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Antibacterial activity of secondary metabolites isolated from *Ficus aurata* (Miq.) Fruits

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ABSTRACT

This research aimed to isolate chemical constituents from ethyl acetate and n-hexane fractions of *Ficus aurata* (Miq.) fruits and investigated their antibacterial activity. Chemical constituents were separated using vacuum liquid chromatography, gravity column chromatography, and thin-layer chromatography. The structures of the isolated compounds 3,4-dihydroxybenzoic acid (1) and β-sitosterol (2) were elucidated using various spectroscopic methods, including UV, IR, and NMR (1H-NMR, 13C-NMR, and DEPT). While, antibacterial activity was done using resazurin microtiter assay. These 2 compounds were first time reported from *F. aurata* fruit. In addition, analysis of the bioassay showed that compound 1 had a notable antibacterial activity against Gram negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATTC 27853), and a gram-positive bacterium (*Staphylococcus aureus* ATCC 23235) with the minimum inhibitory concentration (MIC) value of 0.1563 µg/ml, and it indicated the same MIC as cefadroxil (positive control). Compound 2 has weak antibacterial activity compared to cefadroxil (positive control). The results found that *F. aurata* (Miq.) fruits metabolites have antibacterial activity and show potency as antibacterial agents. Different kinds of bacteria and assay could be needed to investigate and support its antibacterial activity in the future.

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1. Introduction

Ficus is one of the most important plants in the forest ecosystem, because the *Ficus* is used by animals such as birds, orangutans, pigs and tree rats as a food source. In general, people know *Ficus* by the name of banyan, fig/aro, jilabuak or sikalabuak with characteristics on the shape and structure of the fruit called fig or syconium (Nur'aini et al., 2013). The *Ficus* tree's root system is special and suitable for growing in low-lying areas (Ulfah et al, 2015). *Ficus* (Moraceae) is traditionally used as herbs, particularly in rural areas where this plant has been employed as traditional medicine. The use of *Ficus* plant for medical practices was proven safer and cheaper than synthetic drugs (Pranuthi et al, 2014). This plant is commonly used to treat diabetic, liver, dysentery (Gautam et al, 2014), antiinflamatory (Patil and Patil, 2011), cancer, allergic and stress (Dhungana et al., 2011). According to Lushaini et al. (2015), leaves extract of *Ficus variegate* Blume has significant total phenolic, high antioxidant and cytotoxic activity, and people often consume the leaves as it greatly regulated breast milk production. Malaysian have used *F. deltoidea* Jack for injuries, rheumatic, tonic, and diabetic medicines (Bunawan et al., 2014). Joseph and Raj (2011) also enumerated that *F. carica* has pharmaceutical properties for anticancer, antioxidant, antimicrobial, and antifungal activities. Manabo ethnic in Talacogon, Phillipines was applied plant species of *F. nota* as alternative medicines in which its hot water and ethanol extracts were proven for cytotoxic activity that actively against *Arthemia salina* larvae with the LC50 991.00 ppm and 852.22 ppm, respectively (Arquion et al., 2015). Phytochemical investigation of dried fruit *F. carica* has been characterized to contain polyphenols, flavonoid, alkaloid and saponin which contributed of high antioxidant activity using ABTS and FRAP methods while GC-MS analysis of this plant shows vitamin E, β-amyrin, stigmasterol, campesterol, oleic acid, isoamyl laurate dan ϒ tocopherols components (Soni et al., 2014). Moreover, *F. aurata* plant is a species of *Ficus* which still less-reported, the leaves and fruits of *F. aurata* have been known for high antioxidant activity (Nurhamidah et al., 2015). Figure of *F. aurata* fruits is shown on Fig. 1. Based on Nurhamidah et al. (2018), hexane extracts from *F. aurata* leaves and fruit showed very strong cytotoxic activity using Brine Shrimp Lethality Test (BSLT) method, with LC50 at 13.74 µg/ml and 33.10 µg/ml. Santoni et al. (2019), reported the leaves of *F. aurata* contain triterpenoid compounds that can inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. This study aims to explore the potential of Indonesia's natural resources, which has a diversity of plants that can be developed as medicinal plants.

2. Materials and methods

2.1. Plant materials

F. aurata plant was planted at University of Andalas, Padang City, West Sumatra, Indonesia and identified by Herbarium of

Biologi Department, University of Andalas (ANDA) with identification number of 204/K-ID/ANDA/VI/2014.

Fig. 1. *Ficus aurata* (Miq.) fruits

2.2. Instruments

The instruments used were vacuum rotary evaporator (Heidolph WB 2000), oven, vacuum, gravity column chromatography in various size, vacuum liquid chromatography, capillary tube, UV lamp (GL-58 with λ 254 and 365 nm), TLC (silica gel 60 F254) (Merck), micro tube, Melting point (Gallenkamp Melting Point Apparatus) (digital series), UV-Vis (Themo Scientific), FT-IR (Thermo Scientific iS 10), JEOL Delta2-NMR spectrometer 500 MHz (^{1}H NMR, ^{13}C NMR, DEPT), Elisa reader, 96-well plate and centrifuge.

