Isolation and Antibacterial Activity by *in vitro* and *in silico* Approach of 6-Deoxyjacareubin Compound from *Garcinia latissima* Miq. Fruit

Neneng Siti Silfi Ambarwati[1], Berna Elya[2], Amarila Malik[2], Yuditya Artha[2], Islamudin Ahmad[3], Azminah Azminah[4], Muhammad Hanafi[5,6,*], and Hanita Omar[7]

[1]*Faculty of Engineering, Universitas Negeri Jakarta, Jl. Rawamangun Muka, East Jakarta 13220, Indonesia*

[2]*Faculty of Pharmacy, Universitas Indonesia, UI Depok Campus, Depok 16424, West Java, Indonesia*

[3]*Department of Pharmaceutical Sciences, Faculty of Pharmacy, Mulawarman University, Jl. Kuaro, Gn. Kelua, Samarinda 75119, East Kalimantan, Indonesia*

[4]*Faculty of Pharmacy, Universitas Surabaya, Jl. Raya Kalirungkut, Surabaya 60293, East Java, Indonesia*

[5]*Research Centre for Chemistry–Indonesian Institute of Sciences, Kawasan PUSPIPTEK Serpong, Tangerang Selatan 15314, Banten, Indonesia*

[6]*Faculty of Pharmacy, Pancasila University, Jl. Srengseng Sawah, Jagakarsa, South Jakarta 12640, Indonesia*

[7]*Chemistry Division, Centre for Foundation Studies in Science, University of Malaya, Kuala Lumpur, Malaysia*

* ***Corresponding author:***

email: hanafi124@yahoo.com

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Abstract: *The previous research showed that the fraction C from active extract of* Garcinia latissima *Miq. fruit was active against* Bacillus subtilis*. This study aims to isolate and identify the active compound as an antibacterial agent from the fraction C. Fraction C was purified by recrystallization using chloroform and* n*-hexane solvents and then isolated using preparative-thin layer chromatography-silica gel 60 GF_{254} to give a yellow compound. The antibacterial activity was determined using microdilution with thiazolyl blue tetrazolium bromide indicator against* B. subtilis *American Type Culture Collection 6633. The isolate was identified using UV-Vis, IR, MS, Proton Nuclear Magnetic Resonance (^{1}H-NMR) and carbon NMR (^{13}C-NMR), and NMR-2D techniques including HMQC and HMBC. Based on the spectroscopic analysis and literature review, the compound was identified as 6-deoxyjacareubin, which is a new compound from* Garcinia latissima *Miq. The 6-deoxyjacareubin showed antibacterial activity with MIC value of 156.25 ppm and was categorized as a weak antibacterial agent because the MIC value was more than 100 ppm. According to in silico approach to the docking study, 6-deoxyjacareubin showed similar hydrophobic interaction with several amino acid residues including C2565, C2589, G2484, U2590, and U5588 between a native ligand.*

Keywords: *6-deoxyjacareubin; antibacterial;* Bacillus subtilis*;* Garcinia latissima *Miq.*

■ INTRODUCTION

Humans have a dependence on herbal medicines in treating various diseases. Herbal medicines have been used long before the discovery of modern synthetic drugs, as traditional medicines that have always been a part of human traditions and cultures [1]. The sources of traditional medicine can be obtained from plants, including abundant native Indonesian plants [2]. Therefore, these plants need to be investigated further so that native Indonesian plants can continue to be main sources of traditional medicine [2].

One of the important part of medicine for human is their antimicrobial activity [3]. The use of antibiotics has reached an alarming level of resistance. The emergence of toxicity and the reduced effectiveness of using synthetic drugs are other problems that must be

resolved [4]. Therefore, it is necessary to do research to look for compounds from natural ingredients that have antibacterial activity [4]. This study is to test the antimicrobial activity on native Indonesian plants. The qualitative methods of the antimicrobial test include bio-autography and diffusion methods, while the quantitative method of antimicrobial test is performed by the dilution method so that the minimum inhibitory concentration value can be obtained [5].

