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Tyrosinase Inhibitor from Black Rice Bran

MITSUO MIYAZAWA,*,† TERUO OSHIMA,‡ KATSUYA KOSHIO,† YUMI ITSUZAKI,† AND JUN ANZAI†

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka-shi, Osaka 577-8502, Japan, and Real Company, Ltd., 1-3-18, Goshodori, Hyogo-ku, Kobe-shi, Hyogo 652-0885, Japan

The inhibitor of tyrosinase activity in black rice bran was investigated. The methanol extract from black rice bran was re-extracted with hexane, chloroform, ethyl acetate, or water. The ethyl acetate extract had the most potent inhibition against tyrosinase activity by 80.5% at a concentration of 0.4 mg/mL. Inhibitory compound in the ethyl acetate fraction was isolated by silica gel column chromatography, and identified as protocatechuic acid methyl ester (compound **1**) by GC, GC−MS, IR, and ^{1}H and ^{13}C NMR spectroscopy. Compound **1** inhibited 75.4% of tyrosinase activity at a concentration of 0.50 μmol/mL. ID_{50} (50% inhibition dose) value of compound **1** was 0.28 μmol/mL. To study the structure−activity relationship, protocatechuic acid (**2**), vanillic acid (**3**), vanillic acid methyl ester (**4**), isovanillic acid (**5**), isovanillic acid methyl ester (**6**), veratric acid (**7**), and veratric acid methyl ester (**8**) were also assayed.

KEYWORDS: Black rice bran; tyrosinase activity; protocatechuic acid methyl ester; structure−activity relationship

INTRODUCTION

Melanin biosynthesis inhibitory compounds are useful not only for the material used in cosmetics as skin-whitening agents but also as a remedy for disturbances in pigmentation. The color of mammalian skin and hair is determined by a number of factors. The most important factor is the degree and distribution of melanin pigmentation. Tyrosinase (phenol oxidase) (*1*) is known to be a key enzyme for melanin biosynthesis (*2*) in plants, microorganisms, and mammalian cells. Therefore, many tyrosinase inhibitors have been reported and tested as cosmetics (*3*) and pharmaceuticals to prevent overproduction of melanin in epidermal layers. For example, hydroquinone (*4*−*7*), kojic acid (*8*), azelaic acid (*9, 10*), electron-rich phenols (*11*), corticosteroids (*12, 13*), resinoids (*14*, *15*), and arbutin have been utilized as cosmetic agents. Also, tyrosinase is one of the most important key enzymes in the insect molting process, and investigating its inhibitors may be important in finding alternative insect control agents. Furthermore, melanin formation is considered to be deleterious to the color quality of plant-derived food. This broadens the possible use of tyrosinase inhibitors as food additives, in addition to uses as insect control agents and whitening agents.

Black rice (*Oryza sativa* L. Indica), having dark purple-colored grains, is a major rice crop in South Asia and Mainland China. It is broadly known as an enriched rice with medicinal effects. It is superior to robust nourishment and acts as enhancer of spleen, liver, stomach, and intestine, and as a hematopoietic agent in pharmacy. Anthocyanin pigments, cyanidin 3-glucoside and peonidin 3-glucoside, were isolated from this plant (*16*). Various compounds, such as phenolic acids, ferulic acid, vanillic acid, and protocatechuic acid, phenolic acid ester γ-oryzanol, and phytic acid, and inositol were found from rice bran (*17*−*20*). Phenolic acids act as an antioxidant (*21*−*25*), as a radical scavenger (*22*), as an inhibitor of hepatic cytochrome P450 (*26*), as an antibacterial agent (*27*), and also as an antiinflammatory agent (*28, 29*). Besides, they contain a lot of proteins, various amino acids, and vitamins of every kind. As for minerals, they contain 2−3 times as much iron and calcium as polished rice does. In searching for biologically natural products, the inhibitor of tyrosinase activity in black rice bran was investigated.

