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Anticancer Activities of Sesewanua Leaf Extracts (*Clerodendrum fragrans* (Vent.) Willd) Against A549 Lung Cancer Cell

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Abstract

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BACKGROUND: Cancer is one of the leading causes of non-communicable diseases in the world, with about 10 million deaths worldwide in 2020. Lung cancer was the most common type of cancer and the highest cause of death. Therapy for lung cancer can be either conventional therapy or molecular targeted therapy that has many limitations.

AIM: It is, therefore, important to explore new sources of anticancer activity, including those from plants. One plant that is thought to have anticancer activity is Sesewanua (*Clerodendrum fragrans* [Vent.] Willd. Syn. *Clerodendrum chinense* [Osbeck] Mabb., Family Lamiaceae).

METHODS: This research is a laboratory experiment. The sample used is the *C. fragrans* leaves obtained in Malalayang I Timur Village, Malalayang District, Manado City, North Sulawesi Province, while the subjects in this study were A549 lung cancer cells from Cell-Culture Laboratory, Faculty of Pharmacy, Universitas Padjadjaran Bandung. Anticancer activity test was using the MTT tetrazolium assay method. Data in the form of a percentage (%) inhibition of cell proliferation, then determined the value the concentration of 50% proliferation inhibition (IC50) using a computer program online.

RESULTS: The results showed that ethanolic extract, hexane fraction, ethyl acetate fraction, and water-soluble fraction of *C. fragrans* had anticancer activity on A549 lung cancer cells. The smallest IC50 value is indicated by ethyl acetate fraction (191, 165 ppm), which is categorized as moderately active.

Introduction

Cancer is one of the leading non-communicable diseases causing death worldwide. In 2020, cancer caused the deaths of almost 10 million people worldwide [1]. In Indonesia, the prevalence of cancer in all age groups in 2018 was 1.79% and in North Sulawesi Province, at 1.71% [2]. Lung cancer is the most common type of cancer and the highest cause of death in the global population. Recorded in 2020, lung cancer caused the death of 1.8 million people from total cancer death worldwide [1].

The choice of lung cancer therapy depends on the type, stage, health, and age of lung cancer patients, which can be conventional therapy (surgery, platinum-based chemotherapy, and radiotherapy) or molecular targeted therapy [3], [4], [5], [6]. Reportedly, the 5-year survival rate in lung cancer patients receiving conventional therapy is 18% and lower than most other types of cancer [4], [7]. Molecular targeted therapy provides hope with the availability of new drugs to reduce morbidity and mortality from lung cancer. However, this therapy has limitations in the number of drugs, some are still in the clinical trial stage and require appropriate biomolecular diagnostic knowledge

to ensure the success of therapy [4], [6]. It is, therefore, important to explore new sources of anticancer activity, including those from plants.

Since ancient times, plants have been used in cancer treatment and become a source of anticancer compounds with various mechanisms of action [8], [9]. A plant that is thought to have anticancer activity is Sesewanua plants (*Clerodendrum fragrans* [Vent.] Willd. Syn. *Clerodendrum chinense* [Osbeck] Mabb., Family Lamiaceae). The people of North Sulawesi traditionally use Sesewanua (*C. fragrans* [Vent.] Willd) leaves as an anti-inflammation and antipyretic medication [10]. There is no adequate scientific information about the activity of Sesewanua plants as anticancer. Kalonio *et al.* [11] report that there are 12 plants from the genus *Clerodendrum* that have been shown to have *in vitro* and *in vivo* anticancer activities.

Sesewanua (*C. fragrans* [Vent.] Willd) leaves are reported to contain beta-sitosterol, clerosterol, daucosterol, caffeic acid, acteoside, leucoseptoside A, kaempferol, and 5,4'-dihydroxy-kaempferol-7-O-beta-rutinoside [12]. Molecular docking simulation results show that acteoside contained in the genus *Clerodendrum* can bind to the target of cancer proteins so that it has the potential as an anticancer [13].

Kaempferol can inhibit the growth of pancreatic cancer cells Miapaca-2, Panc-1, and SNU-213 [14]. As for beta-sitosterol and daucosterol compounds in benzene extract of leaf *Grewia tiliifolia* showed anticancer activity in A549 lung cancer cells [15]. These things can illustrate that the *Sesewanua (C. fragrans [Vent.] Willd)* leaves are thought to have potential as anticancer.

