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Research Article

ANTIBACTERIAL ACTIVITY AGAINST BACILLUS SUBTILIS AND ANTIOXIDANT PROPERTIES OF METHANOL EXTRACTS FROM GARCINIA LATISSIMA MIQ. LEAVES

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ABSTRACT

Objective: The objective of this study was to identify fractions with the highest antibacterial activity against *Bacillus subtilis* and to determine antioxidant activities and establish the chromatographic fractions as candidate antibacterial and antioxidant agents.

Methods: Extracts were fractionated using column chromatography, and antibacterial activities were assayed by the analyses of inhibition zones and bioautography, as well as by broth microdilution techniques. Antioxidant activities were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Results: The strongest antibacterial activity against *B. subtilis* (ATCC 6633) was observed with fractions B and C obtained in this research, with a minimum inhibitory concentration value of 312.5 µg/mL. The effective percentage (EP) value of crude extract at 10 µg/mL was 29.47±2.01%. Fractions C and D had greater EP values than the crude extract, whereas fraction D had the highest scavenging activity against DPPH free radicals (37.73±1.44%) when used at 10 µg/mL. The half effective concentration of the extract was 23.40 µg/mL, whereas that of the most active fraction D was 19.38 µg/mL and quercetin as positive control was 3.72 µg/mL.

Conclusion: The present data confirm that fractions of methanol extract from *Garcinia latissima* Miq. leaves possess antibacterial and antioxidant activities. These observations may facilitate the development of antimicrobial phytomedicines with a wide spectrum of activities and standardized antioxidant properties.

Keywords: 2,2-diphenyl-1-picrylhydrazyl, *Bacillus subtilis*, Fractions, *Garcinia latissima* Miq., Minimum inhibitory concentration.

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INTRODUCTION

The frequency of life-threatening diseases due to pathogenic microorganisms has risen globally, and it is becoming one of the leading causes of morbidity and mortality in immunocompromised patients [1]. The control of antimicrobial resistance requires the use of better and more effective antibiotics [2], and appropriate antimicrobials with no side effects are urgently needed [1]. As estimated by the World Health Organization (WHO), about 75% of the population in developing countries relies on plant-based traditional medicines [3]. Plants, such as vegetables and medicinal herbs, contain numerous molecules with free radical scavenging and antioxidant properties, such as nitrogen compounds, phenolic compounds, terpenoids, vitamins, and various endogenous metabolites [1]. Many studies have identified new antimicrobial and antioxidant compounds from natural sources, including animals, microorganisms, and plants. However, because botanical flora includes thousands of organisms, numerous medicinal compounds remain unexploited [1].

Previous studies show that fractions of methanol extract from *Garcinia latissima* Miq. leaves are active against *Bacillus subtilis*. At a concentration of 2%, inhibition zones with diameters of 9.9±0.786 mm were obtained with these extracts against *B. subtilis*. These extracts also had minimum inhibition concentration (MIC) values of 10,000 µg/mL and minimum bactericidal concentration values of 20,000 µg/m [4]. As indicated in previous phytochemical screening studies, methanol extracts of *G. latissima* Miq. contain saponin and tannin [4]. In this study, this extract was fractioned using column chromatography with silica gel stationary phase and mobile phases of increasing polarity. Antibacterial activities of the resulting fractions were tested against *B. subtilis*, and antioxidant properties of extracts and fractions were compared. The objective of this study was to identify fractions with the highest antibacterial activity against *B. Subtilis* and to determine antioxidant activities and establish the chromatographic fractions as candidate antibacterial and antioxidant agents.

METHODS

Microorganisms

Antibacterial activities of test substances were determined against *B. subtilis* (ATCC 6633) cells, maintained on an agar slant in a refrigerator at 4°C.

Phytochemical materials

Test materials were obtained during our previous study and included methanol extracts (Fig. 1) of leaves from plants available at the center for plant conservation at Bogor Botanical Garden. In our previous research, we further fractionated methanol extract and an obtained fraction was then named: Fraction B, fraction C, fraction D, fraction E, fraction F, and fraction G which bioautography of active fractions were presented in Fig. 1.

