Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: www.tandfonline.com/journals/iphb20

Antioxidant and Antitumor Promoting Activities of the Flavonoids from *Hedychium thyrsiforme*

Jasril, L. Y. Mooi, A. M. Ali, M. A. Sukari, A. A. Rahman, A. G. Othman, H. Kikuzaki & N. Nakatani

To cite this article: Jasril, L. Y. Mooi, A. M. Ali, M. A. Sukari, A. A. Rahman, A. G. Othman, H. Kikuzaki & N. Nakatani (2003) Antioxidant and Antitumor Promoting Activities of the Flavonoids from *Hedychium thyrsiforme*, Pharmaceutical Biology, 41:7, 506-511, DOI: 10.1080/13880200308951344

To link to this article: https://doi.org/10.1080/13880200308951344

Pharmaceutical Biology
2003, Vol. 41, No. 7, pp. 506–511

Taylor & Francis
Taylor & Francis Group

Antioxidant and Antitumor Promoting Activities of the Flavonoids from *Hedychium thyrsiforme*

Jasril[1,a], L.Y. Mooi[2], N.H. Lajis[1], A.M. Ali[2], M.A. Sukari[3], A.A. Rahman[4], A.G. Othman[1], H. Kikuzaki[5] and N. Nakatani[5]

[1]Laboratory of Phytomedicines, Institute of Bioscience, Universiti Putra Malaysia, Selangor, Malaysia; [2]Department of Biotechnology, Universiti Putra Malaysia, Selangor, Malaysia; [3]Department of Chemistry, Universiti Putra Malaysia, Selangor, Malaysia; [4]Department of Biology, Universiti Putra Malaysia, Selangor, Malaysia; [5]Food Chemistry, Graduate School of Human Life Science, Osaka City University Osaka, Japan; [a]Current address: Department of Chemistry, Faculty of MIPA, Riau University, Pekanbaru, Indonesia

Abstract

Five flavonoids, including 3,7,4′-trimethoxy-5-hydroxyflavone (**1**), 3,4′-dimethoxy-5,7-dihydroxyflavone (**2**), 5,7,4′-trimethoxy-3-hydroxyflavone (**3**), 3,5,7,4′-tetramethoxyflavone (**4**), and 7,4′-dimethoxy-3,5-dihydroxyflavone (**5**), were isolated from the rhizome of *Hedychium thyrsiforme* and assayed for antioxidant and antitumor promoting activities. The antioxidant assays showed that 5,7,4′-trimethoxy-3-hydroxyflavone, 7,4′-dimethoxy-3,5-dihydroxyflavone and 3,4′-dimethoxy-5,7-dihydroxyflavone had strong activities. Only two compounds, 5,7,4′-trimethoxy-3-hydroxyflavone and 7,4′-dimethoxy-3,5-dihydroxyflavone, were found to be strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavengers with fifty percent inhibition concentration (IC_{50}) values of 92 and 119 μM, respectively. Antitumor promoting assays indicated that all the flavonoids showed strong inhibition activity towards Epstein-Barr virus (EBV) activation in Raji cells.

Keywords: *Hedychium thyrsiforme* Smith., antioxidant activity, antitumor promoting activity, flavonoids.

Introduction

Hedychium (Zingiberaceae) is widely distributed in the eastern Himalayas among which few species are found in Southeast Asia (Burkill, 1966). Several species of *Hedychium* are used in eastern traditional medicine. For example, a decoction of the stem near the rhizome of *H. coronarium* J. Koenig may be used as a gargle and the juice of the chewed stem is applied to swelling. A decoction of the roots of *H. longicornutum* Baker is used as a vermifuge and to treat earaches (Burkill, 1966; Perry, 1980). Previous studies on *H. spicatum* Buch. and *H. coronarium* J. Koenig resulted in the isolation of some labdane diterpenes (Sharma et al., 1976; Itokawa et al., 1988; Singh et al., 1991; Nakatani et al., 1994). In our preliminary investigation on the antioxidative potential of the Zingiberaceous species, we found that the crude methanol and dichloromethane extracts of *H. thyrsiforme* Smith showed strong activity in the ferric chloride and 1,1-diphenylpicrylhydrazyl (DPPH) radical scavenging assays. To our knowledge, there has not been any scientific study on this species reported in the literature. We report herein the isolation of the antioxidative components of *H. thyrsiforme* as well as their anti-tumor promoting activity.

