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Assessing cytotoxicity and antiproliferation effects of *Sida rhombifolia* against MCA-B1 and A549 cancer cells

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ABSTRACT

This research aims to determine the cytotoxicity and antiproliferation activities of *Sida rhombifolia* leaves extract against cancer cells MCA-B1, A549, and normal Vero cells. *Sida rhombifolia* leaves were extracted with ethanol using ultrasonication method and fractionated using n-hexane, ethyl acetate, and water. The tested samples were ethanol extract and n-hexane fraction based on the results of cytotoxicity using the Brine Shrimp Lethality Test. The antiproliferation activity test by using Trypan Blue Dye method and the cells harvested after confluence on the third or fourth day and the total cells were calculated by using the Neubauer Hemocytometer. The result showed that the inhibitory activity of ethanol extract at a concentration of 500 ppm is 69.44% with IC_{50} 202.556 ppm on MCA-B1 cancer cells and 69.44% with IC_{50} 276.836 ppm on A549 cancer cells, while the n-hexane fraction at a concentration of 1,000 ppm was 64.13% with IC_{50} 425.969 ppm in MCA-B1 cancer cells and 57.18% with IC_{50} 786.617 ppm on A549 cancer cells. After being tested on normal Vero cells, the inhibition of normal Vero cells proliferation is not more than 1%. This indicates that ethanol extracts and n-hexane fraction are safe for normal cells and analysis by using LC-MS/MS showed a benzazepine compound in the ethanol extract of *S. rhombifolia* is known for its role as antiproliferation. These results indicate that *S. rhombifolia* leaves extract has the potential to be developed as anticancer compounds..

1. INTRODUCTION

Cancer is a disease characterized by uncontrolled cells growth, which has the ability to infiltrate and damage healthy cells in the body that turn into cancer cells. In the process, the cancer cells will continue to evolve with rapid, uncontrolled, keep splitting, and breaking into the surrounding tissues so that it can cause death. Cancer sufferers in Indonesia keep increasing every year, the survivors with age above 40 years have a higher risk. Cancer is not contagious and generally not declining genetically [1]. The cancer is often known by the community as the tumor, whereas not all the tumors are cancerous. The tumor is usually any abnormal lumps. Tumors are divided into two groups, namely, the benign tumor and malignant tumor. Cancer is a generic term for all the types of malignant tumors. Cancer can afflict any one, in every part of the body, and on all of the priesthood. Healing and its treatment are very expensive [2].

Cancer treatments are medically continued treated with surgery, radiotherapy, chemotherapy, and drugs can cause a lot of side effects. External factors that can cause cancer, include radiation, radicals, ultraviolet rays, virus, infection, smoking, and chemicals from food, while the internal factors that cause cancer, include congenital or genetic factors, hormonal factors, psychological factors, and immune [3]. Only a few types of cancer that can be treated, especially if it left untreated while still in an early stage, cancer is also synonymous with expensive medical expenses. Through several studies, it is known that there are many different types of plants as anticancer drugs that are present around us as well as more accessible [1].

Biological natural ingredients derived from plants, animals, and microorganisms have been widely used for human needs, including drugs. Indonesian people are still using herbs for traditional medicine which is often referred to as Herbalism [4]. Indonesia is the second largest Center of biodiversity or biological diversity

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in the world, approximately 80% of the plant resources of tropical forests found in Indonesia [5]. Biodiversity, in General, can also be defined as the diversity of secondary metabolite compounds. Secondary metabolite compounds have more than one functional group so the plants showed a lot of usability and bioactivity because they can interact with more than one target molecule [6]. One of the plants that have promising potential is *S. rhombifolia*. The plant is a shrub that is often found on the roadside. *Sida rhombifolia* included in the family *Malvaceae* that can be grown in the tropical areas, both in the Highlands and lowlands. *Sida rhombifolia* plant can reach a height of 2 m research [7] point out that *S. rhombifolia* plants contain chemical compounds, alkaloids, calcium oxalate, saponins, tannins, phenols, amino acids, essential oils, steroids, phlegmatic, and lubricant substance. Part of *S. rhombifolia* that could be used are leaves, stems, and roots. The compound contained in *S. rhombifolia* plant can be used to cure some diseases, such as scabies, remedies cure ulcers, prevent rheumatism, overcome disease intestinal worms, overcoming diarrhea, kidney stones, and gout.