Fraction B was eluted with n-hexane:ethyl acetate (1:1), fractions C and D were eluted with n-hexane:ethyl acetate (1:3), fraction E was eluted with n-hexane:ethyl acetate (1:9), fraction F was eluted with ethyl acetate:dichloromethane (4:1), and fraction G was eluted with ethyl acetate:dichloromethane (4:1).

Fractionation

The methanol extract (25.41 g) was fractionated using a chromatographic column filled with Silica Gel G60 with a diameter of 45 mm and a height of 330 mm using a 70-230 mesh (E Merck 7734.1000) stationary phase. The mobile phase was a gradient of solvents with increasing polarity (n-hexane, ethyl acetate, and methanol). The flow rate was 20 mL/min, and 100-mL elutes were collected in bottles [5]. The contents of these bottles were assayed using thin-layer chromatography (TLC). The fraction with identical chromatographic patterns was collected, and solvents were evaporated under a flow of air until dry and stored in a refrigerator.

***In vitro* antibacterial assays**

Preparation and standardization of inocula

Bacterial cell suspensions were prepared separately from 24-h Mueller-Hinton agar cultures. Colonies of microorganisms were diluted in 0.9% NaCl to obtain a McFarland standard turbidity of 0.5 using visual assessments and then diluted to approximately 10^6 CFU/ml [1].

Broth microdilution test

To determine MIC values of test substances, broth microdilution assays were performed in 96-well microplates [1]. Assays in 96-well microplates were prepared by adding 50-µL aliquots of test substances to the first wells and three-fold serial dilutions in subsequent wells. Standardized inocula were then added to each well in 50-µL aliquots, leading to a bacterial concentration of approximately 10^6 CFU/mL in a total volume of 100 µL. Serial dilutions produced final concentrations of 20,000, 10,000, 5000, 2500, 1250, 625, and 312.5 µg/mL. The antibiotic gentamicin was used as a positive control as well as dimethyl sulfoxide. Inoculated broths were used in all experiments [6]. The contents of wells were mixed thoroughly, and the microplates were then covered and incubated at 37°C for 24 h [7]. Bacterial growth was monitored colorimetrically using thiazolyl blue tetrazolium bromide (3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide [MTT], BII Life Sciences) assays [8]. MICs were defined as the lowest concentration of test substances that prevented visible growth of microorganisms [9].

Antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays

Radical scavenging activities of extracts and compounds were determined using the stable free radical, DPPH [1]. Test substances from fractions were added at 100 µg/mL and dissolved with DPPH (150 mM) in methanol [10,11]. DPPH radical solution was prepared, and 180-µL aliquots were mixed with 20-µL aliquots of the test sample or reference substance in 96-well microplates [12]. After mixing, plates were incubated at room temperature in the dark for 30 min [13]. Absorbance was then recorded at 516 nm using a Versamax ELISA Microplate Reader (USA) [14]. All tests were conducted in triplicate. Percentage reductions in DPPH radical activity in the presence of test samples were calculated using the following formula [15].

Effectivity percentage (EP) = ([Absorbance of DPPH −Absorbance of mixture]/Absorbance of DPPH)×100, where the mixture contained extract or fraction and DPPH dissolved in methanol.

The half effective concentration (EC_{50}) values were calculated as the concentration of sample required to scavenge 50% of DPPH free radicals and were determined using plots of percentage inhibition versus sample concentration in triplicates [16]. These values were used to identify the most active fractions and extracts.

RESULTS

Fractionation

A total of 14 fractions were eluted from TLC, and these were labeled A, B, C, D, E, F, G, H, I, J, K, L, M, and N. Fractionation results from methanol extracts of *G. latissima* Miq. leaves are presented in Table 1.

Antibacterial properties of chromatographic fractions

The results of inhibition zone assays with 20,000 µg/mL fractions from *G. latissima* leaf extracts are shown in Table 2.

In the inhibition zone assays, fraction C showed the highest antibacterial activity, with an inhibition zone of 8.967±0.208 mm in diameter. Fractions A, H, I, J, K, L, M, and N did not produce significant inhibition zones against *B. subtilis* cultures.

Fractions B and C (MIC, 312.5 µg/mL) showed the highest antibacterial activities against *B. subtilis* (Table 3).