Materials and methods

General

Melting points were determined on a Kofler hot plate and were uncorrected. UV spectra were recorded on a Shimadzu UV-VIS 160 and IR spectra on a Perkin Elmer 1650 FTIR spectrometers. ^{1}H- and ^{13}C-NMR spectra were obtained using a Varian Unity 500 spectrometer at 500 and 125 MHz, respectively, in $CDCl_3$ using TMS as internal standard. Mass spectra were recorded on a Hitachi M-2000 spectrometer with ionization induced by electron impact at 70 eV. α-Tocopherol, *tert*-butyl-4-hydroxyltoluene (BHT) and DPPH were purchased from Wako Pure Chemical Industries.

Accepted: May 15, 2003

Address correspondence to: Prof. Dr. Nordin Hj. Lajis, Institute of Biosciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia. Fax: +603 89423087; E-mail: nhlajis@ibs.upm.edu.my

1388-0209/03/4107-506$22.00

Quercetin was purchased from Sigma Chemical Company. Column and preparative thin-layer chromatography (TLC) utilized Merck silica gel 60 (0.063–0.200 mm) and Merck silica gel 60 PF_{254} containing gypsum, respectively.

Plant materials

The rhizomes of *Hedychium thyrsiforme* were collected from Plant Genetic Resources Centre, Universiti Putra Malaysia in January 2000. The herbarium voucher specimen was identified by A.A. Rahman and deposited at the Herbarium of the Biology Department, Universiti Putra Malaysia.

Extraction and isolation

Ground air-dried rhizomes (800 g) of *Hedychium thyrsiforme* were successively extracted with *n*-hexane and dichloromethane at room temperature. The *n*-hexane extract yielded a yellow solid upon standing and it was recrystallized from *n*-hexane-dichloromethane (3 : 1) mixture to give 235 mg of compound **1**. The *n*-hexane supernatant was further concentrated under reduced pressure to give 3.5 g solid and then fractionated by column chromatography (cc) on silica gel eluting with *n*-hexane followed by dichloromethane and acetone in an increasing polarity manner to give 22 fractions. The fractions were pooled according to their TLC patterns to make five combined fractions (A-E). The combined fraction A was subjected to preparative TLC to give 14 mg of compound **5**. Recrystallization of the combined fraction B and purification of the combined fraction C on a column (Si gel) gave further 142 mg of compound **1**. The concentrated dichloromethane extract was subjected to cc (Si gel) eluting with the same solvent system as in the fractionation of *n*-hexane extract to give 30 fractions which were then combined according to their TLC pattern to give nine fractions (A2-I2). The combined fraction A2 was crystallized to give a further 315 mg of compound **1**. Recrystallization of the combined fraction D2 and the combined fraction F2 from dichloromethane-acetone (3 : 2) gave compounds **2** (326 mg) and **3** (154 mg), respectively. Compound **4** (436 mg) was isolated after purification of combined fraction G2 and H2 on cc (Si gel) followed by preparative TLC.

Antioxidant activity using the ferric thiocyanate (FTC) method

The detection of lipid peroxide and the preparation of solutions were carried out according to the method described by Kikuzaki and Nakatani (1993). The sample solution was prepared by dissolving the 2 mg test sample (final concentration 0.02% w/v), and linoleic acid (0.10 g) in 8.0 ml of 99.6% ethanol, 8.0 ml of phosphate buffer (0.05 M, pH 7.0) and 3.9 ml of distilled water. The vial containing the solution was then placed in the dark at 40 °C. Oxidation of linoleic acid was monitored by the following procedures. To 0.1 ml of sample solution was added 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride ($FeCl_2$) in 3.5% hydrochloric acid, the absorbance of the mixture was measured at 500 nm. The readings were taken every 24 h until one day after the control reached maximum reading.