Pharmacological research on this plant has proven its potential as a bitter taste [8], antibacterial [9], antioxidants [10], analgesics [8], antigout [11], and a hepatoprotector [12]. The antioxidant power of ethanol extract from roots, stems, leaves, and all the parts of the *S. rhombifolia* also have been evaluated through research conducted [10]. The hyperuricemia effect on the male mice by administering ethanol extracts of *S. rhombifolia* leaves at the doses of 50, 100, 200 mg/kg body weight gave results which do not differ significantly by administering allopurinol dose of 10 mg/kg [13]. Acute cytotoxicity test of *S. rhombifolia* leaves has also been performed using a water extract of *S. rhombifolia* given to white mice as a trial on animals. Water extract of *S. rhombifolia* leaves yield a value of 8.5 g/kg of LC_{50} [7]. Based on some of the data above, there has been no testing of cytotoxicity and antiproliferation tests in MCA-B1 and A549 cancer cells. Therefore, it is necessary to conduct cytotoxicity and antiproliferation tests on MCA-B1 and A549 cancer cells as an alternative medicine for natural ingredients that have no side effects on health.

2. MATERIAL AND METHOD

2.1. Plants Material

Sida rhombifolia leaves were collected in Center for the study Biopharmaceutical, Bogor, Indonesia, on February 2018.

2.2. Extraction

Sida rhombifolia plants are washed using running water and dried in the oven with a temperature of 40°C–45°C for 24 hours and *S. rhombifolia* leaves made into 40–60 sized mesh powder using a grinder. Dried leaves powder *S. rhombifolia* leaves extracted by using the ultrasonication method, 50 g simplicia inserted into the beaker and then added 500 ml ethanol 70%. The extract then sonicated at a temperature of 25°C with an ultrasonic frequency of 20 kHz for 25 minutes. The extract of sonication was separated with its filtrate and the filtrate which still contains solvent was concentrated with evaporator at a temperature of 40°C.

2.3. Brine Shrimp Lethality Test (BSLT)

BSLT was carried out to investigate the cytotoxic activity of the extracts and fractions. Brine shrimp (*Artemia salina*) larvae were placed and hatched for 48 hours with constant oxygen supply under the light at room temperature (25°C). The extract (10 mg) was transferred into a test tube containing 50 µl of Tween80 in 10 ml of seawater to produce a stock solution (1,000 ppm). The concentration of extracts was 10, 100, 500, and 1,000 ppm, and experiment for each extract was carried out at triplicates. The surviving larvae were counted after incubation under TL light 14 W for 24 hours. The similar procedure (without adding extracts) was applied as a negative control. The larvae with no movements for 10 seconds were considered dead, while toxicity against larvae was set at LC_{50} less than 1,000 ppm [14]. LC_{50} was determined using SPSS 16.0 for probit analysis at a confidence interval of 95%.

2.4. Antiproliferation Activity Test

Antiproliferation activity was determined using a protocol of [15]. *In vitro* experiment was arranged using MCA-B1 and A549 cancer cells lines obtained from Laboratory of Tissue Culture, Department of Veterinary Clinic, Reproduction, and Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia. The cells lines were cultured in the 24-well microplates at a density of 7×10^5 cells/ml and cultured in Modified Eagle's Medium (DMEM) Dulbecco supplemented with Fetal Calf Serum 10%, antibiotic gentamicin 10%, and fungizone 10%. The extracts were dissolved in a tube containing 100 µl dimethyl sulfoxide in sterile distilled water. Each concentration of extract was added to each well and carried out three repetitions. The concentrations of the extract tested were ethanol extract 500, 400, 300, 200, and 100 ppm, while the extract of n-hexane fraction was 1,000, 800, 600, 400, and 200 ppm. DMEM media were used as negative control, while doxorubicin (100 ppm) was used as positive control. The cancer cells lines were incubated at CO_2 5% and 37°C. The cells were harvested after 3–4 days when the cells grown in the negative control was confluent. They were then stained using Trypan Blue Dye 80:20. The number of cells in each well was counted under the light microscope on 100× magnification using Neubauer Haemocytometer.

