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Antibacterial Activity and Bioautography Polar Fraction of Ethanolic Extracts of Leaves of Soursop (*Annona muricata* L.) Against *Klebsiella pneumoniae* and *Staphylococcus epidermidis*

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Abstract: Soursop (*Annona muricata* L.) has been used as traditional medicine. Soursop as Annonaceae family, contain alkaloids, acetogenins, polyphenols, terpenes and aromatic compounds. This study has the aim to determine the Minimum Inhibitory Concentration (MIC) of the leaf of soursop (*Annonamuricata* L.) ethanol extract polar fraction against to *Klebsiella pneumoniae* and *Staphylococcus epidermidis* and identify the active compound which responsible for the activity. The leaf of Annona was extracted using 96% ethanol by maceration and fractionation with Vacuum Liquid Chromatography (VLC). Antibacterial activity of polar fraction was tasted using solid dilutions method. Polar fraction concentration series of used was 2.5, 3, 3.5, 4 and 4.5% w/v. To determine compounds performed by Thin Layer (TLC) using silica as stationary phase and hexane: ethylacetate (3:7) as the mobile phase. Bioautography using TLC plate was placed inoculated bacteria, antibacterial compounds indicated an inhibition zone. The results showed that polar fraction of ethanol extract of leaves of the soursop has antibacterial activity against *Klebsiella pneumoniae* and *Staphylococcus epidermidis* with MIC were 3 and 3.5% w/v, respectively. Class of compounds that have antibacterial activity against *Klebsiella pneumoniae* and *Staphylococcus epidermidis* were flavonoids, polyphenol, antron and triterpenoid.

Key words: *Annona muricata* L., antibacterial, polar fraction, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, antron

INTRODUCTION

One type of infectious disease is pneumonia which cause by *Klebsiella pneumoniae*. Pneumonia is an acute infectious disease that attacks the lung tissue (alveoli). Pneumonia is a health problem with high mortality rates, not only in developing countries but also in developed countries such as the United States, Canada and European countries. In the United States, for example, there are two million to three million cases of pneumonia per year with an average death of 45,000 people. In Indonesia, pneumonia is the third leading cause of death after cardiovascular and tuberculosis. On the other hand, *Staphylococcus epidermidis* is a common bacterium in human skin and can cause opportunistic infections (attacking individuals with weak immune systems). *Staphylococcus epidermidis* contains a lipase gene that can increase lipase activity and improve structure substrate. This bacterium is usually resistant to many antibiotics. Most of them are effective with germicidal agents such as Vancomycin, Quinolones and Rifampin (McCann *et al*., 2008). To solve the resistant problem, nowadays development of antibacterial from herbal resources, especially, medicinal plant was very important. One of the potential antibacterial from plant was soursop plant (*Annona muricata* L).

The activity of soursop plant has proven as astringent properties (raw leaves and fruit), antibacterial and anticonvulsant. Methanol extract of soursop leaves was shown have antibacterial activity against *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Streptococcus pyogenes, Bacillus subtilis, Salmonella typhimurium, Klebsiella pneumonia* and *Enterobacter aerogenes*. Another study showed that soursop plants contain *Annonaceous acetogenins* which have been published as antitumor, antiparasitic, pesticides, antiprotozoal, antifeedant and antimicrobial activities (Taylor, 2002).

Methanol and water extract of *Annona muricata* leaf have antibacterial activity against *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC8739, *Proteus vulgaris* ATCC13315, *Streptococcus pyogenes* ATCC

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8668, *Bacillus subtilis* ATCC12432, *Salmonella typhimurium* ATCC23564, NCIM *Klebsiella pneumonia* No.2719 and *Enterobacter aerogenes* NCIM No.2340 (Pathak *et al*., 2010). Viera *et al*. (2010) stated that soursop leaf water extract can inhibit the growth of *S. aureus, E. coli* and *Salmonella*.

Based on these data, soursop leaf extract is mostly treated with polar solvent. There was a possibility that the polar fraction of soursop leaf extract has the potency as an antibacterial agent and also contain chemical compound which has antibacterial activity. This research was conducted to determine the antibacterial activity of polar fraction of soursop leaves (*Annona muricata* L.) of ethanol extract with solid dilution method against the bacteria *Klebsiella pneumonia* and *Staphylococcus epidermidis*.

MATERIALS AND METHODS

Plant determination: Determination of soursop leaves (*Annona muricata* L.) was carried out at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta.