Antioxidant activity using the thiobarbituric acid (TBA) method

This test was conducted according to the method of Kikuzaki and Nakatani (1993). The same sample solutions prepared for the FTC method were used. To 2 ml of the sample solution, was added 1 ml of 20% aq. trichloroacetic acid and 2 ml of aq. thiobarbituric acid solution. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant liquid was measured at 532 nm. Antioxidant activity was recorded based on absorbance on the 8th day (final day).

Antioxidant activity using the DPPH free radical scavenging assay

This assay was performed in a 96-well microtitre plate (Nunc). The stock solution of the test samples were prepared at 1 mg/ml concentration in methanol and diluted to the 100 µg/ml (substock solutions). One hundred µl of the substock solution was introduced into a well of the first (A) row of the microtitre plate. Into the respective wells of the following seven rows (row B–H) were then added 80, 60, 40, 20, 10 and 5 µl of the substock solutions. The stock solution in each well was diluted with methanol to make the total volume 195 µl and finally 5 µl of DPPH solution (prepared at 10 mg/ml) was added. The plate was shaken to ensure thorough mixing before placing it in the dark. After allowing it to stand for 30 min, the optical density of the solution was read using an ELISA reader at wavelength 517 nm. The IC_{50} value was determined as the concentration of sample required to scavenge 50% of DPPH free radicals (Lee et al., 1998).

Antitumour promoting activity

Cell line

The Raji cell line was obtained from Dr. Akira Murakami, Kinki University, Japan. The cell line was routinely maintained in RPMI 1640 medium and supplemented with 10% of fetal bovine serum, 10% of penicillin (5000 IU/ml) and streptomycin (5000 µg/ml) at 37 °C in a humidified atmosphere of 5% $C0_2$.

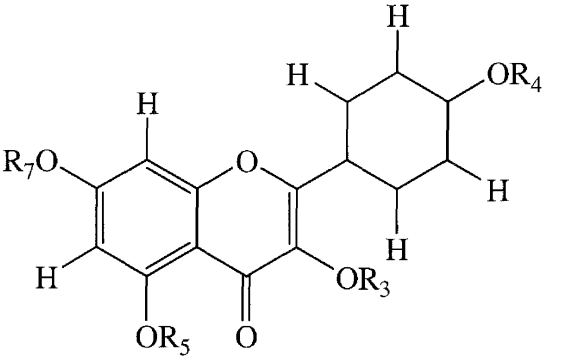
Epstein-Barr virus activation (EBV) assay

The Raji cells (5×10^5 cells/ml) in 1 ml of complete growth medium (RPMI 1640 medium and supplemented with 10% of fetal bovine serum), 10% of penicillin (5000 IU/ml) and

