ORIGINAL ARTICLE

New robustaflavone from *Garcinia latissima* Miq. leave and Its antibacterial activity

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ABSTRACT

Isolation and determination of antibacterial compounds from plants are essential to obtain a new antibacterial as a substitute for conventional resistant antibiotics. This study aims to isolate and identify a new robustaflavone as antibacterial activity from *Garcinia latissima* Miq. leave. In this study, the isolation process was carried out using column chromatography followed by preparative thin layer chromatography (TLC) based on the TLC profile. The fraction D was tested for anti-bacterial *Bacillus subtilis* using the TLC bioautography method. The isolates obtained were then identified using 1H-NMR, 13C-NMR, distortionless enhancement by polarization transfer, heteronuclear single quantum coherence, and heteronuclear multiple bond coherence. The Activity assay of the isolate was performed using the microdilution method. A pure compound obtained the result of the separation process with eluent n-hexane: Ethyl acetate (3:2) with R_f 0.6. This spot follows the spot in the contact bioautographic result of fraction D, the spot with R_f 0.6 gives an inhibition zone. After identifying and purifying the isolate were known as Robustaflavone, this compound has activity against *B. subtilis* with a (minimum inhibitory concentration) value of 2500 ppm. Robustaflavone successfully isolated and identified from *G. latissima* leave and its antibacterial activity.

Key words: Antibacterial, *Bacillus subtilis*, *Garcinia latissima* Miq, minimal inhibitory activity, Robustaflavone

INTRODUCTION

Antibiotics have an essential role in the world of health.[1] Bacterial resistance to antibiotics makes the disease difficult to cure.[2] Therefore, further research needs to be done to get new antibiotics, mainly from plants. Plants are superior because they are a renewable natural resource.[3]

Indonesia is a country that has a very high diversity of plants. The Mangosteen or Clusiaceae family (old name: Guttiferae) is one of the plant members growing in Indonesia.[4] *Garcinia latissima* Miq. is one of the plants of the Clusiaceae tribe. However, scientific data related to this plant is still minimal and has been studied by writers, especially those from Indonesia.

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Isolation from active fractions of natural ingredients is essential to get pure active compounds as natural drugs.[5] Ambarwati *et al.* have reported the activity assay results as antibacterial of the fractions of the ethyl acetate extract of *G. latissima* leave against *Bacillus subtilis*.[6] *B. subtilis* is a gram-positive bacteria that can cause infection.[7] Large amounts of *B. subtilis* infection in the intestine can cause diarrhea.[8] Therefore, this study is needed to get active compounds as antibacterial mainly for *B. subtilis*. Moreover, there has been much antibiotic resistance increasing.

The robustaflavone biflavonoid compound has been isolated from the fruit of the *Nandina domestica* family Berberidaceae,[9] a plant commonly used as a cough medicine and is spread in China, Japan, India, and Korea, *Rhus succedanea* and *Garcinia multiflora*.[10] Meanwhile, the isolation, identification, and antibacterial activity of robustaflavone from *G. latissima* leave has not been reported. Robustaflavone has also been isolated from Rhus succedanea and Garcinia multiflora and shows activity against HIV-1 reverse transcriptase (RT), with an IC50 value of 65 íM.[10]

This study aims to isolate and identify a new robustaflavone as antibacterial activity from *G. latissima* leave.

MATERIALS AND METHODS

Materials

The leaves of *G. latissima* Miq. were obtained and identified by The Center for Plant Conservation Bogor Botanic Gardens, Indonesian Institute of Sciences, Bogor, West Java, Indonesia. The voucher specimen (LFF-09-2015/FF-UI/V/2020) was stored at Laboratory of Pharmacognosy-Phytochemistry, Faculty of Pharmacy, Universitas Indonesia. The bacterial *B. subtilis* American Type Culture Collection 6633 was the collection of Microbiology Laboratory, Faculty of Pharmacy, Universitas Indonesia.

