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Original Article

ISOLATION, IDENTIFICATION, AND ANTIBACTERIAL OF AMENTOFLAVONE FROM *GARCINIA LATISSIMA* MIQ. LEAVES

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ABSTRACT

Objective: This study aimed to isolate and identify the *Bacillus subtilis* antibacterial compound present in the leaves of the *Garcinia latissima* Miq.

Methods: The extracts were obtained by maceration successively, and the active extract was fractionated by column chromatography. The isolation of the most active fraction was performed by open column chromatography and preparative thin-layer chromatography. The isolate antibacterial assay against *Bacillus subtilis* by microdilution method. The compound structure of the isolate was identified by the nuclear magnetic resonance method.

Results: The most active extract of the extracts from *Garcinia latissima* Miq. leaves against *Bacillus subtilis* are the methanol extract. The most active fractions of the fractions from *G. latissima* Miq. Leaves methanolic extract was isolated. The active isolate was identified as Amentoflavone.

Conclusion: *G. latissima* Miq. leaves had the potential to be used as antibacterial medicinal herbs and one of the isolates of *G. latissima* Miq. is amentoflavone (active against *B. Subtilis)*.

Keywords: *Garcinia latissima* Miq., *Bacillus subtilis,* Amentoflavone

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INTRODUCTION

Microbes are the cause of infection in humans [1]. *Bacillus subtilis* is one of the gram-positive bacteria that can cause meningitis, endocarditis, eye infections, and others [2]. Indonesia is a country that is rich in medicinal plants, including antibacterial *B. subtilis* [3]. One of the plants from Indonesia is *G. latissima,* which grows in Maluku and Papua [4]. The previous research showed that 2% *G. latissima* Miq. leaves methanol extract had antibacterial activity against *B. subtilis* with a diameter of inhibition of 9.9 mm [5]. The results of the fractionation of the methanol extract of the leaves of *G. latissima* Miq. yielded 14 fractions (fraction A to fraction N) and fraction B and fraction C had the highest activity with MIC 312.5 µg/ml [6].

Isolation of the most active fraction was carried out to extract and separate the active compound as anti-bacterial *B. subtilis* [7]. Isolated compounds obtained from plants are said to have great potential as antibacterial if they have a MIC value of less than 100 µg/ml [8]. The purpose of this study was to obtain isolates that have the potential as antibacterial *B. subtilis*.

MATERIALS AND METHODS

Plant material, extracts, and fractions

The leaves of the *G. latissima* Miq. plants were obtained from the Bogor Botanical Gardens, West Java, Indonesia, where the plant is cultivated. Determination was carried out at the Center for Plant Conservation Bogor Botanical Gardens, Indonesian Institute of Sciences (LIPI), Bogor, West Java, Indonesia (No.: B-949/IPH.3/KS/III/2017). Extracts (by maceration successively method) and fractions (by open column chromatography method) were obtained from previous studies in Laboratory Pharmacognosy-Phytochemistry at the Faculty of Pharmacy, Universitas Indonesia [6, 9]. The process of fractionation using silica gel as the stationary phase and mobile phases used n-hexane, ethyl acetate, and methanol, in order of increasing polarity [6].

Chemical, bacterial and reagent

Methanolic pro analysis (Smart Lab, Indonesia), Chloroform pro analysis (Smart Lab, Indonesia), Sephadex LH-20 (Merck), Silica gel (Merck), n-Hexane pro analysis (Smart Lab, Indonesia), Ethyl acetate pro analysis (Smart Lab, Indonesia), Acetone pro analysis (Smart Lab, Indonesia), Formic acid (Merck), *Bacillus subtilis* (ATCC 6633), Thin Layer Chromatography-silica gel 60 GF_{254} (Merck), Silika gel G_{60}, (70-230 mesh E Merck 7734.1000), Sephadex® LH-20, bidestilated Aquadest, sterile 0.9% NaCl, ethanol 70%, methanol, nutrient agar, standard solution Mc. Farland III, Thiazolyl Blue Tetrazolium Bromide (Tetrazolium salt) from BBI Life Sciences, Dimethyl Sulfoxide (DMSO) (8.02912-1000 ml, Merck).

Isolation processes method and isolate identification method

The material used for the isolation was the combination of fractions B and C (0.51 g), and the combination of fractions D and E (1.84 g) of methanolic extract of *G. latissima* Miq. leaves [6]. The fractions B and C have a minimum inhibitory concentration (MIC) of 312.5 ppm and the fractions C and D have MIC 625 ppm [6]. First of all, the combination of fraction B and fraction C and the combination of fraction D and fraction E was identified to determine the chromatogram pattern using thin-layer chromatography (TLC). After going through the trials, the eluent that matched TLC was obtained from the combination of isolate B and isolate C using eluent chloroform-acetone-formic acid (70:20:10, v/v) and the combination of fraction D and fraction E using eluent ethyl acetate-methanol (10:1, v/v). After elution, observations were made under ultraviolet light at a wavelength of 254 nm.

