

## The Anticancer Activity of Phytoconstituents of the Stem of *Bouea macrophylla*

Tarso Rudiana<sup>1\*</sup>, Nani Suryani<sup>1</sup>, Dimas D. Indriatmoko<sup>2</sup>,  
Yusransyah Yusransyah<sup>3</sup>, Muhammad A. Hardiyanto<sup>4</sup>,  
Ricky Yohanes<sup>4</sup>, Siti Nurbayti<sup>4</sup>, Eka Nurdiansyah<sup>2</sup>, Hidayatul Fajri<sup>1</sup>,  
Noviany Noviany<sup>5</sup> and Sutopo Hadi<sup>5</sup>

<sup>1</sup>Department of Chemistry Faculty of Science, Pharmacy and Health, Universitas Mathla'ul Anwar, Jalan Raya Labuan-Pandeglang KM 23, Pandeglang Banten 42273, Indonesia.

<sup>2</sup>Department of Pharmacy Faculty of Science, Pharmacy and Health, Universitas Mathla'ul Anwar, Jalan Raya Labuan-Pandeglang KM 23, Pandeglang Banten 42273, Indonesia.

<sup>3</sup>Department of Pharmacy, Sekolah Tinggi Ilmu Kesehatan Salsabila, Jalan Raya Serang-Pandeglang KM 06 No. 33 Serang Banten 42211, Indonesia.

<sup>4</sup>Department of Chemistry Faculty of Science and Technology, Universitas Islam Negeri (UIN) Syarif Hidayatullah Jakarta, Jalan Ir. H. Juanda No. 95, South Tangerang Banten 15412, Indonesia.

<sup>5</sup>Department of Chemistry Faculty of Mathematic and Natural Sciences Universitas Lampung, Bandar Lampung, Lampung 35141, Indonesia.

\*Corresponding Author E-mail: tarso.rudiana@gmail.com

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*Gandaria* (*Bouea macrophylla* Griff) is a typical Asian plant that is commonly found in In-donesia with various secondary metabolite compounds such as phenolic, flavonoid and ter-penoid. The purpose of this study was to isolate secondary metabolites from the stem extract of *B. macrophylla* and determine their activity against cancer cells MCF-7, A549, MDA-MB 231 and HCC-1954. The isolation of the compounds was conducted using various chromatographic techniques, the determination of the chemical structure of the isolates was performed using physicochemical methods including mass spectrometer and nuclear magnetic resonance, the determination of anticancer activity was carried out using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) i.e. MCF-7 and A549 cell lines; and dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for MDA-MB 231 and HCC-1954 cell lines. Four compounds namely stigmaterol (1), fustin (2), garbanzol (3) and methyl galat (4) were successfully isolated from the stem extract of *B. macrophylla*, which was obtained from Serang Regency, Indonesia. These compounds were then tested their anticancer activity against the cancer cells of Michigan Cancer Foundation-7 (MCF-7), human alveolar epithelial cells (A549), human breast cancer cell line-1954 (HCC-1954) and M.D. Anderson-Metastatic Breast-231 (MDA-MB-231). The results of anticancer test indicated that based on the IC50 values for all compounds tested, the compounds 2 and 4 were more active on HCC-1954 cell with IC50 values of  $134.35 \pm 44.62$  and  $153.69 \pm 12.54$   $\mu\text{g/mL}$ , respectively, while the compound 3 was found to be the most active against MDA-MB-231 cell line with IC50 value of  $233.41 \pm 91.57$   $\mu\text{g/mL}$ .

**Keywords:** Anticancer, *B. Macrophylla*, Cytotoxicity, Phytoconstituents.

The *B. macrophylla* (Anacardiaceae) is a high fruit-producing plant rich in antioxidant compounds. It is common in Indonesia, especially

on the islands of Sumatra, Java, Kalimantan and Maluku.<sup>1,2</sup> The methanol extract of *B. macrophylla* fruit has antioxidant activity with an IC<sub>50</sub> value of

16.29 mg/mL.<sup>2</sup> The methanol extract and the fruit skin as well as the fruits of *B. macrophylla* has been reported to be active as antioxidant.<sup>3-7</sup> The fruit of *B. macrophylla* contains compounds of flavonoid class with an antioxidant activity value of IC<sub>50</sub> 2.43 µg/mL, using in vitro 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity method.<sup>1,7</sup> The antioxidant activity has an important correlation with anticancer activity. Kulsum *et al.* (2018) reported that the activity of antioxidants is proportionally correlated with anticancer activity in the test of amla and ginger extract with a probability value under 0.05.<sup>8</sup> Their results indicated that compounds in the extract with an excellent antioxidant activity also have good anticancer activity. Gandaria has been reported to show a good antioxidant activity; thus it makes gandaria has possibility to show a good anticancer activity, too.

Andina and Musfirah reported that the ethanol extract of *B. macrophylla* leaves has strong antioxidant activity with an IC<sub>50</sub> value of 55.83 µg/mL.<sup>9</sup> They also demonstrated that the antioxidant activity of the ethanol extract of the stem bark of *B. macrophylla* with an IC<sub>50</sub> value of 20.03 mg/mL is greater than that of the leaf ethanol extract with an IC<sub>50</sub> value of 55.83 mg/mL.<sup>9</sup> According to Rudiana *et al.* (2018)<sup>10</sup> the ethyl acetate extract from gandaria stems (*B. macrophylla*) has the best antioxidant activity compared to *n*-hexane and methanol extracts with an IC<sub>50</sub> value of 4.89 µg/mL.