This study aims to evaluate the anticancer activity of *Sesewanua* leaves (*C. fragrans [Vent.] Willd*) extracts and fractions against A549 lung cancer cells using the method MTT tetrazolium assay, and determine the 50% inhibition concentration of it.

Methods

C. fragrans leaves collection and processing

Sesewanua leaves (*C. fragrans [Vent.] Willd*) were collected from Malalayang I Timur village, Malalayang District, Manado City, North Sulawesi Province.

Extraction and fractionation

Sesewanua leaves powder (*C. fragrans [Vent.] Willd*) was extracted by maceration method using 70% ethanol solvent. As a result of the extraction process, ethanol extract was obtained in a yield of 27.8%.

Fractionation of the ethanolic extract of the *Sesewanua (C. fragrans [Vent.] Willd)* leaves using a liquid extraction method with n-hexane and ethyl acetate as solvents, respectively [16]. Fractionation results were obtained by 1.03 g n-hexane fraction; ethyl acetate fraction 1.90 g, and water fraction 7.00 g.

Toxicity test with the brine shrimp lethality test (BSLT)

Eggs of *Artemia salina* hatched in artificial seawater (NaCl 3.8%) for 48 h so that adult shrimp called nauplii are obtained. Extracts and fractions were dissolved in DMSO, and diluted with artificial seawater to obtain a concentration of 10; 100; and 1000 µg/ml in 5 ml of solution. DMSO concentration in the solution should not be more than 50 µl/5 ml. A solution of 50 µl of DMSO in 5 ml of artificial seawater was used as a control. A total of ten nauplii were added to the sample solution and control. After 24 h, the number of dead nauplii was observed and the percentage of death was calculated [17], [18]. The LC₅₀ value was calculated using a regression analysis between the log concentration (x) and the probit value (y) using a computer program available online on the website <https://www.aatbio.com/>

tools/linear-logarithmic-semi-log-regression-online-calculator

Anticancer activity using the MTT tetrazolium assay method

A total of 50 µl of cell suspension in RPMI-1640 medium with a density of 5×10^3 /well [19] inserted into a 96-well plate and incubated at 37°C in an incubator flowed CO₂ 5% for 24 h. Furthermore, cell cultures were treated each with extracts and fractions (concentrations of 1000; 100; 10; 1; and 0.1 ppm), cisplatin (100; 10; 1; 0.1; 0.01 ppm), and controls (cells with medium), then incubated at 37°C in an incubator which flowed CO₂ 5% for 24 h. After the incubation period, 10 µL CCK-8 was added to each well and pre-incubated for 3 h. After adding 100 µL of 0.1 N HCl, the absorption was measured at a wavelength of 450 nm and a reference wavelength of 650 nm [20]. The percentage (%) of cell proliferation inhibition was calculated using the equation [21]:

% Proliferation Inhibition =

$$\left(1 - \frac{\text{Absorbance of experimental well}}{\text{Absorbance of negative control well}}\right) \times 100$$

Data analysis

The inhibition concentration of 50% (IC₅₀) value was determined using a computer program online available on the website <https://www.aatbio.com/tools/ic50-calculator> with a value of x = concentration and y value = percentage (%) of proliferation inhibition.

Results and Discussion

Extraction and fractionation

Sesewanua (C. fragrans [Vent.] Willd) leaves powder was extracted by maceration. Ethanol was used as a solvent which was expected to attract all the compounds in the sample. Ethanol with a maximum water content of 30% could extract tannins, polyphenols, polyacetylenes, flavonoids, terpenoids, sterols, and alkaloids from natural products [22].

Preliminary toxicity test with BSLT method

The results of toxicity test extracts, n-hexane, ethyl acetate, and water fraction of *Sesewanua (C. fragrans [Vent.] Willd)* leaves by BSLT method are shown in Table 1.

Data in Table 1 show that the values of LC₅₀ extracts and fractions of *Sesewanua (C. fragrans [Vent.] Willd)* leaves are sequentially ethyl acetate

fraction < n-hexane fraction < ethanol extract < water fraction. Pertiwi *et al.* reported that the ethyl acetate extract from the leaves of plants of the same genus as *C. paniculatum* L. had an LC₅₀ value of 29.182 ppm by BSLT test [23]. The BSLT was a simple bioassay for natural product research, especially for assessing the potential toxicity and biological activity of natural products [23]. According to the Meyer *et al.*, plant extracts were considered toxic if they had LC₅₀ < 1000 ppm [17]. The result in Table 1, extracts and fractions of *Sesewanua* leaves were toxic so that the potential for further investigation is anticancer drugs.

