

ANTIBACTERIAL ACTIVITY OF FRACTIONS OF ETHYL ACETATE EXTRACT OF *GARCINIA LATISSIMA* MIQ. FRUITS**NENENG SITI SILFI AMBARWATI^{1,2}, AMARILA MALIK^{2*}, AGENG TRI LISTARI², NIRWANA², BERNA ELYA², MUHAMMAD HANAFI³**

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Received: 20 April 2017, Revised and Accepted: 13 July 2017

ABSTRACT

Objectives: The emergence of new infections and increase in bacterial drug resistance has created a serious need for the expansion of new antibacterial agents from natural sources. The study was carried out to evaluate the antibacterial activity of fractions of ethyl acetate extract of *Garcinia latissima* Miq. fruits.

Methods: The fractionation was done using a silica gel column and organic solvents as the eluent, i.e., n-hexane, ethyl acetate, and methanol. All fractions were assayed for antibacterial activity, which was done by performing disc diffusion for growth inhibition against *Bacillus subtilis* and *Pseudomonas aeruginosa*. In addition, the growth inhibition activity was also examined by performing bioautography assay using pre-coated silica gel 60 GF 254 plates as the stationary phase. Fractions A-F were eluted using n-hexane:chloroform (1:4), while Fractions G-K were used ethyl acetate:dichloromethane (4:1) as the mobile phase. The plate was visualized by ultraviolet at λ 254 nm and 366 nm, while the other one was contacted with the inoculated agar medium to observe zone inhibition. Further, the minimum inhibitory concentration (MIC) value was determined by performing microdilution.

Results: The result showed that the antibacterial activity of all fractions was more active at inhibiting the growth of *B. subtilis* than *P. aeruginosa*, mainly for Fractions H and J. However, the strongest antibacterial activity was showed by Fractions H and J against *B. subtilis*, MIC 312.5 $\mu\text{g/mL}$ (lower than reference, which is erythromycin antibiotic (25 $\mu\text{g/mL}$), followed by Fraction D against *B. subtilis* MIC 625 $\mu\text{g/mL}$, Fraction K against *P. aeruginosa* MIC 625 $\mu\text{g/mL}$, whereas Fractions C, E, and G against *B. subtilis*, and Fraction E against *P. aeruginosa* also showed low MIC values (1.250 $\mu\text{g/mL}$).

Conclusions: The results indicated that fractions of *G. latissima* Miq. fruit ethyl acetate extracts possessed antibacterial activity. The most active fraction that inhibited the growth of *B. subtilis* was shown by Fractions H and J; these fractions have the potential to be developed as new antibacterial agents.

Keywords: Antibacterial activity, *Garcinia latissima* Miq., Fruit fraction, *Bacillus subtilis*, *Pseudomonas aeruginosa*.

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INTRODUCTION

Infectious diseases have persisted as a major health problem for almost half a century now, and there has been increasing incidence of resistance to currently available antibacterials [1]. There is the need for intensive studies for the possible discovery of new agents with antibacterial potentials [1]. Microorganisms have unfavorable impact on the quality and safety of life [2]. To against these microorganisms widely used chemicals [2]. The occurrence of drug resistance demands new antimicrobial. Therefore, research need to be done to find new antimicrobials, especially from plants.

Since the existence of humans, plants have been used for the drug and a major source of phytochemicals existing in conventional medicine [3]. Ethnobotany studies have described the relationship of cultures and the traditional use of plants [3]. The chemical ingredients of plants play a major role in new cures. Antibiotics were a revolutionary development and way to control pathogens and infectious diseases. However, there are millions of people dependent on these synthetic drugs. Citizens who live in remote places rely on traditional medicines with which they are familiar and trust [4]. About three-quarters of the world's population relies primarily on plants and plant concentrates for their health care [4]. The presence of various constituents of phytochemicals in plant tissue that influence a definite physiological action on the human body caused drug plants have a large role in the discovery of new

drugs. Very few of these chemicals are toxic also [4]. The plant *Garcinia latissima* Miq. is part of the Clusiaceae (Clan, garcinia) family, which consists of 27 genera, about 1,090 species [5]. Many are xanthenes [5]. *G. latissima* Miq. has a broad spectrum of medicinal properties, such as anti-*Bacillus subtilis* and anti-*Staphylococcus aureus* [6].