streptomycin (5000 μg/ml)) containing sodium *n*-butyrate (3 mM), phorbol 12-myristate 13-acetate (0.05 μM) and test substance (5 μl from a stock solution of 200 μg/ml in DMSO) were incubated at 37 °C under 5% CO_2 atmosphere for 48 h in a 24 well-plate. A 100 μl of cell suspension was removed and mixed with an equal volume of 0.4% Trypan blue solution and the percentage of cell viability was determined by counting the viable and nonviable cell using hemocytometer. The treated cells were harvested and aliquot in 1 ml Eppendorf tube and centrifuged at 1000 rpm for 10 min. The cells were washed twice with phosphate buffer saline and finally resuspended in a small volume of phosphate buffered saline (PBS). The cell suspension (15 μl) was dropped on to a Teflon coated slide and allowed to dry at room temperature. The slide was then fixed in ice cold-acetone and kept at –20 °C for 10 min. The slide was dried at room temperature and stored dry at –20 °C until future use. EBV activation was evaluated by the detection of the early antigen (EA) expression in Raji cells. EA expressed in Raji cells were detected by an indirect immunoflourescence method with EA-positive sera from nasophyregeal patient cancer (NPC), followed by fluorescence isothiocynate labeled immunoglobulin G (FITC-labeled IgG). A drop of NPC serum (1 : 20 dilution) was applied onto the fixed cells on the Teflon coated slide. The slide was incubated in a humidified chamber for 30 min at 37 °C. The slide was then rinsed carefully with PBS twice in a staining chamber for 10 min each. Tissue paper was used to remove the excess water without touching the cell surface. Briefly, a drop of FITC-labeled IgG (1 : 30 dilution) was then applied onto the cells and incubated again in a humidified chamber for 30 min at 37 °C. The slide was then rinsed carefully with PBS twice in a staining chamber for 10 min each. The excess water was removed with a tissue paper and a drop of 50% glycerol was added onto the smear. A cover slip was placed on top of the smear and viewed immediately. The fluorescent cells were counted using an inverted microscope (Nikon, Japan). The average of EA induction of the sample-treated cells were compared with the average of a control treated with PMA and sodium *n*-butyrate.

Results and discussion

Five kaempferol flavonoids including 3,7,4′-trimethoxy-5-hydroxyflavone (**1**) (Jaipetch et al., 1983; Voirin, 1983; Kamaya & Ageta, 1990; Dong et al., 1999), 3,4′-dimethoxy-5,7-dihydroxyflavone (ermanin, **2**), (Agrawal, 1989; Nakatani et al., 1991; Voirin, 1983), 5,7,4′-trimethoxy-3-hydroxyflavone (**3**) (Dong et al., 1999), 3,5,7,4′-tetramethoxyflavone (**4**) (Joseph-Nathan et al., 1981; Dong et al., 1999) and 7,4′-dimethoxy-3,5-dihydroxyflavone (**5**) (Agrawal, 1989; Kamaya & Ageta, 1990) were isolated from the rhizomes of *Hedychium thyrsiforme* (Fig. 1). The structures of these compounds were derived from the analyses of their spectral data (HFNMR, IR, UV and MS) as well as their comparison with those found in the literature.



(**1**) R_3 = Me; R_5 = H; R_7 = Me; $R_{4'}$ = Me
(**2**) R_3 = Me; R_5 = H; R_7 = H; $R_{4'}$ = Me
(**3**) R_3 = H; R_5 = Me; R_7 = Me; $R_{4'}$ = Me
(**4**) R_3 = Me; R_5 = Me; R_7 = Me; $R_{4'}$ = Me
(**5**) R_3 = H; R_5 = H; R_7 = Me; $R_{4'}$ = Me

Figure 1. Flavonoids from *Hedychium thyrsiforme*.

All compounds were assayed for antioxidant activities using FTC and TBA methods. These assays represent amounts of peroxides and the degradation products during the oxidation process, respectively, in the initial stages of lipid oxidation (Kikuzaki & Nakatani, 1993). In addition, the compounds were tested for antitumour promotion activity based on their inhibition toward EBV activation in Raji cells.

In the antioxidant activity tests, compounds **3**, **5** and **2** were more active than α-tocopherol but showed comparable antioxidant effects to *tert*-butyl-4-hydroxytoluene (BHT), a potent synthetic antioxidant (Figs. 2 and 3). Compound **1** showed a moderate activity although it still exhibits a greater activity as an antioxidant agent compared to α-tocopherol. Compound **4** was inactive in both assays.

The free radical scavenging activity of the isolated compounds has also been monitored based on scavenging activity of the stable DPPH radicals as shown in Figure 4. In this assay, antioxidant agents can react with the stable free radical of DPPH to produce 1,1-diphenyl-2-picrylhydrazine. The reaction results in the discoloration of the free radical, which was used as the indicator of antioxidant activity by measuring the absorbance at 517 nm (Lee et al., 1998). Only two of the most active compounds (**3** and **5**) in the previous assay were tested for DPPH free radical scavenging activity and found to have the IC_{50} values of 92 and 119 μM, respectively. Their activities were almost identical to that of kaempferol (IC_{50} = 113 μM). However, the activity of compounds **3** and **5** were much lower than the ascorbic acid (IC_{50} = 5.7 μM) and quercetin (IC_{50} = 33.7 μM).