A combination of fractions B, C, D, and E was separated on 30 grams silica gel column chromatography (CC) (305 mm x 20 mm i. d) with n-hexane-ethyl Acetate (85:15-0:100, v/v) and ethyl acetate-methanol (85:15-0:100, v/v) as eluents and yields 2 subfractions (subfraction A and subfraction B). The solvents were used according to the polarity index of each different solvent i.e., n-hexane 0.0, ethyl acetate 4.4, and methanol 5.1, in order of increasing polarity [4]. The subfraction B was purified by Sephadex LH-20 column chromatography with chloroform-methanol (70:30, v/v) as eluents and then use as a preparative thin layer chromatography-silica gel with eluents chloroform-acetone-formic acid (30:20:2, v/v) [10]. From the purification, the isolate was obtained and then was identified using TLC and Nuclear Magnetic Resonance (NMR) spectroscopy that consists of one-dimensional (1D)-^{1}H-NMR, ^{13}C-NMR,

and two-dimensional (2D)-NMR-^{1}H-[13]C Heteronuclear Single-Quantum Correlation (HSQC), and Heteronuclear Multiple-Bond Correlation (HMBC) [4].

Antibacterial activity test method

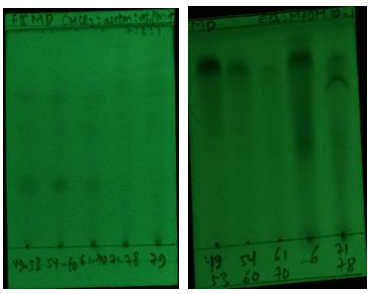
The antibacterial activity of the compound was evaluated against *B. subtillis* ATCC 6633, which was taken from the laboratory of Microbiology, Faculty of Pharmacy, Universitas Indonesia. The bacterial cultures (maintained on an agar slant in a refrigerator at 4 °C) were developed by Mueller-Hinton agar cultures at 37 °C for 24 h [6, 10]. Colonies of microorganisms were diluted in 0.9% NaCl to obtain McFarland standard turbidity of 0.5 using visual assessment and then diluted to more or less 10^6 CFU/ml [6]. The nutrient broth was used for the preparation of the inoculum of the bacteria, and nutrient agar was used for the screening method [6]. The antibacterial activity test against *B. subtilis* was carried out by broth microdilution assay in the 96 well flat bottom tissue sterile culture microplates (Biologix, Shandong, China) using thiazolyl blue tetrazolium bromide (3-(4,5-Dimethylthiazol-2-YI)-2,5-diphenyltetrazolium bromide (MTT) so that the minimum inhibitory concentration (MIC) value (in triplicates) is obtained [6, 10]. The solvent for the MIC determination used DMSO [4]. The antibiotic control used erythromycin, control of culture medium, and positive control of growth of *B. subtilis* was used [4].

The procedure of the MIC determination method is 50 µl of the compound or isolate solution 20,000 parts per million (ppm) to inoculate in three holes horizontally and then diluted with DMSO to three holes beneath it and so on to obtain the compound concentrations of 10,000 ppm, 5,000 ppm, 2,500 ppm, 1,250 ppm, 625 ppm, 312.5 ppm, 156.25 ppm, 78.13 ppm, 39.06 ppm, 19.53 ppm, and 9.77 ppm. Then, each hole was added with 10 µl of *B. subtilis* suspension at a concentration of 10^6 CFU/ml and 40 µl of bacterial growth medium [4]. So that the compound concentration of the assay holes of 5,000 ppm, 2,500 ppm, 1,250 ppm, 625 ppm, 312.5 ppm, 156.25 ppm, 78.13 ppm, 39.06 ppm, 19.53 ppm, and 9.77 ppm, 4.88 ppm, 2.44 ppm [4]. The microplates were incubated at 37 °C for 24 h [4]. Once the incubation period had elapsed, 10 µl of a solution of thiazolyl blue tetrazolium bromide, 0.6 mg/ml was added to the 96 well microplates, which were incubated at 37 °C for 20 m [4].

