

How to Cite:

Barung, E. N. ., Kalonio, D. E., Banne, Y., & Kambuno, N. T. (2022). Anticancer activities of sewewanua leaf extracts (*Clerodendrum fragrans* (Vent.) Wild) againts A549 lung cancer cell. *International Journal of Health Sciences*, 6(S5), 7328–7337.
<https://doi.org/10.53730/ijhs.v6nS5.10383>

Anticancer activities of sewewanua leaf extracts (*Clerodendrum fragrans* (Vent.) Wild) againts A549 lung cancer cell

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Abstract---Cancer is one of the leading causes of non-communicable diseases in the world. In 2015, about 8.8 million cancer deaths worldwide and 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 still has many limitations. 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). 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*. The results showed that ethanol 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*. Further research on the anticancer activity of ethanol extract, hexane fraction, ethyl acetate fraction and water soluble fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) *in vivo* is our recommendation.

Keywords---*Sesewanua* Leaves, Anticancer, MTT Assay.

Introduction

Cancer is one of the leading non-communicable diseases causing death worldwide. In 2015, cancer caused the deaths of 8.8 million people worldwide (WHO 2017). In Indonesia, the prevalence of cancer in all age groups in 2013 was 1.4 ‰ and specifically for North Sulawesi Province, at 1.7 ‰ above the national average (Risksdas 2013). In sequence, the five highest types of cancer that cause death are lung, liver, colorectal, stomach and breast cancer (WHO 2017)

Lung cancer is the most common type of cancer and the highest cause of death in the global population. Data in 2012, lung cancer is the type of cancer that mostly affects men (16.7%) and third in women (8.7%). Lung cancer also causes the most deaths in men (23.6%) and second in women (13.8%) (Forman & Ferlay 2014). Recorded in 2015, lung cancer caused the death of 1.69 million people from a total of 8.8 million cancer deaths worldwide (WHO 2017).

The choice of lung cancer therapy depends on the type, stage, health, and age of lung cancer patients (Bandyopadhyay *et al.* 2015), which can be conventional therapy (surgery, platinum-based chemotherapy and radiotherapy) or molecular targeted therapy (Cagle & Chirieac, 2012; Toschi *et al.*, 2017; Waqar & Morgensztern, 2017). Reportedly, the 5-year survival rate in lung cancer patients receiving conventional therapy is 18% and lower than most other types of cancer (Cagle & Chirieac, 2012; Siegel *et al.*, 2017). 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 (Cagle & Chirieac 2012; Waqar & Morgensztern 2017). It is therefore important to explore new sources of anticancer activity, including those from plants.

Plants since ancient times have been used in cancer treatment and become a source of anticancer compounds with various mechanisms of action (Cragg & Newman 2005; Ashraf *et al.* 2016). One 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* leaves as a antiinflammation and antipyretic medication (Arini & Kinho 2015). There is no adequate scientific information about the activity of *Sesewanua* plants as anticancer. Kalonio *et al.*

(2017) report that there are 12 plants from the genus *Clerodendrum* that have been shown to have *in vitro* and *in vivo* anticancer activities.

Sesewanua leaves are reported to contain beta-sitosterol, clerosterol, daucosterol, caffeic acid, acteoside, leucoseptoside A, kaempferol, and 5,4'-dihydroxy-kaempferol-7-O-beta-rutinoside (Gao *et al.*, 2003). 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 (Gogoi *et al.*, 2017). Kaempferol can inhibit the growth of pancreatic cancer cells Miapaca-2, Panc-1, and SNU-213 (Lee & Kim 2016). Whereas beta-sitosterol and daucosterol compounds in benzene extract of leaf *Grewia tiliaefolia* showed anticancer activity in A549 lung cancer cells (Rajavel *et al.*, 2017). These things can illustrate that the *Sesewanua* leaves are thought to have potential as anticancer.

This study aims to evaluate the anticancer activity of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) extracts and fractions against A549 lung cancer cells using the method *MTT Tetrazolium Assay* (Abdulah *et al.* 2009; Riss *et al.* 2013), and determine the 50% inhibition concentration of it.

Methods

***C. fragrans* Leaves Collection and Processing**

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

Extraction and Fractionation.