2.3. Methodology

2.3.1. Extraction and isolation

The procedures of extraction referred to previous study which was conducted by Abriyani et al. (2014). The fruit of *F. aurata* powder (3.050 g) extracted by maceration with n-hexane, ethyl acetate, and methanol. The weight of crude extracts was 129, 82 and 77 g for n-hexane, ethyl acetate, and methanol extracts. The ethyl acetate extract (30 g) was subjected to vacuum column chromatography and eluted using step gradient polarity with stepwise: n-hexane, n-hexane:ethanol, ethanol, and ethanol:methanol. Each vial was monitored by thin-layer chromatography (TLC), and the same pattern were combined to afford 7 fractions (F1-7). Fraction F4 was purified by column chromatography and eluted using n-hexane:ethanol (7:3) successively, yielded compound 1 (173 mg); Rf 0.27 in n-hexane:ethanol (2:3) and 0.34 in dichloromethane:ethanol (2:3). Meanwhile, the crude extract of n-hexane (30 g) was purified with gravity column chromatography used n-hexane 100% and n-hexane:ethyl acetate. The step of n-hexane was the same as ethyl acetate extract which each vial was monitored by TLC and fractions with the same pattern were combined to afford 7 fractions (F1-7). Fraction F5 was purified by column chromatography with eluents n-hexane:dichloromethane (6:4) successively, and it yielded compound 2 as white solid crystal (49 mg); Rf 0.40 in n-hexane:ethanol (9 : 1) and 0.38 in n-hexane:dichloromethane (2:2).

2.3.2. Antibacterial activity

Chemical constitutes from *F. aurata* fruit were investigated against 2 Gram negative (*E. coli* and *Pseudomonas aeruginosa*) and 2 Gram positive bacteria (*S. aureus* and *Bacillus subtillis*). The procedures of antibacterial activities referred to previous study which was conducted by Sarker et al. (2007). All of the tools were sterilized and bacteria were inoculated into NB (nutrient broth) medium in reaction tubes. Then, the tubes were closed by cotton, following that the incubation at 37°C for 24 h. The OD (optical density) was carried out on 600 nm until the OD_{600} of bacteria had reached 0.8 – 1.0 or equal to 10-6 CFU/ml. Exactly, 270 mg ressazurin was dissolved in 40 ml distilled water to form resazurin solution. Furthermore, the tested solution was conducted using 2 mg of each isolated compound and Cefadroxil (positive control). Then, it dissolved in 20 ml of DMSO until the concentration of the compounds reached 100 ug/ml. The 96-well microplates were prepared and labelled. Well A was filled with 90 µl of NB media and 10 µl of each tested solution (100 µg/ml) and Cefadroxil (100 µg/ml) to obtain 10 µg/ml of end concentration. Two-fold dilution method was conducted by diluting sample and positive control. After diluting steps were taken, 50 µl from well row A was transferred to well row B by diluting 50 µl NB media and took 50 µl solution from well row B. Then, 50 µl solution from well row B was transferred to well row C. This step was repeated until well row H was filled. In addition, 10 µl bacterial suspensions (10-6 CFU/ml) were added to all wells, except one well that contained 50 ul NB media while bacteria tested as negative control. Finally, 10 µl resazurin was filled to all wells as bacterial growing indicator. Microplates were closed and incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) was carried out by analyzing blue wells from the lowest concentration of samples and the positive control.

3. Results and discussion

3.1. Identification of isolated compounds

A brownish powder (1) has been isolated from the fruit of *F. aurata* (Miq.). The melting point of compound **1** was pointed out at 200 – 201°C. UV-Vis analysis (in CH_3OH) showed 2 main peaks, in the wavelength of 259.74 dan 295.12 nm (Figure not shown). This indicated the UV absorption of electronic transition from π to π^* which recognizes as aromatic functional group absorption. Moreover, IR spectroscopy analysis (KBr) showed a wide absorption on $\bar{\upsilon}$ 3447.02 cm^{-1} which indicated the existence of hydroxyl (-OH) group, C=O functional group for $\bar{\upsilon}$ 1675.03 cm^{-1}, the C=C aromatic has been reported from this compound based on $\bar{\upsilon}$ of 1529.42 cm^{-1}, and 1095.77 cm^{-1} refers to C-O bond (Fig. 2). Spectrums analysis from ^{1}H-NMR and ^{13}C-NMR of constitute 1 are shown on (Fig. 3a-b).