RESULTS AND DISCUSSION

Isolation process

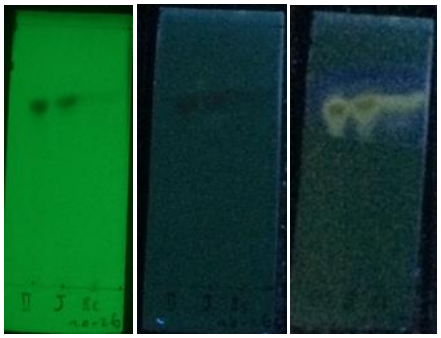
In this study, several of the most active fractions were combined in the antibacterial activity test of *B. subtilis* because the mass of the fractions was very little, and after combining the four fractions (fraction B, fraction C, fraction D, and fraction E) the total fraction mass was 2.35 grams (before doing activity test). The simplification of the number of fractions aims to allow the weight of the fraction to be isolated based on the value of the test results for the same minimum inhibitory concentration on adjacent fractions combined. The combination of these fractions still contains many compounds because the column chromatography has not obtained a perfect separation, the component bands still overlap. This can be seen in the TLC profile of the fractions under ultraviolet (UV) at 254 nm wavelengths (fig. 1).



(A) **(B)**

Fig. 1: The thin layer chromatography (TLC) profile of (A) the combination of fraction B and fraction C and (B) the combination of fraction D and fraction E, of *G. latissima* Miq. leaves methanol extract

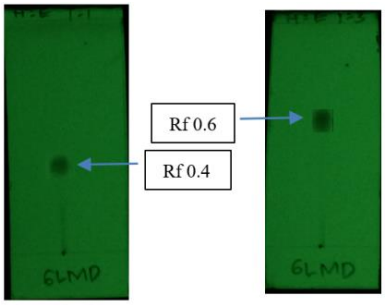
The TLC profile of the isolate with eluents chloroform-acetone-formic acid (30:20:2, v/v) and was visualized under UV at 254 nm (A) and 366 nm (before (B) and after (C) sprayed by $AlCl_3$ spray reagent) can be seen in fig. 2.



(A) **(B)** **(C)**

Fig. 2: The TLC profile of isolate was visualized (A) under UV at 2554 nm, (B) under UV at 366 nm, (C) under UV at 366 nm after sprayed by $AlCl_3$ spray reagent

The TLC profile of the isolate with eluents n-hexane-ethyl acetate (1:1, v/v) and n-hexane-ethyl acetate (1:3, v/v) can be seen in fig. 3.



(A) **(B)**

Fig. 3: The TLC profile of isolate with eluents (A) n-hexane-ethyl acetate (1:1, v/v) and (B) n-hexane-ethyl acetate (1:3, v/v)

After TLC was performed on isolate using n-hexane-ethyl acetate (1:1, v/v) as eluent, the Rf value was 0.4 (fig. 3A). The bioautography results of the combination of fraction B and fraction C of the methanol extract of the leaves of *G. latissima* Miq. Have an inhibitory area of Rf 0.4 [6]. From the TLC results of isolate using n-hexane-ethyl acetate (1:3, v/v) as eluent, an Rf value of 0.6 was obtained. The result of contact bioautography of the combination of fraction D and fraction E can be seen that there is an area of inhibition of bacterial growth in the spot with the Rf of 0.6 [6].

Isolate identification

The results of the measurement of the chemical shift signals (δ)^{1}HNMR of an isolate can be seen in fig. 4, with the expansion of the ^{1}HNMR spectrum in fig. 5.

Based on the ^{1}H-NMR measurement results, at H 7.98 (*dd, J* = 2.0 Hz and 8.6 Hz), 8.19 (*d*, 2.0 Hz) and 7.18 (*d*, 8.6 Hz) indicates the presence of a benzene ring with the ABX system. The presence of 2 doublet protons at H 6.78 (2H, *d*) and 7.68 (2H, *d*) with J = 8.2 Hz indicates the presence of benzene with H *ortho* and symmetric positions (System A2B2). *Meta*-positions appeared at 6.47 (*d*, 2 Hz) and 6.23 (*d*, 2 Hz). The results of the magnetic resonance spectrum of the isolated carbon core can be seen in fig. 4 and its expansion in fig. 5.