Table 1: Percentage of larvae mortality, probit values, and LC₅₀ values due to ethanol extract, n-hexane fraction, ethyl acetate, and water fraction of *Sesewanua* (*Clerodendrum fragrans* [Vent.] Willd) leaves larvae *Artemia salina*

S. No.	Treatment	Concentration (µg/ml)	% Mortality of Larvae	Probit	LC ₅₀ (µg/ml)
1.	Control	-	0.00	0	-
2.	Ethanol Extract	10	3.33	3.12	60.26
		100	76.67	5.74	
		1000	100.00	8.09	
3.	n-Hexane Fraction	10	36.67	4.67	33.88
		100	96.67	6.88	
		1000	100.00	8.09	
4.	Ethyl Acetate Fraction	10	86.67	6.13	26.92
		100	93.33	6.48	
		1000	100.00	8.09	
5.	Water Soluble Fraction	10	10.00	3.72	186.21
		100	33.33	4.56	
		1000	83.33	5.95	

Anticancer activity test with MTT tetrazolium assay method

Activity test results of extract, n-hexane fraction, ethyl acetate, and water fraction of *Sesewanua* (*C. fragrans* [Vent.] Willd) leaves by method MTT tetrazolium assay are shown in Figure 1.

The results were used a computer program online available from the website <https://www.aatbio.com/tools/ic50-calculator> as shown in Figure 2. The IC₅₀ value of ethanol extracts is 436.912 ppm; n-hexane fraction 295.710 ppm; ethyl acetate fraction 191.165 ppm; and the water fraction of 373.783 ppm. The NCI criteria modified by Srisawat *et al.* [24] and stated that the extracts with IC₅₀ ≤ 20 ppm have high activity; IC₅₀ 21–200 ppm, moderate activity; IC₅₀ 201–500 ppm, weak activity; and IC₅₀ > 500 ppm is not active. Based on these criteria, the ethyl acetate fraction of *Sesewanua* (*C. fragrans* [Vent.] Willd) leaves could be said to have moderate anticancer activity. Cisplatin as lung cancer first-line chemotherapy [3] from the results of this study has a high activity because it has IC₅₀ 8294 ppm.

In Figure 2, we can observe changes in A549 lung cancer cell morphology after exposure to cisplatin, extracts and fractions of *Sesewanua* (*C. fragrans* [Vent.] Willd) leaves. Viable cancer cells line was characterized by the formation of a monolayer

adherent cell layer on the base surface of cell culture tubes [25], [26], and dead cells will appear to float in the medium. Changes in the morphology of cancer cells can be used to observe the type of cell death, whether through the mechanism of apoptosis or necrosis [27], [28]. Cisplatin, which includes platinum-based drugs, causes cell death through the mechanism of apoptosis by inducing genotoxic stress which activates a variety of transduction signal [29]. Further research is still needed to determine the mechanism of cancer cell death due to exposure to extracts and fraction of *Sesewanua* (*C. fragrans* [Vent.] Willd) leaves.

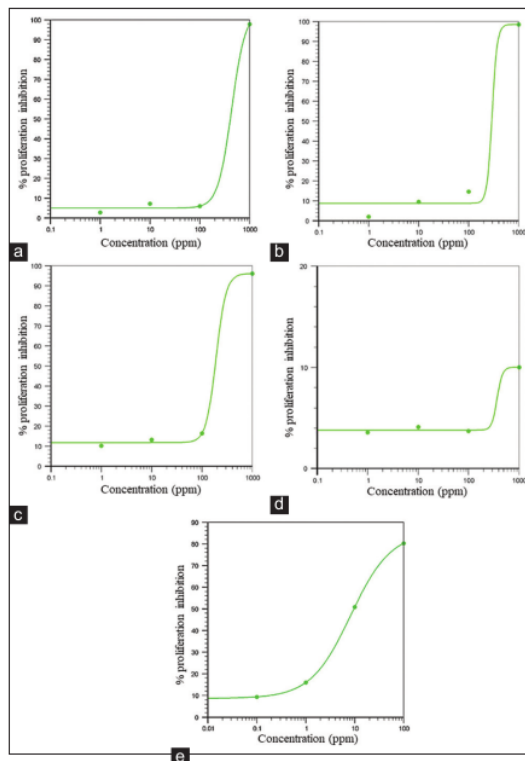


Figure 1: The relationship between the percentage (%) of proliferation inhibition (y) and concentration (x) administration of ethanol extract (a), n-hexane (b), ethyl acetate (c), water (d) of *Sesewanua* (*Clerodendrum fragrans* [Vent.] Willd) leaves, and cisplatin (e) (<https://www.aatbio.com/tools/ic50-calculator> Calculation)

