

## BIOACTIVE COMPOUNDS IN BENGKOANG (*Pachyrhizus erosus*) AS ANTIOXIDANT AND TYROSINASE INHIBITING AGENTS

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### ABSTRACT

In Indonesia, the roots of bengkoang (*Phacyrhizus erosus*) have been used as the excipient for sun screening and skin whitening paste. Since the active compounds exhibiting skin whitening or sun screening effect have not previously been studied, the aim of this study was to identify compounds with antioxidant and tyrosinase inhibitor activities. Soxhlet extraction was used as the method of isolation with petroleum ether as the solvent and it was followed by fractionation using ethyl acetate to obtain three isoflavonoids (i.e. daidzein (**2**); daidzein-7-O- $\beta$ -glucopyranose (**3**); 5-hydroxy-daidzein-7-O- $\beta$ -glucopyranose (**4**)), and a new pterocarpan (i. e. 8,9-furanyl-pterocarpan-3-ol (**1**)) which antioxidant activities (SC<sub>50%</sub> values) of 2.11; 11.86; 0.69 and 7.86 respectively. All compounds showed tyrosinase inhibiting activities with IC<sub>50</sub> values of 4.38; 5.35; 7.49 and 22.20 mM, respectively for compound **4**, **2**, **1** and **3**. These compounds can be used as antioxidant and skin whitening materials.

**Key words:** *Pachyrhizus erosus*, antioxidant, tyrosinase inhibitor, flavonoids

### INTRODUCTION

Bengkoang is a species of *Pachyrizus* and grows naturally in many tropical and subtropical countries such as America and Asia. It is usually consumed directly or sometimes with salt, lemon juice and powdered chilli. In Indonesia, bengkoang roots have been traditionally used as a cosmetics material for centuries empirically. They have been used as skin whitening materials. Yet, the active compounds in bengkoang roots having skin whitening activity have not previously been investigated (Lukitaningsih, 2009).

Skin whitening compounds are in a close relationship with melanin, the major pigment for colour of skin, hair and eye (Briganti *et al.*, 2003). The production of melanin depends on UV light or sun exposure. It is a natural protective mechanism of the skin against too much UV light penetrating the human skin. Too much UV irradiation causes sunburn, disrupts the synthesis of precursors necessary for DNA synthesis and increases the amount of free radicals. Melanin captures free radicals and participates in other oxidation-reduction processes in the human body (Bleehen *et al.*, 1995).

Melanin is classified into two main groups: the black and brown eumelanins which are insoluble in water and the yellow and reddish-brown pheomelanin which is alkali soluble. Both melanins were derived from tyrosine by the same initial step, namely oxidation process at the phenolic system (Bleehen *et al.*, 1995; Parvez *et al.*, 2007; Kobayashi *et al.*, 1994); is starting from the conversion of the *L*-tyrosine to *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and followed by the subsequent oxidation of *L*-DOPA to produce an ortho-quinone (dopaquinone) by tyrosinase. Dopaquinone is further transformed via several reactions to yield brown to black melanin which is responsible for the colour of mammal's skin (Okombi *et al.*, 2006; Lee 2002; Ohguchi *et al.*, 2003; Wang and Hebert, 2006). Another two melanogenic enzymes, tyrosine-related protein1 (TRP1) and tyrosine-related protein2 (TRP2), also named dopachrome tautomerase (DCT) (Solano *et al.*, 1994), are involved in the melanin biosynthesis (Kobayashi *et al.*, 1994; Parvez *et al.*, 2007).

Another strategy for maintaining skin whiteness is to avoid ultraviolet exposure. UV

radiation can also induce the formation of various radicals (Matsuura *et al.*, 2006), primarily reactive oxygen species (ROS) in the skin such as singlet oxygen and superoxide anion, promoting biological damage in exposed tissues via iron-catalyzed oxidative reactions. These radicals play important roles in the activation of tyrosinase in human skin and therefore enhance melanin biosynthesis via induction of the proliferation of the melanocytes. The radicals also cause the damage of DNA. Furthermore, ROS scavengers or inhibitors, such as antioxidant, may reduce hyperpigmentation and can also be used as whitening materials (Wang *et al.*, 2006). Therefore, it is necessary to combine sun screen compounds and antioxidant compounds in cosmetic products to obtain an optimal whitening effect.