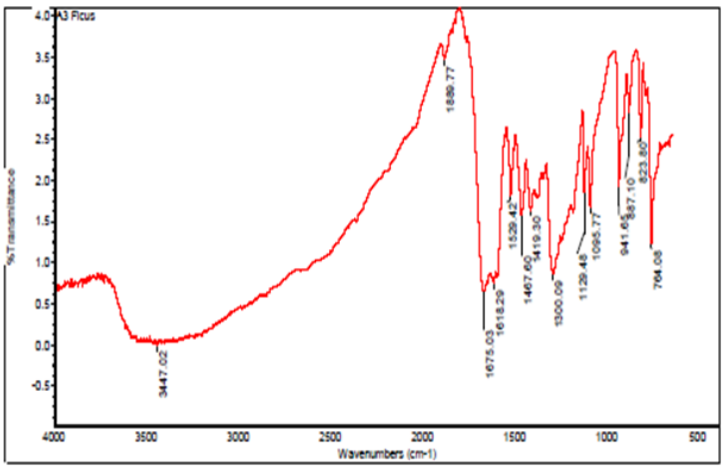
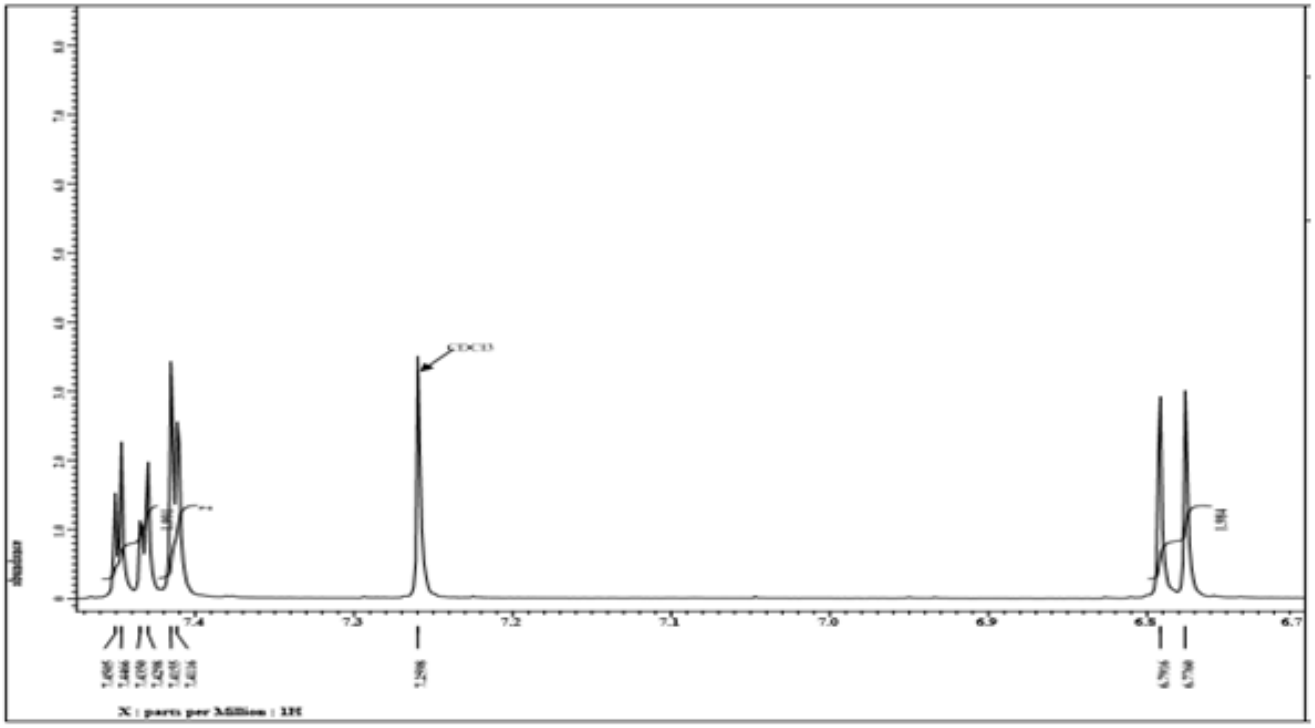


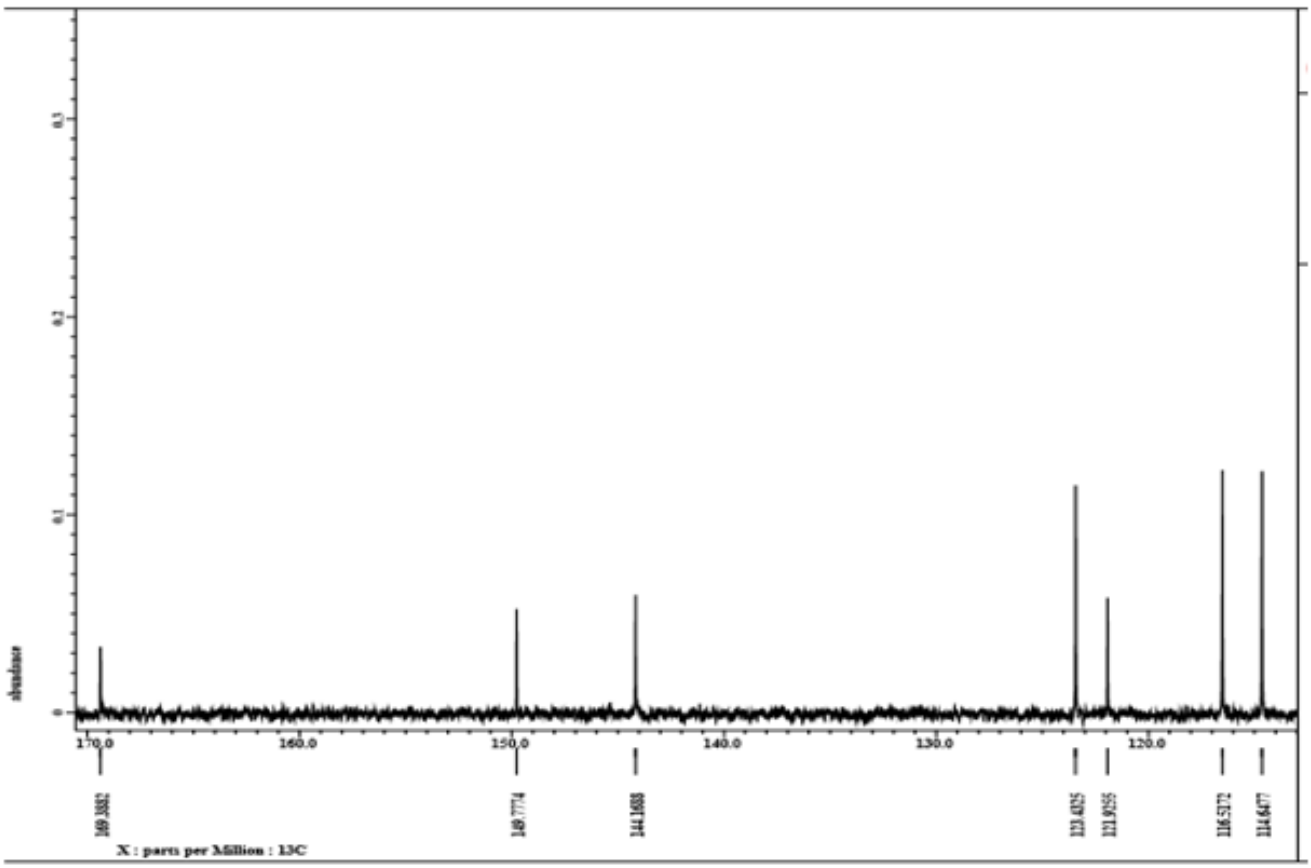
Fig. 2. IR spectrum of compound 1 from the fruit of *F. aurata* (Miq.)