Garcinia latissima Miq. (Clusiaceae) is a plant that grows in tropical and subtropical regions. The fruit of this plant is sweet, sour and rich in nutrition. A previous study showed that 2% of the *G. latissima* Miq. fruit ethyl acetate extract in dimethyl sulfoxide (DMSO) gave inhibition zone diameter against *B. subtilis* of 9.62 mm and the minimum inhibitory concentration (MIC) against *B. subtilis* of the extract was 2500 ppm [6]. The extract was fractionated by column chromatography. Fraction C had the highest activity from 11 fractions (FA-K). The result of the activity test against *B. subtilis* from the previous study of the fraction C 2% in DMSO provided inhibition zone diameter of 9.46 mm and MIC of 1250 ppm [7].

In this study, isolation of fraction C was performed as well as antibacterial test of the isolated result. The presence of pharmacophore groups in the active compound can be used as antibacterial treatment against *B. subtilis* [8]. The positive control that was used as an antibacterial agent against *B. subtilis* was erythromycin that works by inhibiting protein synthesis [9]. The mechanism of erythromycin in the system was observed by the formation of a peptide bond with a ribosomal 50S subunit of bacteria (1:1) thereby inhibiting peptidyl transferase activity present in the 23S rRNA in the 50S subunit [10]. rRNA 23S consists of six domains, two of which are peptidyl transferase sites that are domains V and II connected by 5S rRNA [11]. Erythromycin binds specifically to adenine 2058 in domain V to inhibit the synthesis of a nascent 50S ribosomal subunit from *B. subtilis* [11]. This causes the distribution mRNA to be inhibited and affects the binding of peptidyl tRNA [10]. Molecular docking studies can further find more effective antibacterial agents [8].

6-Deoxyjacareubin isolated from *Calophyllum zeylanicum,* and *Kielmeyera speciosa* woods (Guttiferae) was reported to have antimicrobial activity [12]. 6-Deoxyjacareubin compound has been reported to be isolated from *Calophyllum inophyllum* L. [13]. *Calophyllum inophyllum* stem bark methanol extract acts as an antibacterial agent against *Pseudomonas aeurginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* at 25 μg/mL concentration and against *Bacillus licheniformis*, *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* at 50 μg/mL concentration [14]. Oil from *C. inophyllum* L. has an antibacterial activity against Gram negative bacteria [15].

■ EXPERIMENTAL SECTION

Materials

The fruits of *G. latissima* Miq. were obtained from Bogor Botanical Gardens and has been identified by the center for plant conservation Bogor Botanical Garden, Indonesian Institute of Sciences (LIPI).

The chemical materials used in this study were *n*-hexane, ethyl acetate, methanol, distilled water, glacial acetic acid, thin layer chromatography-silica gel 60 GF_{254}, silica gel G_{60}, H_2SO_4, $AlCl_3$, nutrient agar, cetrimide, Thiazolyl blue tetrazolium bromide, p.a. solvents such as *n*-hexane, ethyl acetate, ethanol, chloroform, dichloromethane, methanol, and acetone.

n-Hexane, ethyl acetate, methanol, and distilled water were from Brataco Chemica, the p.a. solvents were purchased from Smart Laboratory, glacial acetic acid was purchased from Merck, thin layer chromatography-silica gel 60 GF_{254}, silica gel G_{60}, H_2SO_4, $AlCl_3$, nutrient agar, and cetrimide was purchased from Merck, while Thiazolyl blue tetrazolium bromide was purchased from BBI Life Sciences.

Instrumentation

The equipment used in the study were a rotary evaporator, column chromatography equipment, thin layer chromatography equipment, vials and bottles, micro pipet, UV-Vis spectrophotometer, analytical balance, glasswares, CAMAG UV cabinet 4, electric stove,

Memmert oven, refrigerator, infra-red spectrophotometer, nuclear magnetic resonance spectroscopy, High Performance Liquid Chromatography–Mass Spectrometry–Mass Spectrometry, microplate 96-well and Memmert incubator.