Methylated kaempferols showed varying degrees of antioxidant activities. In the assays using FTC and TBA methods, the antioxidant activity followed the order of BHT ~5,7,4′-trimethoxy-3-hydroxyflavone > 7,4′-dimethoxy-3,5-dihydroxyflavone > 3,4′-dimethoxy-5,7-dihydroxyflavone >

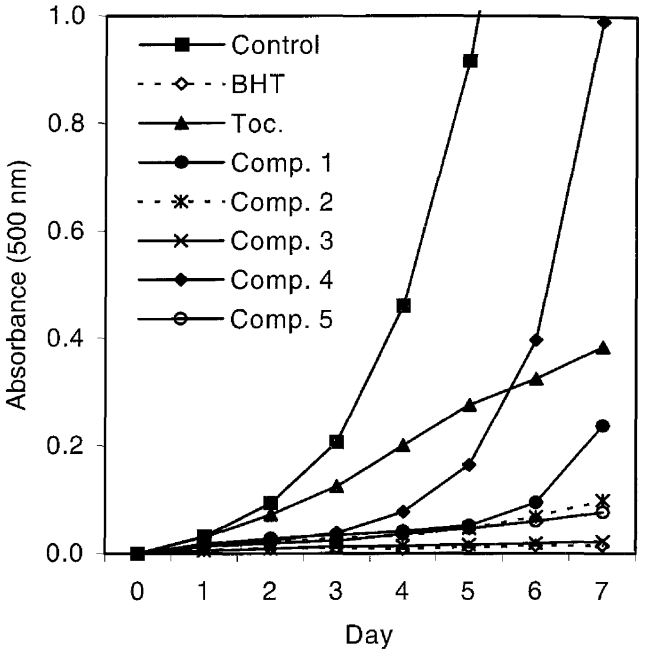


Figure 2. Absorbance values of the flavonoids isolated from *H. thyrsiforme* using the FTC method.

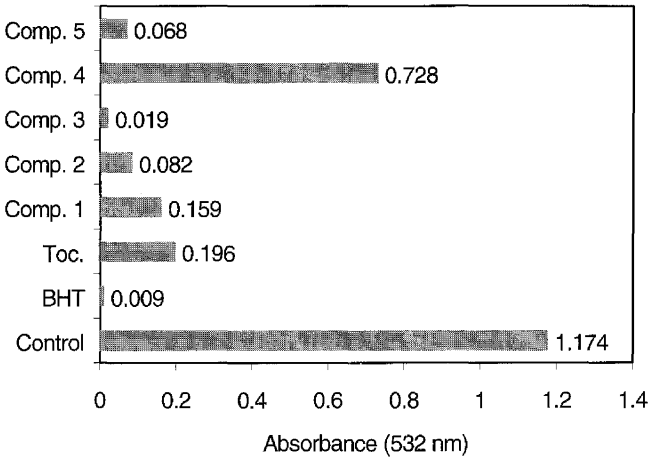


Figure 3. Absorbance values (on the 9th day) of the flavonoids isolated from *H. thyrsiforme* using the TBA method.

3,7,4′-trimethoxy-5-dihydroxyflavone > α-tocopherol > 3,5,7,4′-tetramethoxyflavone. Thus, all compounds except 3,5,7,4′-tetramethoxyflavone are stronger antioxidants than α-tocopherol and only 5,7,4′-trimethoxy-3-hydroxyflavone has an antioxidant activity comparable to that of BHT.