^{1}H-NMR 500 Hz revealed three proton signals at δ H 6.78 ppm (*d*, 1H, 3*J* = 7.8 Hz), δ H 7.41 ppm (*d*, 1H, 4*J* = 1.95 Hz), δ H 7.44 ppm (*dd*, 1H 3*J1* = 7.95 Hz, 4*J2* = 1.95 Hz). 13C-NMR 125 Hz spectrum showed seven carbon signals δ C 114,7; 116.5; 121.9; 123.4; 144.2; 149.8 ppm which referred to aromatics chemical shift

and 169.4 ppm recognized as carboxylate. Furthermore, four quaternary carbon signals on 121.9; 144.2; 149.8; 169.4 ppm and three signals of tertiary carbon from the chemical shift of 114.7; 116.5 dan 123.4 were reported based on DEPT spectrum analysis. Furthermore, spectrum data of ^{1}H-NMR,^{13}C-NMR and DEPT of compound **1** compared to NMR spectroscopy data from previous research (Gurial et al., 2013) are designated on Table 1. Therefore, compound **1** was determined as a phenolic compound 3,4-dihydroxybenzoic acid with molecular formula $C_7H_6O_4$. The chemical structure of the compound is seen from Fig. 4.



(a)



(b)

Fig 3. ^{1}H-NMR (a) and ^{13}C NMR (b) spectra of compound 1 from the fruit of *F. aurata* (Miq.

Compound **2** was obtained as a white needle crystal, there was no spot-on TLC under the UV lamp while it has a purple color after sulfate acid 2 N was added on TLC. Melting point of this compound was 137 – 138°C. Based on the IR and ^{1}H-NMR spectrums (Fig. 5a-b), the compound showed a wide absorption on wavelength number ($\bar{\upsilon}$) of 3432.56 cm^{-1} which indicated the same pattern as compound 1 with hydroxyl (-OH), $\bar{\upsilon}$ of 2936.91 and 2867.37 cm^{-1} refer to stretch C-H bond, the double bonds of C=C were reported from $\bar{\upsilon}$ 1653.87 and $\bar{\upsilon}$ 1465.03 cm^{-1} for cyclic methylene (CH2). In addition, various NMR analysis (13C-NMR dan DEPT) (Fig. 6a-b) identified that compound 2 contained of 49 protons, 29 carbons with three quaternary carbon signals (C-5, C-10 and C-13), nine CH carbon signals (C-3, C-6, C-8, C-9, C-14, C-17, C-18, C-22, and C-25), eleven methylene (C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-20, C-21, and C-23), and six methyl carbons (C-19, C-24, C-26, C-27, C-28 dan C-29).

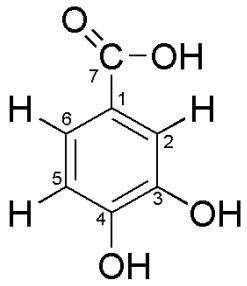


Fig. 4. The structure of 3,4-dihydroxybenzoic acid (compound 1)

Table 1. Chemical shift data (ppm) of ^{1}H NMR, ^{13}C NMR and DEPT of compound 1 and from literature ($CDCl_3$).

