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ANALYSIS OF ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF LEAVES OF FIG (*Ficus carica* L.) FROM CIWIDEY DISTRIC, WEST JAVA, INDONESIA

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ABSTRACT

Leaves of Fig (*Ficus carica* L., Moraceae) contain flavonoids, tannins, phenolic compounds, saponins, alkaloids, steroids, and terpenoids. These compounds create an opportunity to utilize Fig leaves as herbal medicine. This study aimed to analyze the antioxidant and antibacterial activity of *Staphylococcus epidermidis* and *Propionibacterium acnes* of Fig leaves. Antioxidant activity was conducted by the DPPH method, while antibacterial activity was conducted by the agar diffusion method, followed by the microdilution method to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The results showed the IC_{50} values of extract, the fraction of ethyl acetate, *n*-hexane, and distilled water were 78.79, 22.29, 1052.00, and 133.90 µg/mL, respectively. The diameter of the inhibition zone of the extract was higher in *S. epidermidis* compared to *P. acnes*. The antibacterial activity was proportional to the extract concentration. The extract MICs for *S. epidermidis* and *P. acnes* were in the range of 6.25 and 12.5% w/v, while the MBCs value was in the range of 12.5 and 25% w/v. It concluded that the ethyl acetate fraction had the best antioxidant activity compared to the extract, distilled water, and *n*-hexane fraction. The antibacterial activity of the extract was better on *S. epidermidis* than on *P. acnes*.

Keywords: DPPH Method, Microdilution, *Propionibacterium acnes, Staphylococcus epidermidis*, Agar Diffusion

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INTRODUCTION

The Fig (*Ficus carica* L., Moraceae) is a native plant of the Middle East and West Asia but is now cultivated worldwide. Ficus consists of about 750 species. This plant is woody (trees) and has shrubs, mainly in subtropical and tropical areas.[1] The secondary metabolites in Figs include phenolic compounds, phytosterols, organic acids, anthocyanins, triterpenoids, coumarins, and volatile compounds.[2] Various parts of this plant have various pharmacological activities, including antioxidants[3,4] and antibacterial.[5] The literature search shows that Fig products generally come from fruit. This is because the fruit contains the highest polyphenols, flavonoids, and anthocyanins compared to other plant parts.[3] In this study, Fig leaves were used as a source of secondary metabolites, which aimed to increase the economic value of Fig leaves and reduce organic waste. Fig leaves were collected from Ciwidey District, Bandung Regency, West Java, Indonesia.

These Fig leaves contain phenolic compounds, flavonoids, tannins, alkaloids, saponins, terpenoids, and steroids. This Fig leaves extract contains a total phenolic content of 2.52 ± 0.24 mg GAE/g simplicia and a total flavonoid content of 2.03 ± 0.01 mg RE/g simplicia.[6] This study aimed to analyze the antioxidant and antibacterial activity of *Staphylococcus epidermidis* ATCC 12228 and *Propionibacterium acnes* ATCC 1223 of Fig leaves.

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EXPERIMENTAL

Materials

Extract, a fraction of *n*-hexane, ethyl acetate, and distilled water of Fig leaves were obtained from previous studies.[6] Fig leaves were collected from Ciwidey District, West Bandung Regency, West Java, Indonesia. *S. epidermidis* ATCC 12228 and *P. acnes* ATCC 1223 obtained from the Microbiology Laboratory, School of Pharmacy, Bandung Institute of Technology. Vitamin C, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, barium chloride, sulfuric acid, dimethyl sulfoxide (DMSO), and clindamycin phosphate were analytical grade and purchased from Sigma Aldrich (Germany). Mueller Hinton Agar (MHA) and Broth (MHB) were bacteriology grade and purchased from Oxoid (UK).