Procedure

Extraction, fractionation, and isolation processes

The extraction process of *G. latissima* fruits (8.9 kg) by successive maceration (by three solvents: *n*-hexane, ethyl acetate, and methanol) was performed according to the study by Ambarwati et al. [6]. Moreover, the *G. latissima* fruits ethyl acetate extract (157 g) was fractionated. The fractionation was conducted using column chromatography according to the study by Ambarwati et al. [7] and resulted in 11 fractions (A-K).

The material used for the isolation was the fraction C of ethyl acetate extract of *G. latissima* Miq. fruits (1.53 g). Fraction C was separated on silica gel column chromatography (CC) (305 mm × 23 mm i.d) with *n*-hexane/ethyl acetate (100:0–0:100, v/v) as eluents and then purified by recrystallization and thin layer chromatography-silica gel. The recrystallization of fraction C was processed by modifying the non-polar solvent (hexanes) and the semi-polar solvent (ethyl acetate). And then, fraction C was purified further using preparative thin-layer chromatography (*p*-TLC) method. From the purification, 6-deoxyjacareubin was obtained and then identified using thin-layer chromatography with *n*-hexane-chloroform (1:4, v/v) as the mobile system. The retention factor (Rf) value of the isolate obtained from thin-layer chromatography with *n*-hexane-chloroform was 0.4.

Antibacterial activity

The antibacterial activity of the compound was evaluated against *B. subtilis* ATCC 6633 which was taken from the laboratory of Microbiology, Faculty of Pharmacy, Universitas Indonesia. The bacterial cultures were developed by selective nutrient agar at 37 °C for 24 h [1]. The nutrient broth was used for the preparation of inoculum of the bacteria, and nutrient agar was used for the screening method. The antibacterial activity test against *B. subtilis* was carried out by microdilution method using yellow indicator tetrazolium salt (MTT) so that the MIC value (in triplicates) is obtained.

In silico molecular study

Crystal structure of peptidyl transferase was downloaded from the RCSB protein data bank complexed with erythromycin (PDB ID: 1JZY) [16]. The macromolecule enzyme was reduced by removing several of the nonessential residues to minimize the macromolecule size. Docking method was validated by re-docking the co-crystallized ligand (erythromycin) into the same active site of the enzyme using AutoDock Tools, and the results were determined by values of Root Mean Square Deviation (RMSD). Subsequently, the ligand (6-deoxyjacareubin) was optimized by adding charge using Antechamber and docked into the same active site. Gasteiger charge was added to both ligands; free binding energy values were calculated and analyzed. The ligand-residue interaction was visualized using LigPlot to observe involved residue which contributed to the values of binding affinity.

■ RESULTS AND DISCUSSION

The 6-deoxyjacareubin compound obtained was observed as yellow needles crystals (16.8 mg) which were obtained from chloroform solution. The molecular formula was determined to be $C_{18}H_{14}O_5$ from its quasi-molecular ion peak at m/z 311.43 $[M+H]^+$ (molecular-weight or exact mass was calculated 310.0841) in the Liquid Chromatography–Mass Spectrometry spectra which are shown in Fig. 1.

The results of the UV isolate spectrum (Fig. 2) showed that the isolate using chloroform solvent, had the maximum λ absorption, at 308 and 329 nm. The maximum wavelength (329 nm) indicates that the compound contained a conjugated unsaturated ketone. The unsaturated conjugated bond is a single and double bond that alternate each other [17]. The maximum wavelength of 308 nm indicates the presence of a substituted benzene ring, the xanthone framework [18].