MATERIALS AND METHODS

Materials. Black rice bran was obtained from Real Co., Ltd. (Kobe, Japan). Tyrosinase was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). L-Tyrosine, veratric acid, and protocatechuic acid were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Polyoxyethylene nonyl phenyl ether was purchased from Dai-ichi Kogyo Seiyaku Co., Ltd. (Kyoto, Japan). Vanillic acid and isovanillic acid were from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

General Procedure. Gas chromatography (GC) was performed on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (FID). GC−MS was performed on a Hewlett-Packard 5972A series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (DB-5MS, 30 m length × 0.25 mm i.d.). IR spectra were determined with a FT/IR-

* To whom correspondence should be addressed. Telephone: +81-6-6721-2332. Fax: +81-6-6727-4301. E-mail: miyazawa@apch.kindai.ac.jp.
† Kinki University.
‡ Real Co., Ltd.

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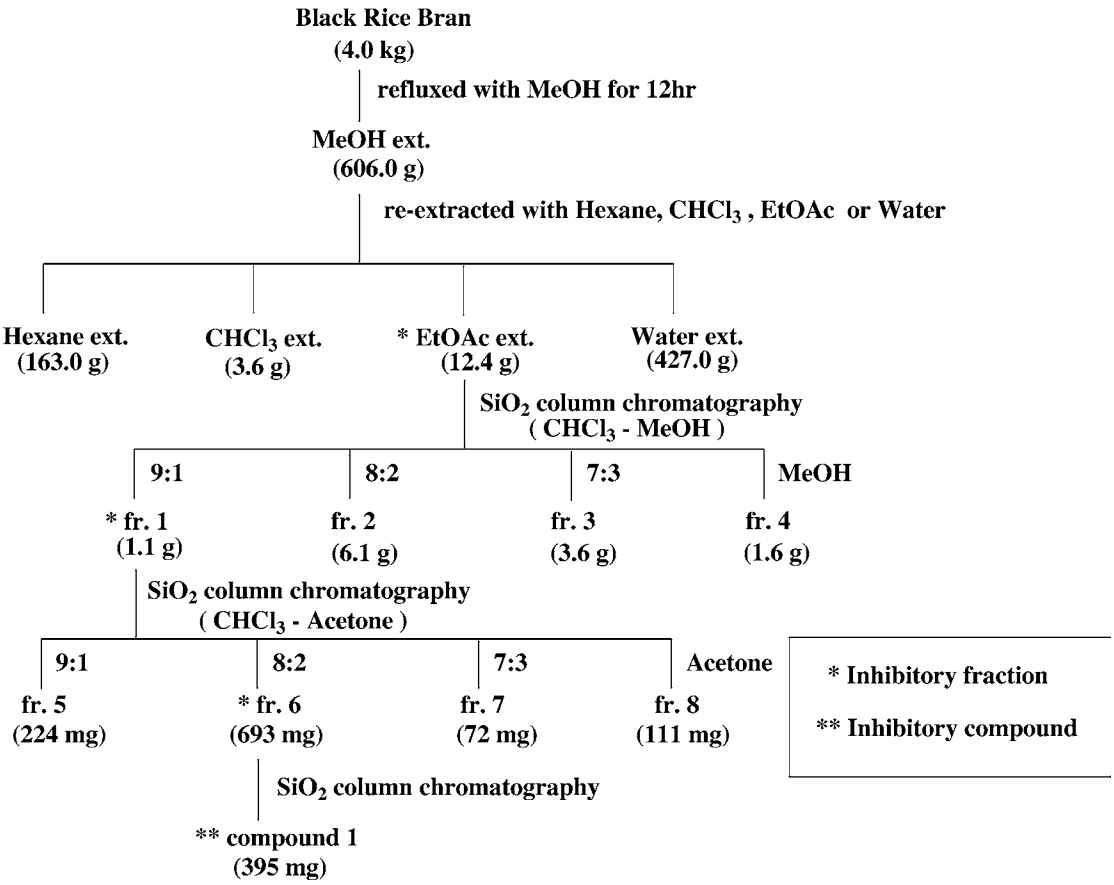

Figure 1. Isolation scheme for the inhibitory compound from black rice bran.