Methylation of the 5-, 7- and 4′-hydroxyl groups did not seem to affect the DPPH radical scavenging activity. This was shown by the activities of 5,7,4′-trimethoxy-3-hydroxyflavone and 7,4′-dimethoxy-3,5-dihydroxyflavone which were comparable in their antioxidant effect to that of kaempferol. These compounds are approximately three- to five-fold less active than quercetin and ascorbic acid, respectively. The results are consistent with previous observation on antioxidative potential of hydroxylated flavonoids (Pietta, 2000).

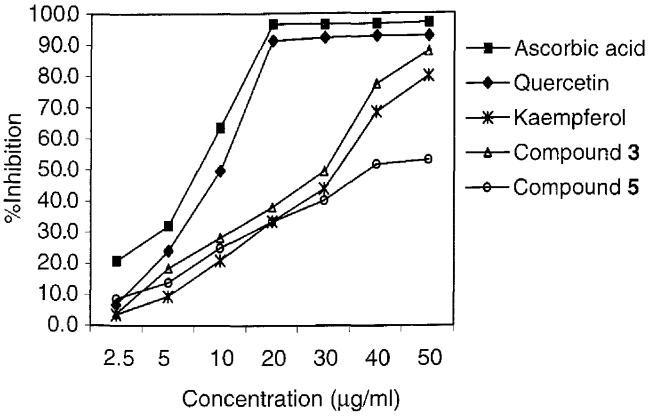


Figure 4. DPPH free radical scavenging activity of compounds **3** and **5** and the standards.

In vitro antitumor promotion was investigated to evaluate inhibitory activity of test compounds toward EBV activation in Raji cells. In this assay, phorbol-12-myristate-13-acetate (PMA) was used as tumor promoter of EBV activation. As shown in Table 1, the inhibitory rate (IR) of each test compound towards the EBV activation was classified into four ranks as follows: +++, strongly active (IR ≥ 70%); ++, moderately active (70% > IR ≥ 50%); +, weakly active (50% > IR ≥ 30%); –, inactive (30% > IR) (Murakami et al., 1998).

All the compounds exhibited strong inhibitory activity at the concentration of 10 μg/ml (Table 1). Compound **3** showed the strongest activity at 0.4 μg/ml with the inhibition rate and cell viability of 69.48 and 91.24%, respectively. However, the other compounds exhibited moderate activity at this concentration. As a reference, the inhibition rate and cell viability of genistein at 1.6 μg/ml was 63.4 and 96.1%, respectively, and that of quercetin was 58.9 and 98.8%, respectively.

The ability of flavonoids to act as antioxidants, possibly through radical scavenging mechanisms, has been well documented. In this study, the correlation of antioxidant with antitumour promoting activities could be implied. Flavonoids have been suggested to be responsible for several biological activities, including antiallergenic, antiviral, antiinflammatory, angiogenesis inhibitor, anticarcinogenic and hepatoprotective action (Middleton et al., 2000). The results of this study may also support the justification on the traditional use of this plant as a remedy for swelling.

Acknowledgements

The authors would like to thank Universiti Putra Malaysia and the Ministry of Science, Technology and Environment under the Program on Intensification of Research in Priority Areas (IRPA: 03-02-04-0043). This study was also financially supported by the Program for Promotion of Basic

Table 1. Antitumor promoting properties of flavonoids isolated from *H. thyrsiforme*.

Compound	Concentration (μg/ml)	Cell viability (%)	Inhibitory rate (%)	Activity rank
1	10.0	85.5	100.0	+++
	2.0	91.4	55.8	++
	0.4	78.2	50.6	++
2	10.0	66.8	74.7	+++
	2.0	89.4	66.8	++
	0.4	85.0	56.7	++
3	10.0	92.2	100.0	+++
	2.0	86.9	78.4	+++
	0.4	91.2	69.5	++
4	10.0	88.6	92.5	+++
	2.0	89.1	77.3	+++
	0.4	85.1	63.6	++
5	10.0	90.6	79.3	+++
	2.0	76.7	67.7	++
	0.4	85.2	53.7	++
Genistein	8.0	100.0	75.2	+++
	1.6	96.1	63.4	++
	0.032	98.8	58.9	++
Quercetin	8.0	97.4	67.8	+++
	1.6	98.8	58.9	++
	0.032	98.9	47.4	+

Research Activities for Innovative Bioscience (BRAIN), Japan.

