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Phytochemical Screening and Cytotoxicity Test of *Sidaguri* Leaf (*Sida rhombifolia* Linn.) Fractionated Extract with Brine Shrimp Lethality Test (BSLT) Method

Skrining Fitokimia Dan Uji Sitotoksisitas Ekstrak Fraksinasi Daun Sidaguri *(Sida rhombifolia Linn.)* dengan Metode *Brine Shrimp Lethality Test*

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ABSTRACT

Traditional medicine can be an alternative in the treatment of cancer. One of the traditional medicines used as herbal ingredients that have the potential to be anticancer is sidaguri (Sida rhombifolia Linn.). The leaves of S. rhombifolia are plants belonging to the family Malvaceae. S. rhombifolia plants contain chemical compounds. This study aims to determine the chemical content of S. rhombifolia extract and potential cytotoxicity based on LC_{50} (Lethal Concentration 50) values in the death of nauplii Artemia salina Leach after administration of ethanol extract and *n*-hexane fraction, ethyl acetate and water. The method used S. rhombifolia leaves that have been in the form of simplisia were extracted with 70% ethanol solvent using the ultrasonication method. Then tested for the content of chemical compounds. Phytochemical screening results show that S. rhombifolia ethanol extract contains alkaloid compounds, flavonoids, saponins, tannins, and triterpenoids. The ethanol extract was further fractionated using solvents *n*-hexane, ethyl acetate and water. The cytotoxicity of S. rhombifolia extract was tested using the Brine Shrimp Lethality Test (BSLT) method using ethanol extract, *n*-hexane fraction, ethyl acetate fraction and toxic water fraction against Artemia salina or less than 1000 ppm, of the four test extracts that had the smallest LC_{50} value were ethanol extract which was 320.15 µg / ml and alkaloid compounds fractionated from ethanol extract of S. rhombifolia leaves *n*-hexane fraction 500.10 µg / ml, ethyl acetate 575.06 µg/ml and water 873.21 µg/ml. It was concluded that the results obtained that ethanol extract is more cytotoxicity compared to fractionation
Keywords : BSLT; Sidaguri; Cytotoxicity

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1. INTRODUCTION

Cancer is a disease that occurs due to uncontrolled and indefinite cell division (Ma'at, 2003). Cancer cells can spread to other parts of the body so that it can cause death (Anderson *et al*, 1991); Medical cancer treatment is usually handled with surgery, radiotherapy, chemotherapy and drugs that cause many side effects. External factors that can cause cancer, namely radiation, free radicals, ultraviolet light, viruses, infections, cigarettes and chemicals from food. While internal factors that cause cancer, namely genetic or congenital factors, hormonal factors, psychiatric factors, and immune (Utari *et al*, 2013). Only some types of cancer can be treated, especially if treated at an early stage, cancer is also synonymous with expensive treatment costs. Through several studies it has been known that there are various types of anticancer medicinal plants that are around us and easier to obtain (Mangan, 2009).

Bionatural materials derived from plants, animals, and microorganisms have been widely used for human needs including medicines. Indonesian people still use plants for traditional medicine commonly referred to as jamu (Lenny, Barus & Sitopu, 2010) Indonesia is the second largest center of biodiversity or biodiversity in the world, where about 80% of tropical forest plant sources are found in Indonesia (Handa, Rakesh and Vasisht, 2006). One plant that has quite promising potential is Sidaguri. This plant is in the form of a shrub that is often found on the roadside. Sidaguri belongs to the family *Malvaceae*, with the Latin name *Sida rhombifolia* this plant can grow in areas with tropical climates, both in the highlands and in the lowlands. The height *S. rhombifolia* can reach 2 meters. Research conducted by Mu'im & Hanani (2011) mentioned S. *rhombifolia* plants contain chemical compounds alkaloids, calcium oxalate, saponins, tannins, phenols, amino acids, steroids, essential oils. The content contained in S. *rhombifolia* plants is used for health, such as scabies, curing ulcers, preventing rheumatism, overcoming helminthiasis, overcoming diarrhea, kidney stones, gout. [8] Pharmacological research that has been conducted on this plant has proven its potential as an anti-inflammatory (Atsana, 2017), antibacterial (Dewi, 2012), antioxidant (Muammar, 2014).