## MATERIALS AND METHODS

### Chemicals and solvent

The chemicals used in the detection and isolation methods were mushroom tyrosinase 4187IU/mg, *L*-DOPA (dihydroxy phenyl alanine), kojic acid (Fluka, Seelze, Germany), dimethylsulfoxide extra pure (Acros® organic, Geel, Belgium), DPPH (1,1-diphenyl-1-picrylhydrazine), catechin, Dulbeco's phosphate buffered saline, (purchased from Sigma Aldrich, Steinheim Germany), ascorbic acid (Sigma Aldrich, Steinheim, Germany), Sephadex LH20 (Aldrich, Steinheim, Germany), Silica gel 60 (particle sizes 0.063-0.200mm, Merck, Darmstadt, Germany), TLC Aluminium sheets, silica gel 60 F254 (layer thickness 0.2mm, Merck, Darmstadt, Germany).

Solvents for separation were petroleum ether, ethyl acetate (Fisher Scientific, Leicestershire, UK), methanol (Merck, Darmstadt, Germany), chloroform, dichloromethane and *n*-butanol (Fluka, Seelze, Germany).

### Equipments

Melting point SMP3 Stuart® apparatus (Staffordshire, UK), Cary 50 Bio UV-Visible spectrophotometer (Varian, California, USA), JASCO FT/IR-6100 Spectrometer (Gross-Umstadt, Germany), Thermo Mixer Comfort 5355 V.2.12 Eppendorf (Hamburg, Germany), ALPHA II-12 Freeze dryer (Osterode, Germany), Bruker Avance 400 NMR spectro-

meter (Rheinstetten, Germany), Shimadzu GC/MS-QP 20105 gas chromatography (Kyoto, Japan), Agilent 1100 series HPLC apparatus (California, USA) equipped by column Zorbax SB-C18 (25cm, i.d. 0.46 cm, 5µm, Agilent, California, USA), UV absorbance detector and ESI-MS detectors, Agilent 1100 series preparative-HPLC (California, USA) equipped by column Zorbax SB-C18 (7µm, 21.2X150 mm, Agilent, California, USA).

### Plant material and extraction

Bengkoang was collected from Purwo-rejo, Central Java, Indonesia on the dry season from July until September on 2007. The roots (45kg) were peeled and washed with water, subsequently dried at 60°C and milled into fine powder. The fine powder (4.75kg) was extracted by Soxhlet using 6L petroleum ether. The residue was extracted using methanol to achieve the semi polar and polar compounds. The extracts were filtered and concentrated in vacuum evaporator. The concentrated methanol extract was added with water and then partitioned with ethyl acetate. The ethyl acetate phase was further concentrated.

### Compound isolation of ethyl acetate extract

The ethyl acetate extract (31.1gram) was subjected to silica gel column chromatography and eluted using the gradient mixture of petroleum ether-ethyl acetate and ethyl acetate-methanol producing 35 fractions with 100mL of eluents.

Fractions 6-11 which had an  $R_f$  value of 0.17 (positive with DPPH) were collected and evaporated. The fractions may contain antioxidant compounds because the spot was able to reduce DPPH. The concentrated fraction was then purified using column sephadex LH-20 chromatography and methanol as an eluent, producing 50 fractions. Fractions 9-15 were further subjected into preparative HPLC and yield 670mg yellow crystal (**1**) that is identified as **8,9-Furanylpterocarpan-3-ol**. Fractions 37-40 from column chromatography with an  $R_f$  value of 0.35 on TLC were collected and purified using sephadex column chromatography giving 19 fractions. Fractions 7-11 of this chromatography had the  $R_f$  value of 0.85

(positive with DPPH) then purified using preparative-HPLC giving 33 mg of **compound 2**. Based on the GC/MS and NMR spectroscopic data, compound **2** was identified as **Daidzein**.

Fractions 17-24 of the first column chromatography with an R<sub>f</sub> value of 0.52 on TLC were collected and evaporated. The concentrated fraction was purified by preparative HPLC giving two compounds. The compound with a retention time of 11.4min (**compound 3**) was assigned to **Daidzein-7-O-β-glucopyranose**, while the other compound having a retention time of 12.6min was assigned to **5-hydroxy-daidzein-7-O-β-glucopyranose (compound 4)**.