470 Plus Fourier Transform Infrared Spectrometer (JASCO Co., Ltd., Japan). Nuclear magnetic resonance (NMR) spectra (δ, J in hertz) were recorded on a JEOL GSX 500 NMR spectrometer (Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan). Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H NMR spectra measured in $CDCl_3$, acetone-d_6, or DMSO-d_6.

Enzymatic Assay of Tyrosinase. Tyrosinase activity using L-tyrosine as the substrate was assayed spectrophotometrically. Phosphorus buffer (1.7 mL of 0.1 M P-buffer (pH 6.8)), 200 μL of 20% polyoxyethylene nonyl phenyl ether, 250 μL of 0.03% L-tyrosine, and 100 μL of test compounds solution (dissolved in DMSO) were added in a test tube and incubated at 37 °C for 10 min. After incubation, 250 μL of tyrosinase solution (3000 units/mL, dissolved in water) was added in the test tube and incubated at 37 °C for 60 min. Enzymatic activity was quantified by measuring the absorbance at 475 nm. Tyrosinase activity was obtained by the following formula:

$$\text{Tyrosinase activity (\%)} = [(A - B)/(\text{Cp} - \text{Cn})] \times 100$$

where A is absorbance of test sample (0.1 M P-buffer, polyoxyethylene nonyl phenyl ether, L-tyrosine and sample solution); B is absorbance of blank (0.1 M P-buffer, polyoxyethylene nonyl phenyl ether, distilled water, and sample solution); Cp is absorbance of positive control (0.1 M P-buffer, polyoxyethylene nonyl phenyl ether, L-tyrosine, and DMSO); and Cn is absorbance of negative control (0.1 M P-buffer, polyoxyethylene nonyl phenyl ether, distilled water, and DMSO)

Fractionation and Isolation of Inhibitory Compound 1. To prepare the inhibitory compound, fractionation of black rice bran was carried out as described in **Figure 1** using the tyrosinase inhibitory activity test as a guide. Black rice bran (4.0 kg) was refluxed with methanol (8000 mL) for 12 h to give a methanol extract (606 g). This extract was suspended in water (3000 mL), and re-extracted with hexane, chloroform, ethyl acetate, or water. Each soluble fraction was concentrated under reduced pressure to give hexane (163 g), chloroform (3.60 g), ethyl acetate (12.4 g), and water (427 g) fractions. The ethyl acetate fraction showed the strongest inhibitory effect (**Figure 2**). The ethyl acetate fraction was divided into fractions 1−4 by silica gel column chromatography with chloroform and methanol as eluents. Fraction 1 showed tyrosinase inhibitory activity and was further divided into fractions 5−8 by silica gel column chromatography with chloroform and acetone as eluents. Fraction 6 showed tyrosinase inhibitory activity and was re-chromatogramed on silica gel to yield 359 mg of compound **1**. Compound **1** was identified as protocatechuic acid methyl ester by GC, GC−MS, IR, and ^{1}H and ^{13}C NMR.