The antioxidant power of ethanol extracts from the roots, stems, leaves, and all parts of the *S. rhombifolia* plant has also been evaluated through research conducted by (Rahman *et al*, 2011) Anti-inflammatory and antihyperuricemia effects were observed by looking at the inhibitory activity of Sidaguri on xanthinoxidase. In this study, simplisia powder was extracted successively by stratified maceration using four types of solvents based on polarity levels, namely petroleum ether, ethyl acetate, n-butanol and 96% ethanol. All extracts can inhibit xanthin oxidase activity with IC_{50} value of 1.71 µg/ml for n-butanol extract, ethyl acetate extract with IC_{50} 2.38 µg/ml, ethanol extract with IC_{50} 4.64 µg/ml, and in petroleum ether extract with IC_{50} 9.52 µg/ml (Lestari, 2012). The selection of the concentration is desirable in order to obtain linear results. This cytotoxicity examination uses the Brine shrimp lethality test (BSLT) method and uses *Artemia salina Leach nauplii*. This method is the beginning of the search for anti-cancer compounds because the results of toxicity tests have a positive correlation with anti-cancer cytotoxicity activity (Meyer *et al*, 1982). The use of the Brine shrimp lethality test (BSLT) method has several advantages compared to other methods, namely easy to do, cheap, fast, quite accurate, does not require aseptic conditions and is reliable (Dachrinus, Oktima & Stanias, 2005; Fajarningsih *et al*, 2006). The data will be analyzed with the SPSS 20.0 probit analysis

program to look for the relationship between the concentration of the test solution and the nauplii mortality response of Artemia salina Leach (Silva *et* al, 2007) and determine the IC_{50} value (Lethal Concentration 50). The tested extract can be said to have a toxic effect if the IC_{50} value is less than 1000 µg/ml [13].

2. MATERIALS AND METHODS

2.1. Determination S. rhombifolia

S. *rhombifolia* samples were determined at the Center for Tropical Biopharmaceutical Studies LPPM IPB, to determine the identity of the sample species.

2.2. Preparation sample

Preparation of S. rhombifolia based on modified method (Ardianti and Kusnadi, 2014). The leaves of S. rhombifolia are washed using running water, dried in an oven at 40-45°C and made a 40 mesh powder using a blender.

2.3. Determination of water content

Water content is determined based on the SNI 01-2891-1992 method. Empty porcelain dishes are dried in an oven at 105°C for 1 hour then cooled in a desiccator for 15 minutes and weighed with an analytical balance. A total of 1 gram of sample was put into a saucer and heated at 105°C for 3 hours and then cooled in a desiccator for 15 minutes and then weighed. The cup containing the sample is weighed and recorded in mass. The drying of the cup containing the sample is repeated until it reaches a constant mass (mass change of not more than 0.003 grams). The moisture content of the sample is calculated based on the following equation:

$$Water\ content\ (\%) = \frac{a-b}{a} \times 100\%$$

Information:
a = mass of cup and sample before drying (g)
b = mass of cup and sample after drying (g)

2.4. Sample Extraction

Extraction of S. *rhombifolia* was performed based on a modified method (Sasongko *et al*, 2017). First, S. *rhombifolia* is dried in the oven at 40°C for 2-3 days. The dry sample is then mashed using a blender. S. *Rhombifolia* simplisia of 50 grams each was put into a beaker and then added 70% ethanol as much as 500 mL of extraction using the ultrasonication method at 25°C with an ultrasonic frequency of 20 kHz for 25 min. The sonicated extract is filtered to separate the filtrate and its residue. The filtrate that still contains solvent is concentrated with an evaporator at 40°C. The yield of the extract obtained is calculated using the formula:

$$Yield = \frac{\text{mass of extract produced}}{\text{mass of simplicia extracted}} \times 100\%$$

2.5. Phytochemical screening

Determination of phytochemical content of samples is carried out based on the modified method (Setiyono & Bermawie, 2014).