Sesewanua leaves powder (*Clerodendrum fragrans* [Vent.] Willd) was extracted by maceration method using 70% ethanol solvent ((Depkes RI 2008). The extraction results were obtained ethanol extract with a yield% of 27.8%. Fractionation of ethanol extract of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) using liquid-liquid extraction method using solvents, respectively n-hexane and ethyl acetate (Zhao *et al.* 2009 with minor modifications). Fractionation results 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)

Method Preliminary toxicity tests with the BSLT method were carried out according to the method stated in Meyer *et al.* (1982) and Kumar *et al.* (2014). Eggs of *Artemia salina* hatched in artificial seawater (NaCl 3.8%) for 48 hours 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. Used as a control solution of 50 µl / 5 ml of artificial seawater. A total of 10 nauplii were added to the sample solution and control. After 24 hours, the number of dead nauplii was observed and the percentage of death was calculated. Calculated values of LC₅₀ using a regression analysis between log concentrations (x) and probit values (y) with computer programs [online](https://www.researchgate.net/publication/338111111) available on the site

<https://www.aatbio.com/tools/linear-logarithmic-semi-log-regression-online-calculator>

Testing Anticancer Activity Using the MTT Tetrazolium Assay Method

A total of 50 μ L of cell suspension in RPMI-1640 medium with a density of 5×10^3 /well (Lee *et al.* 2014) was inserted into a 96-well plate and incubated at 37°C in an incubator flowed CO₂ 5% for 24 hours. Furthermore, cell cultures were treated each with extracts and fractions (concentrations of 1000; 100; 10; 1; 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 hours. After the incubation period, 10 μ L CCK-8 was added to each well and pre-incubated for 3 hours. After adding 100 μ L of 0.1 N HCl the absorption was measured at a wavelength of 450 nm and a reference wavelength of 650 nm (Abdulah *et al.* 2009). The percentage (%) of cell proliferation inhibition was calculated using the equation (Florento *et al.* 2012):

$$\% \text{ Proliferation Inhibition} = \left(1 - \frac{\text{absorbance of experimental well}}{\text{absorbance of negative control well}} \right) \times 100$$

Data Analysis

The 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

Preliminary Toxicity Test with BSLT Method

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

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* leaves (*Clerodendrum fragrans* [Vent.] Willd) larvae *Artemia salina*

No.	Treatment	Concentration (μ g/ml)	% Mortality of Larvae	Probit	LC ₅₀ (μ g/ml)
1	Control	-	0,00	0	--
2	Ethanol Extrac	10	3,33	3,12	60,26
		100	76,67	5,74	
		1000	100,00	8,09	
3	n-Hexan Fraction	10	36,67	4,67	33,88
		100	96,67	6,88	
		1000	100,00	8,09	
4	Ethyl Acetate	10	86,67	6,13	26,92

	Fraction	100	93,33	6,48	
		1000	100,00	8,09	
5	Water Soluble Fraction	10	10,00	3,72	
		100	33,33	4,56	186,21
		1000	83,33	5,95	

Data in Table 1 shows that the values of LC₅₀ extracts and fractions of *Sesewanua* leaves are sequentially ethyl acetate fraction <n-hexane fraction <ethanol extract <water fraction. According to Meyer *et al.* 1982, plant extracts were considered toxic if they had LC₅₀ <1000 ppm, based on Table 1 extracts and fraction of *Sesewanua* leaves were toxic, so that the potential for further investigation as anticancer drugs.

Anticancer Activity Test with MTT Tetrazolium Assay Method

Activity test results of extract, n-hexane fraction, ethyl acetate and water fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) by method *MTT Tetrazolium Assay* (Meyer et al. 1982; Kumar et al. 2014) can be seen in Figure 1.