No.	^{1}H-NMR Compound 1	^{13}C-NMR Compound 1	DEPT	^{1}H-NMR reference	^{13}C-NMR reference
1		121.9	C		122.3
2	7.41, 1H, *d*, ^4J = 1.95 Hz	116.5	CH	7.36, 1H, *d*, *J* = 2.1 Hz	116.9
3		144.2	C		145.2
4		149.8	C		150.3
5	6.78, 1H, *d*, ^3J = 7.8 Hz	114.7	CH	6.80, 1H, *d*, *J* = 8.1	115.5
6	7.44, 1H, *dd*, $^3J_1$ = 7.75 Hz; $^4J_1$ = 1.95 Hz	123.4	CH	7.31, 1H, *dd*, *J* = 8.1; 2.1 Hz	122
7		169.4	C		167.7

Table 2 shows the comparison among compound **2** spectrums from ^{1}H-NMR. Based on the ^{1}H-NMR, ^{13}C-NMR, and DEPT spectrum data of compound **2**, and compared to NMR data of reported compound in Table 2, compound **2** was identified as steroid β-sitosterol with molecular formula $C_{29}H_{50}O$. Structure of β-sitosterol is represented in Fig. 6. In addition, Table 2 shows the comparison among compound **2** spectrums from ^{1}H-NMR, ^{13}C-NMR, and DEPT and reference from Chaturvedula and Prakash (2012).

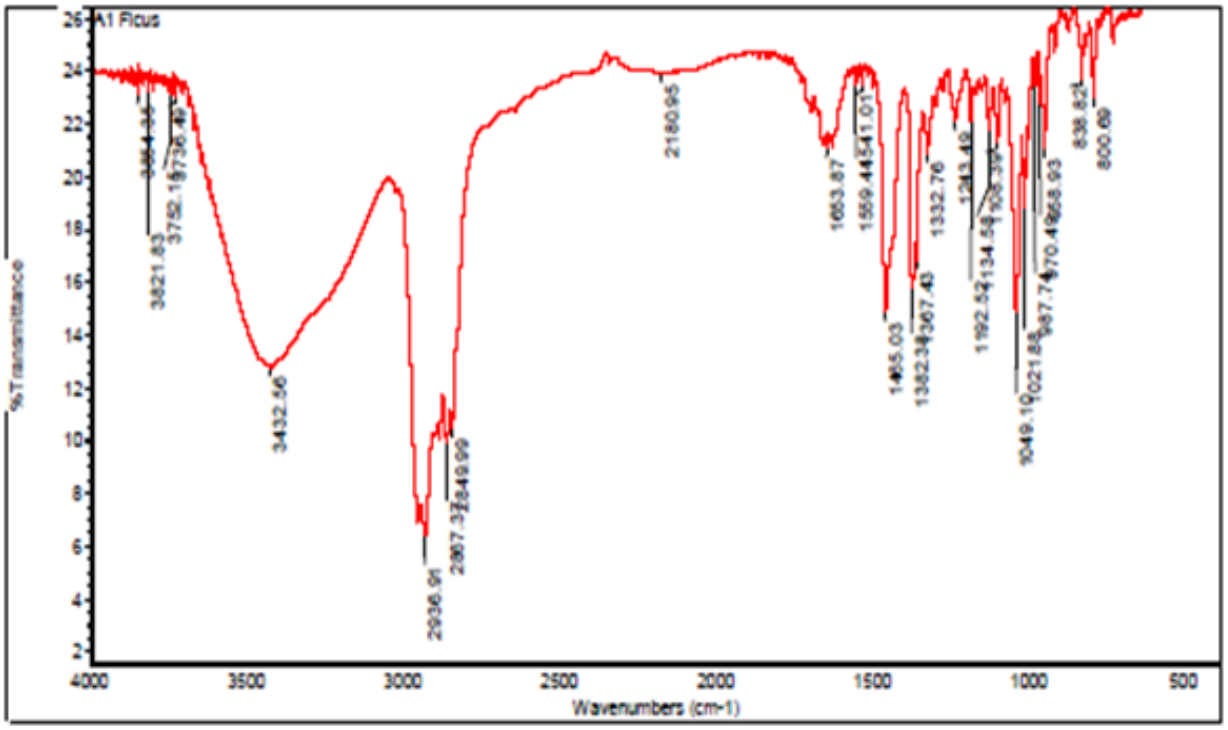
3.2. Antibacterial activity of the isolated compounds

Antibacterial activities of isolated compounds using resazurin microtiter assay (REMA) against *E. coli* and *P. aeroginosa* (Gram negative) and *S. aureus* and *B. subtilis* (Gram positive) were investigated and seen on Fig. 7 and Table 3. Antibacterial activities were determined by the MIC, which was known by color analyzing in 96-wells. The blue color on wells indicated no bacterial grown while bacterial grown were identified by pink color, because there was a reduction of resazurin indicator which made a blue turning to a pink. Total reduction of resazurin which formed resorufin equaled to bacterial grown.