Test alkaloids as much as 10 mg extract dissolved with 2 mL chloroform and 2 mL ammonia, filtrate filtered and accommodated. The filtrate obtained is then added 5 drops of concentrated H_2SO_4. The solution is whipped to form 2 layers. The top layers are then taken and tested with Meyer, Wagner and Dragendorf reagents respectively. The presence of alkaloids in the sample is indicated by the formation of white or yellow deposits in Meyer's reagents, brown to black deposits in Wagner reagents and orange deposits in Dragendorf reagents. Flavonoid test as much as 10 mg sample reacted with 10 mL of water then heated. The mixture is separated and the filtrate is fed with a powder of 0.1 Mg, 1 mL of 5 concentrated HCl and 1 mL of amyl alcohol. A positive test is characterized by the appearance of a red or yellow color on the amyl alcohol layer. Test saponins as much as 10 mg of extract is put into a test tube, then added 10 mL of hot water and then cooled. The

test solution is shaken vertically for 10 seconds, then observed for 10 minutes. The formation of foam 1.10 cm high indicates the presence of saponins in the sample. On the addition of 1 drop of HCl 2N foam does not disappear. The tannin test solution is prepared by reacting 10 mg of the sample with 5 mL of aquades, then heated to boiling for 5 minutes and the filtrate is filtered. A total of 5 mL of test solution is inserted into the test tube, then a few drops of $FeCl_3$ are added. The formation of dark blue or greenish-black indicates the presence of tannins. Test terpenoids and steroids as much as 10 mg extract added 0.5 mL chloroform, 0.5 mL acetic anhydride, and dripped with concentrated sulfuric acid through the tube wall. The result at the second boundary of the solution is formed brownish-red or purple cicin indicates the presence of terpenoids and the result of the solution at the top becomes green or purple indicating the presence of steroids or triterpenoids.

2.6. Fractionation of extracts

Fractionation is carried out on the basis of a modified method (Salni, Marisa and Muti, 2011). Fractionation is carried out with 3 solvents namely *n*-hexane, ethyl acetate, and water with a separate funnel. as much as 1 gram of ethanol extract is dissolved in 100 mL of water, then put into a separate funnel. then into the split funnel added *n*-hexane as much as 100 mL of mixture shaken slowly every 10 minutes for three times and allowed to stand for 30 minutes until two layers of *n*-hexane fraction (top) and water phase (bottom) are formed. Furthermore, the nonpolar phase (*n*-hexane) is separated from the polar phase (water) and accommodated as the *n*-hexane fraction. The polar phase is then inserted into the split funnel and fractionated again using ethyl acetate. The polar phase (water) is further separated from the semipolar phase (ethyl acetate) and then accommodated in a container. The three fractions are evaporated solvent using a vacuum evaporator at 40°C.

2.7. Brine Shrimp Lethality Test (BSLT) Method

Cytotoxicity activity testing was performed on the basis of a modified method (McLaughluin, Rigers & Anderson, 1998). The larvae of A. *salina* shrimp were used as test animals. A. *salina* larvae were placed in Erlenmeyer filled with seawater and hatched for 2×24 hours with a constant oxygen supply and under a 25 Watt TL lamp at room temperature of 25°C. Ten milligrams of extract were added to a test tube containing 50 µL of Tween80 in 10 mL of seawater to obtain a stock solution (2000 µg/mL). This study used concentration variations of 10, 100, 500 and 1000 µg/mL. Each concentration of the extract was tested three times. Incubation is carried out at room temperature under the light of a 25-watt TL lamp for 24 hours, then the number of live larvae is calculated. Negative control is carried out by the same procedure without the addition of extracts. Larvae of A. salina are considered dead if they do not show any movement during 10 seconds of observation. The extract is considered toxic to A. *salina* larvae if it has an LC_{50} (Letha1 Concentration) value of less than 1000 ppm (Meyer *et al*, 1982).

2.8. Data analysis

The research data will be processed and presented in table form. Data from the toxicity test will be analyzed using linear regression analysis using SPSS 23 to determine the LC_{50} value .

