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Preliminary Study on Antifungal Activity of Soursop Leaf Crude Extract (*Annona muricata* L.) towards Fungi Isolated from Bolu Paranggi Cake

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Abstract

Soursop leaf extract (*Annona muricata* L.) contains a many potential active compounds, one of which is antifungal. Bolu Paranggi is a traditional cake from Mandar (West Sulawesi), made from wheat flour and brown sugar so it is at risk of being damaged and overgrown with fungus. This study aimed to extract soursop leaves using double RO (Reverse Osmosis) water solvent with variations in extraction time and the ratio of soursop leaf mass and water volume, identify groups of secondary metabolites compounds from soursop leaf water extract, isolate and identify fungi in Paranggi Bolu cake, and perform antifungal activity test of soursop leaf extract against fungi isolated from Paranggi Bolu cake. Extraction of soursop leaves using double RO water as a solvent with maceration for 24 hours and heating for 15 minutes with a variation of the ratio of soursop leaf mass and water volume of 1:1; 1:1.5; 1:2; 1:2.5; and 1:3. The analysis of the secondary metabolite compound was analyzed using the phytochemical test method and antifungal activity using the agar diffusion method on PDA (Potato Dextrose Agar) media and then measuring the inhibition zone using 6 mm diameter paper discs. The results showed that extraction with the heating method for 15 minutes produced more extract than the 24-hour maceration method at room temperature (RT), where the highest amount of extract was obtained from the ratio of soursop leaf mass and water volume of 1:3. The results of the phytochemical analysis of soursop leaf water extract, both heating and maceration methods, contained flavonoids, alkaloids, terpenoids, steroids and saponins, and antifungal activity tests against *Aspergillus* isolated from Bolu Paranggi showed weak antifungal activity at extract concentrations of 5,000 ppm - 50,000 ppm. with an inhibition zone diameter of 2 mm.

Keywords: soursop leaf extract, antifungal, Bolu Paranggi cake

A. Introduction

Soursop is a plant that grows widely in the tropics and subtropics. Soursop is known by the Latin name *Annona muricata* L. which is a genus of the Annonaceae family (Badrie, et. al., 2010). In Indonesia soursop is found in almost all parts of Indonesia. Almost all parts of the soursop plant have been used traditionally. Soursop fruit has been widely used as food or drink. Soursop leaves have traditionally been used as a remedy for headaches, insomnia, liver, diabetes, hypertension, and anti-swelling (Sousa et. al., 2010).

Taylor (2002) reported that in Jamaica, Haiti and western India soursop fruit juice has been used as a fever medicine, killing parasites and treating diarrhea. In Brazil, soursop fruit juice is used to increase breast milk production for nursing mothers, and to treat diarrhea and dysentery. Soursop seeds have also been used as a vermifuge and anthelmintic against internal and external parasites and worms. In Indonesia, young soursop fruit has been used as a medicine to lower high blood pressure, ripe soursop fruit is used as a hepatitis medicine, and soursop leaves as cough medicine (Mardiana and Ratnasari, 2011).

Several studies have found compounds in soursop such as alkaloids (reticulin, coreximine, coclame, and Annonine) as well as essential oils such as -karoyifene, -kadinen, epi-α-cadinol, and -cadinol (Kossouoh et. al., 2007). The medical effects of soursop plant extracts have also been widely reported. Soursop leaf ethanol extract has been used as an antinociceptive and anti-inflammatory (Sousa et. al., 2010). Water extracts from soursop leaves, seeds, and fruit were reported to kill T47D breast cancer cells (Fidianingsih & Handayani, 2014). Soursop root extract is also reported to contain asimicin, bullatacin, and bullatalicin which are used as acetogenins (Syahida, 2012).

A preliminary biochemical test of soursop leaf extract by Tambunan (2012) showed the presence of compounds in the group of alkaloids, flavonoids, saponins, tannins, gallates, quinones, steroids, essential oils, and coumarins. Permatasari (2013) reported the inhibitory power of soursop leaf extract on the growth of *E.coli* bacteria.

Apart from being anticancer and antibacterial, soursop antifungal activity has also been reported. Albiter (2007) reported that soursop leaf crude extract has potential as an antifungal agent. Abubacker and Deepalakshmi (2013) isolated hexadecanoic acid methyl ester compounds as antifungals in *Alternaria solani* and *Aspergillus enthrocephalus*. Nurjanah (2014) reported that ethanol extract and aqueous extract of soursop leaves had antifungal activity against *Aspergillus* niger.