We conducted prior research on the antimicrobial activity of *G. latissima* fruit ethyl acetate extracts against two positive bacteria (*S. aureus* and *B. subtilis*), two negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and two fungi (*Trichophyton mentagrophytes* and *Candida albicans*). Our results showed that this extract was active against *B. subtilis* and *P. aeruginosa* and inactive against other bacteria. The purpose of this study was to evaluate the antibacterial activity of the fractions of *G. latissima* fruits ethyl acetate extracts against *B. subtilis* and *P. aeruginosa*.

METHODS**Extract material**

G. latissima Miq. fruit ethyl acetate extract from our previous research in the Phytochemistry Laboratory, Faculty of Pharmacy, Universitas Indonesia was used.

Preparation of fractions

We fractioned a portion of the extract of *G. latissima* Miq. fruits (25 g) by filter column chromatography over 250 g of silica gel 60 (S). The

column chromatography was eluted with approximately 2.5 L of the solvents hexane, ethyl acetate, and methanol, in order of increasing polarity, until a clear extract was obtained at the end of the elution [3]. We collected the eluate in 100-mL bottles and subjected each fraction to evaporate. Fractions were stored at 4°C until assayed.

Antimicrobial assay of different fractions

Bacterial strains

We individually tested the various fractions of *G. latissima* Miq. fruit ethyl acetate extract against two bacteria including Gram-positive bacteria (*B. subtilis* ATCC 6633) and Gram-negative bacteria (*P. aeruginosa* ATCC 27853).

We obtained the pure bacterial strains from the Microbiology Laboratory of Pharmacy Faculty, Universitas Indonesia. *B. subtilis* was cultured overnight (for 24 hrs) at 37°C in nutrient agar (Merck/Difco). *P. aeruginosa* was cultured overnight in Cetrimide (Merck). The bacteria colonies were transferred into a sterile loop or cotton swab and mixed well from the fresh agar plate (Diastuti et al., 2014). The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland turbidity standard (Diastuti et al., 2014). This suspension resulted in a suspension containing approximately 1×10^9 CFU/mL [7].

Antibacterial test using the agar diffusion method (disc) and diameter of the inhibition zone

A preliminary evaluation of the antibacterial activity of the different fractions of the *G. latissima* Miq. ethyl acetate extracts was determined using the disc diffusion method [4]. Briefly, all the samples were dissolved in dimethyl sulfoxide (DMSO) [3]. We impregnated the disc (6 mm in diameter) with 5,000 mg/mL fractions (20 µL/disc) placed on the inoculated agar. 1 mL of inoculum of the bacterial strains (10^6 CFU/mL) was added to 4 mL 11 antibiotic medium (Merck). Then, the vortex was used to obtain a homogenous seed layer. The seed layer immediately was poured into Petri dishes (90 mm) already filled with 20 mL of the solid base layer (nutrient agar [Difco]) for *B. subtilis* and Cetrimide for *P. aeruginosa*. Then, 6 mm wells were used and filled with 20 µL of samples (20,000 ppm). The positive reference for bacteria used erythromycin (15 µg/disc) (Oxoid) and gentamycin (10 µg/disc) (Oxoid). The negative control used DMSO. 3 hrs were required for pre-incubated the Petri dishes at room temperature, allowing the complete diffusion of the samples; then, they were incubated at 37°C for 24 hrs [3]. The total diffusion of samples into the nutrient agar was visually perceptible and confirmed.