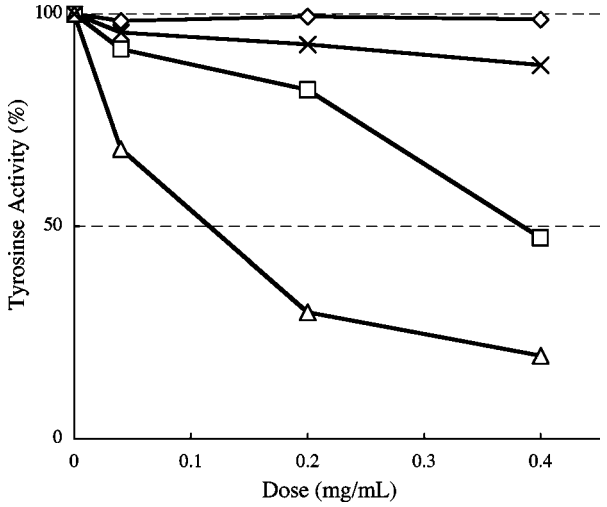

Figure 2. Inhibitory effects of extracts from black rice bran against tyrosinase activity: (◇) effect of hexane extract; (□) effect of chloroform extract; (△) effect of ethylacetate extract; and (×) effect of water extract.

Structures of Compounds. *Esterification of Compounds **4**, **6**, and **8**.* Compounds **3**, **5,** and **7** were prepared by esterification of the vanillic acid (**4**), isovanillic acid (**6**), and veratric acid (**8**) treated with methanol and concentrated sulfuric acid to determine whether esterification of protocatechuic acid (**2**) would affect their ability to inhibit tyrosinase

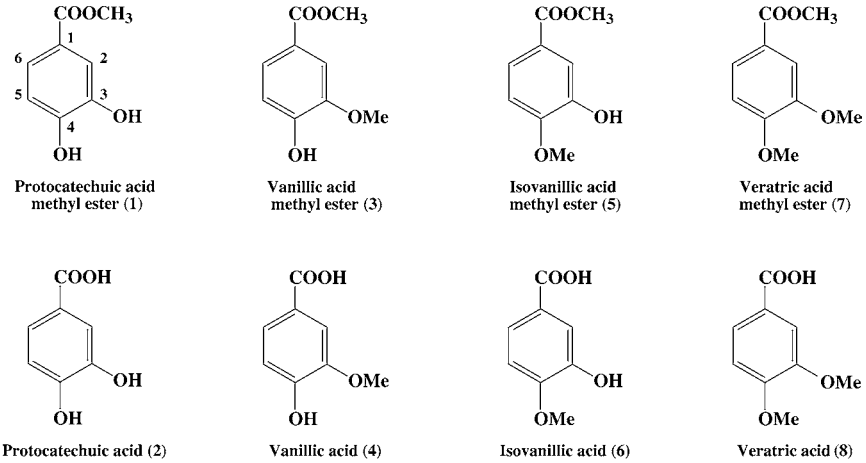

Figure 3. Structures of compounds **1**–**8**.

activity. These structures were identified by GC, GC−MS, and IR. Structures of compounds **1**−**8** are given in **Figure 3**.

Protocatechuic Acid Methyl Ester (1). Compound **1** was a whitish yellow oil. EIMS, *m*/*z* 168 ([M]$^+$, 35.4%), 137 (100%), 109 (25.3%), 81 (18.2%), 63 (11.1%), 53 (18.2%). IR (KBr, ν_{max} cm^{-1}) 3338, 1685, 1604, 1521, 1438, 1301. ^{1}H NMR (500 MHz, acetone-d_6) δ 7.39 (1H, *d*, *J* = 2.0 Hz, H-2), δ 7.34 (1H, *dd*, *J* = 2.0, 8.3 Hz, H-6), δ 6.80 (1H, *d*, *J* = 8.3 Hz, H-5), δ 3.80 (3H, *s*, H-8). ^{13}C NMR (125 MHz, acetone-d_6) δ 166 (−*C*OOCH$_3$), δ 150 (C-4), δ 145 (C-3), δ 123 (C-6), δ 122 (C-1), δ 117 (C-2), δ 115 (C-5), δ 51 (−COO*C*H$_3$). Compound **1** was identified as protocatechuic acid methyl ester (methyl 3,4-dihydroxy benzoate) from these spectral data.