Almost all parts of soursop have been studied, starting from the roots, stems, leaves, and seeds. However, research on the antifungal potential and its application as a preservative in food products is still lacking. So far, food products generally use sodium propionate (CH_3CH_2COOH) to prevent mold and mildew from growing. In addition to sodium and kamium propionate, synthetic antifungals commonly used in food products are benzoic acid and its salts and sorbic acid and its salts. However, the use of this synthetic antifungal has a negative impact if used in inappropriate doses and in long-term use. Several studies on natural antifungals have also been reported. Spirulina extract is used as an antimicrobial in white bread. Ella, et al. (2013) reported the effectiveness of citronella essential oil against *Aspergillus* sp. Sinha (2011) reported that turmeric root and turmeric powder can be used as antibacterial and antifungal in bread.

Food products are susceptible to damage, especially biological damage, one of which is caused by fungi (Nurjanah, 2014). Damage to food is generally caused by the growth of molds such as *Aspergillus*, *Fusarium*, and *Penicillium* (Muhialdin et. al., 2013). Damage by these fungi can reduce the organoleptic value and shelf life of these foods, and can even cause toxic compounds such as aflatoxins produced by *Aspergillus flavus* (Nurjanah, 2104). One type of food that is susceptible to mold is the traditional Mandar cake called Bolu Paranggi. A type of sponge cake made from wheat flour and brown sugar. This cake is quite risky for damage. Paranggi usually used as a food gift from Mandar, but is very easy to damage caused by fungus, so its storage cannot last long.

Soursop leaf extract may have antifungal activity that can be potential as natural ingredient to lengthen the shelf life of the Bolu Paranggi cake. However, the information and experimental data on that is still lacking. Based on the above background, in this study, soursop leaf extraction was carried out using a safe polar solvent, namely water, identified the secondary metabolite compounds in soursop leaf water extract, and tested the antifungal effectiveness of soursop leaf water extract against fungi isolated from traditional Mandar Bolu Paranggi cake.

B. Literature Review

1. *Soursop Leaf*

Soursop leaves contain monotetrahydrofuran acetogenin compounds. For example, ganiothalamicin, anomuricin A and B, 10-one annonacin, uricatosin A and B, and gigantretosin A (Muyassaro, 2014). These compounds can treat diseases such as cancer, and rheumatic pain, inhibit the growth of viruses and bacteria, inhibit gene mutations, treat intestinal worms, treat jaundice (liver), treat coughs, and treat seizures (Dewi and Hermawati, 2013).

Another benefit of soursop leaves is as an antispasmodic and has a calming effect. Soursop leaves are usually consumed in the form of tea. Leaf tea soursop is used as a medicine for inflammation of the mucous membranes of the nose. Soursop leaf decoction is also effective for head lice and bed bugs. Freshly ground leaves are used to treat wounds on the skin (Taylor, 2002). According to Asprey & Thornton (2000), soursop leaves contain flavonoids, alkaloids, fatty acids, phytosterols, myricyl alcohol, and anonol (Purwatresna, 2012).

2. *Food Damage*

The shelf life of food is the time during which the food remains stable and can maintain the desired quality. Food spoilage is unavoidable and can be caused by a variety of factors. One of the causes of food spoilage is due to the growth of micro-organisms in the food. Microorganisms that are important in food microbiology are those belonging to bacteria, molds, and yeasts. Mold is a multicellular fungus that has a filament (mycelium), and its growth on food is easy to see because of its fibrous appearance like cotton (Fardiaz, 1992).

Various types of fungi such as *Aspergillus flavus* and *Penicillium* sp. Very much attack post-harvest foodstuffs, the fungus can produce aflatoxins that are very toxic to consumers. Aflatoxins cannot be neutralized through cooking so efforts to avoid fungal contamination need to be made (Hutasoit et al., 2013).

C. Methodology

1. *Research Design*

Quantitative research methods with experimental research types.

2. *Instruments*

Extract Preparation

Making soursop leaf powder was done by picking fresh soursop leaves and then washing them with running water to remove dirt that sticks. The cleaned leaves were air-dried after washing, then put in the oven at 40^0C. The dried soursop leaves were then mashed using a blender until they become powder, the leaves are mashed to facilitate the extraction process.