#### Identification of isolated compounds

##### New compound **1** (*8,9-Furanyl-pterocarpan-3-ol*)

Yellow crystal; UV MeOH λ<sub>max</sub> 293 nm; MW 280; IR (cm<sup>-1</sup>): 3295, 1624, 1607, 1540, 1494, 1469, 1353, 1306, 1270, 1229, 1144, 1123, 1055, 1018, 960, 843, 826, 764; ESI-MS m/z (% intensity): 281.3 ([M+H]<sup>+</sup>, 100), 123.1 ([C<sub>7</sub>H<sub>7</sub>O<sub>2</sub>]<sup>+</sup>, 100), 121.0 ([C<sub>7</sub>H<sub>7</sub>O<sub>2</sub>]<sup>-</sup>, 100); <sup>1</sup>H NMR (500 MHz, MeOH-d<sub>4</sub>): δ 3.44 (dd, 1H, H<sup>6a</sup>), 3.48 (d, 1H, H<sup>6</sup>), 4.14 (d, 1H, H<sup>6</sup>), 5.49 (d, 1H, H<sup>11a</sup>), 6.17 (d, 1H, H<sup>4</sup>), 6.22 (dd, 1H, H<sup>2</sup>), 6.66 (d, 1H, H<sup>3</sup>), 6.89 (s, 1H, H<sup>10</sup>), 6.95 (d, 1H, H<sup>1</sup>), 7.53 (d, 1H, H<sup>2</sup>), 7.57 (s, 1H, H<sup>7</sup>); <sup>13</sup>C NMR (125 MHz, MeOH-d<sub>4</sub>): δ 124.66 (C<sup>1</sup>), 107.40 (C<sup>2</sup>), 160.53 (C<sup>3</sup>), 97.35 (C<sup>4</sup>), 158.45 (C<sup>4a</sup>), 66.84 (C<sup>6</sup>), 39.73 (C<sup>6a</sup>), 116.90 (C<sup>6b</sup>), 122.89 (C<sup>7</sup>), 122.19 (C<sup>8</sup>), 155.61 (C<sup>9</sup>), 98.87 (C<sup>10</sup>), 153.61 (C<sup>10a</sup>), 79.02 (C<sup>11a</sup>), 118.03 (C<sup>11b</sup>), 145.07 (C<sup>2</sup>), 105 (C<sup>3</sup>)

DEPT data shows that compound **1** has 17 carbons that divided into 3 groups: 9 (-CH), 1 (-CH<sub>2</sub>) and 7 quaternary carbons (C). COSY data shows the correlation of H<sup>3</sup>-H<sup>2</sup> and H<sup>2</sup>-H<sup>1</sup>. HMBC data shows the signal from the correlation between C<sup>2</sup> to H<sup>4</sup>.

##### Compound **2** (*Daidzein*)

Brown powder; m.p. 295-297 °C; MW 254; IR (cm<sup>-1</sup>): 3149, 1621, 1592, 1573, 1514, 1456, 1381, 1307, 1234, 1190, 1094, 1044, 838, 769, 774; EIMS m/z (% intensity): 254 ([M]<sup>+</sup>, 100), 226 (47.2), 197 (7.2), 137 ([C<sub>7</sub>H<sub>6</sub>O<sub>3</sub>]<sup>+</sup>, 100), 118 ([C<sub>8</sub>H<sub>6</sub>O]<sup>+</sup>, 75.1), 108 (32.6), 89 (45.4), 80 (20.5), 63 (35.0), 51 (29.2), 39 (23.7);

<sup>1</sup>H NMR (500 MHz, MeOH-d<sub>4</sub>): δ 6.74 (s, 1H, H<sup>8</sup>), 6.76 (d, 2H, H<sup>3</sup>, H<sup>5</sup>), 6.85 (d, 1H, H<sup>6</sup>), 7.26 (d, 2H, H<sup>2</sup>, H<sup>6</sup>), 7.95 (d, 1H, H<sup>5</sup>), 8.02 (s, 1H, H<sup>2</sup>); <sup>13</sup>C NMR (125 MHz, MeOH-d<sub>4</sub>): δ 153.04 (C<sup>2</sup>), 124.32 (C<sup>3</sup>), 176.57 (C<sup>4</sup>), 128.53 (C<sup>5</sup>), 116.47 (C<sup>6</sup>), 164.62 (C<sup>7</sup>), 103.24 (C<sup>8</sup>), 158.72 (C<sup>9</sup>), 118.23 (C<sup>10</sup>), 125.98 (C<sup>1</sup>), 131.24 (C<sup>2</sup>), 116.24 (C<sup>3</sup>), 159.82 (C<sup>4</sup>), 116.24 (C<sup>5</sup>), 131.42 (C<sup>6</sup>).