Antioxidant activity tests

Antioxidant activity assays were performed using the DPPH scavenging method with a microplate reader (Versamax ELISA Microplate Reader, USA) at wavelength maxima for DPPH of 516 nm. Quercetin was used as a control and showed an IC_{50} of 3.72 µg/mL. Preliminary antioxidant assays of extracts were performed using crude extract and fractions A, B, C, D, E, F, G, H, I, J, K, L, M, and N at 10 µg/mL.

In these experiments, fraction D showed the highest antioxidant activity, as indicated by effectivity percentage (EP) values (Table 4). EP values were 37.73±1.44% and 29.47±2.01% for fraction D and the crude methanol extract, respectively (Table 4). The EC_{50} value of fraction D was 19.38 µg/mL, whereas that of the crude extract was 23.40 µg/mL (Table 5) and that of quercetin was 3.72 µg/mL (Table 5).

Table 1: Fractionation of methanol extracts from *G. latissima* Miq. leaves

Fraction	Bottle number	Fraction weight (g)	Fraction percentage (%)
A	1–5	0.5402	3.30
B	43–70	0.2576	1.58
C	71–77	0.2520	1.54
D	78–84	1.4587	8.92
E	85–112	0.3767	2.30
F	113–118	1.7375	10.62
G	119–142	1.9141	11.70
H	143–148	1.691	10.34
I	131–137	1.673	10.23
J	138–147	1.4374	8.79
K	148–150	0.9471	5.79
L	151–158	1.1978	7.32
M	159–188	2.1875	13.37
N	189–195	0.6893	4.21
Total weight of fraction		16.3599	

G. latissima: Garcinia latissima

Table 2: Inhibition zone assays with 20,000 µg/mL fractions from *G. latissima* Miq. leaf extracts

Fraction	Diameters of inhibition zones (mm) against *Bacillus subtilis*
A	0
B	7.467±0.153
C	8.967±0.208
D	8.133±0.569
E	7.367±0.569
F	7.800±0.100
G	7.100±0.361
H	0
I	0
J	0
K	0
L	0
M	0
N	0
Gentamicin	29.175±0.983

G. latissima: Garcinia latissima

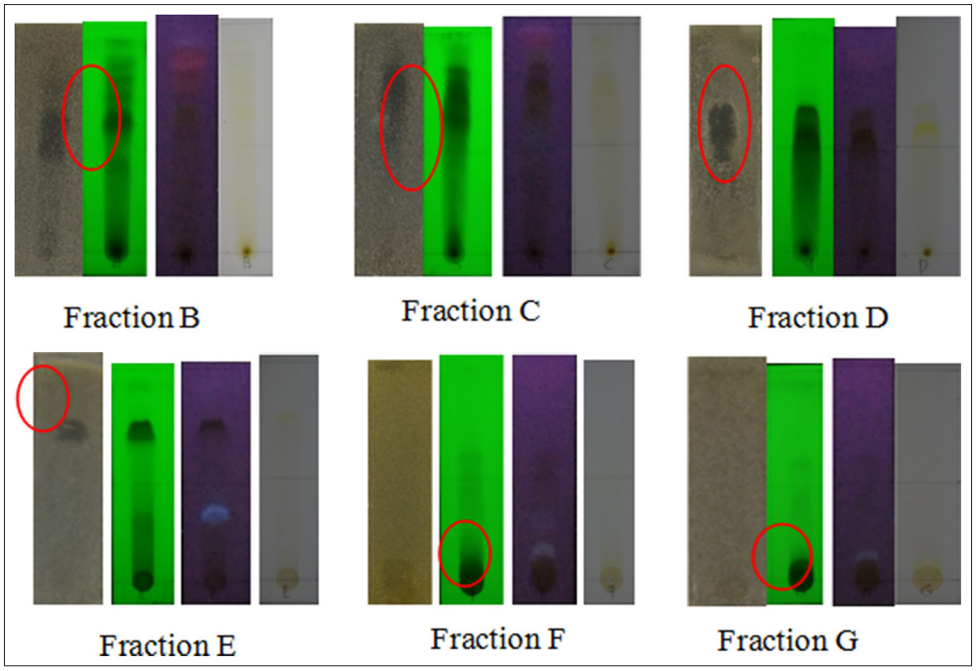

Fig. 1: Bioautography of active fractions from methanol extracts of *Garcinia latissima* Miq. leaves against *Bacillus subtilis* bacteria

