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-ß-glucopyranose (3); 5-hydroxy-daidzein-7-O-ß-glucopyranose (4)), and a new pterocarpan (i.e. 8,9-furanyl-pterocarpan-3-ol (1)) which antioxidant activities (SC₅₀% values) of 2.11; 11.86; 0.69 and 7.86 respectively. All compounds showed tyrosinase inhibiting activities with IC₅₀ 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 chili. 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 phacomelanin which is alkali soluble. Both melanosins 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 (dopquinone) by tyrosinase. Dopquinone 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; Ohiguchi 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 buffered saline, (purchased from Sigma Aldrich, Steinheim Germany), Silica gel 60 (particle sizes 0.063

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 Rf 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 (I) that is identified as 8,9-Furanylpterocarpan-3-ol. Fractions 37-40 from column chromatography with an Rf 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 Rf value of 0.85
Identification of isolated compounds

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

Yellow crystal; UV MeOH $\lambda_{max}$ 293 nm; MW 280; IR (cm$^{-1}$): 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]$^+$), 100, 1231 ([C$_4$H$_4$O$_2$]$^+$), 100); $^1$H NMR (500 MHz, MeOH-d$_4$): $\delta$ 3.44 (dd, 1H, H$_{6''}$), 3.48 (dd, 1H, H$_{4''}$), 4.14 (dd, 1H, H$_{5''}$), 5.49 (d, 1H, H$_{1''}$), 6.17 (d, 1H, H$_2$), 6.22 (dd, 1H, H$_3$), 6.66 (d, 1H, H$_4$), 6.89 (s, 1H, H$_{1'}$), 6.95 (d, 1H, H$_5$), 7.53 (d, 1H, H$_6$), 7.57 (s, 1H, H$_7$); $^1$C NMR (125 MHz, MeOH-d$_4$): $\delta$ 124.66 (C$_7$), 107.40 (C$_2$), 160.53 (C$_5$), 97.35 (C$_3$), 158.45 (C$_4$), 66.84 (C$_6$), 39.73 (C$_8$), 116.90 (C$_9$), 122.89 (C$_7$), 122.19 (C$_6$), 155.61 (C$_5$), 98.87 (C$_4$), 153.61 (C$_2$), 79.02 (C$_1$), 118.03 (C$_3$), 145.07 (C$_8$), 105 (C$_3$).

DEPT data shows that compound 1 has 17 carbons that divided into 3 groups: 9 (-CH), 1 (-CH$_2$) and 7 quaternary carbons (-C). COSY data shows the correlation of H$_3''$-H$_2''$ and H$_2''$-H$_1''$. HMBC data shows the signal from the correlation between C$_2$ to H$_4$.

**Compound 2 (Daidzein)**

Brown powder; m.p. 295-297 °C; MW 254; IR (cm$^{-1}$): 3149, 1621, 1592, 1573, 1514, 1456, 1381, 1307, 1234, 1190, 1094, 1044, 837, 789; ESI-MS m/z (% intensity): 254 ([M+H]$^+$), 100, 226 (47.2), 197 (7.2), 137 ([C$_4$H$_4$O$_2$]$^+$), 100, 118 ([C$_4$H$_4$O$_2$]$^+$), 75.1), 108 (32.6), 89 (45.4), 80 (20.5), 63 (35.0), 51 (29.2), 39 (23.7); $^1$H NMR (500 MHz, MeOH-d$_4$): $\delta$ 6.74 (s, 1H, H$_8$), 6.76 (d, 2H, H$_{1'}$), 6.85 (d, 1H, H$_9$), 7.26 (d, 2H, H$_2''$, H$_5''$), 7.95 (d, 1H, H$_8$), 8.02 (s, 1H, H$_7$); $^1$C NMR (125 MHz, MeOH-d$_4$): $\delta$ 153.04 (C$_7$), 124.32 (C$_6$), 176.57 (C$_5$), 128.53 (C$_4$), 116.47 (C$_3$), 164.62 (C$_2$), 103.24 (C$_1$), 158.72 (C$_6''$), 118.23 (C$_5''$), 125.98 (C$_4''$), 131.24 (C$_3''$), 116.24 (C$_2''$), 159.82 (C$_1''$), 116.24 (C$_3''$), 131.42 (C$_4''$).

COSY data shows the correlations of H$_6''$-H$_8$; H$_2''$-H$_3''$ and H$_6''$-H$_9$. HMBC data shows the correlations between H$_2$ and C$_2''$ (H$_{C_2}$), H$_2$ and C$_4''$ (H$_{C_4}$), H$_2$ and C$_7''$ (H$_{C_7}$), and H$_8$ and C$_5''$ (H$_{C_5}$).