COSY data shows the correlations of H<sup>6</sup>-H<sup>5</sup>; H<sup>2</sup>-3' and H<sup>6</sup>-H<sup>5</sup>. HMBC data shows the correlations between H<sup>2</sup> and C<sup>1</sup> (<sup>3</sup>J<sub>C-H</sub>), H<sup>2</sup> and C<sup>4</sup> (<sup>3</sup>J<sub>C-H</sub>), H<sup>2</sup> and C<sup>9</sup> (<sup>3</sup>J<sub>C-H</sub>), H<sup>5</sup> and C<sup>7</sup> (<sup>3</sup>J<sub>C-H</sub>), H<sup>2</sup> and C<sup>1</sup> (<sup>2</sup>J<sub>C-H</sub>), and H<sup>6</sup> and C<sup>1</sup> (<sup>2</sup>J<sub>C-H</sub>).

##### Compound **3** (*Daidzein-7-O-β-glucopyranose*)

Yellow powder; MW 416; UV (MeOH) λ<sub>max</sub> 260 nm; IR (cm<sup>-1</sup>): 3324, 2921, 1617, 1516, 1445, 1374, 1249, 1070, 1044, 887, 836, 785; ESI-MS m/z (% intensity): 417 ([M+H]<sup>+</sup>, 100), 255 ([M-C<sub>6</sub>H<sub>11</sub>O<sub>6</sub>]<sup>+</sup>, 100); <sup>1</sup>H NMR (500 MHz, MeOH-d<sub>4</sub>): δ 6.78 (d, 2H, H<sup>3</sup>, H<sup>5</sup>), 7.12 (d, 1H, H<sup>6</sup>), 7.16 (s, 1H, H<sup>8</sup>), 7.29 (d, 2H, H<sup>2</sup>, H<sup>6</sup>), 8.06 (d, 1H, H<sup>5</sup>), 8.11 (s, 1H, H<sup>2</sup>), 5.03 (d, 1H, H<sup>1</sup>), 3.36-3.86 (m, 6H, H<sup>2a</sup>, H<sup>3a</sup>, H<sup>4a</sup>, H<sup>5a</sup>, H<sup>6a</sup>); <sup>13</sup>C NMR (125 MHz, MeOH-d<sub>4</sub>): δ 154.04 (C<sup>2</sup>), 124.87 (C<sup>3</sup>), 177.09 (C<sup>4</sup>), 127.14 (C<sup>5</sup>), 115.89 (C<sup>6</sup>), 162.13 (C<sup>7</sup>), 103.79 (C<sup>8</sup>), 157.97 (C<sup>9</sup>), 118.94 (C<sup>10</sup>), 124.87 (C<sup>1</sup>), 130.23 (C<sup>2</sup>), 115.15 (C<sup>3</sup>), 157.19 (C<sup>4</sup>), 115.15 (C<sup>5</sup>), 130.23 (C<sup>6</sup>), 100.42 (C<sup>1a</sup>), 73.41 (C<sup>2a</sup>), 76.42 (C<sup>3a</sup>), 69.93 (C<sup>4a</sup>), 77.00 (C<sup>5a</sup>), 61.10 (C<sup>6a</sup>).

COSY data shows correlations between the protons of H<sup>5</sup>-6<sup>H</sup>, H<sup>2</sup>-H<sup>3</sup> and H<sup>5</sup>-H<sup>6</sup>. HMBC data shows the correlations of H<sup>1</sup>-C<sup>7</sup>, H<sup>3</sup>-C<sup>1</sup> (<sup>3</sup>J<sub>C-H</sub>), H<sup>6</sup>-C<sup>9</sup> (<sup>4</sup>J<sub>C-H</sub>), H<sup>2</sup>-C<sup>1</sup> (<sup>3</sup>J<sub>C-H</sub>), H<sup>2</sup>-C<sup>9</sup> (<sup>3</sup>J<sub>C-H</sub>) and H<sup>2</sup>-C<sup>4</sup> (<sup>3</sup>J<sub>C-H</sub>).