A total of 100 grams of soursop leaf powder was macerated with double RO (Reverse Osmosis) water for 24 hours with a ratio of mass: solvent volume, 1:1; 1:1.5; 1:2; 1:2.5; and 1:3. Macerated for 24 hours. Then the filtrate was separated using a vacuum filter. The extraction results were then concentrated with a rotary evaporator and freeze dryer and then the extract yield was calculated so that the optimum mass and volume ratio was obtained.

Soursop leaf extraction with variations in the mass of soursop leaf powder was carried out by maceration of soursop leaf powder with double RO (Reverse Osmosis) water with a ratio of mass: solvent volume, 1:1; 1:1.5; 1:2; 1:2.5; and 1:3. Heat and simmer at 100^0C for 15 minutes. It is then cooled and the filtrate is separated using a vacuum filter. The extraction results were then concentrated with a rotary evaporator and freeze dryer and then the extract yield was calculated so that the optimum mass and volume ratio was obtained.

Secondary Metabolite Detection

The aqueous extract of soursop leaf is dripped with a dilute solution of ammonia and drops of concentrated sulfuric acid. The formation of yellow color indicates the presence of flavonoids. Another way is done by adding 10% NaOH, the formation of yellow color indicates the presence of flavonoids. Then tested using 5% $FeCl_3$, the blue color indicates the presence of flavonoids.

The alkaloid test was carried out using 3 reagents, namely Meyer, Dragendorf, and Wagner reagents. A positive test for alkaloids was indicated by the presence of a white precipitate, for the Meyer reagent test and Dragendorff's reagent test indicated the presence of orange to red-brown precipitate.

Administration of acetic acid anhydride (AC_2O) as much as 1-2 drops in chloroform extract and as a comparison using concentrated H_2SO_4 (1-2 drops). A color change to red or purplish-

red indicates terpenoids and green or bluish-green for steroids. Terpenoid test (Salkowski Test) by giving concentrated H_2SO_4 to chloroform extract to form 2 layers of liquid phases. The formation of reddish-brown color at the interface layer indicates the presence of terpenoids.

The extract in the test tube was shaken vigorously, the formation of permanent foam (about 15 minutes) and did not disappear with the addition of 1 drop of concentrated HCl indicating the presence of positive saponins.

Aseptic Method

The material was sterilized at the time of making the media using an autoclave for 15 minutes at a temperature of 121^0C and a pressure of 1 atm. The tools that will be used first also need to be sterilized, such as Petri dishes, Erlenmeyer, loop needles, and cotton. Petri dishes and Erlenmeyer that will be used are sterilized by autoclaving for 20 minutes at a temperature of 121^0C and a pressure of 1 atm. While the loop needles are sterilized at the time of going, during, and after use by heating them using a Bunsen fire until they are smoldering. For cotton sterilized by storing in the oven at 47^0C for 24 hours. Before, during, and after use, the work surface (laminar airflow) is cleaned with 70% alcohol using a sprayer and cleaned using a tissue. The air blower in laminar airflow is turned on before and during use to avoid contaminants. In addition, before use, laminar airflow can be sterilized by using a UV lamp that is turned on for a few minutes.

Bolu Paranggi Cooking Method

Brown sugar is melted by heating and using boiling water, then cool. Heat Palekko (cover). Next, mix the eggs, baking powder, TBM, vanilla, and sugar, then beat until evenly distributed. Added brown sugar that has been cooled before. After that, add brown sugar, and flour little by little, while stirring. Heat the mold, then grease it with coconut oil. Pour the mixture into the mold, then heat it, wait 5 minutes then remove it from the mold.

Fungal Isolation

The fungi used in this study were isolated from Bolu Paranggi cake. A total of 1 g of cake was mashed using a mortar and pestle, then dissolved with 9 ml of distilled water to obtain a suspension with a dilution of 10-1. Then 1 ml of suspension with a dilution of 10-1 was dissolved into 9 ml of distilled water to obtain a suspension with a dilution of 10-2 and so on until a suspension with a dilution of 10-4 was obtained. 0.1 ml of each suspension was taken and then inoculated into PDA media and incubated at room temperature (RT) for 3 days. After 3 days, the growing fungal colonies were isolated in a new agar medium to obtain pure isolates. Then it was incubated again for 7 days to be further observed macroscopically and microscopically and to be used in further tests

Antifungal Activity Test

Before testing the antifungal activity of the extract, a preliminary test was carried out to optimize the number of fungi to be used. Mushroom cultures were made first, namely from one mushroom that had been isolated and then inoculated into 100 ml PDA (Potato Dextrose Agar) media and incubated for 3 days. The mushroom culture was taken as much as 0.02 ml; 0.04 ml; 0.06 ml; 0.08 ml and 0.1 ml. Incubated into PDA media, then homogenized using a sterilized bent stirring rod, aiming to even out fungal colonies on solid media. Paper discs with a diameter of 6 mm were immersed in a solution of 5000 ppm each for soursop leaf water extract without heating, soursop leaf water extract with heating, and propionic acid as a positive control. Furthermore, the paper disc containing the extract was placed on the PDA media which already contained the fungus. Incubated and observed changes on days 1, 2, and 3.