3. RESULTS

3.1. Determination results of Sida Rhombifolia samples

Plant determination is carried out to establish the identity of a plant. Determination in this study is important to determine and ensure the type (species) and the purpose of

determination is to ensure the identity of a plant so as to avoid errors in sample selection. The determination of S. rhombifolia in this study was carried out by sending leaf samples to the Center for Tropical Biopharmaceutical Studies LPPM IPB. The results of the determination showed that the sample was S. *rhombifolia* with the scientific name S. *rhombifolia* Linn.

3.2. Water content and yield extract Sida rhombifolia

Determination of water content aims to provide a limit or maximum range of water content in simplisia which is useful for minimizing the growth of microorganisms, so that simplisia can be stored for a long time. Determination of water content is carried out gravimetrically through heating at a temperature of 105°C. Based on the results of water content measurement, the water content of S. *rhombifolia* simplisia was 2.75% with the percentage yield of S. *rhombifolia* ethanol extract from sonicated extraction was 3.78% of the total weight of dry simplisia of 500 grams. The extract that has been obtained is then phytochemical screening to identify the presence of secondary metabolite compounds in the extract.

3.3. Phytochemistry of ethanol extract of Sida rhombifolia

Based on the results of phytochemical tests conducted to identify the compound content in the ethanol extract of S. rhombifolia leaves. The basic principle is to see the change in reaction formed by adding a reagent specific to a particular content (Oktari, Fitmawati & Nery, 2014). From the test results it was obtained that simplisia and leaf extract of S. rhombifolia contain compounds as shown in Table 1.

Table 1 Screening test results phytochemicals *Sida r hombifolia*

Phytochemicals Compound	Extract ethanol *Sida r hombifolia*
Alkaloids	
Dragendrof	+++
Meyer	++
Wagner	++
Flavonoids	+++
Saponin	+++
Tannin	++
Triterpenoids	++

Description : (+++) : Contains more compounds / concentrated colors
(++) : Contained light compounds/colors

3.4. Fractionation extract ethanol Sida rhombifolia

Based on the results of fractionation using 3 solvents of different polarity, namely n-hexane (nonpolar), ethyl acetate (semipolar) and water (polar). Data on the fractionation of ethanol extract with the three types of solvents are presented in Table 2.

Table 2 Percent yield fraction extract ethanol S. *rhombifolia*

Sample	Yield (%)
n- hexane fraction	12.70
Fraction ethyl acetate	14.30
Water	60.46

3.5. Cytotoxicity extract Sida rhombifolia

The method of testing extracts on A. *salina* shrimp larvae known as BSLT is a pre-screening method for anticancer activity. BSLT test was performed on ethanol extract, *n*-hexane fraction, ethyl acetate and S. *rhombifolia* water. The results obtained are then calculated as the LC50 value of the extract, where the concentration of the extract that can cause the death of A. salina is as much as 50% of the number of shrimp larvae. The death of low-level organisms such as A. *salina* can be used as a guiding tool for screening and searching for new bioactive ingredients. The cytotoxic activity of S. rhombifolia leaf extract and fraction against shrimp larvae can be seen in Table 3.

Table 3 Activities cytotoxic extracts and fractions leaf S. *rhombifolia* against *Artemia salina*

Sample	LC_{50}
Extract ethanol	320.15
n-hexane fraction	500.10
Fraction ethyl acetate	575.06
Water	873.21

4. DISCUSSION

4.1. Water Content and Yield Extract Ethanol Sida rhombifolia

Determination of water content of simlipsia is very important to provide a maximum limit of water content contained in S. *rhombifolia* simplisia. This is because a high amount of water can be a growing medium for microorganisms that can damage and change the content of chemical compounds in simplisia. The moisture content requirement of simplisia is based on the applicable standard parameters according to Food and Drug Monitoring Agency (2014) the moisture content of a good simplisia is not more than 10% to prolong storage. Based on the results of measuring the moisture content of S. rhombifolia simplisia, a simplisia moisture content of 2.80% was obtained, this was due to the drying process that was less than optimal or due to lack of heating. This shows that the moisture content of S. *rhombifolia* simplisia has met the requirements for good water content of simplisia and is suitable for use as a herabal material that is eligible for further testing.