##### Compound **4** (*5-Hydroxy-daidzein-7-O-β-glucopyranose*)

Yellow-brown powder; MW 432; UV (MeOH) λ<sub>max</sub> 260 nm; IR (cm<sup>-1</sup>): 3330, 2927, 1651, 1614, 1516, 1444, 1363, 1251, 1177, 1069, 1044, 837, 789; ESI-MS m/z (% intensity): 433.2 ([M+H]<sup>+</sup>, 100), 271 ([M-C<sub>6</sub>H<sub>11</sub>O<sub>6</sub>]<sup>+</sup>, 100); <sup>1</sup>H NMR (500 MHz, MeOH-d<sub>4</sub>): δ 6.76 (d, 2H, H<sup>3</sup>, H<sup>5</sup>), 6.58 (d, 1H, H<sup>6</sup>), 6.39 (s, 1H, H<sup>8</sup>), 7.29 (d, 2H, H<sup>2</sup>, H<sup>6</sup>), 8.03 (d, 1H, H<sup>2</sup>), 4.95 (d, 1H, H<sup>1</sup>), 3.39-3.80 (m, 6H, H<sup>2a</sup>, H<sup>3a</sup>, H<sup>4a</sup>, H<sup>5a</sup>, H<sup>6a</sup>); <sup>13</sup>C NMR (125 MHz, MeOH-d<sub>4</sub>): δ 156.32 (C<sup>2</sup>), 84.32 (C<sup>3</sup>), 174.21 (C<sup>4</sup>), 164.77 (C<sup>5</sup>), 101.64 (C<sup>6</sup>),

163.07 (C<sup>7</sup>), 94.00 (C<sup>8</sup>), 157.68 (C<sup>9</sup>), 115.08 (C<sup>10</sup>), 127.93 (C<sup>11</sup>), 130.16 (C<sup>2</sup>), 115.08 (C<sup>3</sup>), 157.69 (C<sup>4</sup>), 115.08 (C<sup>5</sup>), 130.16 (C<sup>6</sup>), 100.41 (C<sup>1\*</sup>), 73.48 (C<sup>2\*</sup>), 76.61 (C<sup>3\*</sup>), 69.98 (C<sup>4\*</sup>), 77.15 (C<sup>5\*</sup>), 62.43 (C<sup>6\*</sup>)

COSY data show the correlations of H<sup>2</sup>-H<sup>3</sup> and H<sup>5</sup>-H<sup>6</sup>. HMBC data shows the correlations of H<sup>2</sup>-C<sup>1</sup> (<sup>3</sup>J<sub>CH</sub>), H<sup>2</sup>-C<sup>4</sup> (<sup>3</sup>J<sub>CH</sub>), H<sup>2</sup>-C<sup>9</sup> (<sup>3</sup>J<sub>CH</sub>) and H<sup>1\*</sup>-C<sup>7</sup> (<sup>3</sup>J<sub>CH</sub>).

### Assay of antioxidant and tyrosinase activity

#### Assay of antioxidant activity

The antioxidant activity of crude extracts and isolated compounds were evaluated by measuring the scavenging activity assay against DPPH radical with ascorbic acid as the positive control (IC<sub>50</sub> 7.24 ppm) according to Wang *et al.*, (2006) and Dickson *et al.*, (2007). 4mL of 100µM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution in methanol was thoroughly mixed with 1mL of a sample solution at various concentrations. The mixture was kept in the dark for 30min. The absorbance of these solutions was measured at 517nm. The concentration in ppm at which the absorbance decreased to 50% of its initial value was used as the SC<sub>50</sub> value for each test solution. All tests were done in triplicate.

#### Assay of tyrosinase inhibition

Tyrosinase inhibitory activity of crude extracts and isolated compounds was measured according to Hearing (1987) and Rangkadilok *et al.* (2006) with a slight modification using mushroom tyrosinase as the enzyme, L-DOPA as the substrate and kojic acid as the positive control. An aliquot (50µL) of samples in DMSO was mixed with 100µL of 200 IU/mL of mushroom tyrosinase and 100µL of phosphate buffered saline (pH 6.8). The assay mixture was pre-incubated at 37°C for 10min and then 100µL of L-1,4-dihydroxyphenylalanine (L-DOPA) solution 7.6 mM was added. This reaction mixture incubated for 15 min at 37°C. The dopachrome was measured at 475 nm using a UV/Vis spectrophotometer (A). As a blank, DMSO was used (B). As a colour control test, phosphate buffer was used instead of the enzyme tyrosinase (C). The percentage of tyrosinase inhibitions was expressed as a

percentage of inhibition of tyrosinase activity and calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \frac{\{B-(A-C)\}}{B} \times 100\%$$

Kojic acid was used as a standard inhibitor for tyrosinase. All tests were done in triplicate.

## RESULTS AND DISCUSSION

In order to isolate active compounds of the extracts, a bioassay-guided fractionation has been performed with a column chromatography and TLC using DPPH reagent for antioxidant detector. Equal fractions with the same R<sub>f</sub> on TLC showing the antioxidant activity were collected and further analyzed using mushroom tyrosinase to evaluate their tyrosinase inhibitory activity. Compounds having both antioxidant and tyrosinase inhibitory activities, can be developed as skin whitening compounds in cosmetics. Compounds **1**, **2**, **3** and **4** are candidates for the skin whitening compound.