The seed extract of *B. macrophylla* has been reported to have anticancer activity against human hypopharyngeal FaDu (HTB-43), MCF-7 and MDA-MB-231 cells with IC<sub>50</sub> values of 34.36; 59.07; 28.65 µg/mL, respectively.<sup>11</sup> The seed extract of *B. macrophylla* contains pentagalloyl glucose and ethyl gallate compounds, which can inhibit MCF-7 cells through the apoptotic pathway.<sup>11-13</sup> Besides that, the seed extract of *B. macrophylla* can inhibit the growth of leukemia and lung cancer cells with IC<sub>50</sub> values ranging from 3 to 45 µg/mL.<sup>11,12</sup>

The exploration of pure phytoconstituents isolation of the stem of *B. macrophylla* has not been investigated. In our previous work, two compounds, luteolin and naringenin, have been identified in the ethyl acetate extract of *B. macrophylla* stem using liquid chromatography-mass spectrometry. Still, they were not isolated.<sup>14</sup> The previous works on

*B. macrophylla* mostly focused on the chemical content of their extracts.<sup>10-14</sup> The present work aims to study the isolation of secondary metabolites in the stem of *B. macrophylla* and determined the anticancer activity of the compounds isolated against MCF-7, HCC-1954, MDA-MB-231, and A549 cell lines.

## MATERIALS AND METHODS

### Plant Material

The stem of *B. macrophylla* was obtained from Serang District, Banten Province of Indonesia and identified at the Herbarium Bogoriense, a Center for Biological Research, Indonesian Institute of Research, Cibinong with voucher specimen number of 1068/IPH.1.01./If.07/VI/2018.

### General Experiment

Thin layer chromatography analysis was carried out using silica gel on an aluminum layer (Merck Kieselgel 60 F<sub>254</sub>), monitoring TLC under UV lamps 254 and 365 nm. The vacuum liquid chromatography was performed using silica gel 60 G (Merck) as the stationary phase and silica gel 60 (Merck) in chromatography gravity column. The chemical structure of the isolates was determined using spectroscopic techniques including mass spectroscopy (Waters UPLC-MS/MS H-Class TQD), and <sup>1</sup>H- and <sup>13</sup>C-NMR spectroscopy which were obtained with JEOL ECA 500 with frequencies at 500 MHz and 125 MHz, respectively.

### Extraction and Isolation

The stem powder of *B. macrophylla* (7.6 kg) was macerated in stages with *n*-hexane, ethyl acetate, and methanol (Technical, Pha Che, Indonesia) for 3 x 24 hours each using similar procedure available in the literatures.<sup>15, 16</sup> Each extract was tested anticancer activity against MCF-7, HCC 1954, MDA-MB 231, and A549 cell lines. The *n*-hexane extract (21 g) was separated by VLC using the mobile phase *n*-hexane: ethyl acetate: methanol: acetone: methanol in a 10% polarity gradient in such a way that the A-B fraction was obtained. Fraction A was purified by CC using *n*-hexane, ethyl acetate as the stationary phase to produce compound **1** (28 mg).

The ethyl acetate extract (19.90 g) was separated by VLC using methylene chloride: ethyl

acetate: ethanol as the mobile phase to produce the A-K fraction. The G fraction (594.70 mg) was purified by CC using *n*-hexane: MTC: ethyl acetate as the mobile phase to produce compound **2** (5.1 mg). The methanol extract (15 g) was separated by VLC using *n*-hexane: ethyl acetate: methanol in 10% gradient as the mobile phase to obtain the A-J fraction. The G fraction (778.2 mg) was purified by CC using *n*-hexane: ethyl acetate as the mobile phase to produce compound **3** (4.7 mg). Fraction J (7 g) was purified by CC using *n*-hexane: ethyl acetate: methanol in a polarity gradient to give compound **4** (20 mg).

#### Anticancer Activity

The anticancer activity of MCF-7 breast adenocarcinoma (ATCC HTB-22) and A549 Lung Carcinoma (ATCC CCL-185) cell lines were analysed using the MTS assay method which were carried out at The Biological Activity Laboratory, the Central Laboratory, Universitas Padjadjaran, Bandung, Indonesia: the cells were cultured on RPMI media (Sigma-Aldrich) containing 10% FBS and antibiotics, and trypsin-EDTA was added and incubated for 5 minutes. When the growth of cells reached confluent level where the numbers of cell lines were minimum 70%, they were then transferred to 96-microtube well plates, and each of the microtube was added with samples of various concentrations and incubated for 48 hours. The mixture was then added with presto blue gluing as cell staining and incubated for 1-2 hours until a discoloration was observed. PrestoBlue® reagent is reduced by the blue compound resazurin to resorufin, which is pink and very fluorescent. The absorbance measurements were carried out at 570 nm (resorufin) and 600 nm (resazurin) wavelengths using a multimode reader, and cisplatin was used as a positive control and DMSO as a negative control.<sup>17</sup>