The chemical materials for extraction, fractionation, and isolation used n-hexane, ethyl acetate, methanol from PT Duta Pratama Chemika Bogor, Indonesia, and were distilled before used, aqua demineralization (PT Brataco Chemika, Indonesia), pro analysis solvents (n-hexane, ethyl acetate, methanol, chloroform, dichloromethane, acetone) from PT Smart Lab Indonesia, formic acid pro analysis (Merck, Germany), thin layer chromatography (TLC)-silica gel 60 GF_{254} (Merck, Germany) for thin-layer chromatography, silica gel G60 70–230 mesh (Merck 7734.1000, Germany) and Sephadex LH-20 (Merck, Germany) for the stationary phase of column chromatography, silica gel $GF_{254}+_{366}$ (Merck, Germany) for preparative-TLC. The anti-bacterial assay materials used nutrient agar (Merck, Germany), aqua bidestilata, sterilized sodium chloride 0.9%, ethanol 70%, methanol, thiazolyl blue tetrazolium bromide (BBI Life Sciences).

Instrumentation

Column chromatography equipment, a chamber for TLC, vials, and bottles for column chromatography yield collected, Ultra Violet-Visible Spectrometer (Camag, Japan), analytical balance, glasses equipment, the ultraviolet light of 254 and 366 nm, oven, refrigerator, nuclear magnetic resonance spectrophotometry (BRUKER Ascend™ 600 MHz), microplate 96-well.

Extraction and isolation process

The fraction of the ethyl acetate extract of *G. latissima* leaves, which has inhibitory activity against *B. subtilis*, was isolated using conventional column chromatography based on a chromatogram (TLC) pattern on ultraviolet light at 254 nm and 366 nm.[11] If many spots were still detected on the chromatogram pattern, then purification was performed using column chromatography. Silica gel G60 was used as a stationary phase in column chromatography if the fraction is nonpolar, and Sephadex LH-20 was used if the fraction is semi-polar or polar.[12] The mobile phase was determined based on the mobile phase in TLC data using a combination of n-hexane and ethyl acetate to obtain the separated spots with R_f value from 0.25 to 0.35.[13] The LH-20 Sephadex column chromatography using the mobile phase chloroform: Methanol.[14] The results of column chromatography were further refined using preparative-TLC (TLC-P with silica gel $GF_{254 + 366}$).[15,16]

Bioautography assay of fraction D

TLC bioautography assay was performed using bilayer media.[17] The agar media inoculated with *B. subtilis* ATCC 6633 poured into the agar media, which is already stable in the petri dish. The TLC plate containing the fraction spot is affixed to the bilayer layer, solidified for 1 h so that the compound contained on the TLC plate diffuses into the media. Then the TLC plate was removed, then the media was incubated at 37°C for 24 h. Inhibition zones on media showed fraction spot activity against *B. subtilis*.[18]

Identification and structure determination of isolate

The isolate was identified with measure chemical shift signals (δ)^1 H-NMR and ^{13}C-NMR using NMR Spectroscopy, (distortionless enhancement by polarization transfer [DEPT]), (heteronuclear single quantum coherence [HSQC]), and (heteronuclear multiple bond coherence [HMBC]).

Antibacterial activity assay

Antibacterial activity assay of the isolate was performed using the method of microdilution 96 well and thiazolyl blue tetrazolium bromide as an indicator on *B. subtilis* ATCC 6633, and inoculated on nutrient broth media according to some literature (1920),[19-21] with slight modification. Briefly, a 50 µL of isolate (20,000 ppm) were put into a well and each diluted using dimethyl sulfoxide to obtain some different concentration 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. Each well added 10 µL of *B. subtilis* 106 colony forming unit/mL suspension and 40 µL of nutrient broth media. Furthermore, it was incubated at 37°C for 24 h.[1] After that, 10 µL thiazolyl blue tetrazolium bromide 0.6 mg/mL solution and re-incubation at 37°C for 20 min.

RESULTS

Extraction, isolation, and thin layer chromatography -bioautography process

According to our previous study,[6] The fraction D of *G. latissima* leaves ethyl acetate extract has the highest activity against *B. subtilis* compared to other fractions with its minimum inhibitory concentration (MIC) value of 312.5 ppm and the TLC profile as shown in Figure 1a. The TLC result of the isolate with mobile phase n-hexane: ethyl acetate (3:2) demonstrated in Figure 1b. Meanwhile, the Figure 1c showed that contact bioautography profile of fraction D against *B. subtilis*.

Identification of isolates

The ^{1}HNMR chemical shift signals (δ) of the isolate were shown in Figure 2 with tabulation in Table 1. The measurement results of chemical shift signals (δ) ^{13}C-NMR and expansion of isolates showed in Figure 3a (blue) and DEPT in Figure 3b (red).