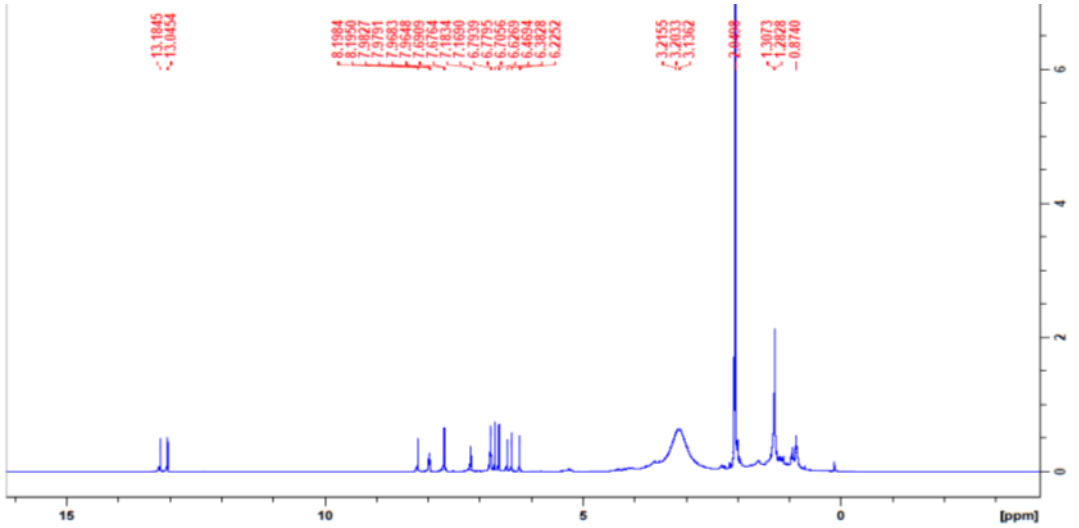


Fig. 4: The nuclear magnetic resonance spectrum of the isolate (acetone-d_6, 600 MHz)

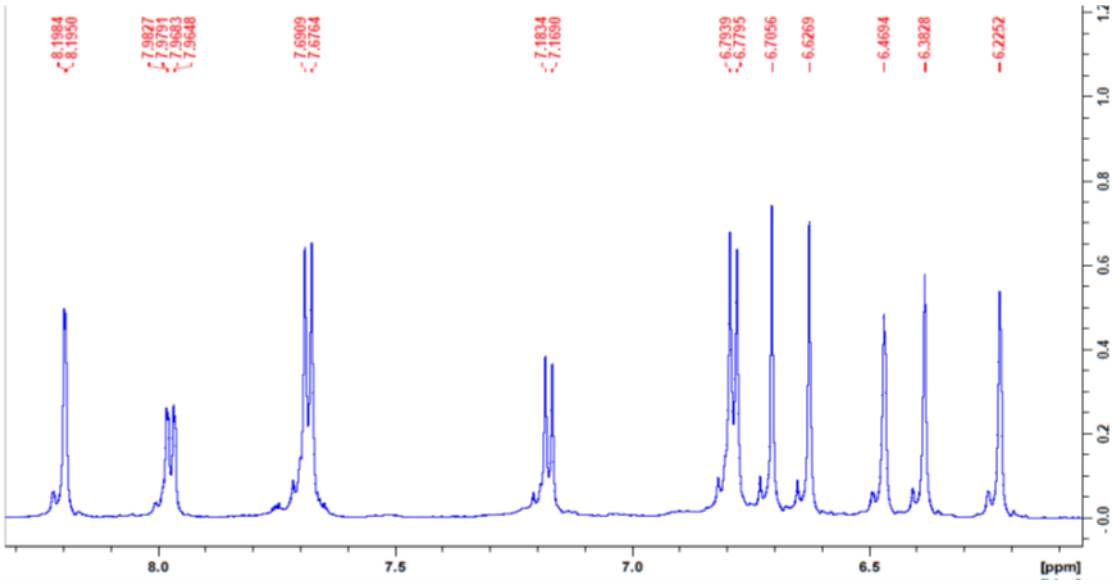


Fig. 5: Proton nuclear magnetic resonance spectrum expansion of isolate (acetone-d_6, 600 MHz)