2.5. The Identification of Active Compounds Method of LC-MS/MS

The identification of active compounds using an instrument of LC-MS/MS-based methods of Kapitan [16] that have been modified. As many as 5 µl sample solution with a concentration of 1,000 µg/ml instrument inserted into the LC-MS/MS with a flow rate of 0.20 ml/minute. Motion phase used was a mixture of Acetonitrile and water, while the silent phase used is column C18. The time analysis done for 22 minutes at a temperature of 40°C separation.

2.6. Statistical Analysis

Data were evaluated using one-way analysis of variance (ANOVA) at $\alpha = 0.05$. The Significance of their variance was verified using Duncan's test.

3. RESULTS AND DISCUSSION

3.1. Brine Shrimp Lethality Test (BSLT)

Cytotoxicity test is a screening of active compounds contained in plant extracts and carried out as a preliminary test before proliferation inhibition test. BSLT was applied to evaluate the toxicity of *S. rhombifolia* extracts that potentially showed antiproliferation activity against cancer cells, and as a promising candidate of anticancer compound drugs [17]. BSLT test results are also known as a method of screening for anticancer compounds from the plants. It means that the higher levels of secondary metabolites in plant toxicity BSLT, represented with an LC_{50} value is getting smaller, then the more potential of the plant to use in anticancer treatment [15]. The observations showed that the mortality rate of larvae of *A. salina* at different concentrations of ethanol extracts and n-hexane fraction, ethyl acetate and water of *S. rhombifolia* leaves, the LC_{50} value and result test of BSLT have a different average number of deaths and deaths percentage (Table 1).

Based on the results of BSLT test (Table 1), it is noted that various concentration ethanol of extract, n-hexane fraction, ethyl acetate fraction, and water against concentration of larvae showed the different influence against death. Data in Table 1 show that the ethanol extract, n-hexane fraction, ethyl acetate fraction, and water are toxic against *A. salina* or less than 1,000 ppm, extracts that have the lowest LC_{50} value is ethanol extract (336.156 ppm) as compared with the other extracts. These data suggest that ethanol fraction extract can be potential as anticancer. The research of Priosoeryanto [15] also reported that an extract showing the activity of cytotoxic in the BSLT if the extracts can cause the death of 50% of the test animals at concentrations of less than 1,000 ppm.

The study indicates that ethanol extracts and n-hexane fraction have very high cytotoxic activity, so might contain suitable secondary metabolite compounds, and the identification of the available components also contained anticancer compounds. Furthermore, testing of antiproliferation activity on the MCA-B1 and A549 cancer cells as well as on normal Vero only focused on ethanol extracts and n-hexane fraction. This is in line with the results reported [18]. The control results by sea water (mortality 0%) indicating the larvae are dying due to toxic compounds in the extracts other than those factors. According to Cahyadi [19] alkaloids and flavonoids, compounds can act as stomach poisoning. Therefore, when such alkaloids and flavonoids compound enters into the body of the larva then it's digestive tract will be disturbed. In addition, these compounds inhibit the receptors of taste in the mouth area of the larvae. The larvae so may fail to taste stimulus consequently it is not able to recognize the food, this leads to starvation of the larvae to death. According to Prabowo [20] reported that stomach toxins affect the metabolism of larvae after consuming poison. Toxins will enter into the body and circulated alongside the blood. Blood-borne toxins that would affect the larval nervous system and then will cause death.