The antifungal activity test was carried out using the agar diffusion method. Mushroom culture as much as 0.02 mL was poured into a petri dish that already contained solidified PDA media. Paper discs with a diameter of 6 mm were immersed respectively into soursop leaf water extract without heating, soursop leaf water extract with heating, and propionic acid as a positive control with a concentration variation of 5000 ppm; 10,000 ppm; 20,000 ppm; 30,000 ppm; and 50,000 ppm. Furthermore, the paper disc containing the extract was placed on the PDA media which already contained the fungus. Double RO water was used as a negative control. Incubated and observed for changes on days 1 and 2. Tests were carried out in triples for each concentration. The apparent clear zone around the paper disc was then measured using a caliper.

3. The technique of Data Analysis

Data analysis was carried out descriptively by looking at the results of the identification of secondary metabolites of soursop leaf extract, including flavonoids, alkaloids, terpenoids, and steroids, as well as saponins. Then proceed with testing the antifungal activity of soursop leaf water extract in vitro.

D. Findings and Discussion

1. Findings

Taking fresh soursop leaves is done in the morning at 8-10 hours when the photosynthesis process of plants runs optimally. Drying is done by aerating at room temperature soursop leaves that have been reduced in size. Soursop leaf powder was extracted by maceration and heating methods. Maceration was carried out with double RO water as the solvent and soaked at room temperature (RT) for 24 hours, while extraction by heating was carried out by soaking soursop leaf powder in double RO water and boiling for 5 minutes. Then filtered using a vacuum filter, concentrated with an evaporator and freeze dryer.

Using the maceration method, a total of 0.713 g of crude extract was obtained from 100 g of soursop leaf powder. Meanwhile, using the heating method, about 1.123 grams of crude extract was produced. The extract obtained from 5 variations of the leaf mass ratio to solvent volume is presented in Table 1.

Table 1. Crude extract obtained from various leaf mass to solvent volume ratios using maceration and heating extraction methods

Leaf mass to solvent volume ratio	Extract weight (grams)	
	Maceration at RT for 24 h	Heating at 100 °C for 15 min
1 : 1	0,713	1,123
1 : 1,5	1,083	1,212
1 : 2	1,105	1,105
1 : 2,5	1,408	1,757
1 : 3	1,661	1,954

Note: All extraction used 100 g of soursop leaf powder as starting material

Based on Table 1, it can be seen that the extraction method using heating for 15 minutes produced more extract than the maceration at RT for 24 hours. This is because the compounds contained in soursop leaves more easily diffuse out of cells at high temperatures. In addition, increasing the ratio of soursop leaf mass to solvent volume increases the amount of extract produced. The relationship between water volume and yield is presented in Figure 1.

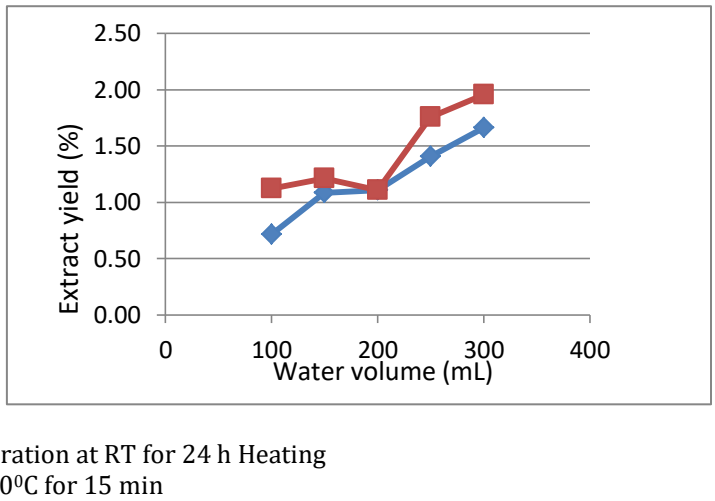

Figure 1. Relationship between water (solvent) volume and Soursop Leaf Extract Yield

In Figure 1, it can be seen that the extract yield percentage resulting from heating is greater than that of maceration at RT. The previous report find that high temperature increases the yield of the extract as well The highest extract yield was found in the extract by heating with a solvent volume of 300 mL per 100 grams of soursop leaf powder, which was 1.85%, while the

lowest yield of 0.71% was obtained from maceration with a solvent volume of 100 mL per 100 grams of soursop leaf powder (Fakhruzy, et al., 2020).