Kalonio *et al.* suggested that there were 12 plants from the genus *Clerodendrum* having anticancer activities both *in vitro* and *in vivo* [11]. Some plants from the Lamiaceae family such as *Pogostemon heyneanus*, *Plectranthus amboinicus* [30], and *Ocimum sanctum* [31] were reported to have anticancer activity. The research data support the results of the present study that extracts and fractions of *Sesewanua* (*C. fragrans* [Vent.] Willd) leaves have potential as anticancer in A549 lung cancer cells.

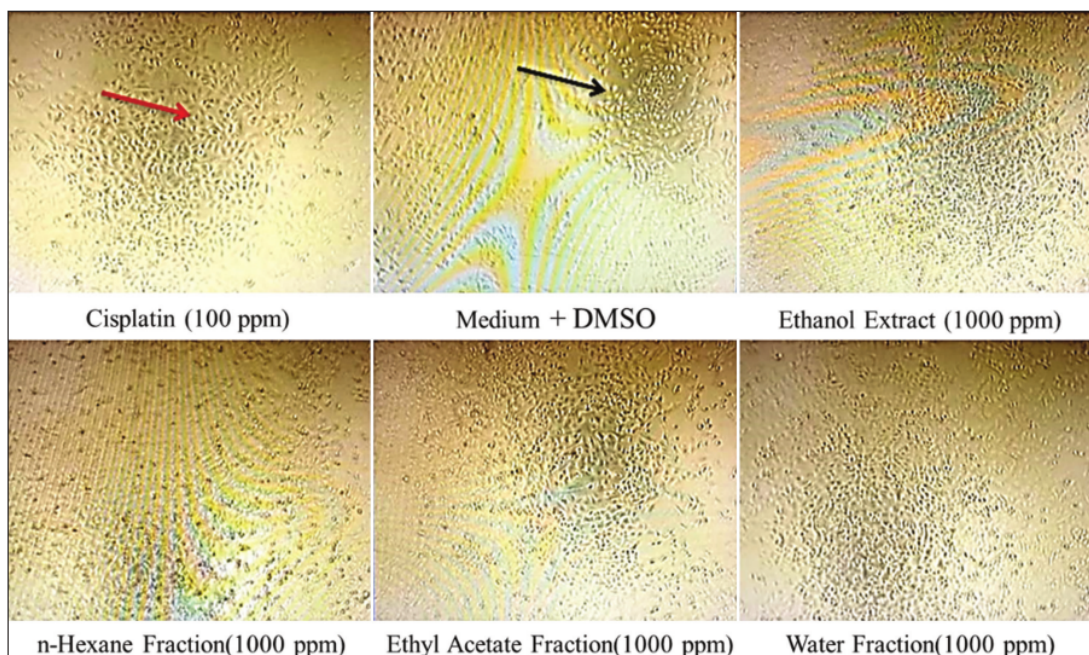


Figure 2: A549 lung cancer cell morphology after exposure to cisplatin, extract and fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd)

Conclusion

Based on the results of the study, ethanol extract, hexane fraction, ethyl acetate fraction, and water soluble fraction of *Sesewanua* (*C. fragrans* [Vent.] Willd) leaves (*C. fragrans* [Vent.] Willd) have anticancer activity on A549 lung cancer cells. The value of IC₅₀ proliferation of A549 lung cancer cells respectively ethyl acetate fraction is lower than hexane fraction, then IC₅₀ value of hexane fraction is lower than water fraction, and IC₅₀ value of water fraction is lower than ethanol extract. The smallest IC₅₀ value is indicated by ethyl acetate fraction (191, 165 ppm), which is categorized as moderately active.

Ethical Approvals

This research met the ethical requirements to be carried out from the Ethics Committee of Politeknik Kesehatan Kementerian Kesehatan Manado, Indonesia.

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