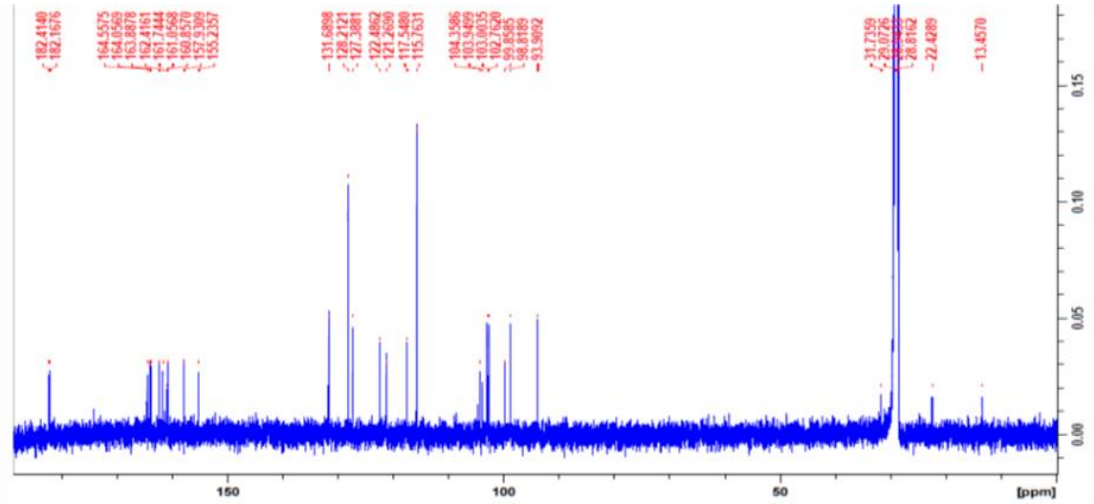


Fig. 6: The magnetic resonance spectrum of the isolate carbon core (acetone-d_6, 150 MHz)

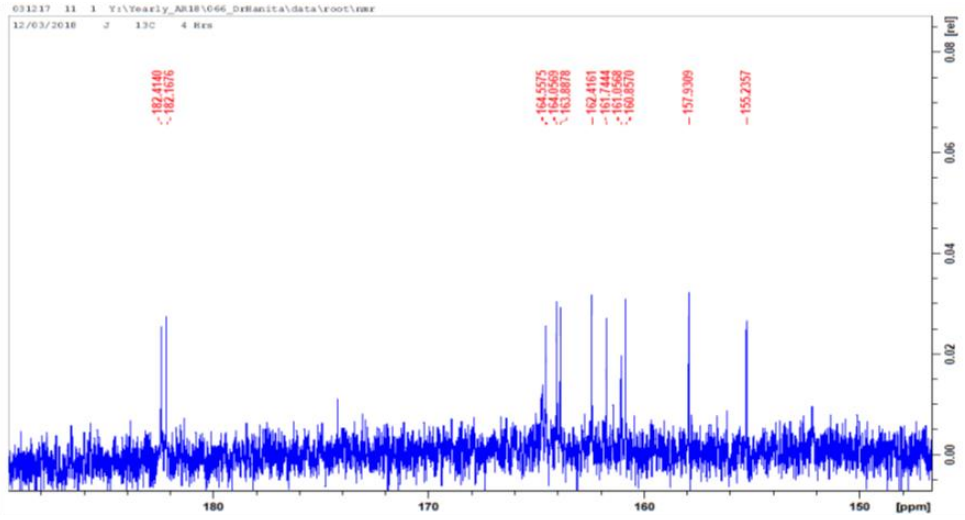


Fig. 7: Expansion of the isolate carbon core magnetic resonance spectrum (acetone-d_6, 150 MHz)

The HSQC result of the isolate is as shown in fig. 8 and fig. 9.

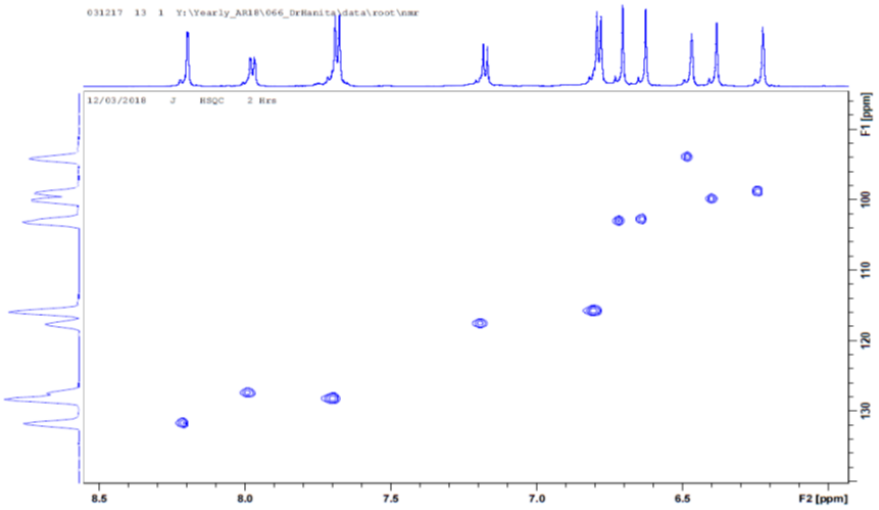


Fig. 8: The magnetic resonance spectrum of the isolate HSQC (acetone-d_6, 600 MHz)