The anticancer activity against HCC 1995 and MDA-MB 231 cell lines was analyzed using the MTT assay method which were carried out at The Culture Cell and Cytogenetics Laboratory, Medical Faculty, Universitas Padjadjaran, Bandung, Indonesia. The HCC-1954 and MDA-MB-231 cells were cultured in RPMI 1640 media (Sigma-Aldrich) containing 10% fetal calf serum, antibiotics, and streptomycin. The cells and media were incubated for 24 hours, and then the cells were then added with samples with various

concentrations and phosphate buffer saline. Furthermore, the mixture was then incubated again for 24 hours, and 3-(4,5-Dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) compound was added to each well containing 15,000 cells and incubated for 2 hours. The MTT reaction was stopped using *n*-hexane, and the absorbance of the reaction was measured using an ELISA reader at a wavelength of 550 nm.<sup>18</sup>

## RESULTS AND DISCUSSION

### The Isolated Compounds

Four compounds were successfully isolated. The separation is guided by spot pattern. The compounds were as follows: Stigmasterol (**1**) (Figure 1): colourless crystal, <sup>1</sup>H-NMR (in CDCl<sub>3</sub>, 500 MHz)  $\delta_{\text{H}}$  (ppm) 1.83 (2H, *m*, H-1); 1.51 (2H, *t*, H-2); 3.53 (1H, *m*, H-3); 2.26 (2H, *d*, *J*=6.5 Hz, H-4); 5.33 (1H, *d*, *J*=4.9, H-6); 1.97 (2H, *m*, H-7); 1.47 (1H, *m*, H-8); 0.95 (1H, *m*, H-9); 1.50 (2H, *m*, H-11); 1.97 (2H, *m*, H-12); 1.05 (1H, *m*, H-14); 1.52 (2H, *m*, H-15); 1.23 (2H, *m*, H-16); 1.15 (1H, *m*, H-17); 0.66 (3H, *s*, H-18); 0.99 (3H, *s*, H-19); 1.97 (1H, *m*, H-20); 1.00 (3H, *d*, *J*=9.7 Hz, H-21); 5.13 (1H, *m*, H-22); 5.12 (1H, *m*, H-23); 1.47 (1H, *m*, H-24); 1.81 (1H, *m*, H-25); 0.81 (3H, *d*, *J*= Hz, H-26); 0.82 (3H, *m*, H-27); 1.15 (2H, *m*, H-28) and 0.78 (1H, *s*, H-29). <sup>13</sup>C-NMR (in CDCl<sub>3</sub>, 125 MHz):  $\delta_{\text{C}}$  (ppm): 36.3 (C-1); 32.5 (C-2); 71.9 (C-3); 42.3 (C-4); 140.9 (C-5); 120.7 (C-6); 32.0 (C-7); 32.0 (C-8); 50.3 (C-9); 36.5 (C-10); 21.4 (C-11); 39.2 (C-12); 42.4 (C-13); 56.9 (C-14); 24.5 (C-15); 29.2 (C-16); 56.2 (C-17); 12.2 (C-18); 19.5 (C-19); 39.9 (C-20); 23.2 (C-21); 138.5 (C-22); 129.4 (C-23); 51.4 (C-24); 31.8 (C-25); 20.0 (C-26); 21.2 (C-27); 25.6 (C-28) and 12.2 (C-29). UPLC-QTOFMS *m/z* 411.24 [M-] (calculated (calcd.) for C<sub>29</sub>H<sub>48</sub>O, *m/z* 412.69).

Fustin (**2**) (Figure 1): yellow amorphous, <sup>1</sup>H-NMR (in acetone-d<sub>6</sub>, 500 MHz)  $\delta_{\text{H}}$  (ppm): 6.40 (1H, *d*, *J*= 2.5 Hz, H-8); 6.62 (1H, *dd*, *J*= 8.5 and 2 Hz, H-6); 6.86 (1H, *dd*, *J*= 8 and 2.5 Hz, H-5'); 6.92 (1H, *td*, *J*= 8 and 2 Hz, H-6'); 7.07 (1H, *d*, *J*= 2.5 Hz, H-2'); 7.72 (1H, *d*, *J*= 8.5 Hz, H-5); 4.98 (1H, *d*, *J*= 12 Hz, H-2) and 4.53 (1H, *d*, *J*= 11.5 Hz, H-3). <sup>13</sup>C-NMR (in acetone-d<sub>6</sub>, 125 MHz):  $\delta_{\text{C}}$  (ppm): 74.0 (C-3); 85.0 (C-2); 103.1 (C-8); 111.8 (C-6); 115.8 (C-2'); 113.1 (C-10); 115.9 (C-5'); 120.9 (C-6'); 129.8 (C-5); 130.1 (C-1'); 145.8