The hydroxyl group (–OH) was detected in the IR spectrum at 3425 cm^{-1} (shown in Fig. 3). The absorption bands at 2900 and 2800 cm^{-1} indicate the vibration of the

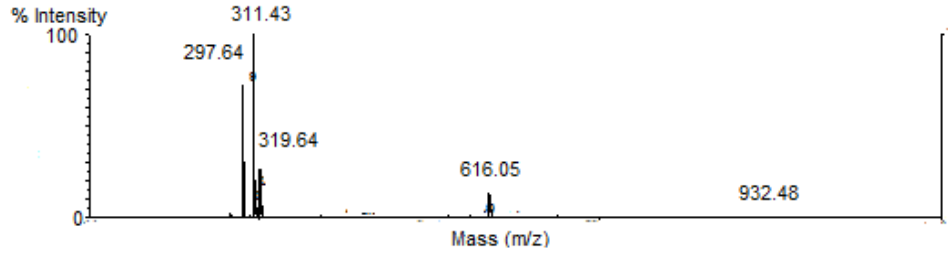

Fig 1. The Liquid chromatography–mass spectrometry spectra of compound 1

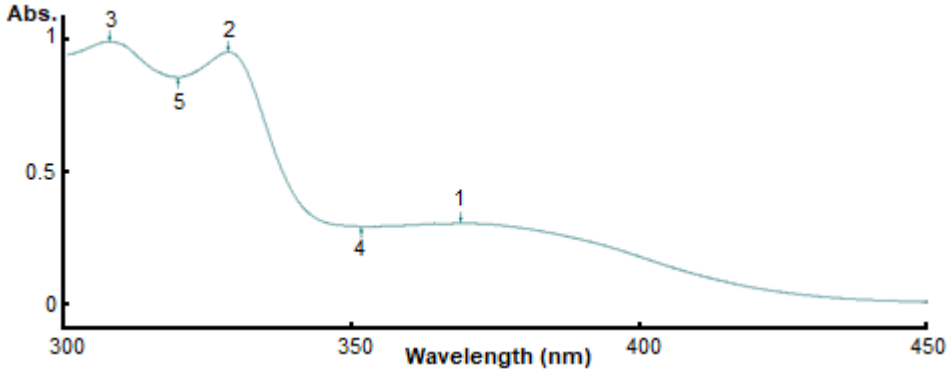

Fig 2. The UV spectrum of compound 1

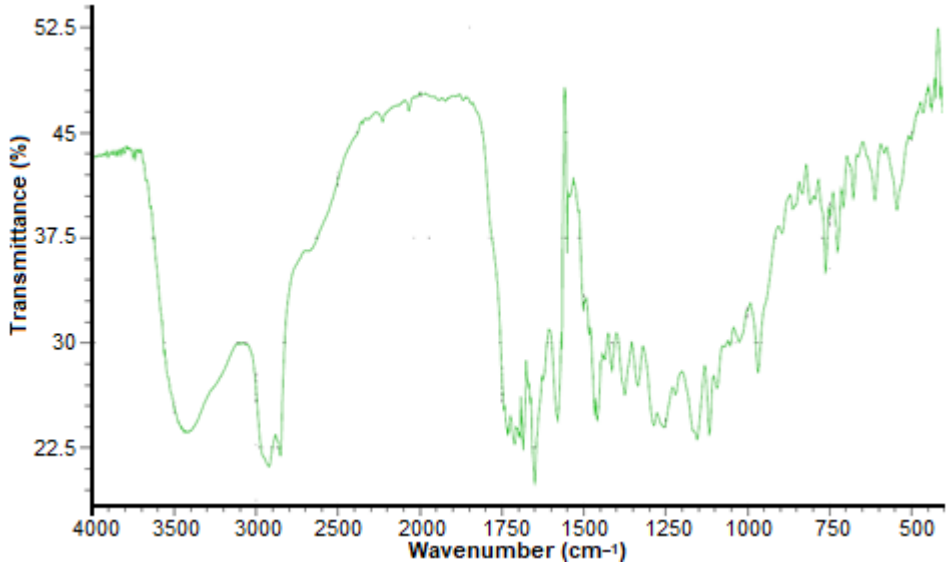

Fig 3. The IR spectrum of compound 1

CH (CH, CH_2, CH_3). The presence of an absorption band at 1680 cm^{-1} indicates the vibration of the phenyl group [18]. From these infra-red spectra data, it is estimated that these compounds contain hydroxyl groups, phenyl groups, and CH, CH_2, or CH_3 groups.

