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Antibacterial Activities of Extract and Fraction From Methanol Extract of Manggis Leaves (Garcinia mangostana) on Bacteria Causing Diabetic Ulcer

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Abstract–Diabetes mellitus (DM) is a degenerative disease due to metabolic disorders with acute to chronic hyperglycemic conditions. The complications of this disease are exacerbated by bacterials infection causing ulcers or sores on feets, hands, knees, back and other parts of the body. The mangosteen plant (*Garcinia mangostana*) was believed as antidiabetic empirically and cured diabetic ulcers. This study aims to determine the comparison of antibacterial activity of extracts and fractions from mangosteen leaves at concentrations 20%, 40%, 60%, 80% and 100% againts bacterias which caused diabetic ulcers by in vitro. The research began with maceration of mangosteen leaves used methanol. The fractions were obtained by vacuum liquid chromatography. The bacterias that cause diabetic ulcers were obtained from ulcer specimens of diabetic ulcer patients. Antibacterial activity was tested using the disc diffusion method with 10% of DMSO as negative control. The positive control was determined by antibacterial sensitivity methods. Antibacterial activity was expressed by the large area of the inhibition zone on media that inoculated which ulcer bacterias and incubated for 24 hours at 37^0 C. The results showed that there were 5 dominant bacteria found in diabetic ulcers. There were *Escherichia spp, Klebsiella spp, Staphylococcus spp, Salmonella spp and Shigella spp*. The antibacterial sensitivity showed that ciprofloxacin had the largest area of inhibition zone compared to Amoxicillin, Ampicillin, Cefotaxim, Gentamycin, Erythromicin and Doxycicline. The results showed that mangosteen leaf extract at 100% concentration had the biggest of antibacterial activity with the largest inhibition zone was 27.10 mm ± 0.1 compared to the other concentration of methanol extract, fractions A, B and C in all concentration series, but smaller than the positive control of ciprofloxacin 5 µg / disk was 35.01 mm ± 0.1.

Keywords–Diabetic Ulcer, Mangosteen Leaves, Antibacterial)

I. INTRODUCTION

Diabetes mellitus (DM) is a degenerative disease caused by a complex metabolism characterized by chronic hyperglycemic conditions [1]. This condition increases the risk of complications such as peripheral neuropathy which is exacerbated by bacterialS infection causing ulcers on the feet, hands, knees, back and other parts of the body [2]. The prevalence of DM in Indonesia grow and increase based on 10.9% blood tests and 1.5% of a doctor's diagnosis [3]. The mortality rate for DM sufferers with ulcers ranges from 17-23% and had amputations ranges from 15-30% [4]. Diabetic ulcer patients in Indonesia has been requiring for a high cost medication of 1.3 million to IDR 1.6 million per month and IDR 43.5 million per year for a patient [5].

The presence of bacterias becomes the initial caused of ulcers which occures when hyperglycemic conditions made bacterias growth up and develop. The bacterias contained in diabetic ulcers were aerobic and anaerobic bacteria`s [6]. Anggriawan [7] reported that aerobic and anaerobic bacterias` in the specimens of diabetic ulcer patients were *Enterobacter sp.* (48%), *Staphylococcus aureus* (18.2%), *Streptococcus spp* (16.8%), and *Pseudomonas sp* (17%). Tahir and Nurwahidah (2019) also reported that gram negative bacterias such as *Proteus mirabilis* group`s became the highest value that infects diabetic ulcers about 20.5% and E. coli about 17.6%. Different types of bacterias caused diabetic ulcers are influenced by trauma, biomechanical abnormalities, limited joint motion, and an increased risk of infection. Treatment of diabetic ulcers is using antibiotics [8]. However, treatment with antibiotics needs to be reviewed because there are bacterias that are resistant to specific antibiotics.

Natural ingredients from plants could be an alternative treatment for diabetic ulcers that had been researched widely, including the treatment and healing of diabetic ulcers using banana leaves combined with seaweed by Juwono and Sudiarto [9], worm extracts by Mardianti et al [10] and turmeric extract by Doddy et al [11]. Previous research of the mangosteen plant (*Garcinia mangostana*) included *mangosteen* peel extract which was able to reduce the sugar level in blood system by alloxan-induced rats at a dose of 500 mg/kg BW [12], Pasaribu et al [13] reported that the ethanol extract of mangosteen rind was able to reduce blood sugar levels in mice at a dose of 100 mg/kg BW. Research on the comparison of the antibacterial activity of the extract and the fraction of the methanol extract of mangosteen leaves against the bacteria that cause diabetic ulcers has never been done, so this research is needed to find alternative solutions for diabetic ulcer treatment or treatment.