The chemical structures of isolated compounds can be found in Figure 1. New compound **1** was isolated from the ethyl acetate extract as yellow crystals. The UV spectrum shows that the compound **1** has a high activity to absorb the UV light at a maximum wavelength 293 nm, therefore it is predicted that compound **1** has many conjugated C-C bonds. This compound has also the potential to reduce the DPPH reagent and exhibits antioxidant activity and tyrosinase inhibitory activity. Therefore, the structure was carefully elucidated. The molecular formula of compound **1**, C<sub>17</sub>H<sub>12</sub>O<sub>4</sub> is determined by ESI-LC/MS m/z [M+H]<sup>+</sup> 281.3. In addition, MS spectra show that the compound **1** is fragmented producing signal m/z at 123 (in positive detection) and m/z at 121 (in negative detection). Its <sup>1</sup>H NMR spectrum exhibits five aromatic protons at δ<sub>H</sub> 7.6 (s, H<sup>7</sup>), 6.9 (d, J=8.06 Hz; H<sup>1</sup>), 6.9 (s, H<sup>10</sup>), 6.2 (dd, J=8.06; 2.20; H<sup>2</sup>), 6.2 (d, J=2.23, H<sup>4</sup>). There are a coupling of *meta*-related protons (H<sup>2</sup>-H<sup>4</sup>) and a coupling of *ortho*-related protons (H<sup>1</sup>-H<sup>2</sup>). The signals at δ<sub>H</sub> 7.5 (d, J=2.25, H<sup>2</sup>) and 6.7 (d, J=2.23, H<sup>3</sup>) are characteristic for *ortho*-related protons in a furan system. The finding was also supported by the correlation of δ 7.5 (H<sup>2</sup>) and δ 6.7 (H<sup>3</sup>) in the COSY diagram.

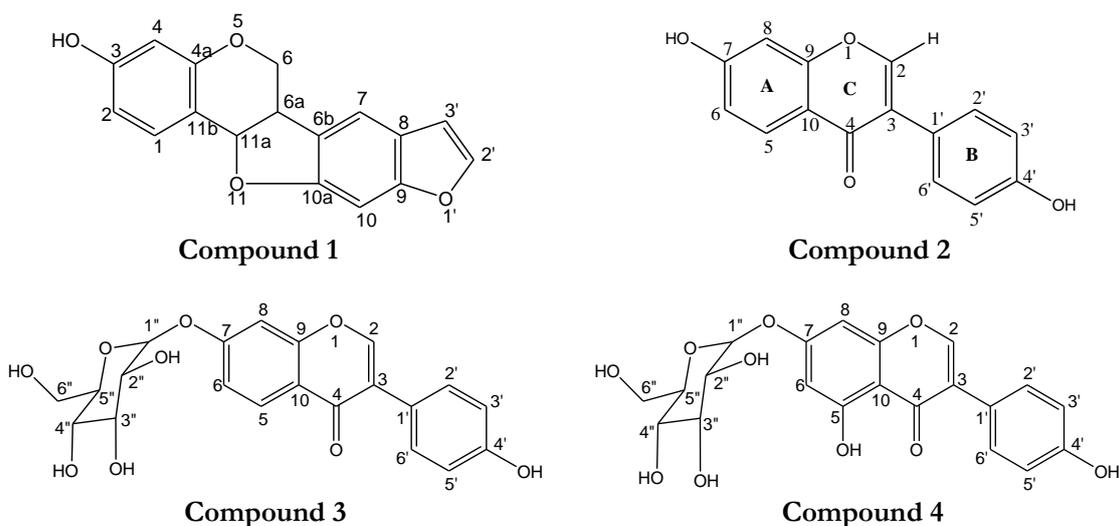


Figure 1. Chemical structure of isolated compounds in ethyl acetate extract of *Bengkoang*