Table 1: Cytotoxic activity of *S. Rhombifolia* extracts and fractions against brine shrimps.

Sample	Lethal concentration (LC50) (ppm)
Ethanol 70% extract	336,156
n-hexane fraction	600,101
ethyl acetate fraction	680,067
Water	973,211
Control	00

3.2. Antiproliferation Activity Test

After the Lethal Concentration (LC_{50}) is known, research continued with the growth inhibition of proliferation test. LC_{50} value of ethanol extracts and n-hexane fraction was used as a test to acquired antiproliferation activity, i.e., ethanol extract 336.156 ppm and n-hexane fraction 600.101 ppm in the middle as a value in the variation of the concentrations for testing antiproliferation activities on MCA-B1 and A549 cancer cells. Determination of the number of cells which survive on cytotoxicity test is performed based on the parameters of membrane damage which was done using the Trypan Blue. If the cancer cells are dead, Trypan Blue will damage the membranes and it will enter into the cells and bind to the protein cells and cells will appear blue. In living cells, because their plasma membrane is still intact then the proteins in the cells will not bind with the Trypan Blue and the cells will be bright.

The data (Figs. 1 and 2) on this research suggests that the influence of ethanol extract and the n-hexane fraction *in vitro* rates can lower the growth of MCA-B1 and A549 cancer cells compared to negative control. This is supported by statistical data using a

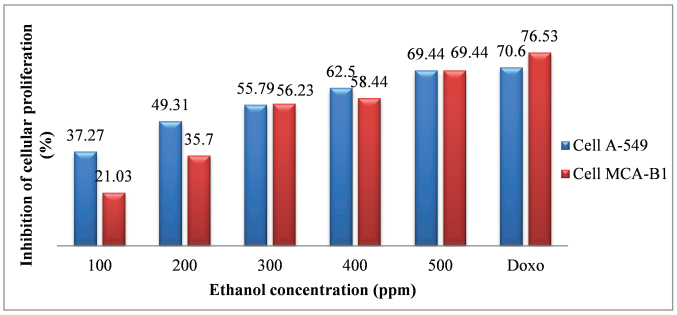

Figure 1: Inhibitory activity of MCA-B1 and A549 cancer cells proliferation by ethanol extract. The data are expressed as a percentage of proliferation inhibition, as compared to the negative control (100%). Level of significance is denoted as follows: $p < 0.05$.

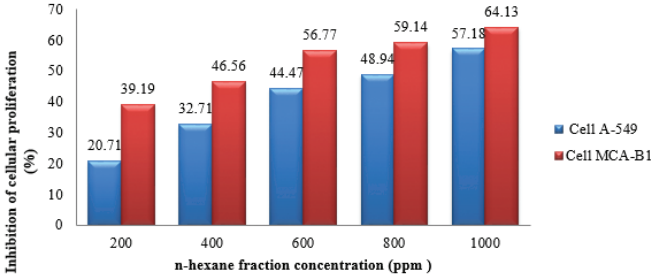

Figure 2: Inhibitory activity of MCA-B1 and A549 cancer cells proliferation by n-hexane fraction. The data are expressed as a percentage of proliferation inhibition, as compared to the negative control (100%). Level of significance is denoted as follows: $p < 0.05$.