II. METHOD

A. *Extraction and Isolation*

The method used maceration process extraction by dilute 2 kg of mangosteen leaves powder with 20 liters of methanolt. Furthermore, the maserate was concentrated by rotary evaporator. Fractionation process used partition method to produce methanol fraction, etil asetat and n hexane fraction. The methanol extract og mangosteen leaves was partitioned with n heksan for 2 times to have n heksan fraction. Then methanol fraction was patitioned with ethyl acetate solvent to

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have ethyl acetat fraction. The ethyl acetate fraction was pretreated using silica gel, then it was fractionated using the silica gel as stationary phase and the mobile phases used n-hexane, ethyl acetate and n butanol. The elution process was carried out with a solvent starting from low polarity, it was n-hexane, then the polarity was increased from n hexane, ethyl acetate to n butanol. The column was sucked dry at each fraction collection. Each fraction was concentrated by rotary evaporator.

B. *Identification of Extract and Fraction*

Extracts and fractions were identified by phytochemicals including steroid/triterpene tests by adding a thick extract sample with acetic acid anhydride and concentrated sulfuric acid (Liebermann Burchard's reagent) to produce blue or blue green color indicating steroids, while red, pink or purple indicates the triterpenoids. Test flavonoids by adding a sample that has been crushed in a mortar with a little of water, transfer in a test tube, add a little of magnesium metal and 5 drops of 2 N HCl, the whole mixture is heated for 5–10 minutes. Then it is filtered in hot condition and allowed to cooling down. amyl alcohol was added to the filtrate, then shaken vigorously. A positive reaction showed of a red color on the amyl alcohol layer (Depkes RI, 1989). The alkaloid test was carried out with the sample in a mortar, alkalized with 1 mL of ammonia, then added chloroform and crushed strongly. The chloroform liquid was filtered, the filtrate is placed in a test tube then added 2 N HCl, shaken, then left until separation occured. In a separate test tube, a reaction is carried out which indicated of alkaloids. The filtrate was added 1 drop of Dragendorff's reagent solution indicated brownish sediment or turbidity, and the remained filtrate was added with 1 drop of Mayer reagent solution indicated white sediment. Saponin test was carried out by adding 1 g of thick extract with warm water, shaked it vertically for 10 seconds then leaved for 10 seconds. Foam formation as high as 1–10 cm which is stable for not less than 10 minutes, indicated of saponins. With the addition of 1 drop of 2 N HCl, the foam does not disappear (Depkes RI, 1995). The Tannin test was carried out with 200 mg of the extract dissolved in 20 ml of hot water and shaked until homogeneous. chilled and added of 3% FeCl3 showed a positive result if a blue-black or brownish green solution is formed.

C. *Preparation and Identification of Specimen Diabetic Ulcer*

Diabetic ulcer specimens were taken by swabbing method with sterile cotton swab for 3 swabbing times then brought to the Microbiology Laboratory of STIKES Bhakti Husada Mulia Madiun using the sample box in a cold state. The specimen was diluted in effendor with a dilution ranging from 10^{-1} to 10^{-7} CFU / uL. The sterilizing process used autoclave to stelilized of media and other equipment sauch as plates, ose, at a temperature of 121^0C for 15 minutes. The media was used NA media made by dissolved 20 g of Nutrient Agar (NA) in 1 liter of distilled water, heated to a boiled. 20 ml of liquid Mac conkay medium was poured into a petri dish and let it solidify. Tsi media was made with 1.5 plus 50 ml aquadest, heated to boiled, SIM media was made with 2 grams of media added with 50 ml of distilled water, heated to a boiled, Simmon citrate media for (citrate) was made with 1.5 grams plus aquadest 50 ml. All media were sterilized using an autoclave at 121^0C for 15 minutes, then poured into a petri dish and allowed to solidify.

Identification of Bacteria in Diabetic Ulcer Specimens was carried out based on the bacteria that grew on Nutrient Agar (NA) and Mac conkay media, then gram staining was carried out. The identification of bacteria was followed by biochemical test with the inoculation of the bacteria in the TSI (Tripple Sugar Iron) medium, SIM (Sulfide Indole Motility), citrate, MR-VP and LIA (Lactose Iron Agar), incubated for 24 hours at 37^0 C.