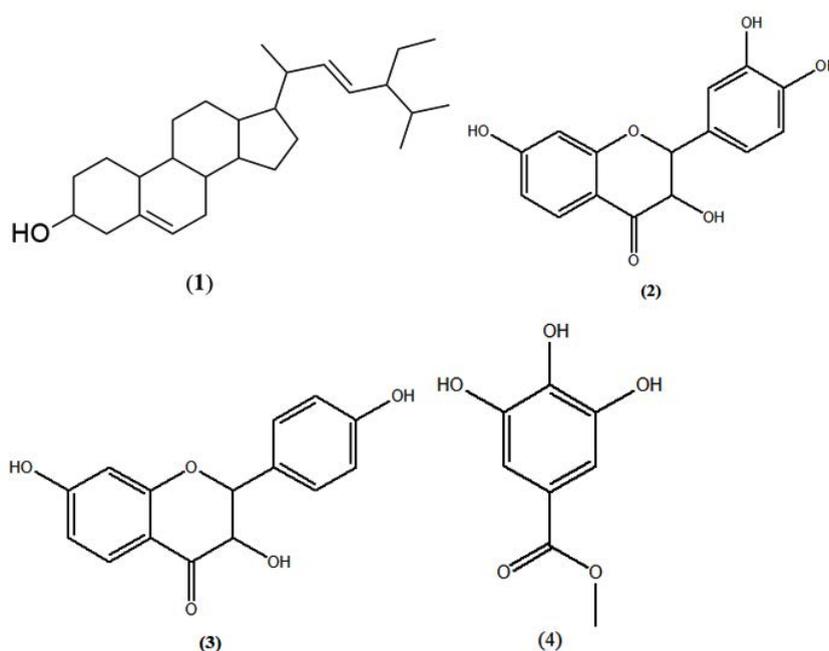
(C-4'); 146.6 (C-3'); 164.6 (C-9); 165.8 (C-7); and 193.2 (C-4). UPLC-QTOFMS  $m/z$  287.28 [M<sup>+</sup>] (calcd. for C<sub>15</sub>H<sub>12</sub>O<sub>6</sub>,  $m/z$  288.25).

Garbanzole (**3**) (Figure 1): yellow needle crystal. <sup>1</sup>H-NMR (in acetone-d<sub>6</sub>, 500 MHz)  $\delta_{\text{H}}$  (ppm): 7.70 (1H, *d*,  $J = x$ , H-5); 6.60 (1H, *dd*, H-6); 6.37 (1H, *d*, H-8); 7.41 (2H, *d*, H-2' and 6'); 6.87 (2H, *d*, H-3' and H-5') 5.02 (1H, *d*, H-2); and 4.45 (1H, *dd*, H-3). <sup>13</sup>C-NMR (in acetone-d<sub>6</sub>, 125 MHz):  $\delta_{\text{C}}$  (ppm): 84.9 (C-2); 73.9 (C-3); 193.3 (C-4); 129.8 (C-5); 111.8 (C-6); 165.9 (C-7); 103.7 (C-8); 164.6 (C-9); 113.1 (C-10); 129.4 (C-1'); 130.4 (C-2' and C-6'); 115.9 (C-3' and C-5'); and 158.

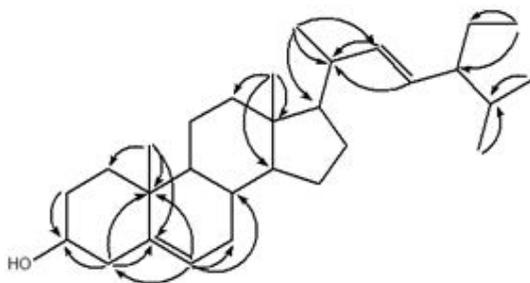
9 (C-4'). UPLC-QTOFMS  $m/z$  273.0559 [M+2] (calculated for C<sub>15</sub>H<sub>12</sub>O<sub>5</sub>,  $m/z$  272.0559).

Methyl gallate (**4**) (Figure 1): yellow needle crystal. <sup>1</sup>H-NMR (in acetone-d<sub>6</sub>, 500 MHz)  $\delta_{\text{H}}$  (ppm): 3.75 ppm (3H, *s*, -OCH<sub>3</sub>); 7.07 (2H, *s*, H-2 and H-6); and 8.19 (1H, *s*, -OH). <sup>13</sup>C-NMR (in acetone-d<sub>6</sub>, 125 MHz):  $\delta_{\text{C}}$  (ppm): 166.3 (C-7); 145.2 (C-3 and C-5); 137.9 (C-4); 120.9 (C-1); 108.9 (C-2 and C-6); and 51.1 (-OCH<sub>3</sub>). UPLC-QTOFMS  $m/z$  183.09 [M<sup>-</sup>] (calcd. for C<sub>15</sub>H<sub>11</sub>O<sub>5</sub>,  $m/z$  184.15).