To identify the classes of secondary metabolites in the soursop extract a preliminary phytochemical test was conducted (Table 2).

Table 2. Secondary Metabolic Classes detected in the Soursop Leaf Extract

Compound Group (test type)	Discoloration or Reaction Indicator		Test results	
	Maceration at RT for 24 h	Heating for 15 min	Maceration at RT for 24 h	Heating at 100 °C for 15 min
Flavonoids:				
a. Concentrated H_2SO_4	Brownish red	Dark yellow	+	+
b. NaOH 10%	Brownish-yellow	Yellow	+	+
c. $FeCl_3$ 5%	Dark blue	Dark blue	+	+
Alkaloids:				
a. Meyer	Yellow	Light brown	+	-
b. Dragendorff	Yellow	Brown	-	+
c. Wagner	Brown	Dark brown	-	-
Terpenoids and steroids	Brown ring	Brown ring	+	+
Saponins	Foam formed	Foam formed	+	+

Note: (+) = detected; (-) = not detected

Based on table 2, it can be seen that the soursop leaf water extract obtained from maceration for 24 hours or from heating for 15 minutes contains the same group of compounds, namely flavonoids, alkaloids, terpenoids, steroids, and saponins. But the extract from heating is thought to have a higher alkaloid content. This can be seen from the color changes produced in the alkaloid test using three types of reagents producing a darker brown color reaction than the extract with 24 hours of maceration (yellow).

a. Fungi grown on Bolu Paranggi cake (5 days) b. Isolated fungi under the microscope (magnification 40×10)

Figure 2. Mushrooms on Bolu Paranggi

Table 3. Inhibitory Effect of Soursop Leaf Extract on the growth of Aspergillus isolated from Bolu Paranggi cake

Aspergillus Volume (mL)	Inhibition zone diameter (mm)			
	P	M	A	AP
0,02	2	2	1	2
0,04	2	2	1	2
0,06	1,5	1,5	1	2
0,08	2	1	1	2
0,1	1,5	1	1	2

P = 5000 ppm soursop leaf extract obtained using heating for 15 min

M= 5000 ppm soursop leaf extract obtained using maceration at RT for 24 h

A= Water

AP = 5000 ppm propionic acid

Table 3 shows that with a volume of *Aspergillus* as much as 0.02 mL to 0.1 mL at an extract concentration of 5000 ppm, both the macerated extract and the heating product showed a weak

antifungal activity indicated by the presence of a clear zone around the paper disc comparable to the inhibition zone by propionic acid. To determine the antifungal activity, it is necessary to test at a higher extract concentration because at 5000 ppm extract concentration the diameter of the inhibition zone is still the same as the positive control (propionic acid). The *Aspergillus* culture volume of 0.02 mL was chosen for further experiment. Further testing of antifungal activity was carried out by using increased concentrations of extract and propionic acid at 5000 ppm, 10,000 ppm, 20,000 ppm, 30,000 ppm, and 50,000 ppm. The test results can be seen in Table 4.

Table 4. Inhibitory Effect of increased Soursop Leaf Extract concentrations on the growth of Aspergillus isolated from Bolu Paranggi

Concentration (ppm)	Inhibition zone diameter (mm)			
	P	**M**	**A**	**AP**
5.000	2	2	1	2
10.000	2	1,5	1	2
20.000	1,5	1,5	1	2
30.000	2	1	1	10
50.000	2	1	1	15

P = Soursop leaf extract obtained using heating for 15 min

M= Soursop leaf extract obtained using maceration at RT for 24 h

A= Water

AP = Propionic acid

While higher concentrations of propionic acid (30,000 ppm and 50,000 ppm) markedly increase the inhibition zone toward Aspergillus growth, the increased soursop extract concentrations did not result in any change compared to the 5000 ppm. Soursop extract may require a much higher concentration for the antifungal activity to manifest.