**Compound 3 (Daidzein-7-O-β-glucopyranoside)**

Yellow powder; MW 416; UV (MeOH) $\lambda_{max}$ 260 nm; IR (cm$^{-1}$): 3324, 2921, 1617, 1516, 1445, 1374, 1249, 1070, 1044, 887, 836, 785; ESI-MS m/z (% intensity): 417 ([M+H]$^+$), 100, 255 ([M-C$_6$H$_5$O$_2$]$^+$), 100; $^1$H NMR (500 MHz, MeOH-d$_4$): $\delta$ 6.78 (d, 2H, H$_3'$, H$_5'$), 7.12 (d, 1H, H$_9'$), 7.16 (s, 1H, H$_8'$), 7.29 (d, 2H, H$_2''$, H$_5''$), 8.06 (d, 1H, H$_6$), 8.11 (s, 1H, H$_7$), 5.03 (d, 1H, H$_7''$), 3.36-3.86 (m, 6H, H$_2''$, H$_3''$, H$_4''$, H$_5''$, H$_6''$, H$_7''$); $^1$C NMR (125 MHz, MeOH-d$_4$): $\delta$ 154.04 (C$_7$), 124.87 (C$_6$), 177.09 (C$_5$), 127.14 (C$_4$), 115.89 (C$_3$), 162.13 (C$_2$), 103.79 (C$_1$), 157.97 (C$_8$), 118.94 (C$_9$), 124.87 (C$_7''$), 130.23 (C$_5''$), 115.15 (C$_4''$), 157.19 (C$_3''$), 115.15 (C$_2''$), 130.23 (C$_1''$), 100.42 (C$_6''$), 73.41 (C$_5''$), 76.42 (C$_2''$), 69.93 (C$_4''$), 77.00 (C$_3''$), 61.10 (C$_8''$).

COSY data shows correlations between the protons of H$_6''$-H$_7''$, H$_2''$-H$_3''$ and H$_6''$-H$_9''$. HMBC data shows the correlations of H$_3''$-C$_2''$, H$_2''$-C$_7''$ (H$_{C_7}$), H$_6''$-C$_9''$ (H$_{C_9}$), H$_2''$-C$_5''$ (H$_{C_5}$), and H$_6''$-C$_3''$ (H$_{C_3}$).

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

Yellow-brown powder; MW 432; UV (MeOH) $\lambda_{max}$ 260 nm; IR (cm$^{-1}$): 3330, 2927, 1651, 1614, 1516, 1444, 1363, 1251, 1177, 1069, 1044, 837, 789; ESI-MS m/z (% intensity): 433.2 ([M+H]$^+$), 100, 271 ([M-C$_6$H$_5$O$_2$]$^+$), 100; $^1$H NMR (500 MHz, MeOH-d$_4$): $\delta$ 6.76 (d, 2H, H$_3'$, H$_5'$), 6.58 (d, 1H, H$_9'$), 6.39 (s, 1H, H$_8'$), 7.29 (d, 2H, H$_2''$, H$_5''$), 8.03 (d, 1H, H$_6''$), 4.95 (d, 1H, H$_7''$), 3.39-3.80 (m, 6H, H$_2''$, H$_3''$, H$_4''$, H$_5''$, H$_6''$); $^1$C NMR (125 MHz, MeOH-d$_4$): $\delta$ 156.32 (C$_7$), 84.32 (C$_6$), 174.21 (C$_5$), 164.77 (C$_4$), 101.64 (C$_3$).
The ure was kept in the 3'-th for a slight modification using 2.23, H 5'-m which the absorbance min 25.08 10' at 475 nm. The dopachrome was measured at 475 nm reaction mix and mixture was pre phosphate buffered DMSO was mixed with 100µL of 200 IU/mL mushroom tyrosinase to evaluate their 4-μ 3ºC. NMR spectrum exhibits five 1H NMR spectrum exhibits five (δ) in the COSY diagram. The finding was also supported by the correlation of δ7.5 (H2) and δ 6.7 (H3) in the COSY diagram.