Compound **1** is a colorless crystal. The UPLC-QTOFMS spectrum for compound **1** has the molecular formula C<sub>29</sub>H<sub>48</sub>O,  $m/z$  412.69. The



**Fig. 1.** Structure of the isolated compound; Stigmasterol (1), Fustin (2), Garbanzole (3), and Methyl gallate (4)



<sup>1</sup>H-NMR spectrum of isolate **1** is similar to the reference compound reported by others<sup>19</sup> and it showed a typical signal for the group of steroid compound in which the signal accumulated in the area below 2 ppm  $\delta_{\text{H}}$  was typical for steroids. Four signals in the  $\delta_{\text{H}}$  1.00 region (3H, *d*,  $J = 9.74$  Hz); 0.81 (3H, *s*); 0.82 (3H, *m*); and 0.79 (1H, *s*) ppm indicated the presence of a methyl signal bound to C-21, C-26, C-27 and C-29, respectively. Furthermore, it is believed that 9 signals indicated the presence of methylene protons in the  $\delta_{\text{H}}$  region of 1.82 (2H, *m*); 1.51 (2H, *m*); 2.26 (2H, *d*,  $J = 6.5$  Hz); 1.97 (2H, *m*); 1.50 (2H, *m*); 1.97 (2H, *m*); 1.52 (2H, *m*); 1.23 (2H, *m*); 1.15 (2H, *m*) ppm, which is binds to C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16 and C-28 respectively. The proton signal from methine is believed to appear at  $\delta_{\text{H}}$  1.47 (1H, *m*); 0.95 (1H, *m*); 1.05 (1H, *m*); 1.15 (1H, *m*); 1, 97 (1H, *m*); 1.47 (1H, *m*); and 1.81 (1H, *m*) ppm bound to C-8, C-9, C-14, C-17, C-20, C-24, and C-25, respectively. There is a typical signal for olefinic protons at  $\delta_{\text{H}}$  5 ppm and there is a signal for oxygenated protons at  $\delta_{\text{H}}$  3 ppm, which is commonly reported in the class of the steroid compound.<sup>19,20</sup> The signal was detected as an oxygenated proton at  $\delta_{\text{H}}$  3.51 (1H, *m*) ppm. The

signal in the region  $\delta_{\text{H}}$  5.13 (1H, *d*); 5.12 (1H, *m*) and 5.15 (1H, *m*) ppm are from the olefinic methine double bond protons attached to C-6, C-20 and C-21, respectively.

Rings A, B, and C consist of six carbon or cyclohexane atoms, and ring D consists of five or cycloheptane. Furthermore, most of the steroids have properties, which includes the oxygen functional group (as = O or OH) at C-3, and contains side groups at C-17, many of which contain double bonds at C-4 - C-5 or C-5 - C-6. The carbon signal that appears in the area above  $\delta_{\text{C}}$  is 100 ppm (140.9; 120.7; 138.5; and 129.4 ppm), which are at C-5, C-6, C-22, and C, respectively. C-23 was confirmed by the presence of two double bonds in the analyzed compound. The double bond signal is reported in the  $\delta_{\text{C}}$  140.9 region; 120.7; 138.5; and 129.4 ppm. The methyl signal is believed to be in the  $\delta_{\text{C}}$  12.2 (C-18) region; 19.5 (C-19); 23.2 (C-21); 20.0 (C-26); 21.2 (C-27) and 12.2 (C-29) ppm. The signal from the methylene group is believed to be present in the region:  $\delta_{\text{C}}$  36.3 (C-1); 32.5 (C-2); 42.3 (C-4); 32.0 (C-7); 21.4 (C-11); 39.2 (C-12); 24.5 (C-15); 29.2 (C-16) and 25.6 (C-28). In addition, the signal in the region 32.0 (C-8); 50.3 (C-9); 56.9 (C-14); 56.2 (C-17); 39.9 (C-20); 51.4 (C-24), and 31.8 (C-25) ppm were believed to indicate the presence of a methine group.

The quaternary carbon group containing 3 signals was predicted in the  $\delta_{\text{C}}$  140.9 (C-5) region; 36.5 (C-10) and 42.4 (C-13) ppm. Figure 1 shows that the methyl proton at position C-29 ( $\delta_{\text{H}}$  - 0.78) correlates with C-26 ( $\delta_{\text{C}}$  - 20.0) and C-28 ( $\delta_{\text{C}}$  - 25.6). The methyl proton at position C-18 ( $\delta_{\text{H}}$  - 0.66) correlates with C-12, C-13, C-14 and C-17 with each value  $\delta_{\text{C}}$ , namely 39.2; 42.4; 56.9; and 56.2 ppm. The methyl proton at position C-19 ( $\delta_{\text{H}}$  - 0.99) correlates with C-1, C-5, C-9, and C-10 with values of  $\delta_{\text{C}}$  - 36.3,  $\delta_{\text{C}}$  - 141  $\delta_{\text{C}}$  - 50.1 and  $\delta_{\text{C}}$

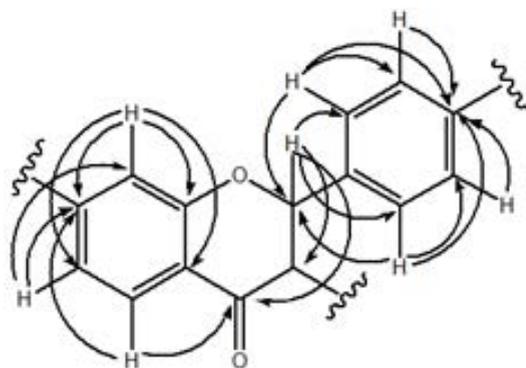


Fig. 4. HMBC correlation of garbanzol

Table 1. Toxicity of extract and compound 1-4

No	Sample	IC <sub>50</sub> (µg/mL)			
		MCF-7	A549	HCC-1954	MDA-MB-231
1	Stigmasterol (1)	1209.25 ± 47.92	5757.60 ± 4173.23	853.53 ± 103.87	553.60 ± 121.35
2	Fustin (2)	377.94 ± 9.17	359.31 ± 3.58	134.35 ± 44.62	5704.28 ± 289.08
3	Garbanzole (3)	248.16 ± 83.12	568.77 ± 98.13	427.05 ± 122.71	233.41 ± 91.57
4	Methyl gallate (4)	351.75 ± 3.80	388.84 ± 9.75	153.69 ± 12.54	311360.41 ± 262865.2