one-way ANOVA analysis that crude extract and the n-hexane fraction significantly ($p > 0.05$) can inhibit the growth of these two types of cancer cells. Ethanol extract can inhibit growth and proliferation of MCA-B1 cancer cells 69.44% as much with the presentation of the value of the Inhibitory Concentration (IC_{50}) value of 202.556 ppm and on A549 cancer cells 69.44% as much with IC_{50} of 276.836 ppm concentration 500 ppm compared to the positive control Doxorubicin that is able to inhibit 70.56%, while in the n-hexane fraction looks different on the second inhibitory activity percentage of cancer cells. On MCA-B1 cancer cells, 64.13% as much with IC_{50} values of presentation 425.969 ppm and on A549 cancer cells 57.18% as much with IC_{50} of 786.617 ppm concentration of 1,000 ppm. The percentage of cancer cell proliferation inhibition increased with increasing concentration of ethanol extract, but there was a difference in percentage inhibition in both types of cancer cells. This data is in accordance with that reported [21] that anticancer activity of extracts depends on the type of cancer cells used. Ethanol extract has good antiproliferation activity against MCA-B1 cancer cells compared to A549 cancer cells; this is presumably because MCA-B1 cancer cells are more sensitive to ethanol extract and S. *rhombifolia* n-hexane fraction than A549 cancer cells. The data showed (Figs. 1 and 2) that ethanol extracts and n-hexane fraction antiproliferation activity inhibits more at the MCA- B1 cancer cells as compared with A549 cancer cells, so MCA-B1 cancer cells are more sensitive to the experimental extract than A549 cancer cells.

In addition, the research is also done by granting ethanol extract, n-hexane fraction and doxorubicin against a proliferation of normal Vero cells. Normal Vero cells cytotoxicity test is performed manually (direct calculation) and counts the number of living cells than control. Normal Vero cells were first isolated from the kidneys of African Green Monkey healthy adults on 27 March 1967 by T. Yasamura and T. Kawabata of the University of Chiba, Japan [22]. The data in Figure 3 showed that the percentage of inhibition of normal Vero cells proliferation is not more than 1%. This indicates that ethanol extracts and n-hexane are safe against normal cells. Based on the Ordinance of the National Cancer Institute NCI 2001 in [23] that an extract of the active anticancer activity expressed in value $IC_{50} < 30$ µg/ml, moderate active in value $IC_{50} \geq 30$ µg/ml, and is said to be inactive when the value of the $IC_{50} > 100$ µg/ml. Figure 3 also shows the presence of inhibitory activity of normal Vero cells proliferation to be 45.93% by doxorubicin. It is clear that conventional chemotherapy drugs are not safe and are toxic in normal cells.

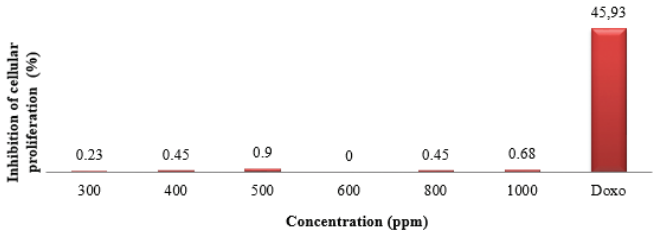

Figure 3: The percentage inhibition of the cells proliferation of normal Vero cells after administering ethanol extracts and n-hexane fraction.

According to Wang et al. [24] reported that doxorubicin can induce apoptosis in normal cells of cattle aortic endothelium. Doxorubicin induces apoptosis in normal endothelial cells through a mechanism mediated by reactive oxygen species (ROS) such as H2O2 and is largely independent of p53 activation. The p53 protein is encoded by the TP53/p53 gene and has a molecular weight of around 53 kilo Dalton. This gene has 11 exons and has a total sequence of 20 pb. p53 protein regulates both repression and activation of a number of downstream genes that play a vital role in cell responses to environmental stress, genotoxic effects, such as alteration of DNA caused by UV radiation, carcinogens, cytotoxic chemotherapy drugs, and damage to microtubules and loss of intercellular contact [25]. Another function of p53 is that it induces growth retention or activates 14-3-3σ (for holding in G2 position), induces apoptosis, and on the other hand mediates the activation of Bax and genes involved in producing reactive oxygen.

3.3. The Active Compounds of Ethanol Extract of *S. rhombifolia*

Ethanol extract of *S. rhombifolia* was analyzed using LC-MS/MS to identify the active compounds contained in the leaves of *S. rhombifolia*. Analysis of LC-MS/MS was performed on ethanol extract which was the best extracts that were able to inhibit the growth of MCA-B1 and A549 cancer cells and did not damage normal cell. The results of the analysis of chromatogram LC-MS/MS *S. rhombifolia* is presented in Figure 4.