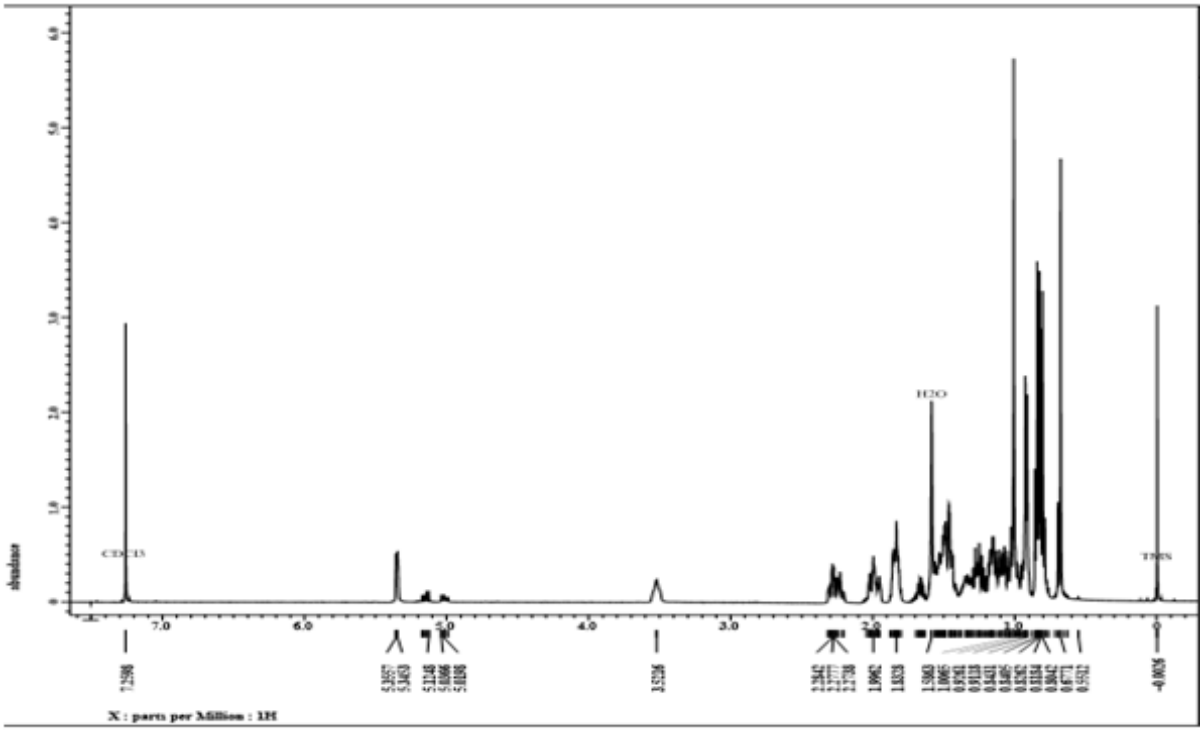
Table 3 represents the result of antibacterial activities of isolated compounds against *E. coli, S. aureus*, *P. aeroginosa*, and *B. subtillis* bacteria tested.

Table 2. ^{1}H-NMR and ^{13}C-NMR chemical shift (ppm) of compound 2 and previous research (in $CDCl_3$)

Position	^{1}H-NMR compound 2	^{13}C-NMR compound 2	DEPT	^{1}H-NMR reference	^{13}C-NMR reference
1		37.44	CH_2		37.5
2		31.85	CH_2		31.9
3	3.52 (*s*, 1H)	72.00	CH	3.5 (*t*, 1H)	72.0
4		42.49	CH_2		42.5
5		14.,94	C		14.9
6	5.35 (*t*, 1 H)	12.,91	CH	5.36 (*t*, 1H)	12.,9
7		32.09	CH_2		32.1
8		32.09	CH		32.1
9		50.31	CH		50.3
10		36.70	C		36.7
11		21.28	CH_2		21.3
12		39.96	CH_2		39.3
13		42.59	C		42.6
14		56.95	CH		56.9
15		26.24	CH_2		26.3
16		28.44	CH_2		28.5
17		56.23	CH		56.3
18		36.34	CH		36.3
19	0.93 (*d*, 3H)	19.22	CH_3	0.93 (*d*, 3H)	19.2
20		34.12	CH_2		34.2
21		24.49	CH_2		26.3
22		46.02	CH		46.1
23		23.25	CH_2		23.3
24	0.84 (*t*, 3H)	12.18	CH_3	0.84 (*t*, 3H)	12.2
25		29.33	CH		29.4
26	0.83 (*d*, 3H)	20.02	CH_3	0.83 (*d*, 3H)	20.1
27	0.81 (*d*, 3H)	19.59	CH_3	0.81 (*d*, 3H)	19.6
28	0.68 (*s*, 3H)	18.97	CH_3	0.68 (*s*, 3H)	19.0
29	1.01 (*s*, 3H)	12.05	CH_3	1.01 (*s*, 3H)	12.0



(a)



(b)