Table 3: MIC of fractions of methanol extracts from G. latissima Miq. leaves against *B. subtilis*

Fraction	MIC (µg/mL)
A	1250
B	312.5
C	312.5
D	625
E	625
F	1250
G	1250
H	2500
I	5000
J	2500
K	5000
L	5000
M	5000
N	5000
Gentamicin	25
DMSO	>5000

B. subtilis: Bacillus subtilis, G. latissima: Garcinia latissima, DMSO: Dimethyl sulfoxide

Table 4: EP values of crude extract and the ensuing fractions of *G. latissima* Miq. leaves at 10 µg/mL

Sample	EP (%)
Extract	29.47±2.01
Fraction A	20.68±3.29
Fraction B	7.93±2.97
Fraction C	29.66±0.80
Fraction D	37.73±1.44
Fraction E	23.83±1.83
Fraction F	18.91±2.85
Fraction G	17.96±1.96
Fraction H	16.76±1.27
Fraction I	9.60±1.45
Fraction J	9.79±2.52
Fraction K	7.83±1.89
Fraction L	15.23±0.95
Fraction M	13.51±0.91
Fraction N	18.82±4.25

EP: Effectivity percentage, *G. latissima: Garcinia latissima*

Table 5: Antioxidant activities of crude extract and fraction D of methanol extracts from *G. latissima* Miq. leaves (EC_{50}, µg/mL)

Sample	EC_{50} (µg/mL)
Fraction D	19.38
Extract	23.40
Quercetin	3.72

G. latissima: Garcinia latissima, EC_{50}: Half effective concentration

DISCUSSION

In vitro antibacterial activities

Crude methanol extract from *G. latissima* leaves and its fractions produced antibacterial activity against *B. subtilis*. The present fractionation process separated extracts into 14 fractions (A–N) with fewer active ingredients than the parent extract, and fractions B and C had greater activity when compared with the other fractions. In the most active fractions, the fractionation process likely reduced the antagonistic effects of contaminating compounds [1].

The relationship between antibacterial activity and contents of active compounds in plant extracts or fractions of *G. latissima* have been investigated in multiple previous studies [1,17-21]. Moreover, phytochemical tests of methanol extracts of *G. latissima* leaves showed the presence of tannin and saponin, which are known phytochemicals with health benefits and biological activities [4]. The MIC of the most active fraction of methanol extracts from *G. latissima* leaves against *B. subtilis* was 312.5 µg/mL. In contrast, the MIC of the crude methanol extract was 10,000 µg/mL [4], indicating a dramatic improvement in the activity.

The present assays of antibacterial activity showed that the chromatographic fractions of methanol *G. latissima* leaf extracts contain compounds that inhibit the growth of *B. subtilis*, with significant increases in areas of inhibition [22]. We also used contact bioautography to qualitatively analyze antibacterial activities [23], and observed zones of bacterial growth inhibition around spots of fractions B, C, D, E, F, and G, indicating the presence of useful agents in these fractions [24]. Moreover, differing localizations of clear zones between fractions indicated that each fraction contained different active compounds [25].

Antioxidant activities of crude extract and its fractions

Reductions of DPPH radical were estimated in terms of EP and for the crude methanol extract of *G. latissima* Miq. leaves, the EP was 29.47±2.01%. Fractions C and D had higher EPs of 29.66±0.80% and 37.73±1.44%, respectively.

Antioxidant activities of natural materials have been widely studied and reviewed [1]. In the present study, we showed that the crude extract had free radical scavenging activity, but the most active fraction D had a lower EC_{50} value. Fraction D had a stronger antioxidant activity than crude extract; the antioxidant activity of quercetin was much greater (EC_{50}=3.72 µg/mL).

The effects of quercetin are reportedly influenced by the degree to which it can prevent oxidation of other molecules, which also neutralize free radicals both actively and passively [1]. As reported in previous studies, *G. latissima* extract contains saponins and tannins, and these active compounds likely contribute to the antioxidant activities [1,4].

CONCLUSION

We confirm that *G. latissima* Miq. leaves have antibacterial and antioxidant properties. Our data contribute to the standardization of antimicrobial phytomedicines with a wide spectrum of activities from *G. latissima* Miq. leaf extract fractions.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest

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