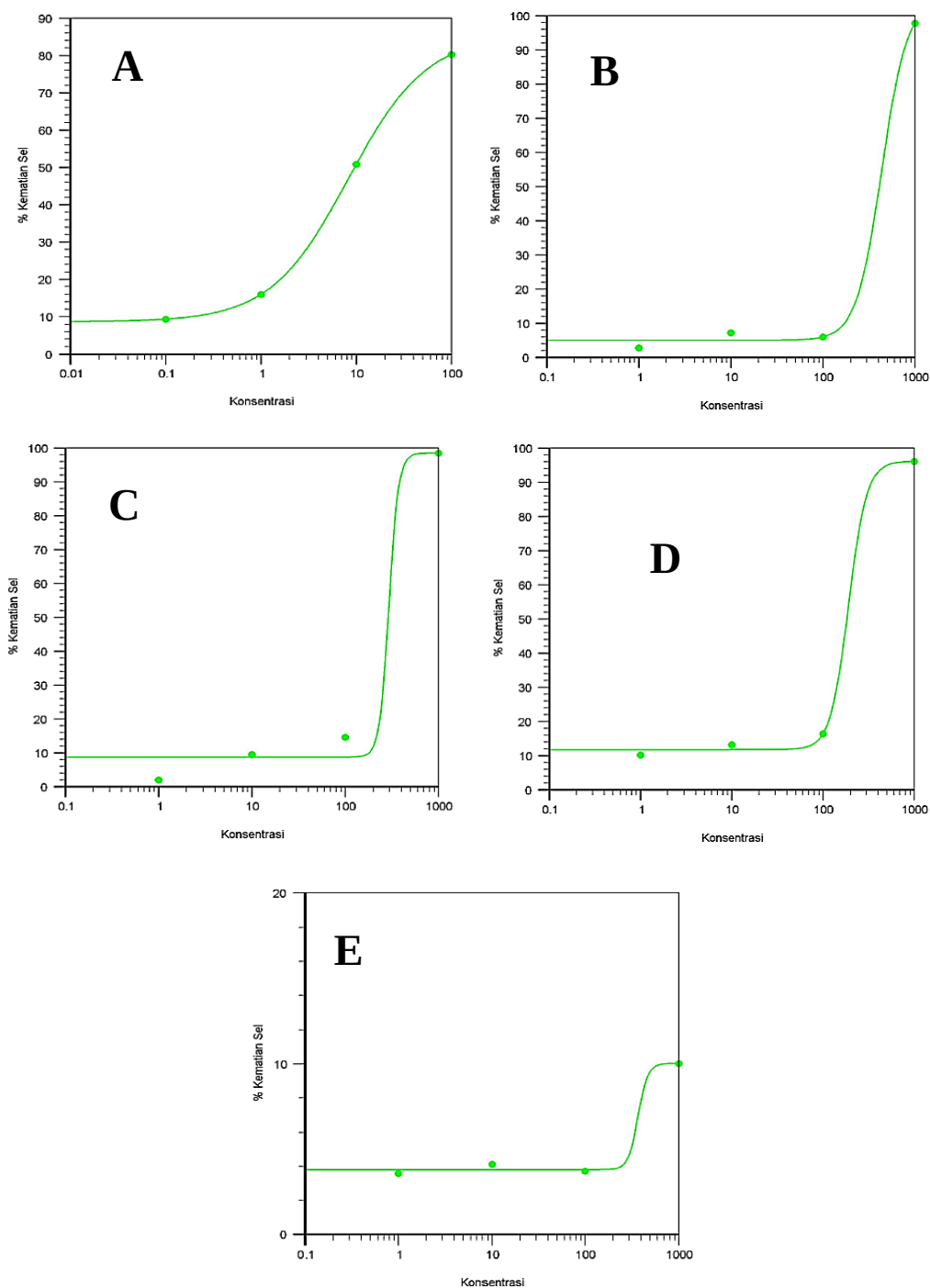


Figure 1. The relationship between the percentage (%) of proliferation inhibition (y) and concentration (x) administration of ethanol extract (A), n-hexane (B), ethyl acetate (C), water (D) of *Sesewanua* leaves (<https://www.aatbio.com/tools/ic50-calculatorCalculation>)

Results using computer program online available on the site <https://www.aatbio.com/tools/ic50-calculator> as can be seen in Figure 2, the IC_{50} 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. Based on the NCI criteria and Geran *et al* modified by Srisawat *et al.* (2013) which states that extracts with $IC_{50} \leq 20$ ppm have high activity; IC_{50} 21-200 ppm, moderate activity; IC_{50} 201-500 ppm, weak activity; and $IC_{50} > 500$ ppm is not active, then the ethyl acetate fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) can be said to have moderate anticancer activity. Cisplatin as lung cancer first-line chemotherapy (Bandyopadhyay *et al.* 2015; KPKN 2016), from the results of this study has a high activity because it has IC_{50} 8,294 ppm.