Preparation of polar fraction of soursop leaf ethanol extract: Soursop leaves are dried using solar heat and covered with black cloth. After drying, the leaves are powdered using a blender to expand the surface of the simplicia. Simplicia of soursop leaves were macerated using 96% ethanol. The maceration results were filtered to obtain filtrate, the ethanol filtrate was concentrated using a rotary evaporator vacuum to obtain an extract. Ethanol extract of soursop leaf was fractionated using vacuum column chromatography. Vacuum column chromatography with silica gel as stationary phase and mobile phase using multilevel polarity gradient. The extract was impregnated with silica impreg (2×the weight of impreg) then put into a column and eluted with multilevel polarity gradient mobile phase, the ratio of hexane: ethyl acetate were 9:1, 8:2, 7:3 and 6:4. Fractionation was carried out 3 times, then the results of the polar fraction were concentrated using a vacuum rotary evaporator.

Antibacterial activity test with solid dilution method: The antibacterial activity test using MH media. The amount of media weighed for each liter for MH media is 64 g, BHI media is 37 g while BHI DS media is made twice, namely 74 g for 1 L. Bacteria *Klebsiella pneumoniae* and *Staphylococcus epidermidis* were taken from the stock of bacteria and then streaked on MH media. Then, it was incubated at 37°C for 24 h. After the bacteria grew then was stored at 4°C as a stock of bacteria.

The bacteria *Staphylococcus epidermidis* and *Klebsiella pneumoniae* were each taken from the stock, suspended in a 2 mL BHI liquid medium, incubated at 37°C for 24 h. After that 100 μL was taken and added into 2 BHI mL media. Then incubated for 3-5 h and the concentration was compared to the McFarland standard (108 CFU/mL) by add sterile distilled water to obtain the same turbidity as the McFarland standard was used. Final bacterial concentration was 10^6 CFU/mL, taken from 50 μL then diluted to 5 mL of BHI media.

The controls used for the antibacterial activity test of polar fraction of soursop leaves (*Annona muricata* L.) ethanol extract against *Klebsiella pneumoniae* and *Staphylococcus epidermidis* consisted of: media control: MH media, control bacteria: MH+bacterial media, control of suspending agent: MH +bacterial + DMSO media.

The polar fraction of *Annona muricata* L. leaves ethanol extract was prepared at 20% as stock concentration (5 g of extract extracted with 100% to 20 mL of DMSO suspending agent). A series of concentrations were prepared for *Staphylococcus epidermidis* and *Klebsiella pneumoniae* were 2.5; 3; 3.5; 4 and 4.5%.

The polar fraction concentration series that has been made, each coupled with MH media is shaken until, it is truly homogeneous, then compacted in a slanted position. Furthermore, if the MH medium which has been mixed with the extract has been solid as much as 25 μL of bacterial suspension that has been made is equivalent to 10^6 CFU/mL dripped into the media, then incubated 18-24 h at 37°C. Then bacterial growth was observed. The smallest level that can inhibit bacteria is called the Minimum Inhibitory Concentration (MIC).

Thin layer chromatography: The polar fraction of soursop leaf ethanol extract was dissolved in methanol, then it was sprayed on the TLC plate namely silica gel GF254 as a stationary phase, then eluted with a mobile phase Hexane: Ethyl acetate (3:7). Observation under UV light with a wavelength of 254 and 366 nm as well as spray detection of Sitroborat, Liebermann-Burchard, $FeCl_3$ and KOH.

Bioautography: Bioautography method was used to detect active compounds that have antibacterial activity. The TLC result plate placed on the surface of MH media which has been inoculated with bacteria for 20 min. After that it was incubated for 24 h at a temperature of 37°C. If the spots on the TLC plate have antibacterial activity, the presence of diffusion of active compounds will form a clear zone which is a zone of inhibition.

RESULTS AND DISCUSSION

Yield of extraction and fractionation: Previously obtained soursop (*Annona muricata* L.) ethanol extract results from the initial weight before extraction of 2000.1 g obtained final weight of 598.66 g, the and yield of 29.91% was obtained.

The polar fraction separation from the ethanol extract of the stationary soursop leaves used was silica which had previously been activated to reduce the water content in silica and will increase its activity. To get a good fractionation result, the mobile phase optimization is carried out. Based on the optimization results, the mobile phase used is a mixture of ethyl acetate: hexane with multilevel polarity gradient which is 1:9, 2:8, 3:7, 4:6 and finally with ethanol of 150 mL each. Fractionation was carried out 3 times to get more yields. Fractionation results of soursop leaf ethanol extract from an initial weight of 150 g obtained the final weight of the polar fraction of 9.41 g with a yield of 6.27%.

Antibacterial activity test: The method used in the polar fraction activity test of soursop leaf ethanol extract against *Klebsiella pneumoniae* and *Staphylococcus epidermidis* is a solid dilution method. Solid dilution method can provide homogeneity between the media and the test material, thus the contact with bacteria is more effective. The MIC results were obtained by observing the presence or absence of bacterial growth after incubation for 18-24 h at 37°C. To help dissolve the polar fraction, the ethanol extract of soursop leaves was used 100% DMSO, then, the extract could be distributed evenly in the medium. The concentration of the polar fraction of the soursop leaf extract to be tested is 2.5; 3; 3.5; 4 and 4.5%. The bacteria *S. epidermidis* and *K. pneumoniae* were not inhibited by growth in the presence of 100% DMSO suspending agent.