RESULTS AND DISCUSSION

A methanol extract of black rice bran was fractionated as described in **Figure 1** to identify tyrosinase inhibitory compounds in this rice species. To obtain dose−response data, test samples were evaluated at dose levels of 0.4, 0.2, and 0.04 mg/mL. The ethyl acetate extract exhibited an inhibitory effect on tyrosinase activity (**Figure 2**). To prepare the inhibitory compound, fractionation of the ethyl acetate extract was carried out as described in **Figure 1**. Finally, inhibitory compound **1** (395 mg) was isolated from black rice bran. Compound **1** was identified as protocatechuic acid methyl ester (methyl 3,4-dihydroxy benzoate) by GC, GC−MS, IR, and ^{1}H and ^{13}C NMR spectroscopy.

The inhibitory effect of compounds protocatechuic acid methyl ester (**1**), protocatechuic acid (**2**), vanillic acid (**3**), vanillic acid methyl ester (**4**), isovanillic acid (**5**), isovanillic acid methyl ester (**6**), veratric acid (**7**) and veratric acid methyl ester (**8**) against tyrosinase activity are shown in **Figure 4**. Compounds **1** and **2** inhibited 75.4 and 60.1% of the tyrosinase inhibitory activity at a concentration of 0.50 μmol/mL, and their ID$_{50}$ (50% inhibition dose) values were 0.28 and 0.42 μmol/mL, respectively. However compounds **3**−**8** did not inhibit tyrosinase activity.

Melanin synthesis in mammals proceeds from the amino acid L-tyrosine through a series of enzymatic and chemical steps initiated by tyrosine hydroxylation to yield DOPA and DOPA oxidation to L-DOPA quinone. Both reactions are catalyzed by tyrosinase (monophenol monooxygenase), a melanocyte-specific copper-containing glycoprotein located within specialized organelles called melanosomes. In this study, we investigated tyrosinase inhibitor from black rice bran. As a result, the inhibitor of tyrosinase activity in black rice bran was clearly identified as protocatechuic acid methyl ester (**1**). Compound **1** inhibited 75.4% of tyrosinase activity at a concentration of 0.5

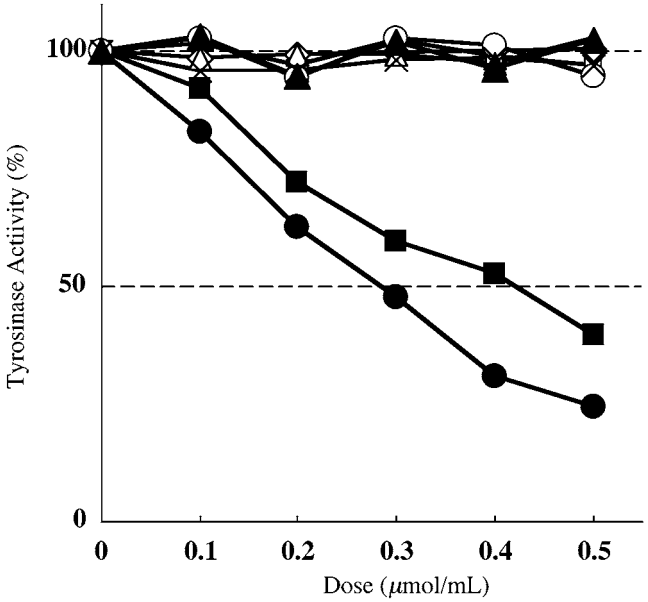

Figure 4. Inhibitory effects of compounds **1**–**8** against tyrosinase activity in black rice bran: (●) effect of compound **1**; (■) effect of compound **2**; (□) effect of compound **3**; (◊) effect of compound **4**; (○) effect of compound **5**; (△) effect of compound **6**; (▲) effect of compound **7**; (×) effect of compound **8**.