Minimum inhibitory concentration (MIC)

The MIC of plant fractions was evaluated by the microdilution method [2]. The fraction solutions were transferred in 80% DMSO, v/v (20,000 ppm, 50 µL) into 96 well plates in the first row. Then, 50 µL of nutrient broth was added to all test wells. Seven-fold serial dilutions were performed using a micropipette such that each well had 50 µL of the test material in serially descending concentrations. Finally, 50 µL of bacterial suspension (10^6 µL of assay bacterial inoculum of approx. 10^6 CFU/mL and 40 µL nutrient broth) was added to each well. The fraction solution concentrations were 5,000, 2,500, 1,250, 625, 312.5, 156.25, and 78.125 ppm from top to bottom. Each plate had a set of controls: A column with a broad-spectrum antibiotic as a positive control, and a column with 100% DMSO (v/v) solution as a negative control. The plates were prepared in triplicate and incubated at 37°C for 24 hrs, respectively. Then, 10 µL of MTT salt solution was added to each well (BBI Life Sciences) prepared by dissolving 6 mg of MTT in 10 mL of sterile distilled water. Visual assessment was done to see whether there was any discoloration. Their growth was marked by color changes from yellow or colorless-to-purple or pink. The lowest concentration in which no color change appeared was taken as the MIC value [4].

Bio-autography

In 1946, Goodall and Levi combined the paper chromatography method with contact bioautography to detect different penicillins for their determination. After that, Fischer and Lautner introduced thin-layer

chromatography (TLC) in the same field. This technique combines TLC with both biological and chemical detection methods. Several works have been done on the screening of organic extracts, mainly plant extracts, for antibacterial activity by TLC-bioautography.

This research used the agar diffusion technique or contact bioautography [8]. In addition, known as the agar contact method, it is the least-employed one of the techniques. It involves the transfer by diffusion of the antibacterial agent from the chromatogram (TLC) to an agar plate previously inoculated with the microorganism tested. After 1 hr to allow diffusion, the chromatogram is removed, and the agar plate is incubated. The growth inhibition zones appear in the place where the antibacterial compounds come into contact with the agar layer [8].

Silica gel TLC 60 GF 254 plates were used. Plant extracts (200 mg/mL) were applied (5 µL). The chromatogram was developed using n-hexane:chloroform (1:4) for Fractions C-F and ethyl acetate:dichloromethane (4:1) for Fractions G-K as the solvent. TLC plates were eluted in duplicate, and one set was used as the reference chromatogram. Chromatograms were visualized by ultraviolet irradiation (254 and 366 nm) and vanillin/sulphuric acid 2% spray reagent. The other sets were used for bioautography [9].

Bioautography was performed with *B. subtilis* and *P. aeruginosa* exhibiting high sensitivity to the fractions. For bioautography, the solvent of the TLC plates was completely removed. TLC plates were placed face down on agar containing an aliquot of an overnight culture and incubated at 37°C [10].

RESULTS AND DISCUSSION

The antibacterial activity of fractions of ethyl acetate extract of *G. latissima* Miq. fruits against *B. subtilis* and *P. aeruginosa* was determined. To perform a rapid screening study of potential antibacterial activity, a disc diffusion assay followed by bioautography is the best combined method, in addition to MIC [3]. The inhibition zone results are presented in Table 1.

The disc diffusion assay results showed that fractions of *G. latissima* Miq. fruit ethyl acetate extract exhibited strong growth inhibition activity against *B. subtilis* with diameter, i.e., 12.283 ± 2.2420 mm and 11.108 ± 0.3800 mm by Fractions E and D, respectively. Strong activity against *P. aeruginosa* was exhibited as well, but by Fraction K only with an inhibition zone diameter of 16.100 ± 7.6254 mm.