D. *Antibacterial Activity*

Antibacterial activity was tested using the disc diffusion method with 10% of DMSO as negative control. The positive control was determined by antibacterial sensitivity methods. Antibacterial activity was expressed by the large area of the inhibition zone on media that inoculated which ulcer bacterias and incubated for 24 hours at 37^0 C. The sensitivity test was carried out antibiotics to determine which antibiotics had high sensitivity for inhibit the growth of bacteria which from the isolation of diabetic ulcers. The Antibiotics used for sensitivity test were Amoxicillin (10 ug), Ampicillin (10 ug), Ciprofloxacin (5 ug), Cefotaxim (30 ug), Gentamycin (10 ug), Erythromicin (15 ug) and Doxycicline (30 ug) per disk. Antibacterial activity of extracts and fractions from methanol extract of mangosteen leaves using the disc diffusion method by in vitro with negative control was 10% of dimethyl sulfoxide (DMSO) and the samples tested were extracts and fractions of mangosteen leaves at concentration of 20%, 40%, 60%, 80% and 100%.

III. RESULT

The leaves of mangosteen (*Garcinia mangostana*) are obtained from mangosteen plantations in Dagangan village of Madiun Regency that had about 20 kg of fresh leaves. The fresh mangosteen leaves obtained for 9.3 kg of dried mangosteen leaves. It was be powder using a blender and obtained about 4.86 kg. Extraction by maceration resulted of methanol extract for about 287.2 grams with a yield of 14.6%. The amount of yield depends on the solubility of the bioactive components. This is because the solubility of bioactive component in this solvent depend on its ability to had formed with hydrogen bonds (Khopkar, 1990). Several factors that could affected the yield was extraction time, extraction method, sample size, storage, and the ratio of the number samples to the amount of solvent. The yield value can be interpreted by the amount of active compound content. The high level of bioactive compounds contained in extract was indicated by the amount of extract yield.

The solvent used for maceration is methanol. According to Ishmael et al (2004) in Ningsih (2017) methanol was chosen as a solvent because it had high polarity so that it is able to dissolve most of the compounds in the simplicia that can be extracted in methanol. Low polarity solvents attracted less active extracts than mixtures of ethanol and methanol or methanol alone. ECC fractionation of mangosteen leaves methanol extract resulted ethyl acetate fraction which was fractionated by KCV using n hexane, ethyl acetate and n butanol as solvents to produce fraction groups that were grouped based on thin layer chromatography (TLC) profiles. TLC monitoring used a stationary phase of silica gel 60 F254 and a mobile phase of ethyl acetate: formic acid: water (8: 0.5:

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0.5), emphasized by ammonia vapor, seen under UV light of 254 nm and 366 nm.

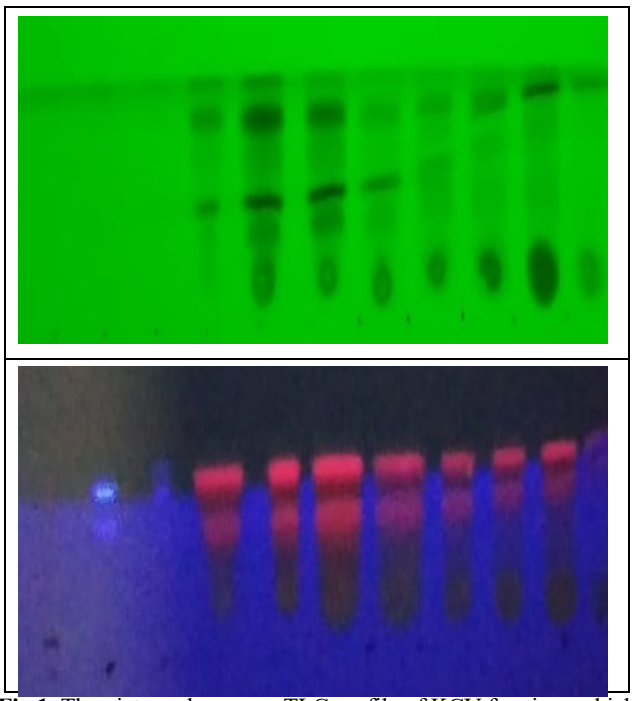

Fig 1. The picture above was TLC profile of KCV fractions which had been seen under light of UV 254 and 366. (a) TLC UV 254 profile, (b) TLC UV 366 profile

In the TLC profile above, spots appear to indicated metabolites compound in the CVC fraction. The spots were calculated for the Rf value and grouped according to their profile. The following was the data.