Antioxidant Activity Assay

Each 1 mL of vitamin C (2, 4, 6, 8, and 10 µg/mL), extract, a fraction of ethyl acetate and distilled water (25, 50, 100, 200, and 400 µg/mL), and *n*-hexane fraction (100, 200, 400, 800, and 1600 µg/mL) was added with 2 mL of 40 ppm DPPH solution. All mixture was incubated at room temperature for 30 min and stored in a dark room. The absorbance was measured by a spectrophotometer at 517 nm with 96% ethanol as a blank. The graph of concentration against absorbance was made to obtain a linear regression equation. Antioxidant activity was expressed as a percentage of inhibition and calculated by the formula (1).[7,8]

$$\% \text{ inhibition} = \frac{\text{Abs DPPH} - \text{Abs sampel}}{\text{Abs DPPH}} \text{ x } 100\% \qquad (1)$$

Determination of Antioxidant Activity Index (AAI)

AAI value was calculated by the formula (2). The AAI category of an extract is AAI < 0.5 means low antioxidant activity, AAI of 0.5-1 means moderate antioxidant activity, AAI of 1-2 means strong antioxidant activity, and AAI > 2 means very strong antioxidant activity.[9]

$$\text{AAI} = \frac{\text{concentration of DPPH } (\frac{\mu g}{mL})}{\text{IC50 } (\frac{\mu g}{mL})} \qquad (2)$$

Preparation of *S. epidermidis* and *P. acnes* Culture

Pure cultures of *S. epidermidis* ATCC 12228 and *P. acnes* ATCC 1223, were inoculated on slanted MHA and incubated for 24 h at 37 °C. Next, bacterial colonies were inoculated into 5 mL of sterile 0.9% NaCl. The turbidity of the bacterial suspension was compared to a 0.5 Mc Farland solution which is equivalent to $1.5x10^8$ CFU/mL. The 0.5 McFarland solution was prepared from a mixture of 1.175% barium chloride solution and 1% sulfuric acid solution in a ratio of 0.05: 9.95.[10]

Antibacterial Activity Assay

A petri dish containing MHA and 20 µL of bacterial suspension was allowed to solidify. The agar was perforated with a perforator (diameter = 7.18 mm) and filled with 50 µL of 25% clindamycin phosphate as a positive control, 1% DMSO as a negative control, and extract of 30, 40, 50, and 60%. The plates were incubated for 18-24 h at 37 °C. Each inhibition zone was measured using a caliper.[10]

Determination of Minimum Inhibitory Concentration (MIC)

The tube containing 1 mL of MHB was added with 1 mL of 50% extract, to obtain a concentration of 25%. A total of 1 mL of this solution was transferred into a tube containing 1 mL of MHB, to obtain a concentration of 12.5%. Microdilution was carried out to obtain concentrations of 6.25, 3.125, 1.5625, and 0.78125%. The negative control was MHB and the positive control was 25% clindamycin phosphate. All tubes were added with 0.1 mL of the test bacteria suspension and incubated for 18-24 h at 37 °C. Observation of turbidity by comparing with negative control and positive control. The MIC value was determined based on the smallest extract concentration indicating inhibition of bacterial growth.[10]

Determination of Minimum Bactericidal Concentration (MBC)

The determination of MBC was conducted using the agar plate method. A total of 0.1 mL was taken from the MIC tube, then dripped on the MHA surface, and incubated for 18-24 h at 37 °C. The MBC value is the lowest concentration where the number of colonies 0 or 99% can kill the initial bacterial colonies.[10]

RESULTS AND DISCUSSION

Antioxidant Activity Assay

The DPPH method is a sensitive, fast, and easy method to determine the antioxidant activity of plant extracts.[11] DPPH is a compound that has nitrogen free radicals. The reaction between DPPH and antioxidant compounds (H-A) is caused by the ability of antioxidants to convert DPPH as a stable-free radical into DPPH-H (non-radical form).[12] The maximum wavelength of the DPPH solution is 517 nm, following previous studies.[7,8] This wavelength is the wavelength of the green color (500-520 nm) as the transmitted color.[13] Antioxidant activity was observed by the color change of the DPPH solution. The DPPH solution changed from purple to yellow.[7,8] The color intensity of extracts or antioxidant compounds indicates antioxidant potential in terms of the ability to donate hydrogen.[12] Vitamin C as standard, represents both synthetic and natural antioxidants. Vitamin C scavenges reactive oxygen species to fight cellular damage from oxidative stress, by protecting proteins from alkylation by electrophilic lipid peroxidation products.[14] Vitamin C is a strong reducing agent and an excellent source of electrons for free radicals that seek electrons to regain their stability. Vitamin C can donate electrons to free radicals and stop their reactivity.[15] Antioxidant compounds derived from plants, including Fig leaves, protect the body from disease-inducing free radicals with little or no side effects. Antioxidant compounds reduce oxidative stress by stopping oxidative chain reactions, thereby inhibiting oxidative damage.[16]