Results of the analysis (Table 2) by using LC-MS/MS can be suspected that chemical components contained in metabolites largely determine anticancer properties of *S. rhombifolia* leaves in ethanol extracts. Allegedly, 22 compounds were suspected having anticancer potential. Benzazepine compounds had the highest peak allegedly had a role in inhibition of the proliferation of cancer MCA-B1 and A549 cells. One of the compound from LC-MS/MS analysis alleged had strong contribute as antiproliferation was benzazepine from alkaloid group that appeared on retention time 8.49 seconds. Chromatogram spectrum results in that retention time allegedly showed a great similarity with benzazepine compounds with 99.99% similarity percentage with the highest peak of which was suspected of having a role in the inhibition of the proliferation of MCA-B1 and A549 cancer cells. Reported that benzazepine caspase substrates can inhibit proliferation of leukemia cancer cells HL-60 *in vitro* independently through apoptosis [26], so that

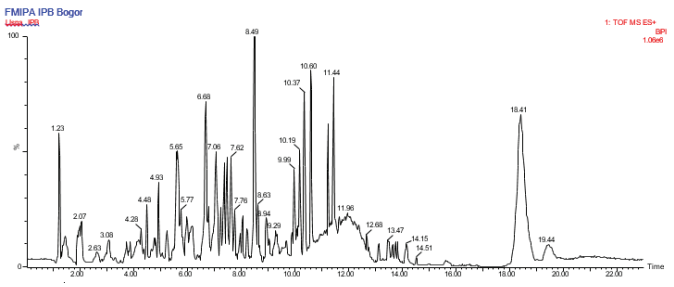

Figure 4: Chomatogram LC-MS/MS ethanol extrac romatogram of *Sida rhombifolia*

Table 2: The active compounds of ethanol extract S. *rhombifolia*.

Pick	Retention time (minutes)	Molecular weight	Compounds name	Similiarity (%)
1	1.47	123.0917	4-Dimetillaminopiridin	99.30
2	2.07	136.0619	Adenin	99.51
3	2.63	150.0911	P-Dimetilaminobenzaldehid	100
4	3.08	96.0448	2-Piridon	99.99
5	3.88	194.1185	Butamben	47.03
6	4.28	146.0601	Benzoilacetonitrile	95.32
7	5.25	350.1593	Erucifoline	38.24
8	5.65	427.2828	Scopadiol	99.93
9	5.77	497.3117	Muristerone A	94.92
10	7.06	465.3215	(2alpha,3beta,5alpha,6beta,14xi)-Spirostan-2,3,5,6-tetrol	94.87
11	7.76	237.1849	Butilated hydrosimetilphenol	99.96
12	8.49	256.1336	Benzazepin	99.99
13	8.63	221.1902	Butilated hidrositoluene	99.96
14	8.94	431.3146	Hecogenin	94.59
15	9.29	272.2213	6-(nonanoilamino)asam hesanoik	99.83
16	9.99	353.2301	Dinoprostone	98.02
17	10.37	284.1283	p-Coumaroiltiramine	99.47
18	10.60	403.1397	Nobiletin	81.30
19	11.24	373.1281	Sinensetin	73.46
20	13.47	121.0393	Benzofurazan	100
21	14.15	123.0915	2,3-Diamonotoluene	99.81
22	19.44	141.1132	Metenamine	99.97

it can serve as a basis in the development of the ethanol extracts of *S. rhombifolia* as herbal antiproliferation ingredients.

4. CONCLUSION

Ethanol extracts and n-hexane fractions have antiproliferation activity against MCA-B1 and A549 cancer cells *in vitro*. Antiproliferation activity increase due to the increase in the concentration of the extract. *Sida rhombifolia* leaves do not damage normal Vero cells and analysis by using LC-MS/MS showed benzazepine compound in ethanol extract of *S. rhombifolia* is known for its role as antiproliferation and it has the potential to be developed into one of the anticancer ingredients.

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