-37.9, respectively. The methyl protons at position C-26 ( $\delta_{\text{H}} - 0.81$ ) and position C-27 ( $\delta_{\text{H}} - 0.82$ ) correlate with C-25 ( $\delta_{\text{C}} - 31.8$ ). In the methyl proton  $\delta_{\text{H}} - 0.79$ , the C-29 position correlates with C-28 and C-24 with  $\delta_{\text{C}}$  values of 25.6 and 52.4 ppm, respectively. The position of the hydroxyl group is strengthened and proven by the correlation at C-3 with methylene protons at positions C-2 ( $\delta_{\text{H}} - 1.51$ ) and C-4 ( $\delta_{\text{H}} - 2.26$ ) with carbon C-3 with values ( $\delta_{\text{C}} - 71, 9$ ). The position of the proton group ( $\delta_{\text{H}} - 2.26$ ) at C-4 also strengthens the position of the hydroxyl group, which correlates with C-3, C-5, C-2 and C-10 with the respective values of  $\delta_{\text{C}}$  71.9, 140, 9, 32.5 and 36.5 ppm. The important HMBC correlation of 1 is shown in Figure 2.

Compound 2 is a yellow amorphous solid. The 6-proton aromatic signal appears at  $\delta_{\text{H}}$  6.40 (1H, *d*,  $J = 2.5$  Hz) and 6.62 (1H, *dd*,  $J = 8.5$  Hz; 2 Hz),  $\delta_{\text{H}}$  6.86 (1H, *dd*,  $J = 8$  Hz; 2.5 Hz), 6.92 (1H, *td*,  $J = 8$  Hz; 2 Hz), and 7.07 (1H, *d*,  $J = 2.5$  Hz), 7.72 (1H, *d*,  $J = 8.5$  Hz). This characterization is in agreement to reported values [21]. According to Mabry *et al.*,<sup>22</sup> the proton signal in the range of 6 to 8 ppm with a pattern of *ortho* and *meta* coupling values indicated that the isolates obtained are in the flavonoid group. The proton signal in the range 6.0 to 6.7 ppm appears up to 2 signals at  $\delta_{\text{H}}$  6.40 (1H, *d*,  $J = 2.5$  Hz) and 6.62 (1H, *dd*,  $J = 8.5$  Hz; 2 Hz), which indicates the characteristics of the

H-8 and H-6 protons in ring A. Furthermore, one proton signal appears at a shift of 7.72 ppm (1H, *d*,  $J = 8.5$  Hz). The spectral patterns showing the proton matchmaking characteristics of the AB system were observed at  $\delta_{\text{H}}$  6.62 (1H, *dd*,  $J = 8.5$ ; 2 Hz) and 7.72 (1H, *d*,  $J = 8.5$  Hz) indicated the specificity for protons at H-6 and H-5. Protons H-6 and H-5 have *ortho* correlation with each other. In the range of 6.7 to 7.7 ppm there were 3 signals, namely at  $\delta_{\text{H}}$  6.86 (1H, *dd*,  $J = 8$  Hz; 2.5 Hz), 6.92 (1H, *td*,  $J = 8$  Hz; 2 Hz), and 7.07 (1H, *d*,  $J = 2.5$  Hz).

The proton characteristics of the ABX system are shown in this shift. The three signals indicate the proton signal for ring B with each signal representing H-5', H-6', and H-2' and the three signals are *ortho* and *meta* correlated with each other. The H-3 proton on the flavone produced a single sharp signal at a shift of about  $\delta_{\text{H}}$  4.53 ppm. H-2 signal appear at a shift of approximately  $\delta_{\text{H}}$  4.9 ppm. This signal appears in pairs with the H-3 proton signal that appears at a shift of about  $\delta_{\text{H}}$  4.2 ppm.<sup>22</sup> The spectrum of isolate 2 shows the flavonoid pattern of the dihydroflavonoid type where the signal appears at  $\delta_{\text{H}}$  4.98 (1H, *d*,  $J = 12$  Hz) as H-2 and  $\delta_{\text{H}}$  4.53 (1H, *d*,  $J = 11.5$  Hz) as H-3. The results of the analysis on the <sup>13</sup>C-NMR spectrum showed that isolate 2 had 15 carbon signals. The results of the DEPT 135° NMR analysis