Table-1: Antioxidant Activity and AAI of Vitamin C, Extract, and Fractions (n = 3)

Compounds	Concentration (µg/mL)	Absorbance	% of inhibition	Linear regression equation	IC_{50} value (µg/mL)	AAI value
Vitamin C	2	0.433 ± 0.004	49.65	y = 5.7625x + 38.725 R^2 = 0.9983	1.95	20.51
	4	0.325 ± 0.011	62.20			
	6	0.221 ± 0.005	74.30			
	8	0.138 ± 0.003	83.95			
	10	0.031 ± 0.005	96.40			
Extract	25	0.465 ± 0.007	45.93	y = 0.0679x + 44.575 R^2 = 0.9924	78.79	0.51
	50	0.438 ± 0.002	49.06			
	100	0.418 ± 0.006	51.39			
	200	0.371 ± 0.005	56.86			
	400	0.234 ± 0.017	72.27			
Distilled water fraction	25	0.521 ± 0.008	39.41	y = 0.0877x + 38.256 R^2 = 0.9951	133.90	0.30
	50	0.495 ± 0.014	42.44			
	100	0.449 ± 0.003	47.79			
	200	0.367 ± 0.002	56.97			
	400	0.325 ± 0.006	72.67			
Ethyl acetate fraction	25	0.441 ± 0.003	48.72	y = 0.0632x + 48.591 R^2 = 0.9908	22.29	1.79
	50	0.408 ± 0.017	52.55			
	100	0.383 ± 0.012	55.46			
	200	0.329 ± 0.003	61.74			
	400	0.228 ± 0.006	73.48			
n-Hexane fraction	100	0.620 ± 0.002	27.79	y = 0.0219x + 26.878 R^2 = 0.9922	1204.47	0.03
	200	0.589 ± 0.007	30.81			
	400	0.542 ± 0.002	36.99			
	800	0.469 ± 0.002	45.58			
	1600	0.334 ± 0.003	61.16			

The correlation coefficient values were in the range of 0.9908 to 0.9983 (Table-1). These values indicate the response of the visible spectrophotometer according to the measured concentration.[17] Extract, ethyl acetate fraction, and distilled water fraction of Ciwidey Fig leaves contain phenolic compounds, flavonoids, tannins, alkaloids, saponins, terpenoids, and steroids. While the *n*-hexane fraction only contains alkaloids and steroids.[6] These results indicate that the secondary metabolites in Fig leaves were polar (tannins, phenolic compounds, flavonoids, and saponins), semipolar (flavonoids and alkaloids), and non-polar

(alkaloids and steroids). The flavonoids, alkaloids, and steroids have various structures, so these groups were distributed to polar, semi-polar, and even non-polar fractions. The category of antioxidant activity of the ethyl acetate fraction (22.29 µg/mL) was very strong, extract (78.79 µg/mL) was strong, distilled water fraction (133.90 µg/mL) was medium, and the *n*-hexane fraction (1204.47 ug/mL) was very weak.[18] Polyphenol compounds as primary antioxidants inactivate radicals through the mechanism of (1) hydrogen atom transfer and (2) single electron transfer. In mechanism (1), the antioxidant reacts with the free radical, by transferring hydrogen atoms, through homolytic cleavage of the O-H bond. In mechanism (2), antioxidants provide electrons to donate to free radicals. Both mechanisms result in radical stabilization.[19] Tannins are polyphenolic compounds, so like many polyphenols, they exhibit antioxidant activity. Tannins suppress the formation of hydroxyl radicals.[20] The activity and chemical properties of flavonoids depend on their structural class, degree of hydroxylation, substitution and other conjugation, and degree of polymerization.[21] Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions. The most reactive hydroxyl groups (7-OH in flavones or 3-OH in flavonols) in flavonoids are generally glycosylated. Glycosylation increases solubility in aqueous cell environments, protecting reactive hydroxyl groups from autoxidation.[22] Many alkaloids showed *in vitro* antioxidant activity by the DPPH method. Some of them have antioxidant activity that is similar to or more active than standard antioxidants. The number of aromatic hydroxyl groups is the main determinant of this activity.[23] Saponins consist of aglycone units bound to one or more carbohydrates. The aglycone or sapogenin unit consists of a sterol or triterpene unit. In steroidal saponins and triterpenoids, the carbohydrate is bound to the C3 of the sapogenin.[24] Steroids with a hydroxyl group have antioxidant activity. Antioxidant activity depends on steroid dose (dose-response relationship).[25] The antioxidant and prooxidant activities of terpenes are highly dependent on concentration. At high concentrations, terpenes act as prooxidant compounds, while at low concentrations, terpenes act as antioxidant compounds.[26]

