effect of Brazilin on Apoptosis. Treatment of A549 cell with 20µg/mL of brazilin within 24 hours increase the event of early apoptosis (Q4). Addition of 40µg/mL increase the number of A549 cells undergoing late apoptosis (Q2). In addition to apoptosis, the addition of brazilin also increase the number of cell undergoing necrosis (Q1).

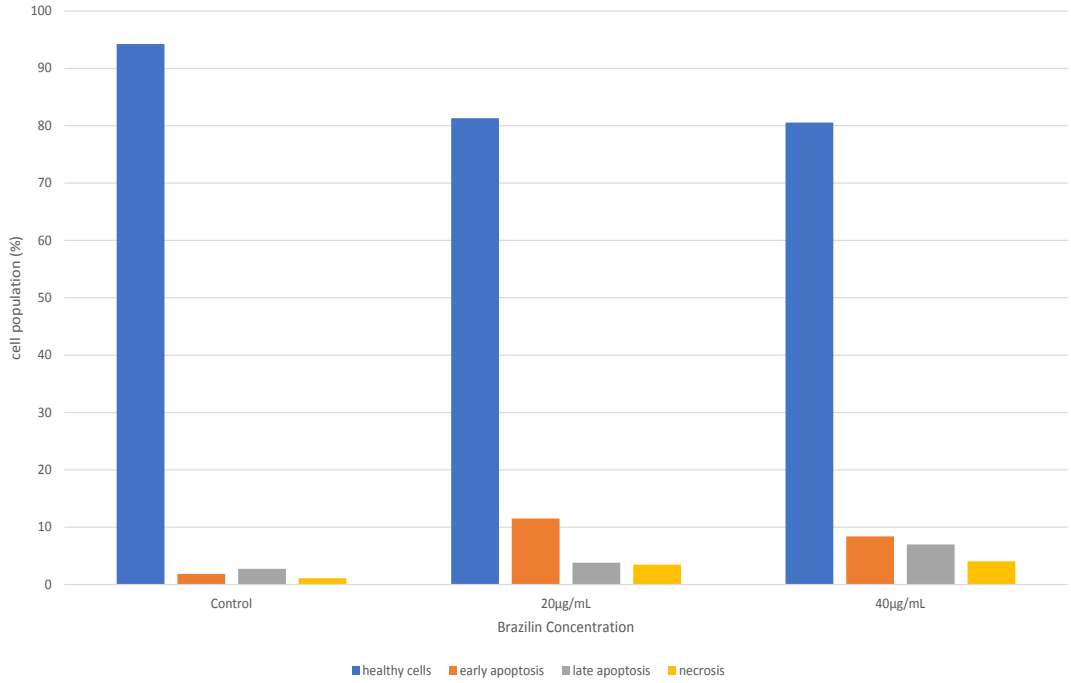

Figure 4. The Effect of Brazilin on Apoptosis. Treatment with 20µg/mL and 40µg/mL brazilin on A549 cells for 24 hours significantly increased the percentage of cells undergoing early and late apoptosis.

cell underwent early and late apoptosis after treatment with 20 µg/mL of brazilin were 11.4% ± 0.14% and 3.2% ± 0.84% respectively. The progression from early apoptosis into late apoptosis can be observed in the cell treated with 40 µg/mL of brazilin. The proportion of cells underwent early and late apoptosis after 24 hours treatment with 40 µg/mL of brazilin were 8.6% ± 0.28% and 6.7% ± 0.42% respectively. A proportion of necrotic cells (3.7% ± 0.35% and 3.6%± 0.46%) were also observed in the cell treated with 20 µg/mL and 40 µg/mL.

Immunocytochemistry analysis

The examination of protein expression was conducted by counting the overall percentage of the cell positively stain for the evaluated protein (p53, caspase-9, caspase-3). Figure 5. showed the microscopic feature of the expression of p53, caspase-9, and caspase-3 after 24 hours administration of brazilin in dose 0 µg/mL, 10 µg/mL, 20