Table 1: ^{1}H-NMR dan ^{13}C-NMR spectrum of isolate

Serial number	^{13}CNMR*	δ ^{1}H-NMR (*m*, *J* in Hz)
2	163.6	-
3	102.8	6.63 (*s*)
4	182.2	-
5-OH	161.8	13.05 (*s*)
6	93.9	6.45 (*d*, *J*=1.6)
7	166.4	
8	93.90	6.23 (*d*, *J*=1.9)
9	158.8	-
10	104.2	-
1′	121.1	-
2′	131.6	8.21 (*d*, *J*=1.8)
3′	105.0	-
4′	160.5	
5′	128.2	7.70 (*d*, *J*=8.5)
6′	127.2	7.96 (*dd*, *J*=2.3 and 8.6)
2′′	163.6	-
3′′	102.6	6.36 (*s*)
4′′	182.1	-
5′′	161.5	13.18 (*s*)
6′′	104.6	-
7′′	163.5	
8′′	102.8	6.71 (*s*)
9′′	157.9	-
10′′	105.0	-
1′′′	123.0	-
2′′′ and 6′′′	118.2	6.78 (2H, *d*, *J*=8.8)
3′′′ and 5′′′	128.2	7.16 (2H, *d*, *J*=8.6)
4′′′	155.2	

CNMR: 150 MHz, H-NMR: 600 MHz

The HSQC of [Figure 4a] shows a direct correlation between δ_C 102.8 with δ_H 6.63 (s) and between δ_C 93.9 with δ_H 6.45 (*d*). There is a direct correlation between δ_C 131.6 with δ_H 8.21 (*d*) and the correlation between δ_C128.2 with δ_H 7.70 (*d*). Also, there is a direct correlation between δ_C 127.2 with 7, H 7.96 (*dd*) and a correlation between δ_C 102.6 with δ_H 6.36 (*s*). The complete position and chemical shift demonstrated in Table 1. Based on the data results, the isolate was estimated as robustaflavone [in Figure 4b].

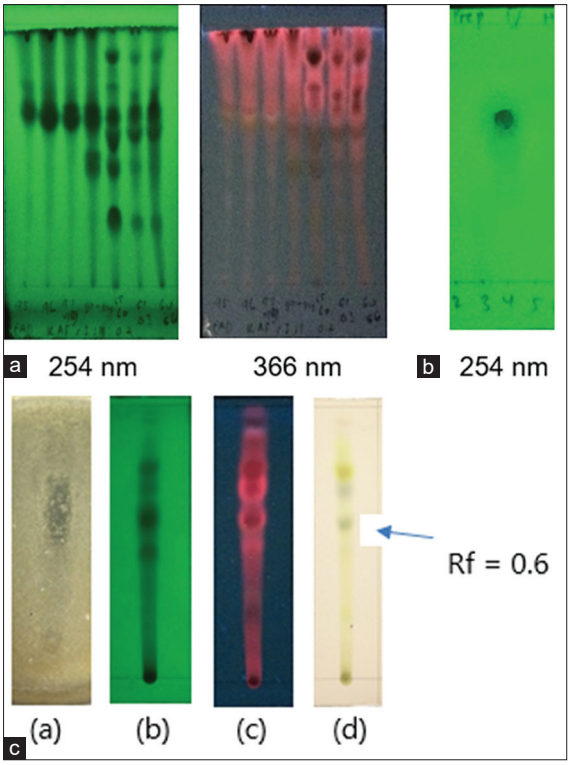

Figure 1: Thin layer chromatography profile of (a) Fraction D with mobile phase chloroform: Acetone: Formic acid (3:1:0.2); (b) Isolate (GLED2) using mobile phase *n*-hexane: Ethyl acetate (3:2); and (c) Contact bioautography profile of fraction D against *B. subtilis* (a) the inoculated agar medium after the thin layer chromatography plate was removed (b) inspected thin layer chromatography under UV 254 nm (c) inspected thin layer chromatography under UV 366 nm (d) Inspected thin layer chromatography under visible light. (b) shows the pure compound using eluent n-hexane and ethyl acetate (3:2), having an Rf value of 0.6. This Rf value follows the bioautography result of the fraction D extract of ethyl acetate leaves (c). In the thin layer chromatography contact bioautographic assay, the eluted and dried thin layer chromatography plate transferred to Petri plate containing the agar medium has been inoculated with *B. subtilis* for approximately one hour and then incubated for 24 hours. Inhibition zones were detected in the spot with an Rf value of 0.6 on agar medium in a petri dish ((a) of c) after incubation, and the thin layer chromatography plate was removed. This showed that the isolate was active against *B. subtilis*