Determination of AAI

Table-1 showed antioxidant activity based on AAI. The category AAI of ethyl acetate fraction was strong, the extract was medium, and distilled water and *n*-hexane fractions were low.[9] The category of antioxidant activity based on the AAI value was lower than the IC_{50} value. This was due to differences in the calculation method. AAI value shows a more convincing value because it is not influenced by the DPPH concentration. This was because of the various DPPH concentration (range of 30-40 µg/mL) in different studies, so a more universal calculation is needed.

Antibacterial Activity Assay

Clindamycin phosphate, as a positive control, is FDA-approved to treat septicemia, intra-abdominal infections, lower respiratory tract infections, gynecological infections, bone and joint infections, and skin infections.[27] Clindamycin prevents the formation of peptide bonds, thereby inhibiting protein synthesis by reversibly binding to the 50S ribosomal subunit. It can be a bacteriostatic or bactericidal antibiotic, depending on the organism, site of infection, and concentration.[28] The cell wall specificity of *S. epidermidis* is teichoic acid consisting of glycerol with phosphodiester bonds bound to peptidoglycan and glycerol teichoic acid glucosyl in the structural component compared to other bacteria. *S. epidermidis* known as coagulase-negative and Gram-positive, is one of five important microorganisms found on human skin and mucosal surfaces.[29] The presence of *S. epidermidis* in high numbers in human skin microflora, extensive colonization of epithelial cells, and various virulence factors can be considered the main causes of nosocomial infection.[30] *P. acnes*, a Gram-positive anaerobic bacterium, is the main resident of normal human skin and dominates the pilosebaceous unit.[31] *P. acnes* is the most common cause of opportunistic infections and is associated with a variety of apparently different conditions including the skin disease acne vulgaris.[32]

Table-2: Antibacterial Activity of Extracts against *S. epidermidis* and *P. acnes* (n = 3)

Extract (%)	Inhibition zone (mm)	
	S. epidermidis	*P. acnes*
30	15.5 ± 0.361	16.56±0.683
40	17.03 ± 0.775	17.43±0.465

50	18.60 ± 0.507	18.35±0.218
60	19.98 ± 0.104	18.81±0.208
Clindamycin phosphate (25%)	21.16 ± 0.486	20.46±1.039

There was a significant difference between the extract concentration and the inhibition zone for *S. epidermidis* (p-value = 5.03 x 10^{-7}) and *P. acnes* (p-value = 1.59 x 10^{-4}). The increased inhibition zone was proportional to the extract concentration (Table-2). There was a significant difference between the inhibition zone with bacterial species (p-value = 2.75 x 10^{-11}). *S. epidermidis* and *P. acnes* had different responses to the secondary metabolites of Fig leaves which have antibacterial activity. Differences in cell wall structure determine penetration, binding, and antibacterial activity.[33] This causes differences in the inhibition of *S. epidermidis* and *P. acnes*.

Determination of MIC

The MIC test was conducted with the microdilution method and aimed to determine the lowest concentration of Fig leaves extract that could inhibit the growth of *S. epidermidis* and *P. acnes*. The lowest concentration of Fig leaves extract which can inhibit the growth of *S. epidermidis* and *P. acnes* was between 6.25 and 12.5% (Table-3). This was because of the 12.5% extract, there was the growth inhibition activity of *S. epidermidis* and *P. acnes*. While in the 6.25% extract, there was the growth of *S. epidermidis* and *P. acnes*, which was indicated by the turbidity. The results of the MIC test were continued to determine the MBC value.

