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Bioautography of Ethanol extract from *Carica papaya* leaves for antimicrobial activity against *Staphylococcus aureus, E. coli* and *Bacillus subtillis*

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ABSTRACT

In the present study, we explore the use of papaya leaves ethanol extract as an antibacterial using bioautography method. Bioautography of papaya leaf ethanol extract against *Staphylococcus aureus*, *E. coli* and *Bacillus subtillis* with n-butanol, acetic acid, distilled water with the ratio 4: 1: 5 yielded the dissolved compound of tannin. Furthermore, the tannins contained in papaya leaves has moderate antibacterial properties.

Key words : *Bioautografi, Ekstrak ethanol, Staphylococcus aureus, E. coli and Bacillus subtillis*

Introduction

Medicinal plants that are still underutilized have a lot of potencies for research and development to find useful compounds as antimicrobials. The use of herbal medicine, one of which is papaya, is expected to be an alternative for the community to avoid the side effects of using antibiotics. The part of papaya that is generally consumed by people is the fruit as well as the leaf part. The bitter taste of papaya leaves has various benefits, such as for treating diarrhea and skin diseases like acne. Papaya leaves contain ample secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins, and various kinds of enzymes like papain. This alkaloid or saponin compound dominantly contributes to the bitter taste of papaya leaves. These compounds could act as antioxidants, antibacterial, anti-cancer and anti-inflammatory. Hence, supporting papaya to endure its environmental conditions (Tuntun, 2018).

The antibacterial activity of papaya leaves has been shown effective against gram-positive bacteria, namely *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, as well as gram-negative such as *Escherichia coli* and *Klebsiella pneumonia* (Suresh *et al*., 2008). *Staphylococcus aureus* can cause various infections but can also be commensal (Helina *et al.*, 2020). *Bacillus subtillis* plays a role in reducing protein, starch and pectin in the body and can lead to food poisoning, meningitis and eye infections (Suresh *et al*., 2008). Have conducted research on the antibacterial activity of papaya leaves and proven to have antibacterial effects against gram-positive bacteria, one of which is *Bacillus subtilis*. *Staphylococcus* causes acne skin infection. In acne, *Staphylococcus* and *Corynebacterium lipases* release fatty acids from fat and cause tissue irritation. Apart from these causes, *Staphylococcus aureus* can also adhere to cells, spreads in tissues, forms abscesses, produces external enzymes or exotoxins, fights host defences, and is resistant to various antibiotic therapies (Suresh *et al.,* 2008).

Previous research has shown the inhibitory effect of papaya leaf extract against *Escherichia coli,* but the active component is still unknown. One of the methods to determine the active antibacterial components is bioutography. This method combines the use of thin layer chromatography (TLC) with the response of tested microorganisms based on the activity of analytes in the form of antibacterial, antifungal and antiprotozoa or commonly known as thin layer chromatography - bioautography. This method is commonly used to detect the antibacterial activity of plant extracts and essential oils (Jesionek *et al.,* 2013). One of the advantages of this method is the efficiency in detecting the presence of antimicrobial compounds based on the location of the spots in TLC plate even though they are in a complex mixture, making it possible to isolate the active compound (Yuliani *et al.,* 2011). In the present study, specific identification of papaya leaf content was carried out to determine its antibacterial properties.

Materials and Methods

Carica papaya leaves extraction

150 g of *Carica papaya* leaves dry powder was immersed in an airtight glass jar with 450 ml n-hexane, stirred slowly every day to maintain the homogeneity. After three days, the solvent was squeezed and filtered out using Whatman filter paper number 42. The macerated leaf powder then shade-dried and soaked again using ethanol as described previously. The crude extracts were filtered with the same method and stored in a bottle. The leftover leaf powder was rinsed with ethanol and filtered until the filtrate looked transparent. All of the filtrates were put together in the same bottle as the extract. The extract and filtrate were then evaporated for two hours in a rotary evaporator apparatus with a temperature of 60^{o} and rotation of 80 rpm. The obtained extract was weighed and stored in a desiccator before being used for fractions identification by the bioautography method and the phytochemical screening.

b. Fraction identification using bioautography

Bioautography test was started by applying each concentration of ethanol extract to the pre-marked bottom of the TLC plate using micropipettes. The chromatographic chamber was prepared with the developing solvent contained n-butanol, acetic acid, distilled water with the ratio 4:1:5 prior to plate insertion. Following the development at room temperature until the solvent reached the top, the plate was allowed to dry and observed for the spots under UV light. The detected spots were marked using a carbon pencil. Bioautography test was conducted by overlaying the dried plate onto NA medium seeded with the culture of *Staphylococcus aureus, E. coli* and *Bacillus subtillis* for 15-30 minutes. The medium was then incubated for 24 h at 35ºC. Inhibition zone was measured using callipers and recorded for each extract concentration.

Phytochemical screening of ethanol extract by color test

1. Alkaloid test
   Concentrated ethanol extract (2 ml) was added with 5 ml of HCl 2N, then 3 drops of dragendr of reagent were put into to observe the formation of orange precipitation.
2. Steroid test
   Papaya leaf ethanol extract (2 ml) was added with 0.5 ml of chloroform, followed by 0.5 ml of glacial acetic acid. Then, 2 ml of concentrated H_2SO_4 was added through the tube wall; if a greenish-blue ring is formed then it was declared positive for steroids
3. Triterpenoid test
   2 ml of concentrated papaya leaf ethanol extract was added with 0.5 ml of chloroform, followed by 0.5 ml of glacial acetic acid. Then, 2 mL of concentrated H_2SO_4 was added through the tube wall, if a brown/violet ring is formed then triterpene positive
4. Tannin test
   Ethanol extract from papaya leaves (1 ml) was added with 7 drops of $FeCl_3$ 10%. Tannin content was confirmed if dark blue or greenish-black color observed.
5. Saponin test
   Ethanol extract from papaya leaves (10 ml) was shaken vertically for 10 seconds and rested for the next 10 seconds. Saponin content was confirmed if the foam is formed after adding a

drop of HCl 2N.