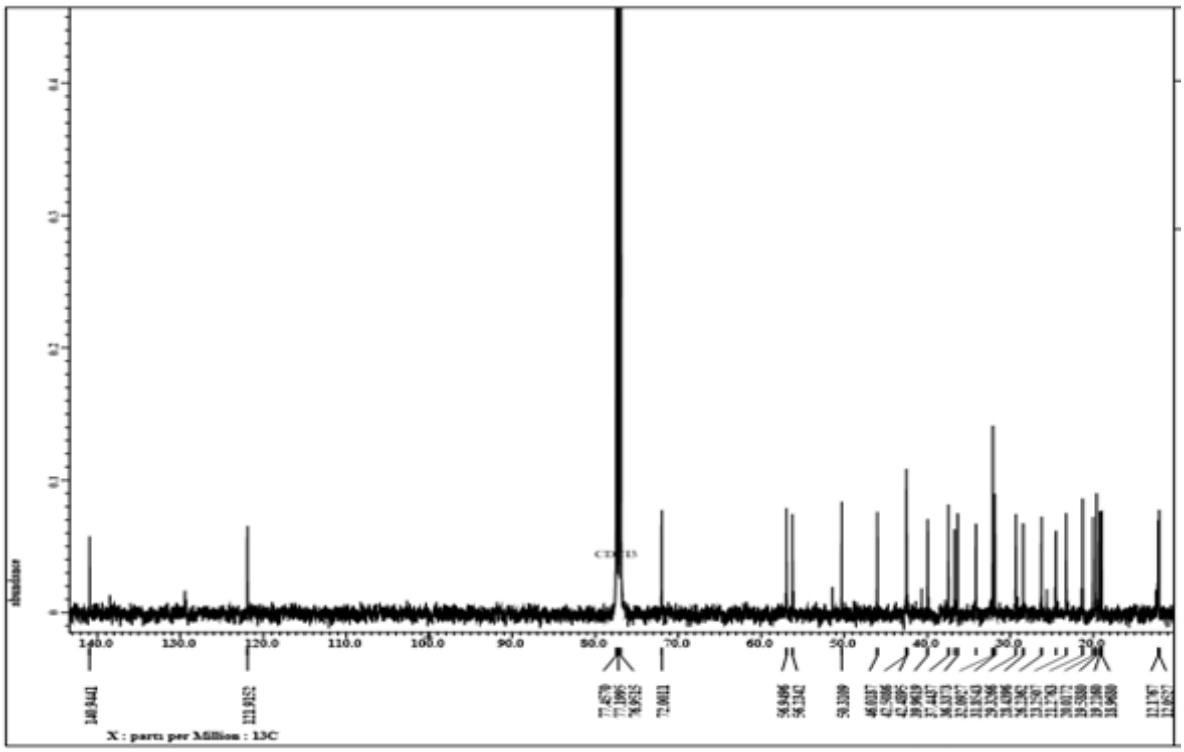
Fig. 5. IR (a) and ^{1}H-NMR (b) spectra of compound 2 from the fruit of *F. aurata* (Miq.)

Table 3. Antibacterial activities of isolated compounds

Bacteria	Minimum Inhibitory Concentration (MIC) µg/ml		
	3,4-dihydroxybenzoic acid (compound 1)	β-Sitosterol (compound 2)	Cefadroxil (positive control)
E. coli (-)	0.1563	0.3125	0.1563
S. aureus (+)	0.1563	0.6250	0.1563
P. aerogionsa (-)	0.1563	0.6250	0.1563
B. subtillis (+)	0.3125	1.250	0.1563

The results showed that 3,4-dihydroxybenzoic acid concluded as an intensive antibacterial activity against both Gram positive and Negative bacteria, which was proven by the MIC of 0.1563 µg/ml (same as the positive control of cefadroxil), except for a *B. subtilis*. Based on the chemical structure, 4-dihydroxybenzoic acid has a benzene molecule containing OH moieties which may contribute to its antibacterial property. As mention before, 3,4-dihydroxybenzoic acid was determined as a phenolic compound. According to Lizárraga-Velázquez et al. (2020), the mechanism of antibacterial activity of phenolic

compounds is related to the damage of the bacteria cell membrane, which is process involves modifying the membrane permeability leading to the loss of cell wall integrity and changes in intracellular functions. While, β-sitosterol is one of phytosterols which the structure similar to the cholesterol and represent a diverse group of triterpenes (Gylling and Simonen, 2015; Uddin et al, 2018). According some researcher, their antibacterial activity has related to their hydrophobicity (Valdivieso-Ugarte et al, 2019; Nazzaro et al, 2017; Pandey et al, 2017). It can cause the wall and membrane of bacterial cells will be unalterable failure, which initiate to a leakage of proteins, DNA molecules, and RNA of the bacterial cells (Meng et al., 2016; Montironi et al., 2016). Furthermore, cefadroxil as a control in this study is a para-hydroxy derivative of cephalexin, which widely used as antibacterial drug. Cefadroxil inactivates penicillin-binding proteins (PBPs) located on the bacterial cell wall. Inactivation of PBPs can affected to the bacterial cell wall strength and rigidity, then causes cell lysis (Auda et al., 2009; Tsuji et al, 1981).



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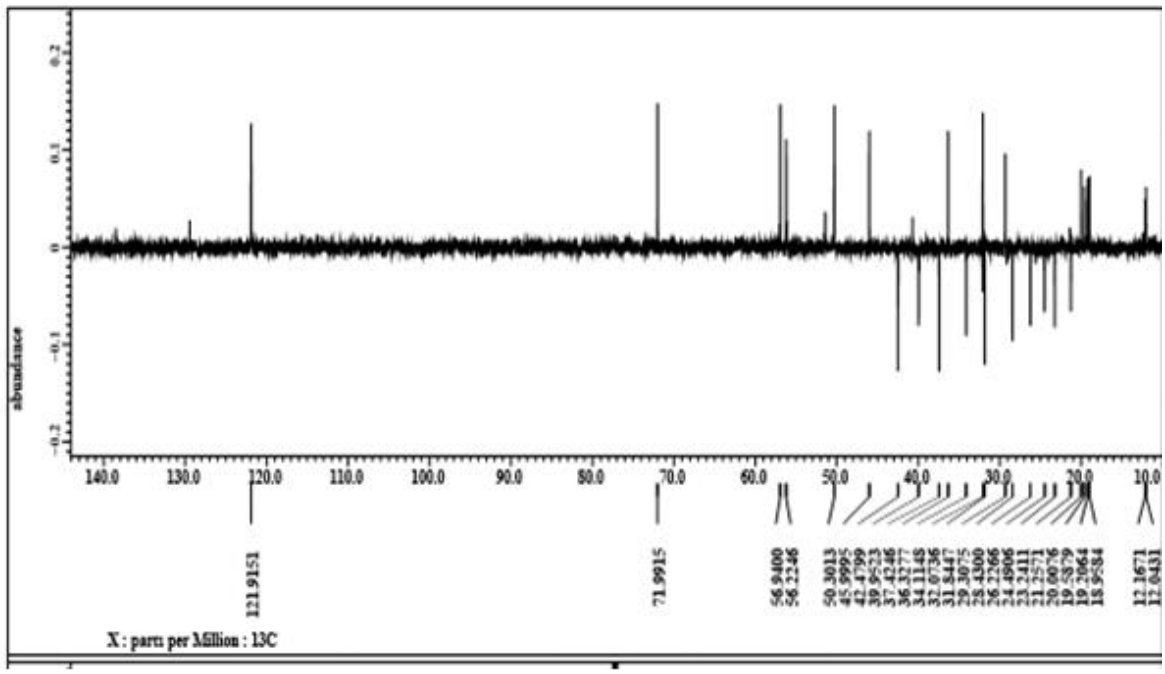