The ^{1}H-NMR spectrum showed a specific peak in the aliphatic region at δ_H 1–2 ppm (Fig. 4). There is also a peak that characterizes a xanthone derivative of a dimethyl group present at δ_H 1.49 (s, 6H) and two doublet protons (as a chromene) at δ_H 5.78 (d, J = 10.4 Hz) and δ_H 7.06 (d, J = 10.4 Hz) [19]. A coupling constant J value (in Hz) in NMR proton provides information in measuring the interaction of proton pairs. Protons in an ortho relationship show large coupling (8–12 Hz, but normally about 10); protons with a meta relationship show a small coupling (2–6 Hz). Furthermore, there is one singlet

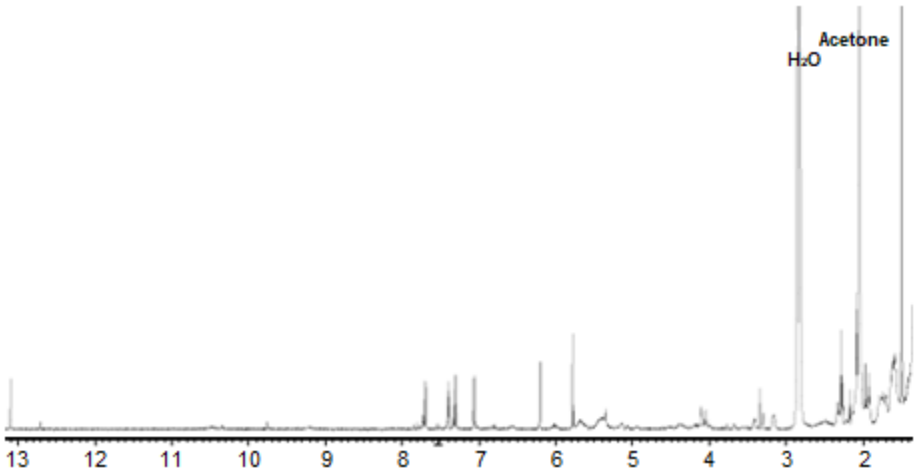

Fig 4. The spectrum of H-NMR

Table 1. ^{1}H-NMR and ^{13}C-NMR data compound 1 compared with 6-deoxyjacareubin

	Compound 1 (in acetone-d6)		6-Deoxyjacareubin (in acetone-d6) [19]	
No	δ ^{13}C-NMR	δ ^{1}H-NMR (m, J in Hz)	δ ^{13}C-NMR	δ ^{1}H-NMR (m, J in Hz)
1	116.0	7.0 (dd, 8.4; 2.0)	116.7	7.66 (dd, 7.8, 1.6)
1a	122.6	-		-
2	121.9	7.31 (t, 8, 4)	122.6	7.27 (t, 7.8)
3	122.6	7.39 (dd, 8.4, 2.0)	122.6	7.35 (dd, 7.8, 1.6)
4	146.8	-	144.3	
4a	147.5	-		
5	99.9	6.21 (s)		6.40 (s)
5a	164.2	-		-
6	153.0	-	159.5	-
7	104.5	-	106.3	-
8	162.3	13.1 (s)	162.7	13.33 (s)
9	182.5	-		-
9a	102.7	-		-
10	116.5	7.06 (d, 10.4)		6.69 (d, 10.1)
11	128.4	5.75 (d, 10.4)		5.76 (d, 10.2)
12	78.3			-
13, 14	28.2	1.50 (6H, s)		1.48 (6H, s)

proton at δ_H 6.21 (s) and aromatics with an ABC system appearing at δ_H 7.39 (dd); 7.31 (t) and 7.71 (dd). The presence of particular groups in the highly downfield region of δ_H 13.10 (s), is also a characteristic that this compound is thought to be a xanthone derivative [19]. The ^{1}H-NMR and ^{13}C-NMR data compound were compared with 6-deoxyjacareubin (chromenoxantone group) [20]: see Table 1. We concluded this structure to be as described in Fig. 5.