According to previous study, fractions with an inhibition zone diameter over 11 mm are assumed to have strong antimicrobial activity; while 6-11 mm and <6 mm are categorized as moderate and weak, respectively [11]. The antibiotic used in this study was carried out as

Table 1: Zone of inhibition of *G. latissima* Miq. fruit ethyl acetate extract fractions^a

Fractions	Diameter of inhibition zone (mm) ^b	
	<i>B. subtilis</i>	<i>P. aeruginosa</i>
A	0	0
B	0	0
C	9.458±1.5239	0
D	11.108±0.3800	0
E	12.283±2.2420	9.316±5.4862
F	10.392±1.4489	8.242±0.4706
G	9.992±0.3412	10.583±0.591
H	9.135±0.7134	9.408±0.4620
I	0	8.542±0.1242
J	6.725±0.8239	8.692±0.8351
K	0	16.100±7.6254
Antibiotic standard	23.58±0.988	17.92±0.549

^aValues are mean±SD of six separate experiments, ^bdiameter of the inhibition zone (mm) includes the disc diameter (6 mm). *P. aeruginosa*: *Pseudomonas aeruginosa*, *B. subtilis*: *Bacillus subtilis*, *G. latissima*: *Garcinia latissima*

a positive control for the assay performed. There is no consensus on the acceptable level of inhibition for natural products when compared with antibiotics standards [12]. Hence, the antibacterial potential of the fractions can be assumed as good, indeed, although the zone diameters obtained are much lower than results of antibiotics. The Gram-positive bacteria (e.g., *B. subtilis*) are known to be more sensitive to plant active extracts than Gram-negative bacteria (e.g., *P. aeruginosa*). This finding, as suggested by Ohadoma *et al.*, indicates that the sensitivity to each group of bacteria is based on the fact that the cell wall of Gram-positive bacteria is less complex than other groups and does not have the effect of the natural sieve against large molecules [2]. The results for the MIC are presented in Table 2.

The results of the MIC assay showed that fractions of *G. latissima* Miq. ethyl acetate extract indicated moderate activity against *B. subtilis* with the lowest MIC values (312.5 ppm by Fractions H and J). The fractions of *G. latissima* Miq. ethyl acetate extract showed weak activity against *P. aeruginosa* with the lowest MIC values (625 ppm by Fraction K). Fernandez *et al.* reported that the strength of antibacterial activity can be categorized using MIC values. Fractions that showed values of MIC at concentrations of <100 ppm were classified as having good antibacterial activity [13]. Values of MIC from 100 to 500 ppm and from 500 to 1,000 ppm were classified as moderate and weak, respectively, while those over 1,000 ppm were considered inactive [13]. In general, the antibacterial activity of the tested fractions in this study was set to be comparable with the reference antimicrobe erythromycin against *B. subtilis* and gentamycin against *P. aeruginosa*.

The results indicated that fractions of ethyl acetate showed moderate inhibition activity against *B. subtilis* and weak inhibition activity against *P. aeruginosa*. The fractions showed varying degree of inhibitory effects. The results revealed that the most susceptible bacterial strain was *B. subtilis* to Fractions H and J. Medicinal plants are an effective source of both traditional and modern medicines [14]. Herbal medicine has been shown to have real utility, and about 80% of the rural population depends on it for primary healthcare [14]. The evaluation of the antibacterial potentials of various plants is imperative, as infections are known to be caused by microorganisms, especially genitourinary infections [14]. The MIC tests result of different fractions showed that *B. subtilis* more vulnerable than *P. aeruginosa*. The plant fractions were more efficient against Gram-positive than Gram-negative organisms [14]. The MIC of the plant's fractions for Gram-negative organisms was >1,000 µg/mL, except Fraction K with a value 625 ppm.

Tetrazolium salt used in this MIC microdilution assay is a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is converted to purple formazan by the dehydrogenases enzyme of living microorganisms [15]. This reaction is a reduction reaction, and the purple to show the bacteria growth [15]. Makes this assay reliable for visual observation. The bioautography results show that the growth of the bacteria tested, *B. subtilis*, was inhibited by a higher number of inhibition bands, indicating a higher number of active compounds compared to *P. aeruginosa* (Figs. 1 and 2). Some of the compounds, which were separated by TLC previously, showed that they could act against both bacteria. The compounds that have activity against *B. subtilis* and *P. aeruginosa* may possess broad antibacterial action; they may even be general metabolic toxins that could be toxic to animals as well [16]. Bioautography is an antibacterial activity test that is not quantitative. This test showed that the fractions of TLC could separate into some compounds, which have antibacterial activity. The absence of bioactivity in some fractions of fruit ethyl acetate extracts allow for synergistic and additive interactions so that they are active [16].