Dried S. *rhombifolia* simplisia is powdered by blending so that the sample size becomes smaller. A smaller sample size can expand the contact and increase its interaction power with the solvent. Samples with powder size will make it easier for solvents to enter and bind to compounds in the sample. The simplisia powder was further extracted using 70% ethanol solvent. The choice of ethanol as a solvent is based on its relatively non-toxic nature and is able to dissolve metabolite compounds optimally (Azis, Febrizky, & Mario, 2014). The extraction method used in this study was sonication extraction. Sonication extraction is an extraction method by utilizing the help of ultrasonic waves generated by the sonicator. This method has advantages over other extraction methods because it can extract metabolites in a material faster and produce a yield that is not much different from other conventional extractions (Jos, Pramudono & Aprianto, 2011). During the extraction process, the sonicator will emit ultrasonic waves. The resulting ultrasonic vibrations will cause the solvent to easily diffuse into the cell, so that the cell undergoes lysis. As a result of cell lysis, the content of metabolite compounds contained in the sample tissue can be deposited into the solvent.

Based on the extraction results using ultrasonication method, the yield percentage of S. *rhombifolia* ethanol extract was 3.78%. The low percentage of yield is thought to come from the comparison between the amount of solvent and simplisia that is not right. According to Meiyanto *et al* (2008), the amount of solvent in the extraction process is one of the factors that greatly determines the percentage of yield, this is because the more the amount of solvent used in extraction, the greater the contact between the material and the solvent so that it has the potential to increase the percentage of yield. Another factor that affects the low yield percentage is the fast length of extraction time. The extraction process that takes place too quickly can allow extraction is not optimal so that it can indirectly have an impact on the percentage of yield produced.

4.2. Phytochemistry of Ethanol Extract of Sida rhombifolia

Phytochemical screening is the initial stage to qualitatively identify the content of plant secondary metabolites. The basic principle is to see the changes in reactions formed by adding a reagent that is specific to a particular content. In addition, this test is also intended to prove that the difference in the location of the region and rainfall from the origin of this plant will show a difference in the composition of secondary metabolites contained in it. Estimation of the class of compounds that play a role in inhibiting cell proliferation can be done with this test. Secondary metabolite compounds commonly found in plants are flavonoids, alkaloids, tannins, saponins, steroids, and terpenoids. Based on previous studies conducted Okoli *et al* (2007), the results of phytochemical screening of S. *rhombifolia* ethanol extract as a whole showed positive results against all tests (table 1). This is in accordance with the statement Okoli *et al* (2007) that the content of secondary metabolites of a plant is strongly influenced by geographical location, altitude and rainfall from which the plant originates. According to Dulay and Decastro (2016), the content of secondary metabolite compounds present in plants is a very important component in their biological activities related to treatment. Some secondary metabolite compounds such as alkaloids, flavonoids, terpenoids, tannins and saponins are said to have biological activities that can act as antimicrobial, antioxidant, anticancer, antiallergic and anti-inflammatory. This is supported by the results from Fartyal and Kumar (2016) which proves that some metabolite compounds such as alkaloids, flavonoids and steroids also have a very important role in the world of medicine, which can be used as natural anticancer agents.

4.3. Fractionation of S. rhombifolia Ethanol Extract

Fractionation is carried out with the aim of separating secondary metabolite compounds in S. *rhombifolia* ethanol extract by using solvents based on polarity properties, namely *n*-hexane solvents, ethyl acetate and water, Polar solvents will dissolve compounds that are polar, while nonpolar solvents will dissolve compounds that are nonpolar, as well as semipolar solvents (Arifianti, Oktarina & Kusumawati, 2014). The selection of these three types of solvents is based on their ability to dissolve compounds secondary metabolites with optimal. Before fractionation, ethanol extract is first dissolved in water until it blends to facilitate fractionation. The dissolved ethanol extract is then put into the split funnel and added *n*-hexane to it for fractionation. When *n*-hexane is inserted into the split funnel, both mixtures of solution form 2 layers. The same thing also happens when the fraction of water fractionated with *n*-hexane is fractionated again using ethyl acetate. The formation of both layers in the solvent mixture is caused by differences in density and polarity between solvents. The fractionation process is carried out by shaking the solvent mixture so that the metabolite compounds from the ethanol extract of S. *rhombifolia* will be partitioned into each solvent according to polarity. The fractionation results that still contain solvent are then evaporated so that a pure fraction is obtained which is expressed in % yield. Based on the results of fractionation that has been done, the water fraction of S. *rhombifolia* has a higher yield percentage than the ethyl acetate fraction and the *n*-hexane fraction (Table 2). The high percentage of yield in the water fraction compared to other fractions is suspected that the secondary metabolites contained in the ethanol extract of S. *rhombifolia* tend to be polar, so that almost all chemical components are more dissolved in water solvents than ethyl acetate and *n*-hexane.