References

Agrawal PK (1989): *Carbon-13 NMR of Flavonoids*. New York, Elsevier, pp. 161–168.

Burkill IH (1966): *A Dictionary of the Economic Products of the Malay Peninsula*, Vol. 1. Ministry of Agriculture and Cooperatives, Kuala Lumpur, pp. 1146–1148.

Dong H, Gou YL, Cao SG, Chen SX, Sim KY, Goh SH, Kini RM (1999): Eicosenones and methylated flavonols from *Amomum koenigii*. *Phytochemistry 50*: 899–902.

Itokawa H, Morita H, Takaya K, Motidome M (1988): Diterpenes from the rhizomes of *Hedychium coronarium*. *Chem Pharm Bull 36*: 2682–2684.

Jaipetch T, Reutrakul V, Tuntiwachwuttikul P, Santisuk T (1983): Flavonoids in the black rhizomes of *Boesenbergia pandurata*. *Phytochem*istry *22*: 625–626.

Joseph-Nathan P, Abramo-Bruno D, Torres MA (1981): Structural elucidation of polymethoxyflavones from shift reagent proton NMR measurements. *Phytochemistry 20*: 313–318.

Kamaya R, Ageta H (1990): Fern constituents: cheilanthenetriol and cheilanthenediol. Sesterterpenoids isolated from the leaves of *Aleuritopteris khunii*. *Chem Pharm Bull 38*: 342–346.

Kikuzaki H, Nakatani N (1993): Antioxidant effects of some ginger constituents. *J Food Sci 58*: 1407–1410.

Lee SK, Mbwambo ZH, Chung HS, Luyengi L, Gamez EJC, Mehta RG, Kinghorn AD, Pezzuto JM (1998): Evaluation of the antioxidant potential of natural products. *Combinatorial Chemistry & High Throughput Screening 1*: 35–46.

Middleton Jr. E, Kandaswami C, Theoharides T (2000): The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and anticancer. *Pharmacol Rev 52*: 673–751.

Murakami A, Morita H, Safitri R, Ramlan A, Koshimizu K, Ohigashi H (1998): Screening for *in-vitro* anti-tumour promoting activities of edible plants from Indonesia. *Cancer Detection and Prevention 22*: 516–525.

Nakatani N, Jitoe A, Masuda T, Yonemori S (1991): Flavonoid constituent of *Zingiber zerumbet* Smith. *Agric Biol Chem 55*: 455–460.

Nakatani N, Kikuzaki H, Yamaji H, Yoshio K, Kitora C, Okada K, Padolina WG (1994): Labdane diterpenes from rhizomes of *Hedychium coronarium*. *Phytochemistry 37*: 1383–1388.

Perry LM (1980): *Medicinal Plants of East and Southeast Asia*, The MIT Press.

Pietta P-G (2000): Flavonoids as antioxidants. *J Nat Prod 63*: 1035–1042.

Sharma SC, Tandon JS, Dhar MM (1976): 7-Hydroxyhedychenone, a furanoditerpene from *Hedychium spicatum*. *Phytochemistry 15*: 827–828.

Singh S, Gray AI, Skelton BW, Waterman PG, White AH (1991): (+)-14β-Hydroxylabda-8(17),12-dieno-16,15-lactone[(+)-isocoronarin-D]: A new diterpene from *Hedychium coronarium* (Zingiberaceae). *Aust J Chem 44*: 1789–1793.

Voirin B (1983): UV spectral differentiation of 5-hydroxy- and 5-hydroxy-3-methoxyflavones with mono-(4′), di-(3′,4′) or tri-(3′,4′,5′)-substituted B rings. *Phytochemistry 22*: 2107–2145.