Humanity urgently needs new antibiotics to fight bacterial diseases. Many antibiotics are known to be powerful, but no longer effective against these and other infections. The situation is mostly related to increasing bacterial resistance to commonly used antibiotics [15]. Chromatography is the perfect tool for analyzing plant constituents. The chromatographic method that is ideal for this type of connection

Table 2: MIC values of ethyl acetate fraction extracts of *G. latissima* Miq. fruits against *B. subtilis* and *P. aeruginosa* (ppm)

Fractions	<i>B. subtilis</i>	<i>P. aeruginosa</i>
A	>5,000	>5,000
B	>5,000	>5,000
C	1,250	>5,000
D	625	>5,000
E	1,250	1,250
F	2,500	>5,000
G	1,250	5,000
H	312.5	2,500
I	>5,000	2,500
J	312.5	2,500
K	>5,000	625
Antibiotic standard	25	0.39

P. aeruginosa: *Pseudomonas aeruginosa*, *B. subtilis*: *Bacillus subtilis*, *G. latissima*: *Garcinia latissima*

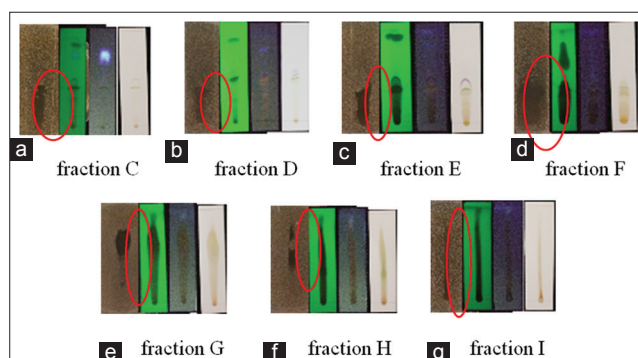


Fig. 1: (a-g) Thin-layer chromatography Si60 F254 plates - contact (indirect) bioautography of fractions of *Garcinia latissima* Miq. ethyl acetate extract against *Bacillus subtilis*

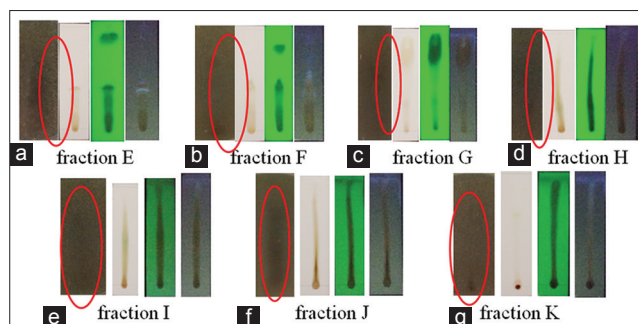


Fig. 2: (a-g) Thin-layer chromatography Si60 F254 plates - contact (indirect) bioautography of fractions of *Garcinia latissima* Miq. ethyl acetate extract against *Pseudomonas aeruginosa*

is thin-layer chromatography (TLC). TLC-bioautography (TLC-B) is a combination of TLC with biological assay such as antibacterial. Thin-layer chromatography-contact bioautography (TLC-CB) is one of three variants of a TLC-B method (the others are direct and agar overlay bioautography), a method of separation was performed using TLC, then tested the activity, and observed visually after incubation. The antibacterial activity could be attributed to the effects of bioactive phytochemical constituents, such as flavonoids [1].

CONCLUSION

This study demonstrated that fractions of ethyl acetate extract of *G. latissima* Miq. fruits possessed various degrees of antibacterial activity. It is concluded that ethyl acetate fractions of *G. latissima* Miq.

fruits possessed moderately antibacterial activity. Therefore, this could serve as a potential source of industrial drugs useful in some bacterial infections. To obtain the active ingredients of *G. latissima* Miq. fruit fractions, we need further research to isolate the active compound and will likely need to synthesize the active compounds as well.

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