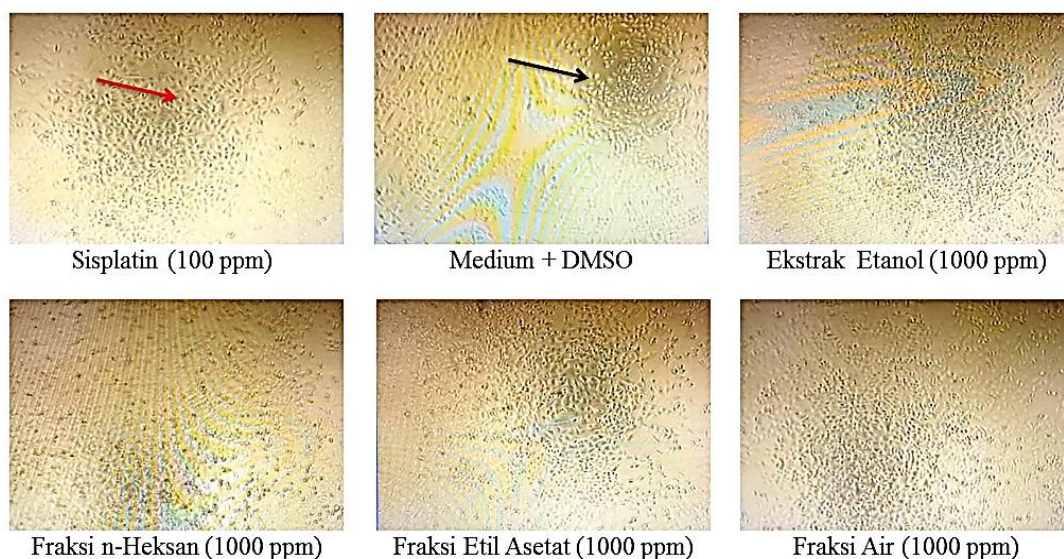


Figure 2. A549 lung cancer cell morphology after exposure to cisplatin, extract and fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd).

In Figure 3, we can observe changes in A549 lung cancer cell morphology after exposure to cisplatin, extracts and fractions of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd). Viable cancer cells line are characterized by the formation of a monolayer adherent cell layer on the base surface of cell culture tubes (Foster *et al.* 1998; Shanmugapriya *et al.* 2016), 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 (Majno & Joris 1995; Muniaraj *et al.* 2018). 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 (Dasari & Tchounwou 2014). To determine the mechanism of cancer cell death due to exposure to extracts and fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) still need further research.

Kalonio *et al.* (2017) suggested that there were 12 plants from the genus *Clerodendrum* having anticancer activities both *in vitro* and *in vivo*. Some plants

from the Lamiaceae family such as *Pogostemon heyneanus*, *Plectranthus amboinicus* (Rai *et al.* 2016), and *Ocimum sanctum* (Iqbal *et al.* 2017), are also reported to have anticancer activities. The research data supports the results of the current study that extracts and fractions of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) have potential as anticancer in A549 lung cancer cells.

The content of phytochemical compounds in plants has contributed to its pharmacological activity. *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) are reported to contain flavonoids such as kaempferol (Gao *et al.* 2003), these compounds reported in other studies have anticancer activity in Miapaca-2, Panc-1, and SNU-prostate cancer cells 213 (Lee & Kim 2016). Steroid compounds such as beta-sitosterol and daucosterol, as contained in the plant *Grewia tiliaefolia*, showed anticancer activity in A549 lung cancer cells (Rajavel *et al.* 2017). Some of these data support the results of this study that extracts and fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) have potential anticancer activity.

Conclusion

Based on the results of the study it can be concluded that ethanol extract, hexane fraction, ethyl acetate fraction and water soluble fraction of *Sesewanua* leaves (*Clerodendrum fragrans* [Vent.] Willd) have anticancer activity on A549 lung cancer cells. The value of inhibition concentration of 50% (IC50) proliferation of A549 lung cancer cells respectively ethyl acetate fraction <hexane fraction <water fraction <ethanol extract. The smallest IC50 value is indicated by ethyl acetate fraction (191,165 ppm), which is categorized as moderately active.

Acknowledgments

This study was funded by the Manado Health Polytechnic through Riset Pembinaan Tenaga Kesehatan Grand skema Hibah Bersaing in 2018.

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