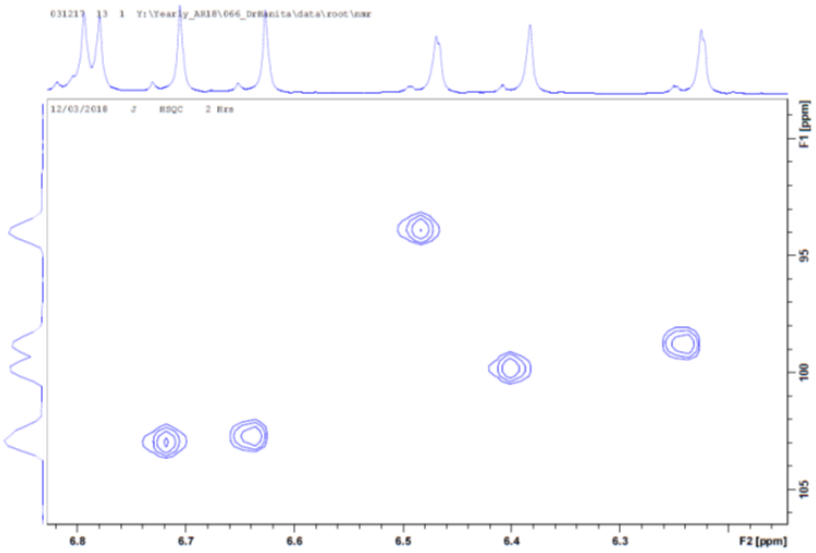


Fig. 9: The magnetic resonance spectrum expansion of the isolate HSQC (acetone-d_6, 600 MHz)

Based on the data on the value of chemical shear, splitting pattern, and HSQC and compared with the above compounds, this compound is a biflavonoid. The complete chemical shear value compared to the literature can be seen in table 1.

Table 1: Tabulated of the chemical shift of ^{1}H-NMR spectra of the isolate compared to literature [11]

	The isolate		**Amentoflavone**			**The isolate**		**Amentoflavone**	
C	δc*	**δH** (m, *J* in Hz)	δc*	**δH** (m, *J* in Hz)	**C**	**δc***	**δH** (m, *J* in Hz)	δc*	**δH** (m, *J* in Hz)
2	163.9	-	164.1	-	2''	162.4	-	164.3	-
3	103.0	6.65 (*s*)	103.2	6.60 (*s*)	3''	102.8	6.71 (1H, s)	102.8	6.59 (1H, *s*)
4	182.2	-	181.9	-	4''	182.4	-	182.2	-
5-OH	164.06	13.05	161.6		5''-OH	163.8	11.85	160.8	
6	98.8	6.23 (*d*, 2.04)	98.8	6.18 (*d*)	6''	99.9	6.41	99.1	6.38 (1H, *s*)
7	164.6		163.9		7'	161.1		161.9	
8	93.9	6.47 (*d*, 2.04)	94.2	6.40 (*d*)	8''	104.4	-	104.1	-
9	155.2	-	157.6	-	9''	157.9	-	154.7	-
10	104.1	-	104.0	-	10''	104.0	-	104.0	-
1'	121.3	-	120.3	-	1'''	127.4	-	121.4	-
2'	131.6	8.19 (*d*, 2.0)	127.9	7.95 (*d*, 1.5)	2'''	128.2	7.68 (2H, d, 8.2)	128.3	7.54 (2H, *d*, 8.0)
3'	117.6	-	121.7	-	3'''	115.8	6.78 (2H, d, 8.8)	116.0	6.72 (2H, *d*, 8.0)
4'	160.9		159.6		4'''	163.9		161.1	
5'	117.6	7.18 (*d*, 8.6)	116.4	7.89 (*d*, 8.0)	5'''	115.8	6.78 (2H, d, 8.2)	116.0	6.72 (2H, *d*, 8.0)
6'	127.4	7.98 (*dd*, 8.6 and 2.3)	131.6	7.12 (*dd*, 8.0 and 1.5)	6'''	128.2	7.68 (2H, *d*, 8.2)	128.3	7.54 (2H, *d*, 8.0)

*The H and C correlation are determined by the HSQC, By looking at the ^{1}H-NMR chemical shift value, the ^{13}C-NMR chemical shift value, and the HSQC correlation, the possible structure of the isolate is as shown in fig. 10 below.