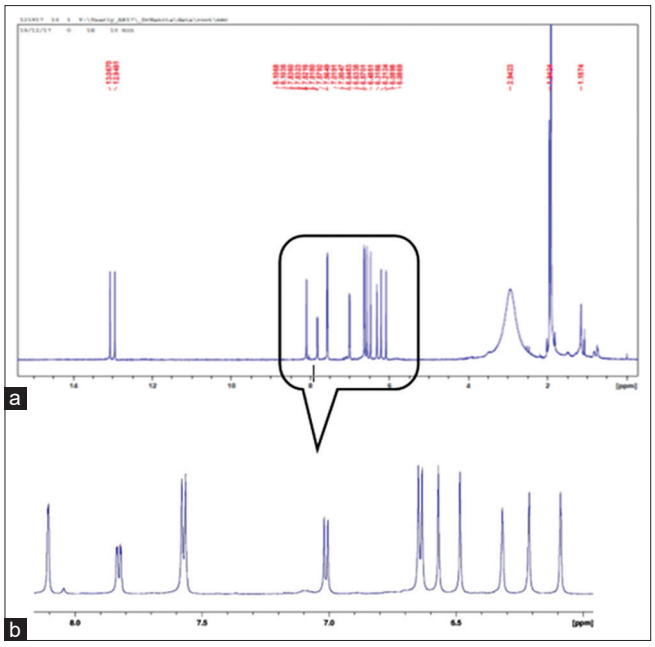

Figure 2: The proton magnetic resonance spectrum (a) and expansion (b) of isolate (Acetone-d_6, 600 MHz)

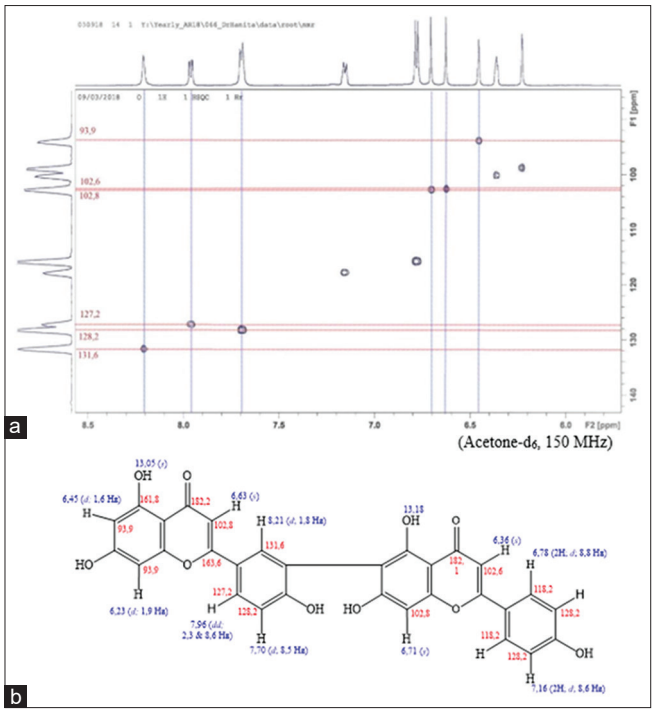

Figure 4: Heteronuclear single quantum coherence spectrum (a) and the structure design-based on heteronuclear single quantum coherence spectrum (b) of isolate

Besides, it is also confirmed by HMBC spectrum data as in Figure 5a, mainly the correlation between δ_H 8.21 (*d*) with δ_C at δ_C 104.6 [Figure 5b], which proves the existence of a bond between the two flavonoids.