(b)

Fig. 6. ^{13}C-NMR (a) and DEPT (b) spectra of compound 2 from the fruit of *F. aurata* (Miq.)

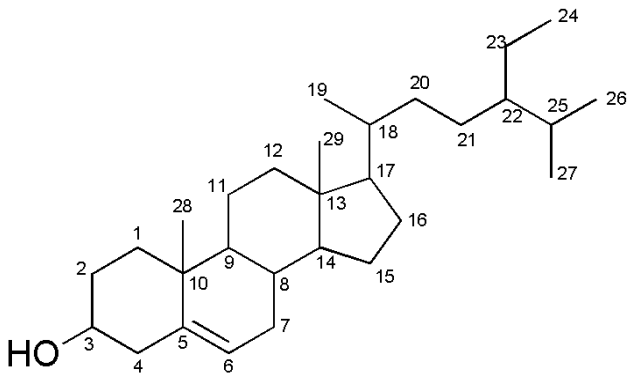

Fig. 7. The structure of β-sitosterol (compound 2)

Some researchers reported that it was shown antibacterial activity against some pathogenic bacteria. The phenolic compounds found in raisin extract (Yousef et al., 2018), and solvent fraction of *H. tiliaceus, P. tectorius fruits, H. formicarum* tuber (Andriani et al., 2017a; Andriani et al., 2017b; Andriani et al., 2019) had strong antibacterial activity. While, as according to Puupponen-Pimia et al. (2001), different bacterial species will show different sensitivity to phenolic compounds. However, β-Sitosterol confirmed as a lower antibacterial activity because the MIC indicated the higher number than 3,4-dihydroxybenzoic acid to all bacteria. Antibacterial potency of β-Sitosterol has reported clearly by some researchers. Previous study has reported that β-Sitosterol (isolated from root barks of *Malva parviflora*) has been shown to possess antibacterial activity against *S. aureus* and *E. coli* (Ododo et al., 2016). Elsewhere, antibacterial activity of β-Sitosterol against *E. coli, M. smegmatis, P. aeruginosa, S. aureus* and some different kinds of bacteria has also reported by Fadipe et al. (2015). They have reported that this compound showed strong activity especially against *E. coli* and *M. smegmatis*.

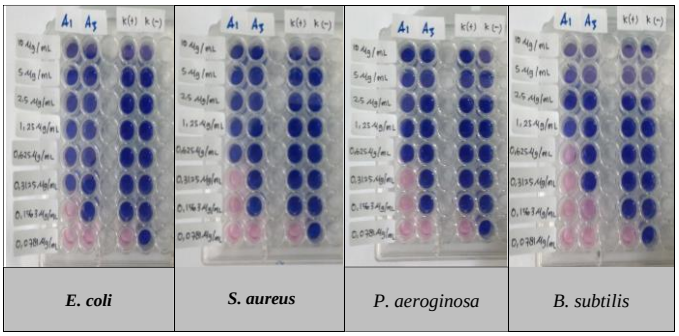

Fig. 8. Antibacterial activity of isolated compounds against *E. coli, S. aureus, P. aeroginosa,* and *B. subtilis* (A1 = β-Sitosterol; A3= 3,4-dihydroxybenzoic acid; K(+)= positive control (Cefadroxil); K(-)= negative control. Sample concentration was prepared by two folds dilution from 10 µg/ml to 0.078 µg/ml

4. Conclusion

Based on UV, IR and NMR spectrum characterization (^{1}H, ^{13}C, DEPT) of secondary metabolite compounds that isolated from *Ficus aurata* (Miq.) was obtained as 3,4-dihydroxybenzoic acid (**1**) and β-Sitosterol (**2**). The results of antibacterial activity tests on the two compounds showed that compound **1** had very good antibacterial activity against Gram negative bacteria (*E. coli* and *P. aeruginosa*) and Gram positive bacteria (*S. aureus*) compared to cefadroxil (positive control). Compound **2** has very weak antibacterial activity compared to positive control.

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Conflict of interest

Authors declare there are no any conflict of interest.

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