μmol/mL, and the ID$_{50}$ (50% inhibition dose) value was 0.28 μmol/mL. To study the structure−activity relationship, compounds **2**−**8** were also assayed. Compound **2** inhibited 60.1% of tyrosinase activity at a concentration of 0.50 μmol/mL, and the ID$_{50}$ value was 0.42 μmol/mL. However compounds **3**−**8** did not inhibit tyrosinase activity. These results indicate that a hydroxyl group at the C-3 and C-4 position is the most important factor for inhibition of the tyrosinase activity. Compound **1** showed stronger inhibition compared with compound **2**. These results indicated that a methylesterification of carboxyl group of compound **1** is an important factor for inhibition of the tyrosinase activity.

Our data imply that the depigmenting effect of black rice bran components likely involves an inhibition of tyrosinase activity. To substantiate that possibility, further investigation will be accomplished by carrying out an in vitro study of the black rice bran components with defined cultured cells. Investigations of the possible therapeutic use of these compounds for the treatment of malignant melanoma, and their chemical and structure−activity relationship are in progress in our laboratory.

LITERATURE CITED

(1) Mayer, A. M. Polyphenol oxidase in plants − Recent progress. *Phytochemistry* **1987**, *26*, 11−20.

(2) Sanchez-Ferrer, A.; Rodriguez-Lopez, J. N.; Garcia-Ganovas, F.; Garcia-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, 1−11.

(3) Maeda, K.; Fukuda, M. In vitro effectiveness of several whitening cosmetic components in human melanocytes. *J. Soc. Cosmet. Chem*. **1991**, *42*, 361−368.

(4) Arandt, K. A.; Fitzpatrick, T. B. Topical use of hydroquinine as a depigmenting agent. *J. Am. Med. Assoc*. **1965**, *194*, 117−119.

(5) Fitztrick, T. B.; Arandt, K. A.; El-Mofty, A. M. M. A. Hydroquinone and psoralens in the therapy of hypermelanosis and vitiligo. *Arch. Dermatol*. **1966**, *93*, 589−600.

(6) Kligman, A. M.; Willis I. A new formula for depigmenting human skin. *Arch. Dermatol*. **1975**, *111*, 40−48.

(7) Bleehen, S. S. Skin bleaching preparations. *J. Soc. Cosmet. Chem*, **1977**, *28*, 407−412.

(8) Mishima, Y.; Hatta, S.; Ohyama, Y.; Inazu, M. Induction of melanogenesis suppression: Cellular pharmacology and mode of differential action. *Pigment Cell Res*. **1998**, *1*, 367−374.

(9) Breathnach, A. C.; Nazzaro-Porro, M.; Passi, S. Azelaic acid therapy in disorders of pigmentation. *Clin. Dermatol*. **1989**, *7*, 106−119.

(10) Verallo-Rowell, V. M.; Verallo, V.; Graupe, K.; Lopez-Villafuerte, L.; Garcia-Lopez M. Double-blind comparison of azelaic acid and hydroquinone in the trearment of melasma. *Acta Dermatol. Venereol*. **1989**, *143*, 58−61.

(11) Jimbow K. *N*-acetyl-4-S-cysteaminylphenol as a new type of depigmenting agent for the melanoderma of melasma patients. *Arch. Dermatol*. **1991**, *127*, 1528−1534.

(12) Neering, H. Treatment of melasma (chloasma) by local application of steroid cream. *Dermatologica* **1975**, *151*, 349−353.

(13) Kanwar, A J.; Dhar, S.; Kaur, S. Treatment of melasma with potent topical corticosteroids. *Dermatology* **1994**, *188*, 170.

(14) Griffiths, C. E. M.; Finkel, L. J.; Ditre, C. M.; Hamilton, T. A.; Ellis, C. N.; Voorhees, J. J. Topical tretinonin (retinoic acid) improves melasma: A vehicle-controlled clinical trial. *Br. J. Dermatol*. **1993**, *129*, 415−421.