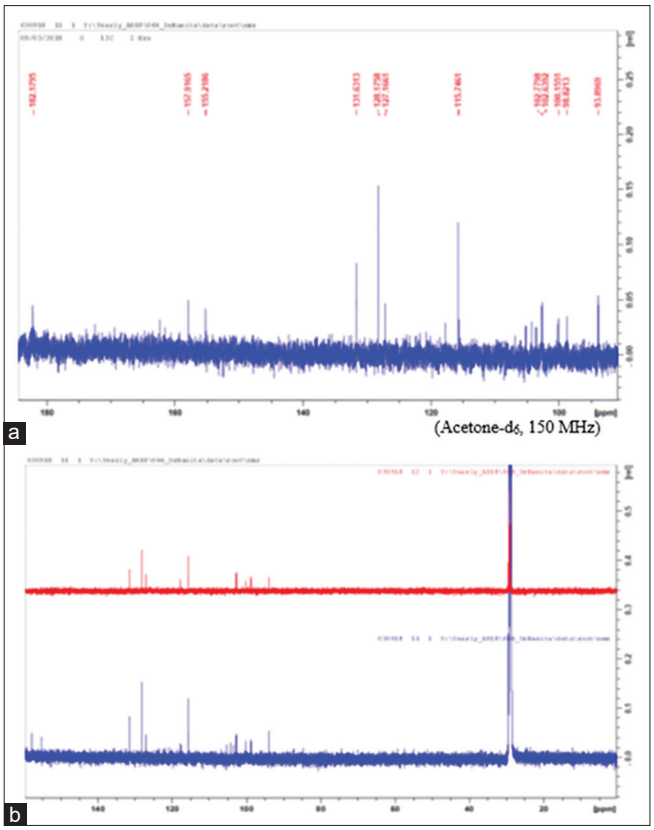

Figure 3: Carbon magnetic resonance spectrum (a) and Carbonmagnetic resonance spectrum (blue) and distortionless enhancement by polarization transfer (red) (b) of isolate

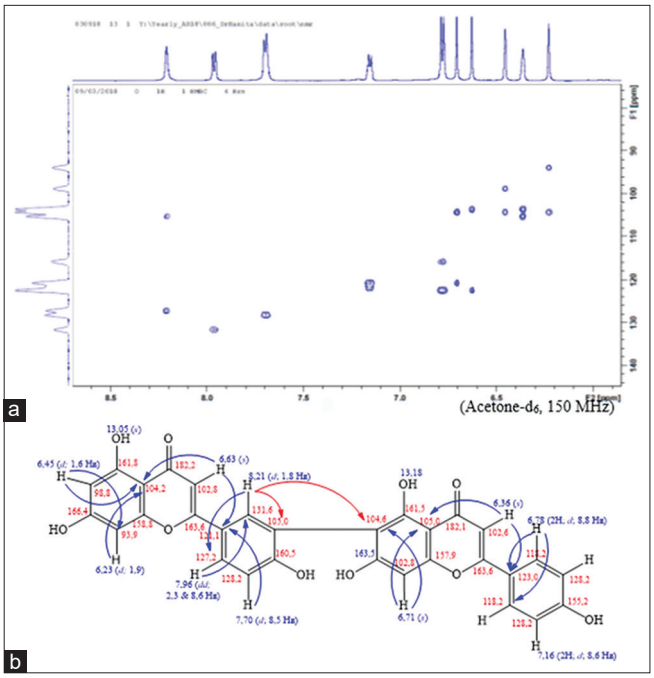

Figure 5: The heteronuclear multiple bond coherence spectrum (a) and the structure design based on the heteronuclear multiple bond coherence spectrum (b)

Antibacterial activity assay on *Bacillus subtilis*

The antibacterial activity assay against *B. subtilis* by microdilution method from this isolate (robustaflavone), with the MIC value, was 2500 ppm [as shown in Figure 6].

DISCUSSION

A total of 0.81 g of this D fraction was further purified using column chromatography with the stationary phase Sephadex LH-20 with the mobile phase of chloroform: Methanol (8: 2) and collected every 10 mL.[22] The stationary phase of Sephadex LH-20 was used because it is lipophilic so that it was expected to be able to separate isolates from chlorophyll. the fraction component will move based on the mobile phase's movement through the stationary phase, and small molecules will be trapped in the gel matrix while the larger molecules will be outside the gel matrix and go faster.[23] This method (Sephadex LH-20 gel) also involves the mechanism of adsorption, partitioning, the possibility of ion exchange, and sometimes the most abundant molecule will be eluted first, and the smallest molecule will be eluted last and vice versa.[24] This chromatography has been widely used to remove confounding pigments such as chlorophyll which tend to be larger and more lipophilic than the secondary metabolite of the plant.[25] From this process, isolates were produced, which after further purification using TLC-partition (TLC-P) and carried out TLC obtained the chromatogram pattern.