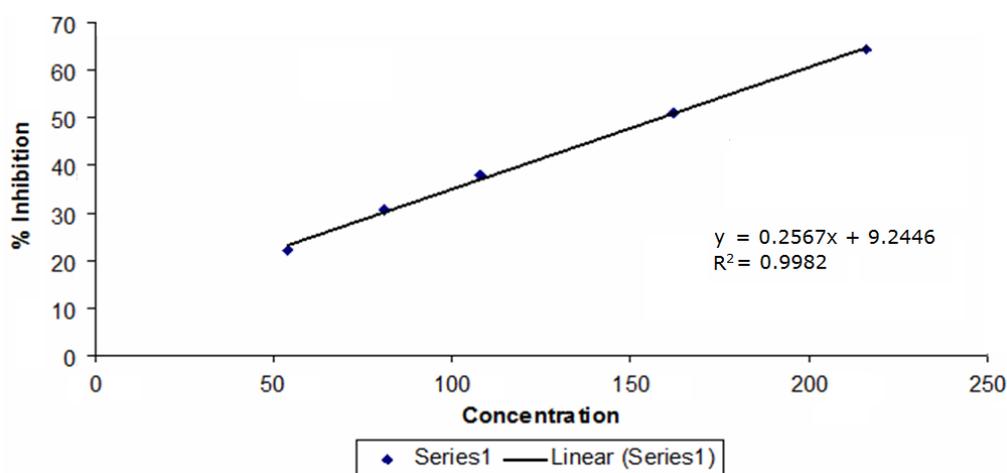


Figure 2. Concentration-tyrosinase inhibition (%) curve of *Bengkoang* crude extracts

Four protons appeared at  $\delta_H$  4.1 (d,  $J=9.68$ ,  $H^6$ ), 3.5 (d,  $J=10.30$ ,  $H^6$ ), 3.4 (dd,  $J=5.63$ ; 2.85,  $H^{6a}$ ), 5.5 (d,  $J=6.71$ ,  $H^{11a}$ ) are characteristic for  $-O-CH_2-CH-CH-O$ . This fact was also supported by  $^{13}C$  NMR spectra. From the DEPT spectrum, we know that the compound **1** has 17 carbons that divided into 3 groups: 9 (-CH), 1 (-CH<sub>2</sub>) and 7 quaternary carbons (C). The presence of hydroxyl group is indicated by band at  $3295\text{ cm}^{-1}$  in IR spectrum. In addition, the IR spectrum shows main bands at 1607, 1469,  $1493\text{ cm}^{-1}$  (-C=C- aromatic) and  $1084\text{ cm}^{-1}$  (-C-O-C-). Based on the NMR assignments, IR spectrum and the fragmentation pattern above,

compound **1** is identified as **8,9-furanylpterocarpan-3-ol**.

Compounds **2**, **3** and **4** were obtained from the ethyl acetate extract and identified by spectral data as isoflavonoid groups. The  $^1H$  NMR spectra of the compounds **2**, **3** and **4** show the signals in aromatic region with the pattern typical for isoflavonoids. In addition, the HMBC diagrams of these compounds reveal significant correlations between  $H^2$  and  $C^{1'}$  ( $^3J_{CH}$ ),  $H^2$  and  $C^4$  ( $^3J_{CH}$ ),  $H^2$  and  $C^9$  ( $^3J_{CH}$ ) that confirm the presence of the isoflavone skeleton. This finding is in accordance with Falco *et al.*, (2005).

Table I. The SC<sub>50</sub> of antioxidant activity of crude extract and isolated compounds

Name	SC <sub>50</sub> value (Mean ± SD)
Standard	
Ascorbic acid	0.041±0.001mM
Extract	
Ethyl acetate	175.06±3.28ppm
Isolated compounds	
Compound 1	2.113±0.001mM
Compound 2	11.86±0.23mM
Compound 3	0.697±0.002mM
Compound 4	7.857±0.069mM

Table II. The IC<sub>50</sub> of tyrosinase inhibitory activity of crude extract and isolated compounds

Name	IC <sub>50</sub> value
<b>Standard</b>	
Kojic acid standard	0.070±0.001mM
<b>Extract</b>	
Ethyl acetate	158.13±1.36ppm
<b>Isolated compounds</b>	
Compound 1	7.19±0.11mM
Compound 2	5.35±0.03mM
Compound 3	22.20±0.27mM
Compound 4	4.38±0.01mM

The NMR and mass spectra of compound **2** are in accordance with **daidzein** data reported by Shimoda *et al.*, (2008), Santos *et al.*, (2006) and Setchell and Welsh (1987). The <sup>1</sup>H NMR spectrum of compound **3** shows that the chemical shift values of the proton H<sup>6</sup> and H<sup>8</sup> are relatively upfield and it supports that the substituent attached of C7 is not a hydroxyl group but a β-glucopyranose. The presence of β-glucopyranose is supported by a β-anomeric proton signal at δ 5.03 ppm (1H, d, J = 7.4 and δ<sub>C</sub> of 100.42 ppm and also by the other group signals at δ 3.38 – 3.86 ppm. This finding is in accordance with Shimoda *et al.*, (2008). The compound **3** is identified as **daidzein-7-O-β-glucopyranose**. The <sup>1</sup>H and <sup>13</sup>C NMR of compound **4** are different from the signal of **3** without hydroxyl at position C<sup>5</sup>. The chemical shift value of H<sup>6</sup> in molecule **4** was relatively downfield because of the presence of –OH at C<sup>5</sup>. The NMR spectra of **4** are in accordance with the data of a hydroxylated daidzein reported by Murthy *et al.*, (1986) and Shimoda *et al.*, (2008). Thus, the compound **4** is elucidated as 5-hydroxy-daidzein-7-O-β-glucopyranose.