Table 1. Fractions profile by TLC (Thin Layer Chromatography)

Fraksi	Nilai Rf 1	Nilai Rf 2	Kelompok
1	0	0	A
2	0	0	
3	0	0	
4	0,36	0,68	B
5	0,36	0,64	
6	0,41	0,65	C
7	0,41	0,64	
8	0,41	0,64	
9	0,41	0,65	
10	0,41	0,65	
11	0,42	0,65	

The results of phytochemical identification were obtained based on the table above. There were methanol extract contains two kind of metabolites compound such as alkaloids, flavonoids, tannins, saponins and polyphenols. The ethyl acetate fraction contains alkaloids, flavonoids and polyphenols. Meanwhile, then hexane fraction contains steroids. The results of phytochemical identification can be seen in the table below:

Table 2. Result of Phytochemistry Identification

Senyawa	Metode	EM	FEA	FNH
Alkaloid	Dragendorff	+	+	-
Flavonoid	Serbuk Mg, HCl pekat	+	+	-
Tanin	$FeCl_3$	+	-	-
Saponin	Pembentukan Busa	+	-	-
Polifenol	$FeCl_{3\ 5\%}$	+	+	-
Steroid/ Terpenoid	*Lieberman Burchard*	-	-	+

The identification of bacteria from of Diabetic Ulcer specimen was carried out by isolation where the ulcer specimen was diluted 7 times with NaCl, then inoculated on Nutrient agar and Mac conkay media. Furthermore, gram staining was carried out to produce bacteria with the following morphology.

Fig 2. The result of gram stain of bactery that isolation from specimen diabetic ulcer

Based on the result of the gram stain above, it showed that the bacteria contained in the diabetic ulcer specimen have a pink to red stem shape and color. Hidayat (2011) stated that gram-positive bacterial cells added with crystal violet dye will turn purple because they absorb the dye. When added with lugol and washed with alcohol, it will remain purple because the bacterial cells strongly bind the crystal violet-lugol complex. Whereas Gram negative bacteria will become colorless when added with crystal violet because gram negative bacteria have a thin peptidoglycan layer when added with safranin it will be absorbed and gram negative bacterial cells will be red. The results of biochemical identification of bacteria are as follows:

Tabel 3. Result of Biochemistry test

No	Jenis Uji	Isolat				
		I	II	III	IV	V
1	Motilitas	+	+	-	+	-
2	Indol	-	-	-	+	+
3	MR	-	-	-	-	+
4	VP	+	+	+	-	+
5	TSIA	-	-	+		
	Sukrosa	-	-	+	+	+
	Laktosa	+	-	+	-	+
	Glukosa	+	-	+	+	+
	Gas	+	-	+	+	+
	H_2S	-	-	-	+	+
6	Simmon citrat	-	+	+	+	+
7	LIA	+	-	+	+	-

Based on the biochemical test, there found 5 dominant bacteria in the diabetic ulcer specimen were isolate I was Enterobacter spp, isolate II Shigella spp, isolate III Klebsiella spp, isolate IV Pseudomonas spp and isolate V Salmonella spp. Before the antibacterial activity test was

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carried out, antibiotic sensitivity test was carried out against bacteria that cause diabetic ulcers. The results of the aantibiotic sensitivity test against bacteria that cause diabetic ulcers are as follows:

Table 4. Result of Sensitivity of Antibiotics

No	Antibiotik (disk)	Rata-rata zona hambat
1	Amoxicillin 10 ug	7,03 ± 0,06
2	Ampicillin 10 ug	0 ± 0
3	Ciprofloxacin 5 ug	35,1 ± 0,1
4	Cefotaxim 30 ug	25,03 ± 0,05
5	Gentamycin 10 ug	15,0 ± 0
6	Eryithromycin 15 ug	6,07 ± 0,06
7	Doxycicline 30 ug	20,63 ± 0,64

Based on the results of the sensitivity test to the antibiotics above it showed the antibiotics which had the largest zone to inhibited bacteria caused diabetic ulcer was Ciprofloxacin 5 ug/disk compared to others. The results of antibacterial tests against bacteria that cause diabetic ulcers are as follows:

Table 5. Result of antibacterial activity

No	Kelompok Uji	Rata –rata Zona Hambat (mm)
1	K (-) DMSO 10 %	0
2	K (+) Ciprofloxacin 5 µg/disk	35,10 ± 0,1
3	Ekstrak metanol 20 %	5,0 ± 0
4	Ekstrak metanol 40 %	10,13 ± 0,11
5	Ekstrak metanol 60 %	15,07 ± 0,11
6	Ekstrak metanol 80 %	20,07 ± 0,06
7	Ekstrak metanol 100 %	27,01 ± 0,1
8	Fraksi A 20 %	0 ± 0
9	Fraksi A 40 %	0 ± 0
10	Fraksi A 60 %	0 ± 0
11	Fraksi A 80 %	0 ± 0
12	Fraksi A 100 %	2,1 ± 0,1
13	Fraksi B 20 %	5,03 ± 0,06
14	Fraksi B 40 %	7,07 ± 0,11
15	Fraksi B 60 %	11,10 ± 0,10
16	Fraksi B 80 %	13,03 ± 0,06
17	Fraksi B 100 %	15,13 ± 0,15
18	Fraksi C 20 %	0 ± 0
19	Fraksi C 40 %	0 ± 0
20	Fraksi C 60 %	0 ± 0
21	Fraksi C 80 %	2,9 ± 0,1
22	Fraksi C 100 %	3,93 ± 0,06

Based on the results of the antibacterial activity test above, it was found that the methanol extract of mangosteen leaves had the potential to inhibit the growth of bacteria that cause diabetic ulcers at a concentration of 20% was 5.0 mm ± 0.40% was 10.13 mm ± 0.11, 60% of was 15.07. mm ± 0.11, 80% was 20.07 mm ± 0.06 and 100% was 27.01 mm ± 0.1. At a concentration of 100% the mangosteen leaf methanol extract was able to inhibit the growth of bacteria that cause diabetic ulcers with the largest inhibition zone area of 27.01 mm ± 0.1.

The mangosteen (Garcinia mangostana) leaf methanol extract fraction from CVC also had potential as antibacterial at other concentrations. Based on the results of the fractionation by coloumn vacuum chromatography, 11 fractions were grouped into 3 large fractions were fractions A, B and C. The three large groups of fractions were tested for their antibacterial activity at concentrations of 20%, 40%, 60%, 80% and 100%. In the three large groups fraction, fraction B at concentration of 100% was the largest to inhibit the growth of diabetic ulcers bacteria for about 15.13 mm ± 0.15 compared to fraction A, fraction B and fraction C in all concentration series. So it can be concluded that methanol extract has the best antibacterial activity compared to the other three fractions.

Based on the test results for the antibacterial activity of methanol extract, fraction A, fraction B, and fraction C, it can be seen that the best antibacterial activity is produced by a concentration of 100%. This is because the higher the concentration of an antimicrobial substance, the greater the antimicrobial activity (Ningsih et al, 2017). The antibacterial mechanism of secondary metabolite compounds were alkaloids could inhibited the growth of bacterials by disrupting the integrity of peptidoglycan constituent components in bacterial cells (Rahman et al., 2016). Flavonoids can inhibit nucleic acid synthesis, inhibit cell membrane function and inhibit energy metabolism (Rahman et al., 2016). Saponins increase cell membrane permeability so that the membrane becomes unstable and results in cell hemolysis. Tannins work by deactivating bacterial adhesins, inhibiting the work of enzymes, inhibiting protein transport to the cell sheath. (Rahman et al, 2016). Polyphenols denature and protein coagulation causes protein precipitation and denaturation causing protein coagulation and cell membrane lysis (Wulandari, 2009). Steroids cause leakage in bacteriological liposomes by interacting with membrane phospholipids causing decreased membrane integrity and altered cell membrane morphology causing brittle cells and lysis (Thresia, 2016)

IV. CONCLUSION

Based on the results of this study, it can be concluded that the methanol extract of mangosteen leaves at concentration of 100% had the greatest antibacterial activity with an inhibition zone about 27.01 mm ± 0.1 compared to methanol extract at a concentration of 20%, 40%, 60%, 80%, Fraction A, Fraction B and Fraction C in all concentration series. Suggestions for further research are the isolation and identification of bacteria from diabetic ulcer specimens using biochemical, catalase, and PCR tests.

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Based on the results of this study, it can be concluded that the methanol extract of mangosteen leaves at concentration of 100% had the greatest antibacterial activity with an inhibition zone about 27.01 mm ± 0.1 compared to methanol extract at a concentration of 20%, 40%, 60%, 80%, Fraction A, Fraction B and Fraction C in all concentration series. Suggestions for further research are the isolation and identification of bacteria from diabetic ulcer specimens using biochemical, catalase, and PCR tests.

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