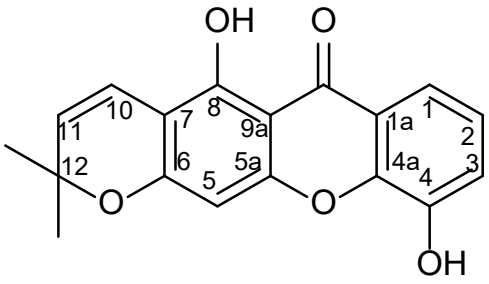

Fig 5. The structure of compound 1

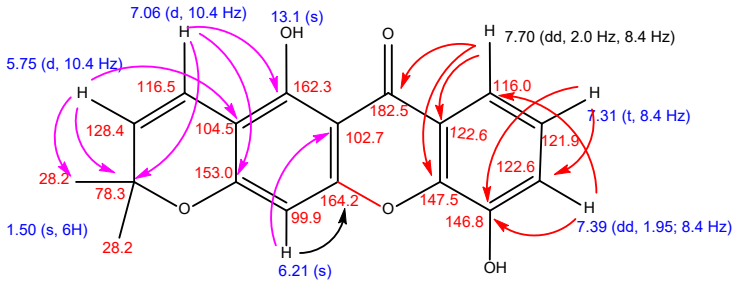

Fig 6. The HMBC correlation the compound 1

The assignments of all carbons were confirmed by the HMBC experiment. In the HMBC spectrum, the long-range correlation from the aromatic proton signal at δ_H 5.75 (d, J = 10.4 Hz) to the carbon signals at δ_C 28.2 (13,14-CH_3), 78.3 (C-12), 104.5 (C-7) indicated 6,6-dimethyl-3,6-dihydro-2H-pyran at C-7 of the phenol. The cross-peaks between 6.21 (1H, s), δ_C 102.7 (C-9a) and 164.2 (C-5a) suggested the connection of but-1–ene in the phenolic unit. The position of 6,6-dimethyl-3,6-dihydro-2H-pyran was determined by the correlations of the proton signal at δ_H 7.06 (1H, d, J = 10.4 Hz) with the carbon signals at δ_C 78.3 (C-12), 153.0 (C-6), and 162.3 (C-8). The position of the phenolic group was determined by the correlations of the proton signal at δ_H 7.31 (1H, t, J = 8.4 Hz) with the carbon signals at δ_C 122.6 (C-3), 146.8 (C-4) and the correlations of the proton signal at δ_H 7.39 (1H, dd, J = 2.0, 8.4 Hz) with the carbon signals at δ_C 116.0 (C-1) and 146.8 (C-4). The cross-peaks between 7.70 (1H, dd, J = 2.0, 8.4 Hz) and δ_C 122.6 (C-1a), 147.5 (C-4a), and 182.5 (C-9) suggested the connection of the phenolic group with the pyran unit. The HMBC correlation is thoroughly illustrated in Fig. 6.

Antibacterial Activity

The test result of the antibacterial activity with microdilution method of 6-deoxyjacareubin is shown in Fig. 7. The MIC of 6-deoxyjacareubin and erythromycin against *B. subtilis* respectively were 156.25 and 25 ppm. The 6-deoxyjacareubin MIC value was greater than the positive-controls (erythromycin) MIC value, which means that the sensitivity of 6-deoxyjacareubin against *B. subtilis* was lower than erythromycin.

The mechanism of inhibiting bacteria by antibacterial compounds is by damaging the cell walls of the bacteria.