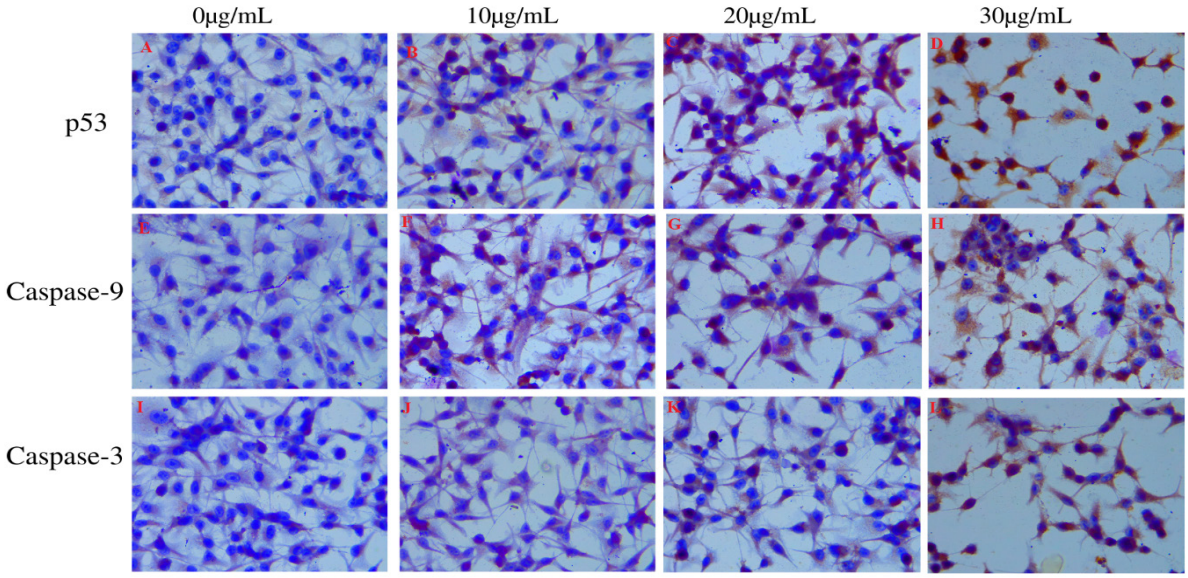

Figure 5. The Effect of Brazilin on the Expression of p53, Caspase-9, and caspase-3 on A549 Cell Line. Treatment with brazilin on A549 cells during 24 hours increased the expression of p53, Caspase-9, and Caspase-3 in dose dependent manner. Nuclear staining for p53 remarkably observed after 24 hours treatment using 20µg/mL and 30µg/mL brazilin. Similarly, the expression of Caspase-9 and Caspase-3 increased linearly with the dose of brazilin.

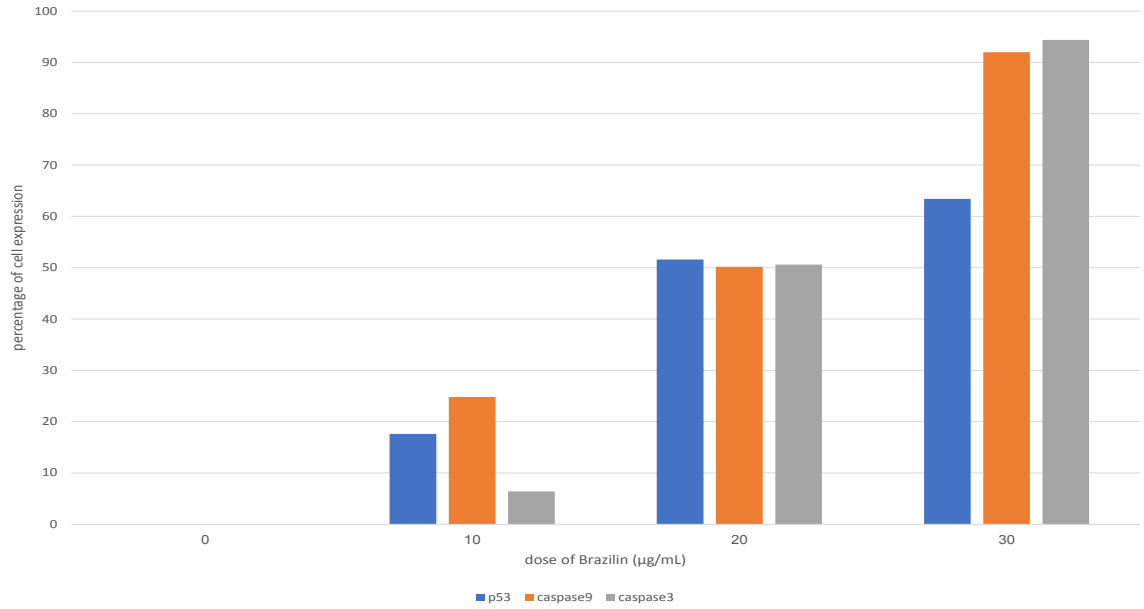

Figure 6. The Effect of Brazilin on p53, caspase-9, and caspase-3 Expression. Treatment with brazilin on A549 during 24 hours significantly increased the expression of p53 ($p<0.001$) and caspase-9 and Caspase-3 ($p<0.001$) in dose dependent manner.