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 Rf 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 bounds. 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, C17H14O4 is determined by ESI-LC/MS m/z [M+H]+ 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 1H NMR spectrum exhibits five aromatic protons at δH 7.6 (s, H1), 6.9 (d, J=8.06 Hz; H1), 6.9 (s, H1'), 6.2 (dd, J=8.06; 2.20); H2'), 6.2 (d, J=2.23, H2'). There are a coupling of meta-related protons (H2-H3) and a coupling of ortho-related protons (H1-H2). The signals at δH 7.5 (d, J=2.25, H2) and 6.7 (d, J=2.25, H3) are characteristic for ortho-related protons in a furan system. The finding was also supported by the correlation of δ7.5 (H2) and δ 6.7 (H3) in the COSY diagram.
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$_2$) and 7 quaternary carbons (C). The presence of hydroxyl group is indicated by band at 3295 cm$^{-1}$ in IR spectrum. In addition, the IR spectrum shows main bands at 1607, 1469, 1493 cm$^{-1}$ (-C=C- aromatic) and 1084 cm$^{-1}$ (-C-O-C). Based on the NMR assignments, IR spectrum and the fragmentation pattern above, compound 1 is identified as 8,9-furanyl-pterocarpan-3-ol.

Compounds 2, 3 and 4 were obtained from the ethyl acetate extract and identified by spectral data as isoflavonoid groups. The $^1$H 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$^1$ ($J_{C1-H2}$), H$^2$ and C$^4$ ($J_{C4-H2}$), H$^2$ and C$^0$ ($J_{C0-H2}$) that confirm the presence of the isoflavone skeleton. This finding is in accordance with Falco et al., (2005).
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 1H NMR spectrum of compound 3 shows that the chemical shift values of the proton H6 and H8 are relatively upfield and it supports that the substituent attached of C7 is not a hydroxyl group but a ß-glucopyranose. The presence of ß-glucopyranose is supported by a ß-anomeric proton signal at δ 5.03 ppm (1H, d, J = 7.4 and δc 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-ß-glucopyranose. The 1H and 13C NMR of compound 4 are different from the signal of 3 without hydroxyl at position C5. The chemical shift value of H6 in molecule 4 was relatively downfield because of the presence of –OH at C5. 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-ß-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 (IC50 7.24 ppm or 0.04 mM). The corresponding SC50 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-ß-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 autoxidation to dopachrome, an orange to red

Table I. The SC50 of antioxidant activity of crude extract and isolated compounds

<table>
<thead>
<tr>
<th>Name</th>
<th>SC50 value (Mean ± SD)</th>
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<tbody>
<tr>
<td>Standard</td>
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</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.041±0.001mM</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>175.06±3.28ppm</td>
</tr>
<tr>
<td>Isolated compounds</td>
<td></td>
</tr>
<tr>
<td>Compound 1</td>
<td>2.113±0.001mM</td>
</tr>
<tr>
<td>Compound 2</td>
<td>11.86±0.23mM</td>
</tr>
<tr>
<td>Compound 3</td>
<td>0.697±0.002mM</td>
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<tr>
<td>Compound 4</td>
<td>7.857±0.069mM</td>
</tr>
</tbody>
</table>

Table II. The IC50 of tyrosinase inhibitory activity of crude extract and isolated compounds

<table>
<thead>
<tr>
<th>Name</th>
<th>IC50 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
</tr>
<tr>
<td>Kojic acid standard</td>
<td>0.070±0.001mM</td>
</tr>
<tr>
<td>Extract</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>158.13±1.36ppm</td>
</tr>
<tr>
<td>Isolated compounds</td>
<td></td>
</tr>
<tr>
<td>Compound 1</td>
<td>7.19±0.11mM</td>
</tr>
<tr>
<td>Compound 2</td>
<td>5.35±0.03mM</td>
</tr>
<tr>
<td>Compound 3</td>
<td>22.20±0.27mM</td>
</tr>
<tr>
<td>Compound 4</td>
<td>4.38±0.01mM</td>
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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 IC₅₀ 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-glucopyranosid has the greatest inhibition activity, followed by daidzein, 8,9-furanyl-pterocarpan-3-ol and daidzein-7-O-β-glucopyranosid, respectively. The decrease of the activities from daidzein to daidzein-7-O-β-glucopyranosid 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-glucopyranosid 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-glucopyranosid is also a glycoside having an extra hydroxyl group at position C⁴ beside the -OH group at position C⁶. 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 C⁷ 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-glucopyranosid; 5-hydroxyl-daidzein-7-O-β-glucopyranosid), and (8,9)-furanyl-pterocarpan-3-ol showed interesting antioxidant and tyrosinase inhibitory activities. The SC₅₀ (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-glucopyranosid, 5-hydroxy-daidzein-7-O-β-glucopyranosid and 8,9-furanyl-pterocarpan-3-ol, respectively. In addition, the IC₅₀ 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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