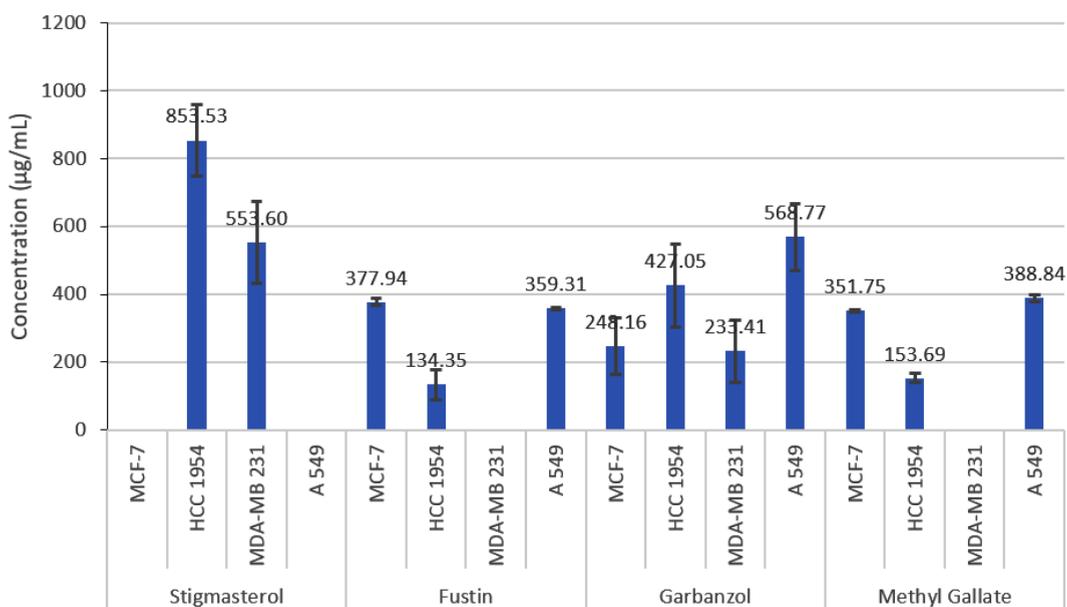


Fig. 5. The comparison of anticancer activity test for all compounds isolated

showed 8 signals of methine carbon ( $\delta_c$  74.0; 85.0; 103.1; 111.8; 115.8; 115.9; 120.9; 129.8 ppm) and there were 7 carbon signals quaternary ( $\delta_c$  113.1; 130; 1; 145.8; 146.6; 164.6; 165.8; 193.2 ppm). The  $^{13}\text{C}$ -NMR spectrum shows a signal at a shift below 100 ppm, namely at  $\delta_c$  85.0 and 74.0 ppm as a characteristic of saturated carbon  $sp^3$  which binds to electronegative atoms such as oxygen.<sup>23</sup> The carbon signal at 193.2 ppm shift is characterized by carbonyl carbon, which has a shift range between 185-220 ppm.<sup>23</sup> The 2D NMR COSY spectrum of  $^1\text{H}$ - $^1\text{H}$  correlation shows that there is a correlation between H-3 ( $\delta_H$  4.53 ppm) with H-2 ( $\delta_H$  5.00 ppm) and H-5 ( $\delta_H$  7.72 ppm) with H-6 ( $\delta_H$  6.62 ppm). This confirms that the basic structure of the isolate is a flavonoid with 2 protons in ring A and ring B, each of which is correlated. The results of the 2D NMR HMQC spectrum analysis showed that there were 8 correlations between the proton and the carbon signal. The correlation shows a direct bond between protons and carbon, namely the proton signal  $\delta_H$  4.53 (H-3); 5.00 (H-2); 6.40 (H-8); 6.62 (H-6); 6.86 (H-5'); 6.92 (H-6'); 7.07 (H-2'); 7.72 (H-5) ppm, respectively correlated with carbon at  $\delta_c$  74.0 (C-3); 85.0 (C-2); 103.6 (C-8); 111.8 (C-6); 115.8 (C-5'); 120.9 (C-6'); 115.9 (C-2'); and 129.8 (C-5) ppm. The important HMBC correlation of 2 was shown in Figure 3.

The  $^1\text{H}$ -NMR spectrum of isolate **3** showed that there were 5 proton signals in the aromatic region of 6-8 ppm. The isolates are believed to be a flavonoid class compound as the data obtained are close to the reported values.<sup>24, 25</sup> The proton signal  $\delta_H$  6.60 ppm was assumed to be H-6, *ortho* and *meta* coupling correlating with the proton signal  $\delta_H$  7.70 (H-5) and 6.37 ppm (H-8), respectively. The proton characterization of ABX is shown in the typical shear of ring A of the flavonoid framework for H-6, H-5 and H-8. Meanwhile, the proton signal  $\delta_H$  7.41 is assumed to be H-2'/ H-6', which has an *ortho* coupling correlating with the proton signal  $\delta_H$  6.87 that has an *ortho* coupling value (H-3'/H-6'). The  $^{13}\text{C}$ -NMR and DEPT 135 analysis showed that there were 7 methine carbon signals ( $\delta_c$  74.0; 84.9; 103.7; 111.8; 115.9; 129.8 and  $\delta_c$  130.4 ppm) and 6 quaternary carbon signals ( $\delta_c$  113.1; 129.4; 158.9; 164.6; 165.9 and  $\delta_c$  193.3 ppm). In the spectrum, a signal appears at 193.3 ppm, which indicates the type of carbonyl carbon (C = O). The  $^{13}\text{C}$ -NMR spectrum shows that