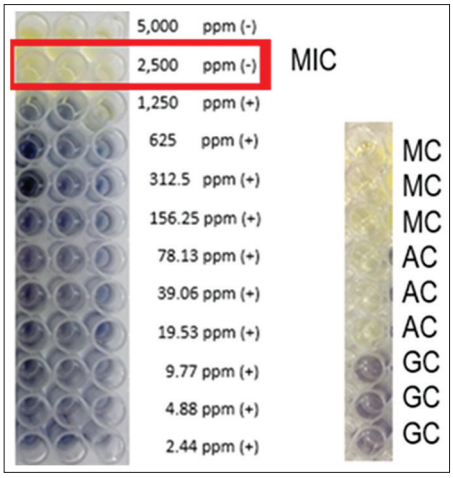

Figure 6: Test results of minimum inhibitory concentration isolates (robustaflavones) using the microdilution method with the tetrazolium salt indicator (3-(4,5-dimethyltiazol-2-il)-2,5-diphenyltetrazolium bromide), where: minimum inhibitory concentration is minimum inhibitory concentration, MC is media control, AC is antibiotic control, GC is germ control, (–), not bacterial growth; (+) there is bacterial growth

Based on the results of ^{1}H-NMR measurements showed that some aromatic functional groups indicate a biflavonoid, the presence of chemical shift at 63H 6.63 (s) aromatic rings with 1 H, and at δ_H 6.45 (d; 1.6 Hz) and 6.23 (d, 1.9 Hz) are aromatic rings with 2H in the meta position. In chemical shift at δ_H 7.96 (*dd*, *J* = 8.6 and 2.3 Hz), 7.70 (*d*, 8.5 Hz) and 8.21 (*d*; 1.8 Hz) was 3H aromatic rings with ABX system. Based on this spectrum, this isolate is thought to be a flavonoid derivative. Other chemical shifts indicate the presence of 3 benzene rings at δ_H 6.71 (s); 6.36 (s) and aromatics with A2B2 systems appear at δ_H 6.78 (2H, *d*, 8.8 Hz) and 7.16 (2H, *d*, 8.6 Hz). Besides that, there is a particular group, which is in the more downfield areas at δ_H 13.05 (*s*) and 13.18 (*s*), indicating the presence of two phenol groups (-OH) that form hydrogen bonds with carbonyl groups (= C = O).

Robustaflavone [Figure 7] has been previously isolated from the bark of *Ochna schweinfurthiana* F. Hoffm,[26] *Rhus succedanea*, and *Garcinia multiflora*.[10] Meanwhile, this compound has also isolated from *Selaginella labordei*[27] and *Selaginella tamariscina*.[28]

Lin *et al*. have reported that Robustaflavone (from *Rhus succedanea*) has activity inhibiting the growth of hepatitis B virus with EC_{50} 0.25 μM.[29] Robustaflavone has also been reported to inhibit the action of the HIV-1 RT enzyme.[30]

CONCLUSION

In conclusion, a biflavonoid compound (robustaflavone)

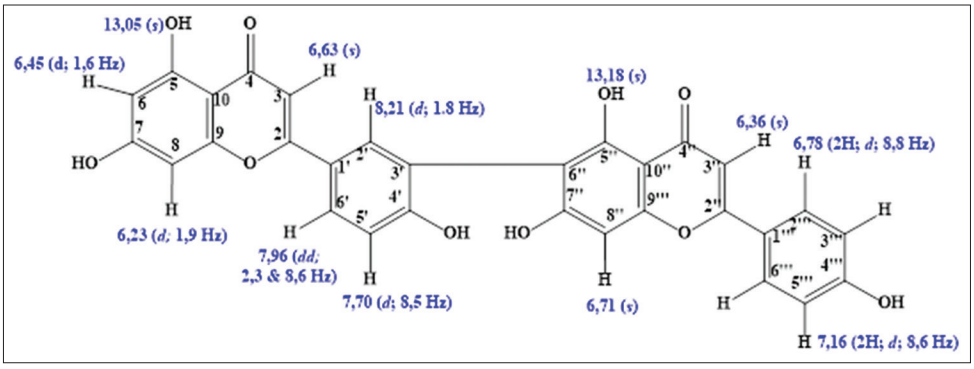

Figure 7: Robustaflavone

has been isolated from the most active fraction of ethyl acetate extract from *G. latissima*. The robustaflavone has an antibacterial activity for *B. subtilis* with a MIC value of 2500 ppm.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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