Table-3: MIC of Extract against *S. epidermidis* and *P. acnes* (n = 3)

Extract concentration (% b/v)	Growth of	
	S. epidermidis	*P. acnes*
Negative control	+	+
Positive control	-	-
25	-	-
12.5	-	-
6.25	+	+
3.125	+	+
1.5625	+	+
0.78125	+	+

Note: (+) there were colonies; (-) there were no colonies

Determination of MBC

The difference between MIC and MBC was that MIC is the lowest concentration of an antimicrobial that can inhibit the growth of microorganisms, while MBC is the lowest concentration of an antimicrobial that can kill microorganisms. The MBC test aimed to determine the lowest concentration with 0 or 99% colony count that could kill the initial bacterial colonies. In the 12.5% extract, there was still growth of *S. epidermidis* and *P. acnes*. Meanwhile, the 25% extract did not find the growth of *S. epidermidis* and *P. acnes* bacteria. So, the MBC of Fig leaves extract was at a concentration between 12.5 and 25%. Secondary metabolites are important sources of novel and effective antibacterial compounds. These compounds were developed to combat drug resistance, due to the emergence of drug-resistant bacterial phenotypes.[34] Phenolic compounds have various chemical structures, so there are many possible mechanisms of antimicrobial activity. The extract contains a mixture of different polyphenol groups, so there is a multidimensional activity. The interaction between polyphenols in the extract can affect the mechanism of action. Phenolic compounds interact with bacterial cell walls, causing cell wall disruption and the release of cellular contents. Cell wall damage decreases the cell's resistance to unfavorable conditions, such as high or low osmotic pressure. Gram-negative bacteria are more resistant to phenolic compounds. This may be related to the presence of a lipophilic outer membrane containing high levels of phospholipids. This makes the cell wall impermeable to some macromolecules.[35] Polyphenols can destroy the outer membrane of Gram-negative bacteria, leading to increased membrane permeability.[36] There are four mechanisms of antibacterial activity of flavonoids, i.e. inhibition of nucleic acid synthesis,[37] inhibition of cytoplasmic membrane function,[38] inhibition of energy metabolism,[39] inhibition of cell membrane synthesis and aggregation effect on all bacterial cells.[40] There is a relationship between flavonoid activity and its structure, such as the number and position of hydroxyl or methoxy groups. The hydroxyl groups at C5 on the A ring

and C4 on the B ring and the methoxy groups at C3 and C8 on the A ring of flavonoids enhance the inhibitory effect of flavonoids. While, the hydroxyl group at C6 on ring A, at C3 and C5 on ring B, and at C3 on ring C or methoxy groups at C3 on ring B reduce flavonoid activity. A study on the quantitative structure-activity relationship showed that flavonoid activity was associated with molecular hydrophobicity and the OH group located at position 3 in the C-ring.[41] Tannins exhibit a high antimicrobial effect. The antibacterial activity of tannins depends on pH, temperature, type of solvent/matrix, and time of action.[42] Tannins are multidentate ligands that can bind to proteins, through hydrophobic interactions and hydrogen bonds, to inhibit bacterial metabolism. The antibacterial effectiveness of tannins is due to the ability of tannins to pass through the bacterial cell wall to the internal membrane and interfere with cell metabolism, leading to cell destruction. In Gram-positive bacteria, the activity of tannins is rapid. However, in Gram-negative bacteria, the rate is slower due to the presence of a two-layer membrane.[43] The biological properties of saponins depend on the aglycone structure and/or the number of sugar units.[44] The saponin's mechanism against eukaryotic cells is attributed to their cell membrane permeabilization properties by complexing them with cholesterol.[45] Saponins, which have surface active properties, can enter the lipid bilayer, bind cholesterol, form domains enriched with cholesterol-saponin complexes, and finally lyse the cells.[46] Triterpenes or steroids as glycosidic skeletons, and sugars to form branching chains make saponins have lipophilic and hydrophilic properties. There are the characteristics of surfactants, which lower the surface tension in an aqueous solution and form micelles.[47] The mechanism of antibacterial activity of alkaloids is related to their chemical structure.[48] Alkaloids inhibit bacterial growth through various mechanisms, including affecting DNA function, inhibition of protein synthesis, modification of bacterial cell membrane permeability, damage to cell membranes and cell walls, inhibition of bacterial metabolism, and inhibition of efflux pumps.[49]

CONCLUSION

The antioxidant activity of the extract and the fraction of ethyl acetate, distilled water, and *n*-hexane of Fig leaves were 78.79; 22.29; 133.90; and 1204.47 µg/mL, respectively. Fig extract has antibacterial activity against *S. epidermidis* and *P. acnes*. The MIC value was in the range of 6.25 and 12.5%, while the MBC value was in the range of 12.5 and 25%.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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