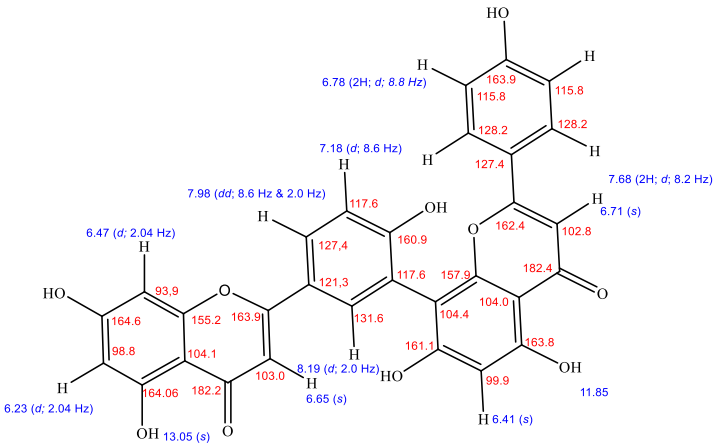


Fig. 10: The correlation between H and C of the HSQC of the isolate with their chemical shear values, the possibility of the isolate compound for amentoflavone was confirmed by the result of 2D HMBC NMR measurements in fig. 11 and fig. 12.

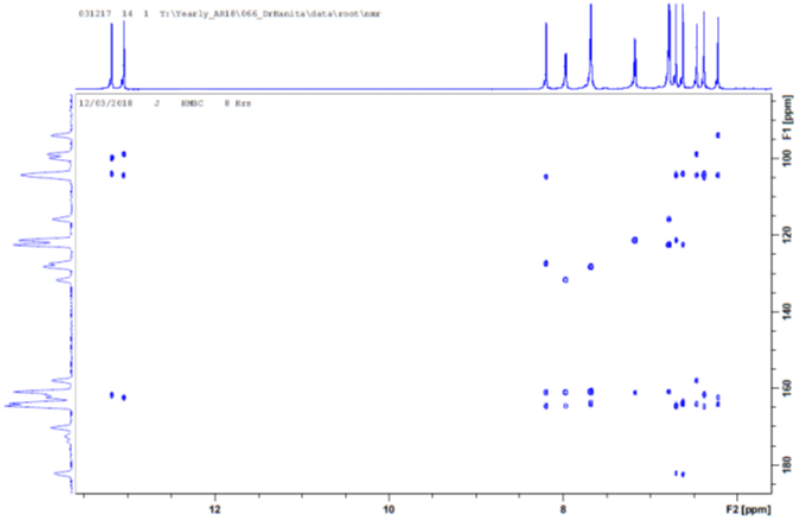


Fig. 11: The isolate HMBC magnetic resonance spectrum (acetone-d$_6$, 600 MHz)

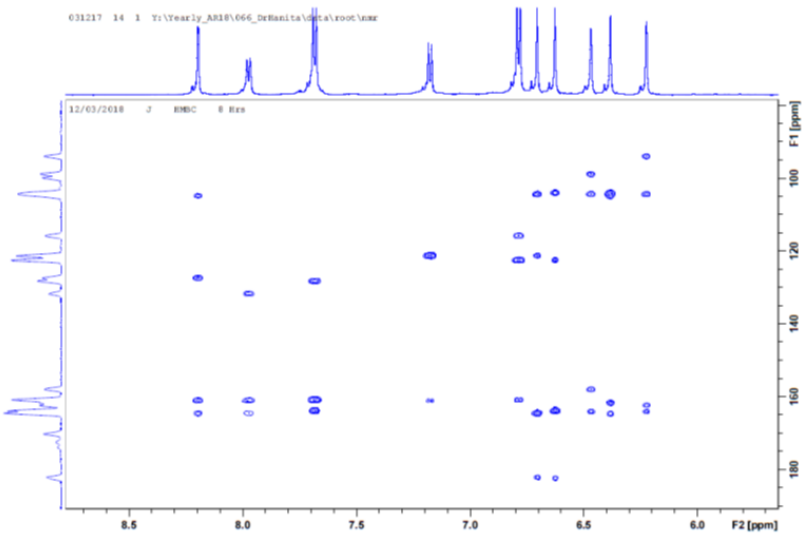


Fig. 12: The magnetic resonance spectrum expansion of the isolate HMBC (acetone-d_6, 600 MHz), From the result of the HMBC magnetic resonance spectrum above, the HMBC correlation is depicted in fig. 13.