6. Glycoside test
   Ethanol extract from papaya leaves (0.1 ml) was heated directly on the open fire, then added with 5 mL of concentrated anhydrous acetic acid and 10 drops of concentrated H_2SO_4. A blue-green color showed a positive result of glycoside content.
7. Flavonoid test
   Dried ethanol extract from papaya leaves (1 ml) was added with 3 drops of concentrated acetic acid, concentrated boric acid powder, concentrated oxalate acid fine powder, then heated on water bath while adding 10 mL of concentrated ether. An intensive yellow color under UV light 366 nm showed positive flavonoid result.

Results and Discussion

Following the extraction process, 5 g of dry extract was produced, which was then followed by bioautography test. The detected spots on the TLC plate observed under UV 366 light have three Rf value which is 0.46; 0.63 and 0.93 shown in purple (Figure 1). Based on the reference compound, it can be confirmed that the separated compound in the TLC plate is tannin. The eluent used to separate the compounds contained in papaya leaf extract (n-butanol, acetic acid, water) was able to provide the best separation because of the high polarity.

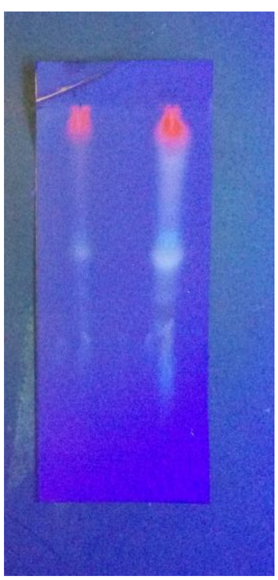

Fig. 1. TLC plate observation under UV light with a wavelength of 366 nm

Besides, according to the phytochemical screening using the color test, it shows that the papaya leaf ethanol extract demonstrated positive results on tannins and other four compounds of steroids, triterpenes, saponins, flavonoids, but negative for alkaloids, glycosides (Table 1). This result is in line with the study from Hong and Kim (2010) stated that papaya leaves contain chemical compounds such as alkaloids 1300-4000 ppm, flavonoids 2000 ppm, tannins 5000-6000 ppm, dehydrocarpaine 1000, and pseudocarpaine 100 ppm.

Table 1. The result of the ethanol extract of papaya leaf color test

No	Color test	Result
1	Alkaloid	-
2	Steroid	+
3	Triterpene	+
4	Tannin	+
5	Saponin	+
6	Glycoside	-
7	Flavonoid	+

The chemical compounds in the extract were determined by the solvent used during the extraction process which was n-hexane and ethanol. The former was first used to dissolve the chlorophyll contained in papaya leaves thereby preventing other chemical compounds from being dissolved in the further extraction process. Meanwhile, ethanol usage aims to dissolve the designated chemical compounds. Ethanol has polar properties allowing to enter through the cell wall into the cell cavity to dissolve the active substance. Due to the different concentration between the solution outside the cell and the active substance inside, then the diffusion process occurs until the equilibrium has reached.

The TLC plate was further tested for its sensitivity against *Staphylococcus aureus*, *E. coli*, and *Bacillus subtillis*. The results showed with 100% tannin extract the inhibition zone formed in an average of 8.9 cm, 8.74 cm and 7.32 cm for *Staphylococcus aureus*, *E. coli*, and *Bacillus subtillis*, respectively (Figure 2). All of these were categorized as a moderate category, according to Davis and Stout (1971). This is because the structure of the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtillis* has a simpler structure, which is only composed of a thick layer of peptidoglycan and teichoic acid. These layers consist of water-soluble polymers, making it easier for polar

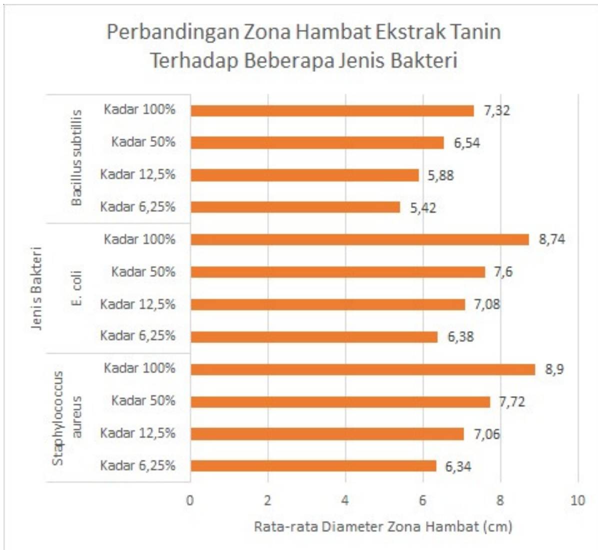

Fig. 2. Diagram Pertumbuhan Bakteri Dari Isolasi Plat KLT pada Media Uji Terhadap Bakteri *Staphylococcus aureus, Bacillus subtillis* and *E. coli*

antibacterial compounds, such as phenolic compounds (flavonoids and tannins) to penetrate into cells (Sudarwati, 2018). Tannins have properties as chelating with spasmolytic effects which can shrink cell walls or cell membranes so that they interfere with the permeability of the cells themselves. Due to disruption of permeability, cells cannot carry out living activities so that their growth is stunted or even dies (Ajizah, 2004).

Conclusion

Using the bioautography method, the identification of secondary metabolites in papaya leaves was confirmed tannin content with a moderate antibacterial category.

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