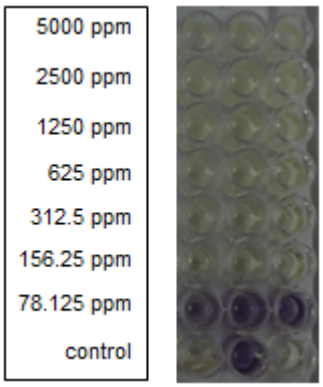

Fig 7. The result of MIC the compound (6-deoxyjacareubin) test using the microdilution method with an indicator of tetrazolium salt (MTT)

Molecules of nucleic acid and protein are also changed after the damaging of the cell wall. The damage also inhibit the works of enzymes, by inhibiting the synthesis of nucleic acid and protein from the bacterial cells which leads to its death [21].

The test of MIC isolate against *B. subtilis* bacteria resulted with a MIC value of 156.25 ppm, while the value of MIC from the ethyl acetate extract of the fruit (fraction C) is 1250 ppm (obtained from previous research). Those results show that the isolate is more active than the fraction. The isolate is a polyphenol compound that can inhibit hydrolytic enzymes (protease) which are found in microbes [22]. The protease enzyme is a proteolytic enzyme that catalysts the termination of peptide bonds in proteins that has a role in cell growth [23].

6-Deoxijacareubin is a chromenoxanthone compound [24]. The xanthone compound is a compound that has a tricyclic aromatic system with an anthraquinone base structure and is also known as an anti-bacterial agent [25]. Xanthone compounds contain

Table 2. Calculated estimated binding free energy and inhibition constant in docking of 6-deoxyjacareubin to erythromycin

No	Compound	Binding free energy		Inhibition constant	
1	Erythromycin (redocking)	RMSD 2.00 Å		RMSD 2.00 Å	
		-5.75 kcal/mol		61.27 μM	
2	6-Deoxyjacareubin	Best rank	Best cluster	Best rank	Best cluster
		-5.66 kcal/mol	6/10 = -5.66 kcal/mol	70.87 μM	70.87 μM

two benzenes with O atoms and ketones, have lipophilic properties and some of these compounds have been studied as anti-*B. subtilis* [26].

The isolate with a MIC value of 156.25 ppm shows that anti-bacterial activity against *B. subtilis* is low since the value of MIC > 100 ppm [27]. This also corresponds with the fraction C activity from the fruit's ethyl acetate extract that has a value of 1250 ppm. The value from fraction C shows that it has a low activity against *B. subtilis* since the value of MIC > 625 ppm [27].

In Silico

The ligand stability is defined by RMSD value obtained from docking studies. Ligand stability is determined by RMSD value which describes the different coordinates to the best docking score. In this study, the ligand exhibited RMSD value of below 2.00 Å. Thus it shows that by using reduced macromolecule, the ligand was able to maintain the coordinate stability by showing similar coordinates within 10 runs of docking. Further molecular dynamic study is required to understand the properties of reduced macromolecule [28].

The affinity and inhibition constant (k_i) were -5.75 kcal/mol and 61.27 μM, respectively. 6-Deoxyjacareubin resulted in lower affinity compared to the cocrystal structure of erythromycin with binding energy and inhibition constant of -5.66 kcal/mol and 70.87 μM, respectively. Docking results of 6-deoxyjacareubin showed interaction with several residues in concordance to the residues bonded with erythromycin. These residues included U2588, C2589, G2484, and U2590, indicating similar activity of 6-deoxyjacareubin with erythromycin. The table of calculated estimated binding free energy and inhibition constant in the docking of 6-deoxyjacareubin to erythromycin can be seen in Table 2 [17].