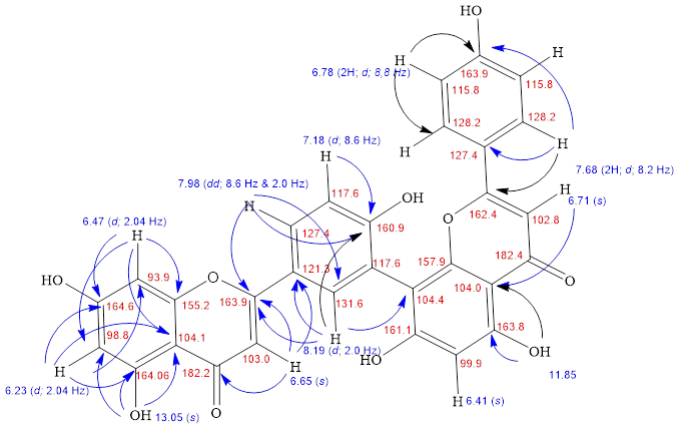


Fig. 13: The HMBC correlation of the isolate and their chemical shear values

Thus, this compound is strongly suspected to be amentoflavone (fig. 14), which is supported by HMBC correlation data, especially the correlation between H 8.19 (d) and C at C 104.4, which proves the presence of a bond between the two flavonoids, at that position, as shown in fig. 13 and the literature, however, it still needs to be confirmed with Liquid Chromatography-Mass Spectrometry (LCMS) data and supported by data from the literature.

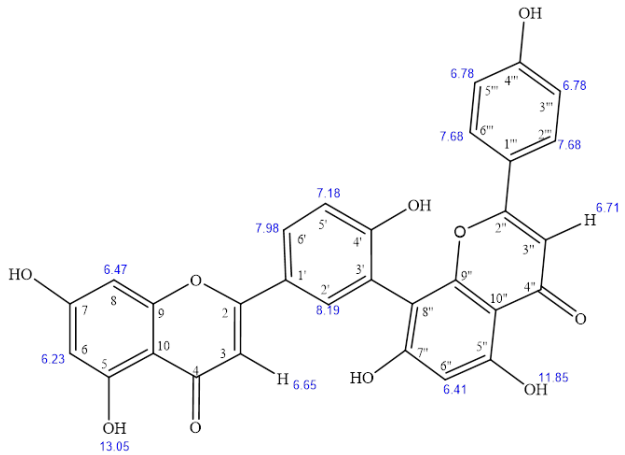


Fig. 14: The alleged isolate compound (Amentoflavone)

Thus, this compound is strongly suspected to be an amentoflavone, which is supported by the predicted value of the proton NMR chemical shift, the carbon NMR, the HSQC, the HMBC. However, it still needs to be confirmed with the LCMS data and supported by data from the literature. Amentoflavone has been isolated previously from the bark of *Ochna schweinfurthiana* F. Hoffm [12], from Rhussuccedanea and *Garcinia multiflora* [13]. Amentoflavone has also been isolated from *Selaginella labordei* [14]. and *Selaginella tamariscina* [15]. Amentoflavone has another name 8-biflavone.

Antibacterial activity test against *Bacillus subtilis*

Amentoflavone has been reported to have an inhibitory effect on the phospholipase A2 enzyme activity can inhibit the cyclooxygenase enzyme of the guinea-pig pest without effecting lipoxygenase, a strong anti-inflammatory in rat ear induced by croton oil, and a strong analgesic [16]. Amentoflavone have also been reported to have antibacterial activity as *Enterococcus faecium* ATCC 19434 (MIC 8 ppm), *Staphylococcus aureus* ATCC 25923 (MIC 4 ppm), *Streptococcus mutans* ATCC 3065 (MIC 32 ppm), *Escherichia coli* O-157ATCC 43895 (MIC 8 ppm), *Escherichia coli* ATCC 25922 (MIC 16 ppm), and *Pseudomonas aeruginosa* ATCC 27853 (MIC 8 ppm) [17].

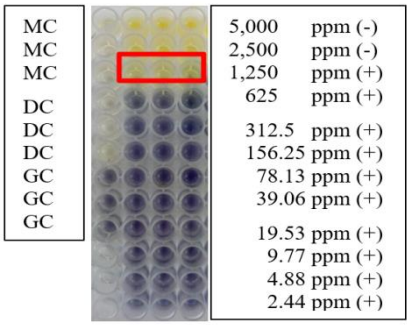


Fig. 15: Result of the isolate (Amentoflavone) MIC test using the microdilution method with the indicator tetrazolium salt, Caption: MC, media control; DC, drug control; GC, growth control; (-), not bacterial growth; (+) there is bacterial growth

From the result of the antibacterial activity test against *B. subtilis* using the microdilution method, the MIC value of the GLMD isolate (amentoflavone) was 1,250 ppm (fig. 15).

CONCLUSION

From this study, it can be concluded that a flavonoid compound, namely Amentoflavone, has been isolated from the methanol extract of the leaves of *G. latissima* Miq. With the MIC value for *B. subtillis* is 1,250 ppm.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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