4.4. Cytotoxicity of S. rhombifolia Extract

The Brine Shrimp Lethality Test (BSLT) method is widely used to bioascertain the bioactivity of an extract. Cytotoxicity test of secondary metabolite compounds in S. *rhombifolia* extract using BSLT method using *Artemia salina* shrimp larvae. This method is an inexpensive screening method and does not require a certain specialization in its implementation and this method is a 95% confidence method to observe the toxicity of a compound in plant extracts and does not require a code of ethics in testing.

This test aims to determine an extract has the ability to inhibit the growth of cancer cells and undergo further procedures in the process of finding anticancer drugs (Kumar *et al*, 2013; McLaughlin, Rogers & Anderson, 1998) stating that the method of testing the extract on A. *salina* shrimp larvae known as BSLT is a pre-screening method for anticancer activity. A. *salina* larvae are thought to represent zoological organisms for in vivo mortality testing. The test results conducted to compare the mortality test for A. *salina* and the proliferative inhibition test for carcinoma showed a positive correlation of the toxicity of the test compound to both types of tests. The BSLT test was carried out by observing the mortality rate caused after being given an extract to shrimp larvae after incubating for 1 x 24 hours. The results obtained are then calculated as the LC_{50} value of the extract, where the concentration of the extract that can cause *Artemia salina* death as much as 50%. Based on the LC_{50} value in Table 3, the highest level of toxicity was found in ethanol extract, *n*-hexane fraction, ethyl acetate fraction and lowest toxicity level in water fraction. The smaller the LC_{50} value indicates the higher the content of bioactive compounds from a natural material.

An extract is categorized as active and has a toxic effect if it can cause 50% death of A. salina at a concentration of less than 1000 ppm and is non-toxic if it has a concentration of more than 1000 ppm and the death of Artemia SalinaL each shrimp larvae due to the presence of secondary metabolite compounds that are toxic, toxic compounds present in the extract can enter through the mouth of Artemia SalinaLeach shrimp larvae and be absorbed into the The digestive tract occurs absorption process through the cell membrane. After the absorption process continued with the distribution of toxic compounds into the body of Artemia SalinaLeach shrimp larvae, and a process of metabolic reaction damage occurred (Meyer *et al*, 1982). The anatomical structure of the body of *Artemia salina* L. shrimp larvae at the naupli stage is still very simple, consisting of layers of skin, mouth, anthena, digestive tract or digestion that is still simple, and prospective thoracopods. Drastic changes in the concentration gradient between inside and outside the cell cause toxic compounds to spread well to the body of Artemia SalinaLeach shrimp larvae. The effects of metabolic damage caused by rapid occurrence can be detected within 24 hours, causing 50% death of *Artemia salina L*. shrimp larvae (Dachrinus, Oktima & Stanias, 2005).

5. CONCLUSIONS

Based on the results of the research that has been carried out can be concluded as follows:

1. Secondary metabolites contained in ethanol extract in the leaves of S. rhombifolia by phytochemical screening method are alkaloids, flavonoids, saponins, tannins, and triterpenoids.
2. Ethanol extract of S.rhombifolia leaves has a potential toxicity with an LC_{50} value of 320.15 µg/mL. Alkaloid compounds fractionated from ethanol extract of S.rhombifolia leaves Fraction *n*-hexane 500.10 µg / mL, ethyl acetate 575.06 µg / mL and water 873.21 µg / mL results obtained that ethanol extract is more cytotoxic than fractionation.

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