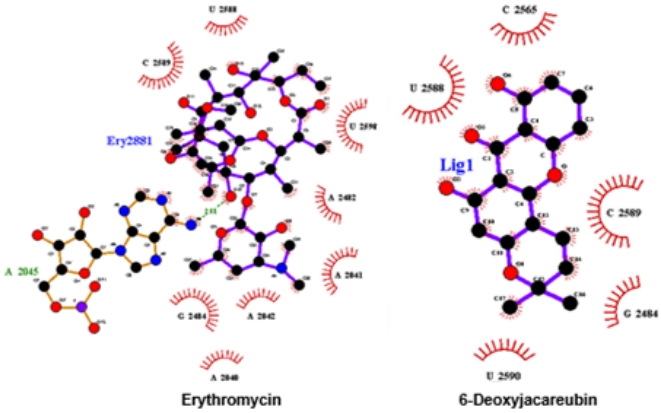

Fig 8. The interaction between erythromycin and 6-deoxyjacareubin with amino acids on the A2042 PDB receptor using the LigPlot program was illustrated

Erythromycin involved hydrogen bonding with A2042 which was not shown on deoxyjacareubin. The hydrogen bonding might have contributed to erythromycin affinity which was slightly higher than deoxyjacareubin. Both ligands exhibited similar hydrophobic interaction with several residues. The interaction between erythromycin and 6-deoxyjacareubin with amino acids on the A2042 PDB receptor using the LigPlot program is illustrated in Fig. 8.

■ CONCLUSION

Deoxyjacareubin compounds were isolated from the ethyl acetate extract of *G. latissima* Miq. fruit, and acted as an antibacterial agent against *B. subtilis* with a MIC value of 156.25 ppm. According to the docking study, 6-deoxyjacareubin showed similar hydrophobic interaction with several amino acid residues (including C2565, C2589, G2484, U2590, and U5588) between a native ligand.

■ ACKNOWLEDGMENTS

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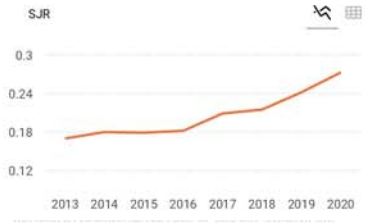

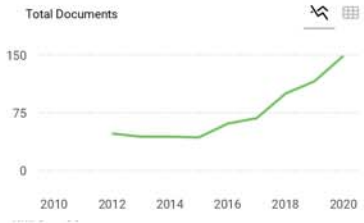

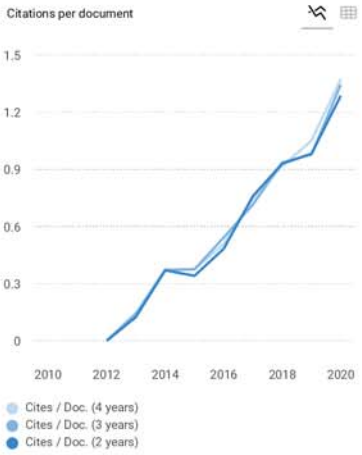

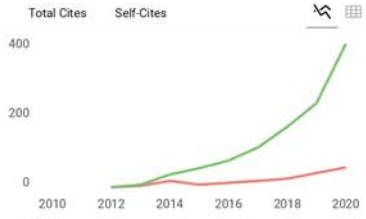

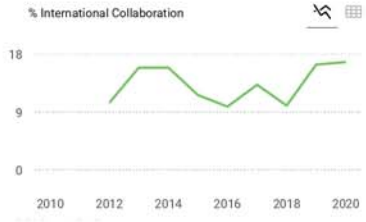

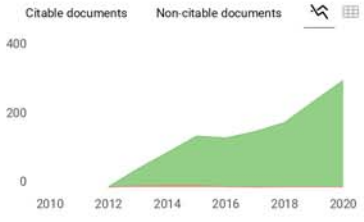

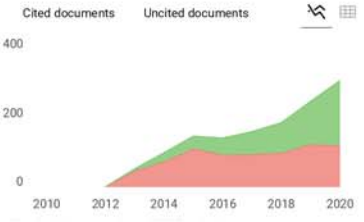

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