there are 10 carbon signals, which ranges from 100-167 ppm that are believed to be aromatic carbon. Furthermore, HMQC 2D spectrum shows a direct correlation between protons and carbon. The correlation signal appears up to 7 signals at  $\delta_H$  4.55 (H-3); 5.03 (H-2); 6.37 (H-8); 6.60 (H-6); 6.87 (3'/5'); 7.41 (2'/6'); and 7.70 (H-5) ppm with carbon at  $\delta_c$  signal 73.9 (C-3), respectively; 84.9 (C-2); 103.7 (C-8); 111.8 (C-6); 115.9 (C-3'/5'); 130.4 (C-2'/6'); and 129.8 (C-5) ppm. 2D HMBC analysis was performed, and the structure of the isolate was believed to be a flavonoid. This is reinforced by the signal that appears at  $\delta_c$  5.04 ppm (C-2), which correlates with  $\delta_c$  73.9 (C-3); 193.3 (C-4); 129.4 (C-1'); 130.4 (C-2'/6'). This signal shows the correlation between the C and B rings of flavonoids. The important HMBC correlation of **3** was shown in Figure 4.

Based on the  $^1\text{H}$ -NMR spectrum of compound **4**, there is  $\text{CH}_3$  (-OCH<sub>3</sub>), which is oxygenated at  $\delta_H$  3.75 ppm (3H, *s*). A typical aromatic signal appears at  $\delta_H$  7.07 (2H, *s*) with a symmetrical plane, hydroxy proton (-OH) appears at  $\delta_H$  8.19 (1H, *s*). Seven carbon signals that are a C = O signal at  $\delta_c$  167.9 ppm (indicating the presence of carbon ester), one signal indicates the presence of aromatic carbon (C-OH) at  $\delta_c$  145.2 ppm, at  $\delta_c$  137.9 ppm indicates aromatic carbon (C-OH),  $\delta_c$  120.9 contains aromatic carbon (C-C),  $\delta_c$  108.9 ppm contains aromatic carbon (C-H), at  $\delta_c$  51.1 indicates the presence of -O-CH<sub>3</sub>. These values are in agreement with reported values available in the literature.<sup>26</sup>

#### Anticancer Activity of Extracts and Compounds 1-4

The anticancer activity against MCF-7 and A549 was measured using the MTS assay method, while the anticancer activity of HCC-1954 and MDA-MB 231 cells was measured using the MTT assay method and the results of the anticancer activity test are shown on the Table 1 and the comparison of their IC<sub>50</sub> values are shown in Figure 5.

All compounds **1** – **4** were assayed for their anticancer property against MCF-7, A549, HCC-1954 and MDA-MB231 cell lines. All isolated compounds exhibited moderate anticancer activity against almost cell lines tested. Compounds **2** and **4** were more active on HCC-1954 cell with IC<sub>50</sub> values of  $134.35 \pm 44.62$  and  $153.69 \pm 12.54$   $\mu\text{g}/$

mL, respectively than other isolated compounds. It presumably that the anticancer activity increase with the absence or with the decreasing number of hydroxyl groups.<sup>27</sup> Additionally, among all isolated compounds, compound **3** gave the most active in the anticancer activity test against MDA-MB-231 cell line with IC<sub>50</sub> value of 233.41 ± 91.57 µg/mL. While compounds **2** and **3** demonstrated respectable anticancer activity against MCF-7, A549, and HCC-1954 cell lines with IC<sub>50</sub> values ranging from 134.35 ± 44.62 to 568.77 ± 98.13 µg/mL. The compounds **2** and **3** are flavanones containing a chiral carbon on chroman-4-one ring unit which is flexible. According Woo *et al.*,<sup>28</sup> the wide range of the flavanone bioactivity may be due to its chiral structure. In addition, the carbonyl group at C-4 on the flavan skeleton is very important for anticancer activity.<sup>27</sup>

However, the structure-activity relationship study is required to provide better understanding of their anticancer activity. The results of the anticancer test for the compounds isolated in this work are lower compared to other compounds reported by others both in the synthetic compounds such as organotin (IV) carboxylates<sup>29</sup>,<sup>30</sup> or other isolated compounds from other plants<sup>31</sup>,<sup>32</sup> although the cell lines used were different. However, the results reported in this work are believed still very important results in attempts to find new candidate for anticancer drugs.

## CONCLUSIONS

Four compounds namely stigmasterol (1), fustin (2), garbanzol (3), and methyl gallate (4) were successfully isolated from the stem of *B. macrophylla*. These compounds were well characterized and the characterization data obtained were similar to the known compounds previous published. These compounds were tested for their anticancer activities against 4 cell lines. The result showed based on the IC<sub>50</sub> values of compounds 2 and 4 were more active on HCC-1954 cell with IC<sub>50</sub> values of 134.35 ± 44.62 and 153.69 ± 12.54 µg/mL, respectively. The compound 3 was the most active against MDA-MB-231 cell line with IC<sub>50</sub> value of 233.41 ± 91.57 µg/mL.

### Data availability

Data can be made available upon request from the corresponding author.

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