After incubation, the polar fraction of soursop leaf ethanol extract against *S. epidermidis*has MIC value 3.5% while the polar fraction of soursop leaf ethanol extract against *K. pneumoniae* show MIC value of 3%.

The minimum inhibitory concentration in this study for polar fractions gave MIC values 3 and 3.5% against *Klebsiella pneumoniae* and *Staphylococcus epidermidis*. When compared to non-polar fractions, semi polar and ethanol extract, the polar fraction showed a smaller MIC value. The results of non-polar and semi-polar fractions were 3.5 and 4%, respectively, for *Klebsiella pneumoniae* and *Staphylococcus epidermidis* and for ethanol extract showed MIC values, respectively, 3.25 and 3.5% of *Klebsiella pneumoniae* and *Staphylococcus epidermidis*. The polar fraction of ethanol extract showed the smallest MIC value which contained chemical compounds flavonoids or tannins, antrons, polyphenols and triterpenoids which might have antibacterial activity.

Research on the antibacterial activity of soursop leaf extract was conducted by Pathak *et al*. (2010) with a method for using cup methanol and plants from India, at a concentration of 0.9% able to inhibit the growth of *Klebsiella pneumoniae* bacteria by 19 mm. Research on Annonaceae contains alkaloids, acetogenin, amino acids, carbohydrates, proteins, fats, polyphenols, essential oils, terpenes and aromatic compounds (Vega *et al*., 2007). Research conducted by Saraswathy *et al*. showed that the methanol extract of soursop leaves had antibacterial activity against *S. aureus,E. coli, Proteus vulgaris, S. pyogenis, Bacillus subtilis, Salmonella typhimurium, Klebsiella pneumonia* and *Enterobacter aerogenes*. This difference depends on the controlled variables, namely the difference in the use of the method, the solvent used and the place where the plant grows affects the quality of the extract of the plant.

Thin layer chromatography analysis: To determine the chemical content in the polar fraction of soursop leaf ethanol extract, the extract was first dissolved with methanol. The solution is then dropped in the silica phase of GF254 and eluted with the mobile phase of hexane: ethyl acetate (3:7) v/v. Spray reagents used include Liebermann Burchard, KOH, Sitroborat and $FeCl_3$. Spots that have Rf 0.23 and 0.66 on observations of UV light 366 nm with spray reagent Lieberman Burchard looks pink which is a triterpenoid. Detection by Sitroborate reagent was observed in 366 nm UV rays, visible yellow and greenish yellow spots at Rf 0.56 and 0.61 are tannin or flavanoid compounds. Spots with Rf 0.15, 0.49 and 0.69 show yellow color with KOH spray reagent which means it contains Antron or Antronol. $FeCl_3$ spray reagent was seen with green spots on Rf 0.72 indicating the presence of polyphenol compounds (Table 1).

Chemical compounds that have been mentioned contained in soursop plants are steroids, cardiac glycosides and tannins (Pathak *et al*., 2010). Research on Annonaceae contains acetogenin alkaloids, amino acids, carbohydrates, fat proteins, polyphenols (including flavonoids), essential oils, terpenes and aromatic compounds (Vega *et al*., 2007). Chemical compounds that are possibly to have antibacterial activity in this study are flavonoids, polyphenols, antrons and triterpenoids.

Bioautography test results: Polar fraction of soursop leaf ethanol extract showed a clear zone in MH media containing *K. pneumoniae* and *S. epidermidis*. When

Table 1: TLC analysis KLT of polar fraction of Sursop leaves *Annona muricata* L.

		Detection						Compound
Spot	Rf	UV254	UV366	LB	Sitroborat	KOH	$FeCl_3$	Chemicals
1	0.15	Dark	-	-	-	Yellow	-	Antron or (not Antronatau)
2	0.23	Dark	-	Pink	-	-	-	Triterpenoid
3	0.49	Dark	Violet	-	-	Yellow	-	Antron or (not Antronatau)
4	0.56	Dark	-	-	Yellow	-	-	Taninatau Flavonoid
5	0.61	Dark	-	-	Yellow	-	-	Tannin or Flavonoid
6	0.66	Dark	-	Pink	-	-	-	Antron or (not Antronatau)
7	0.69	Dark	-	-	-	Yellow	-	Antron or (not Antronatau)
8	0.72	Dark	-	Pink	-	-	Green	Polifenol Blue

Wagner and Bladt (1996)