#### Antioxidant activity assay

The antioxidant activity of the crude extracts and some of the isolated compounds were evaluated by means of scavenging activity assay using DPPH radical and ascorbic acid as a positive control (IC<sub>50</sub> 7.24 ppm or 0.04 mM). The corresponding SC<sub>50</sub> value can be seen in the table I.

The scavenging activity of the ethyl acetate extract was 175.06±3.28. The isoflavonoid compounds, daidzein and daidzein-7-O-β-glucopyranose contain phenol groups which are responsible to their antioxidant activity. According to Jayaprakasha *et al.*, (2003), the antioxidant activity of some natural products depends on the presence of polyphenols which may act as reductors.

#### Tyrosinase Inhibition Assay

The catalytic action of tyrosinase enzyme was the conversion of tyrosine with oxygen to give DOPA which was then converted to dopaquinone and water. Subsequently, dopaquinone was converted through autooxidation to dopachrome, an orange to red

pigment with the value of maximum absorbance at 475nm.

The results provided in figure 2 demonstrate that the ethyl acetate extract shows a significant correlation between the concentration and the tyrosinase inhibitory activity. In cosmetic fields, the ethyl acetate extract can be developed as a natural skin-whitening agent. The IC<sub>50</sub> value of crude extracts and the isolated compounds are displayed in table II.

Comparison of the tyrosinase inhibition potency of isoflavonoids reveals that 5-hydroxy-daidzein-7-O-β-glucopyranose has the greatest inhibition activity, followed by daidzein, 8,9-furanyl-pterocarpan-3-ol and daidzein-7-O-β-glucopyranose, respectively. The decrease of the activities from daidzein to daidzein-7-O-β-glucopyranose may be caused by the glucopyranosyl substituent providing steric bulk. The result is in accordance with findings reported by Chang (2007). The active site of enzyme tyrosinase consisted of two copper atoms that are each coordinated with three histidine residue (Mirica *et al.*, 2005). The compounds having phenol or diphenol group can form a chelate complex with copper in the enzyme and thus irreversibly inactivate the tyrosinase. Daidzein-7-O-β-glucopyranose has only one phenol group because the other phenol groups bond to a glucose molecule which forms a weak complex resulting in a lower inhibitory activity.

The compound of 5-hydroxy-daidzein-7-O-β-glucopyranose is also a glycoside having an extra hydroxyl group at position C<sup>4</sup> beside the -OH group at position C<sup>7</sup>. However, the tyrosinase inhibition activity is still greater than that of daidzein compound. The hydroxyl group (-OH) at position 4 adjacent to the carbonyl group (-C=O) enables this compound to form a strong chelation with the copper of the active site enzyme, so that the inhibition power is greater than the aglycon molecule of daidzein. The molecule of 8,9-furanyl-pterocarpan-3-ol has only one hydroxyl -OH at position C<sup>7</sup> and no carbonyl group (-C=O), thus the activity is much lower than the aglycon daidzein.

## CONCLUSION

Three isolated isoflavonoids (daidzein, daidzein-7-O-β-glucopyranose; 5-hydroxy-daidzein-7-O-β-glucopyranose), and (8,9)-furanyl-pterocarpan-3-ol showed interesting antioxidant and tyrosinase inhibitory activities. The SC<sub>50</sub> (mM) values of isolated compounds were 11.86±0.23; 0.697±0.002; 7.857±0.069; 2.113±0.001, for daidzein, daidzein-7-O-β-glucopyranose, 5-hydroxy-daidzein-7-O-β-glucopyranose and 8,9-furanyl-pterocarpan-3-ol, respectively. In addition, the IC<sub>50</sub> values (in mM) to inhibit tyrosinase of compounds were 5.35±0.03; 22.20±0.27; 4.39±0.01; 7.18±0.11; 0.198±0.004; 1.21±0.02, respectively.

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