2. Discussion

Fidianingsih and Handayani (2014) reported the extraction of antioxidants from soursop leaves using the ultrasonic bath method. The best results were shown at a material: solvent ratio of 1:10 (w/v) with an extraction time of 20 minutes. The effect of solvent volume was seen in both maceration treatments at ordinary temperatures and in extraction by heating. The addition of solvent volume increased the yield of soursop leaf crude extract. The increased contact between soursop leaf powder and the solvent causes more metabolites to be released from the cells.

Suwandi (2015) conducted a phytochemical test on the ethanolic extract of soursop leaves. The test results showed that the ethanolic extract of soursop leaves contained flavonoid compounds, alkaloids, terpenoids, steroids, and quinones and did not contain shampoos. The group of flavonoid compounds and alkaloids is a group of compounds that have a lot of bioactivities, both antibacterial, antifungal, anticancer, anti-inflammatory, and antioxidant.

Morphologically the fungus can be determined by looking at the shape of its structure using a microscope, thus identification and clarification can be determined visually the fungus is seen as cotton or colored or colorless threads called mycelia and spores. Mycelia are formed by the presence of hyphae, both septa, and non-septate. Fungi are divided into several families, including Moniliaceae (*Aspergillus*, *Penicillium, Trichothecium, Geotrichum, Monilia, Sporatrichum, Botrytis*, etc.), Dematiaceae (*Cladosporium, Helminthosporium*, etc. (Kusnadi, 2003).

Nurjannah (2004) isolated mushrooms from a steamed sponge and found that the fungus found in the steamed Bolu Paranggi was *Aspergillus niger*. In this study, fungi isolated from Paranggi Bolu Paranggi were compared with the literature and it was found that the type of fungus in Paranggi cake was dominated by *Aspergillus* sp (Figure 2). Bolu Paranggi that is stored for approximately 5 days will grow mold. This fungus is very easy to grow and develop on Bolu Paranggis because the Bolu Paranggi contains enough food for the growth of the fungus. Likewise on this PDA media. The microscopic form of the fungus Aspergillus sp. namely the spread of mycelia colonies in this fungus spreads in all directions, the shape of the mycelia is like cotton, the mycelia density in this fungus is very tight, and the color of the mycelia colonies is white and gradually becomes greenish. The shape of the conidia of this fungus is usually the

same round but oval, the color of the conidia is black, the hyphae are septate, and usually, the color of the hyphae is black to green. *Aspergillus* sp can grow at a temperature of 35-37 degrees Celsius (optimum) and requires sufficient oxygen.

The flavonoid compounds contained in soursop leaf extract belong to the phenol group which can function as antifungals. Phenol compounds work in cells, especially denaturing cell proteins and damaging fungal cell walls. Damaged cell walls cause no energy reserves, thus inhibiting the growth of fungal hyphae.

The antifungal activity test was carried out using the agar diffusion method (Nurjannah Modification 2014) where the fungus was inoculated on Potato Dextrose Agar (PDA) media, then homogenized using a sterilized bent stirring rod, aiming to even out the fungal colonies on solid media. Next, the test was carried out where each 6 mm paper disc was immersed into 1 mL of soursop leaf water extract with a concentration of 5000 ppm. The positive control used 5000 ppm propionic acid while the negative control used double RO water which had been sterilized. Then the incubation process was carried out for 48 hours at a temperature of 25^0C, observed for the presence of a clear zone around the paper disc and the diameter of the inhibition was measured using a caliper. The use of propionic acid as a positive control because propionic acid and its salt are synthetic preservatives that are widely used to prevent mold growth in bakery products and the like.

E. Conclusion

Soursop leaf extraction with double RO water as a solvent with 15 minutes of heating produced more extract than 24 hours of maceration at RT, the ratio of soursop leaf mass to the water volume of 1:3 resulted in the highest amount of extract. The results of the phytochemical test of soursop leaf extract, indicate that the extracts contains flavonoids, alkaloids, terpenoids, steroids, and saponins. The fungus isolated from the Bolu Paranggi is Aspergillus. Soursop leaf extract at concentrations of 5,000 ppm to 50,000 ppm had weak antifungal activity against *Aspergillus* with an inhibition zone diameter of 2 mm. Future research are needed to: (1) profile the secondary metabolites/compounds contained in soursop leaf extract; (2) analyze the antifungal activity using higher extract concentration > 50,000 ppm, and (3) study the use of soursop leaf extract to improve the shelf life of Bolu Paranggi cake.

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