(15) Kimbrough-Green, C. K.; Griffiths, C. E. M.; Finkel, L. J.; Hamilton, T. A.; Burengo-Ransby, S. M.; Ellis. C. N.; Voorhees, J. J. Topical retinoic acid (tretinoin) for melasma in black patients: A vehicle-controlled clinical trial. *Arch. Dermatol*. **1994**, *130*, 727−733.

(16) Ryu, S. N.; Park, S. Z.; Ho, C.-T. High performance liquid chromatographic determination of anthocyanin pigments in some varieties of blackrice. *Yaowu Shipin Fenxi* **1998**, *6*, 729−736.

(17) Hudson, E. A.; Dinh, P. A.; Kokubun, T.; Simmonds, M. S. J.; Gescher, A. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol., Biomarkers Prev.* **2000**, *9*, 1163−1170.

(18) Das, P. K.; Chaudhuri, A.; Kaimal, T. N. B.; Bhalerao, U. T. Isolation of γ-Oryzanol through Calcium Ion Induced Precipitation of Anionic Micellar Aggregates. *J. Agric. Food Chem.* **1998**, *46*, 3073−3080.

(19) Singh, R. P.; Khanna, R. K.; Mathur, A.; Srivastava, R. Isolation of oryzanol concentrate from rice bran oil. *J. Oil Technol. Assoc. India* **2000**, *32*, 55−58.

(20) Kaimal, T. N. B. Phytic acid and myo-inositol from ricebran. *J. Oil Technol. Assoc. India* **1998**, *30*, 183, 189, 191, 193, 195.

(21) Hadafi, A.; Ismaili Alaoui, M.; Chaouch A.; Benjilali, B.; Zrira, S. Antioxidant activity and phenolic acid content in rosemary (*Rosmarinus officinalis* L.) and myrtle (*Myrtus communis*). *Riv. Ital. EPPOS* **1998**, 325−340.

(22) Martin, T. S.; Kikuzaki, H.; Hisamoto, M.; Nakatani, N. Constituents of Amomum tsao-ko and their radical scavenging and antioxidant activities. *J. Am. Oil Chem. Soc.* **2000**, *77*, 667−673.

(23) Masella, R.; Cantafora, A.; Modesti, D.; Cardilli, A.; Gennaro, L.; Bocca, A.; Coni, E. Antioxidant activity of 3, 4-DHPEA-EA and protocatechuic acid: A comparative assessment with other olive oil biophenols. *Redox Rep.* **1999**, *4*, 113−121.

(24) Wang, M.; Li, J.; Shao, Y.; Huang, T.-C.; Huang, M.-T.; Chin, C.-K.; Rosen, R. T.; Ho, C.-T. Antioxidative and cytotoxic components of highbush blueberry (*Vaccinium corymbosum* L.). *Phytochem. Phytopharm.* **2000**, 271−277.

(25) Watanabe, M.; Ohshita, Y.; Tsushida, T. Antioxidant Compounds from Buckwheat (*Fagopyrum esculentum* Moeench) Hulls. *J. Agric. Food Chem.* **1997**, *45*, 1039−1044.

(26) Baer-Dubowska, W.; Szaefer, H.; Krajka-Kuzniak, V. Inhibition of murine hepatic cytochrome P450 activities by natural and synthetic phenolic compounds. *Xenobiotica* **1998**, *28*, 735−743.

(27) Nia, R.; Adesanya, S. A.; Okeke, I. N.; Illoh, H. C.; Adesina, S. K. Antibacterial constituents of *Calliandra haematocephala*. *Niger. J. Nat. Prod. Med*. **1999**, *3*, 58−60.

(28) Ahn, J. S.; Kwon, Y. S.; Kim, C. M. Anti-inflammatory constituents of *Polygonum bistorta*. *Saengyak Hakhoechi* **1999**, *30*, 345−349.

(29) El-Seedi, H. R. Coumarins, benzoic acids and terpenoids from *Palicourea demissa*. *Rev. Latinoam. Quim*. **1999**, *27*, 13−16.

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