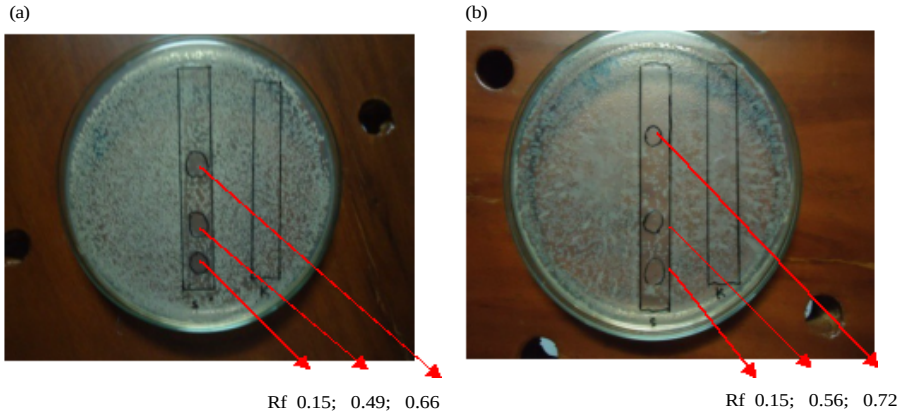

Fig. 1: The result of bioautography of polar fraction of etanol extract of *Annona muricata* L. againts *K. pneumoniae*: a) and *S. epidermidis* and b) Clear zone on *K. pneumoniae*on Rf 0.66 which triterpenoid, 0.49 and 0.15 were antron or antronol; meanwhile on *S. epidermidis* which Rf 0.72 was polifenol, Rf 0.56 was flavonoid or tanin and Rf 0.15 was antron or antronol

observed in MH media, the growth of *K. pneumoniae* was clear at Rf 0.15, 0.49 and 0.66, possibly the presence of triterpenoid compounds at Rf 0.66. While at Rf 0.15 and 0.49 it is possible to have antron or antronol compounds. The growth of *S. epidermidis* looks clear at Rf 0.72, 0.56 and 0.15, the possibility of polyphenol compounds at Rf 0.72 and at Rf 0.56 tannins or flavonoids and 0.15 are compounds that are possible for antron or antronol (Fig. 1).

The results of the polar fraction of soursop leaf ethanol extract showed that the chemical compounds which have antibacterial activity were polyphenols, flavonoids, antron or antronol and triterpenoids. Triterpenoid antibacterial mechanism by producing a membrane that interferes with the lipophilic component of the bacterial cell wall. Flavonoids work to shed bacterial cell walls by complex bonding on the cell wall and dissolve the constituents of bacterial cell walls while antronand antronol which are anthraquinone derivatives have antibacterial effects by changing cell morphology and damaging the outer structure of bacteria. Polyphenols are phenol derivatives whose mechanism of action is antibacterial by denaturing and coagulating proteins (Cowan, 1999).

Another study on nonpolar fraction of soursop leaf extracts of compounds that showed antibacterial activity were flavonoids, antron, antronol and polyphenols. Semipolar fraction of soursop leaf ethanol extract which showed antibacterial activity, namely flavonoids or tannins and triterpenoids, anthraquinone and polyphenols. Based on these data, each fraction in soursop leaf ethanol extract was different in the number of compounds that showed antibacterial activity.

The polar fraction of ethanol extract was the most potent antibacterial soursop leaves which have a MIC 3 and 3.5%, respectively, for *Klebsiella pneumonia* and *Staphylococcus epidermidis*. Compared to ethanol extract which has MIC of 3.25 and 3.5%, respectively for *Klebsiella pneumonia* and *Staphylococcus epidermidis* and nonpolar and semipolar fractions which have MIC 3, 5 and 4% of *Klebsiella pneumonia* and *Staphylococcus epidermidis*. Based on these results the polar fraction showed the highest activity compared to the nonpolar, semipolar and extract fractions. The variety of compounds in the polar fraction responsible for antibacterial activity

is probably the most potential as an antibacterial compared to nonpolar, semipolar and extract fractions. For further investigation, the identification and isolation of antibacterial compounds found in the polar fraction of soursop leaves (*Annona muricata* L.) extract is needed. Partitions need to be done to get specific compounds that are more appropriate for bioactivity testing.

CONCLUSION

Polar fraction of soursop leaves (*Annona muricata* L.) ethanol extract had antibacterial activity against *Klebsiella pneumoniae* and *Stapylococcus epidermidis* with Minimum Inhibitory Concentration (MIC) were 3 and 3.5%, respectively.

Group of compounds from polar fraction of soursop leaf ethanol extract which have antibacterial activity against *Klebsiella pneumoniae* and *Staphylococcus epidermidis* are possibly flavonoid, polyphenol, antron or antronol and triterpenoid compounds.

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