µg/mL, and 30µg/mL. The administration of brazilin on A549 cancer cell line increased the expression of p53, caspase-9, and caspase-3 in dose dependent manner. Table 2. and Figure 6. summarized the percentage of p53, caspase-9, and caspase 3 expression resulted from A549 cells treatment with brazilin. The treatment of A549 cells with 10 µg/mL, 20 µg/mL , and 30 µg/mL of brazilin increased the expression of p53 protein (17.6% ± 4.3%, 51.6% ± 6.2%, and 63.4%±1.7%) respectively compared to control cells. The same effect was also observed in the expression of caspase-9 and Caspase-3. Brazilin with the dose of 10 µg/mL, 20 µg/mL , and 30 µg/mL increased the expression of Caspase-9 (24.8% ± 3.88%, 50.2% ± 5.5%, and 90% ± 2.7%). The expression of caspase 3 were 6.4% ± 1.3%, 50.6% ± 3.1%, and 94.4% ± 2.3% for the cells treated with 10 µg/mL, 20 µg/mL, and 30µg/mL of brazilin respectively.

Discussion

Cancer is a complex disease which requires many treatment strategies. The potency of cancer chemotherapeutic agent are indicated by some parameters including the capacity to inhibit cancer cell proliferation, induce cell differentiation and apoptosis (Gezici and Şekeroğlu, 2019). Despite many molecular pathways which can be the target of treatment strategies, the main purpose of cancer therapy is to eliminate cancer cells from the body. The death of cancer cells through apoptosis becomes the final goal of treatment strategies.

This study evaluated the potency of brazilin isolated from Caesalpinia sappan wood to induce apoptosis in Non-Small Lung Carcinoma cell line (A549). Brazilin showed a dose dependent growth inhibitory activity on A549 cancer cell line. Brazilin in 43µg/mL concentration is capable of inhibiting 50% A549 cells growth during 24 hours treatment as compared to the growth of control cells. Furthermore, treatment of A549 cells with 20 µg/mL of Brazilin during 24 hours induced early apoptosis. Apoptosis is a cascade of events leading to a programed cell death. Apoptotic programed of cell death can be initiated by extracellular or intracellular signals. The extracellular signal activated apoptosis by binding with death receptors on the cell surface. On the other hand, the intracellular apoptotic signal was generated by intracellular stress which induce the mitochondrial membrane damage. The treatment of cancer cells with cytotoxic agents will induced intracellular stress leading to intrinsic apoptosis (Pfeffer and Singh, 2018; Jan and Gul-e-Saba, 2019).

The involvement of caspase in cell death distinguished apoptosis from other type of cell death (Pfeffer and Singh, 2018; Jan and Gul-e-Saba, 2019). To examine whether the death of A549 induced by the cell treatment with brazilin occurred through apoptosis, the expression of p53, caspase-9, and caspase-3 were evaluated using immunocytochemistry. Brazilin increased the expression of p53 protein in dose dependent manner. Accumulation of p53 on nuclear cells will promote the transcription of Bax and in contrary suppressed the transcription of BCL-2. The increased ratio of Bax/BCL-2 initiates the activation of apoptosis. Bax plays its role by increasing the permeability of mitochondrial membrane to release cytochrome C. The release of cytochrome C from mitochondrial intermembrane space into cytoplasm initiate the formation of apoptosome (Jan and Gul-e-Saba, 2019).

This study also showed that treatment of A549 cell with brazilin during 24 hours increased the expression of caspase-9 and caspase-3 in dose dependent manner. The increase of caspase-9 and caspase 3 supported the apoptotic mechanism induced by brazilin. Caspase-9 is one of initiator caspase activated through intrinsic

apoptotic pathway. The inactive form, procaspase-9, combines with cytochrome C and Apaf1 to form apoptosome. Apoptosome will induce the activity of caspase-9 to activate caspase-3 signaling cascade (Pfeffer and Singh, 2018). This cascade will end up with the death of cancer cells.

The data of this study supported previous study on the potential anti-cancer activity of Caesalpinia sappan. Another compound of Caesalpinia sappan wood, sappanchalchone, was reported to induce apoptosis on colon cancer cell line. Sappanchalchone induced apoptosis on HCT116 and SW480 by activating caspase signaling cascade (Seo et al., 2020). Kwak et al., (2021) also reported that 3-deoxysappanchalcone induce apoptosis on human esophageal cell by activating ROS-induce mediated apoptosis. Those reported studies indicated that natural compounds which are originating from the daily consumed plant, could give beneficial use to complement the standard lung cancer therapy. Considering all of the data, this study concludes the potency of brazilin as a cancer chemotherapeutic agent targeting on apoptosis.

Author Contribution Statement

Suyatmi Suyatmi performed the research design, experimental work, and writing the manuscript. Ambar Mudigdo, Bambang Purwanto, and Dono Indarto shared equal contribution on the research design and the manuscript proof reading, Fikar Arsyad Hakim and Dyah Ika Krisnawati contributes in data analysis and manuscript writing.

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Ethic Statement

All the experimental works were approved by The Health Research Ethic Committee of Faculty of Medicine, Universitas Sebelas Maret under the Ethical Clearance Letter No:023/UN27.06.6.1/KEPK/EC/2020.

Conflict of interest

The authors state there is not any conflict of interest related to the publication.

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