# DPPH Free Radical Scavenger Components from the Fruits of *Alpinia rafflesiana* Wall. ex. Bak. (Zingiberaceae)

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The methanol extract of the dried ripe fruits of *Alpinia rafflesiana* was investigated for its DPPH free radical scavenger constituents. 2′,3′,4′,6′-Tetrahydroxychalcone (**7**), which has never been isolated from natural sources was found to be most active as a DPPH free radical scavenger with the $IC_{50}$ value of 55 $\mu$M. Other known compounds isolated from this species include 5,6-dehydrokawain (**1**), flavokawin B (**2**), 1,7-diphenyl-5-hydroxy-6-hepten-3-one (**3**), (−)-pinocembrin (**4**), cardamonin (**5**) and (−)-pinostrobin (**6**). The DPPH free radical scavenger compounds were detected using TLC autographic analysis. The percentage inhibition of DPPH free radical scavenging activity was measured on isolates (**5**–**7**) using colorimetric analysis.

*Key words: Alpinia rafflesiana*, Free Radical Scavenger Activity, 2′,3′,4′,6′-Tetrahydroxychalcone

## Introduction

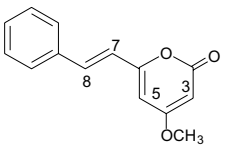
In continuing our research efforts on the anti-oxidative constituents of the Zingiberaceae family, we have selected *Alpinia rafflesiana* for further investigations based on the previous anti-oxidant activity screening results (Habsah *et al.,* 2000). Several species of *Alpinia* have been reported to contain several types of flavonoids including chalcones, flavanones, proanthocyanidin, flavonols and flavones (Sirat and Jamil, 1999; Masuda *et al*., 2000), sesquiterpenes (Miyazawa *et al*., 2000), labdane diterpenes (Sy and Brown, 1997; Sirat and Jamil, 1999), diarylheptanoids (Miyazawa *et al*., 2000; Ali *et al*., 2001), and kava pyrone (Mpalantinos *et al*., 1998; Sirat and Jamil, 1999).

The rhizomes of *A. rafflesiana* have been reported to contain four compounds including 1,7-diphenyl-5-hydroxy-6-hepten-3-one (**3**), flavokawin B (**2**), 5,6-dehydrokawain (**1**) and methyl cinnamate (Sirat *et al*., 1996). There has not been any report on the phytochemical and biological activity study of the fruits of this species. We wish to report the isolation of the new 2′,3′,4′,6′-tetrahydroxychalcone (**7**) and the DPPH free radical scavenging activity of the constituents isolated from the fruits of *Alpinia rafflesiana*.

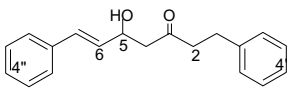
## Results and Discussion

Compounds **1**–**6** (Fig. 1) were identified by spectral data (UV, MS, IR, $^1$H NMR and $^{13}$C NMR) and by comparison with literature values of 5,6-dehydrokawain (**1**) (Itokawa *et al*, 1981; Sirat *et al*., 1996), flavokawin B (**2**), 1,7-diphenyl-5-hydroxy-6-hepten-3-one (**3**), (−)-pinocembrin (**4**) (Sirat *et al*., 1996), cardamonin (**5**) (Kuroyonogi *et al*., 1983) and (−)-pinostrobin (**6**) (Liu *et al*., 1992).

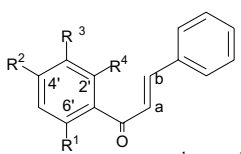
The structure of 2′,3′,4′,6′-tetrahydroxychalcone (**7**) was established by comparison of spectral data with several related 2′,3′,4′,6′-tetrasubstituted chalcones (Panichpol and Waterman, 1978). The molecular formula was determined to be $C_{15}H_{12}O_5$ by HREIMS which showed a $[M]^+$ peak at *m/z* 272.0680. The $^1$H NMR spectrum showed a pair of doublets integrated for one proton each at $\delta$ 7.74 (1H, d, $J$ = 16.1 Hz, H-$\beta$) and $\delta$ 8.23 (1H, d, $J$ = 16.1 Hz, H-$\alpha$), which indicated that the two protons were in *trans* configuration. The peaks res-



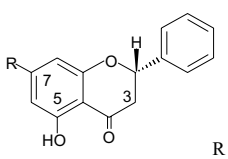
5,6-Dehydrokawain



1,7-Diphenyl-5-hydroxy-6-hepten-3-one



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| Flavokawin B | OH | $OCH_3$ | H | $OCH_3$ |
| Cardamonin | OH | OH | H | $OCH_3$ |
| 2',3',4',6'-Tetrahydroxychalcone | OH | OH | OH | OH |



| | R |
|---|---|
| (-)-Pinocembrin | OH |
| (-)-Pinostrobin | $OCH_3$ |

Fig. 1. The constituents isolated from *A. rafflesiana*.

onating as a multiplet at $\delta$ 7.42 (3H, H-3, H-4 and H-5) and $\delta$ 7.63 (2H, H-2 and H-6) were consistent with an unsubstituted phenyl ring. A singlet peak appearing at $\delta$ 5.88 (1H, H-5′) indicated the presence of tetraoxygenated phenyl carbon atoms, and was supported by four peaks [$\delta$ 164.9 (C-2′, C-6′), 165.0 (C-3′), 165.4 (C-4′) and 165.5 (C-6′)] in the $^{13}$C NMR spectrum. The $^1$H-$^1$H COSY spectrum showed that this proton (H-5′) does not have any correlation with other protons. According to these observations the compound contains an unsubstituted phenyl ring, a tetrasubstituted phenyl ring and a pair of *trans*-olefinic protons. The HMBC correlations further confirmed the structure of the compound **7** as 2′,3′,4′,6′-tetrahydroxychalcone (Fig. 2).

The chalcones isolated from *Alpinia* have so far been restricted to flavokawin B, cardamonin and

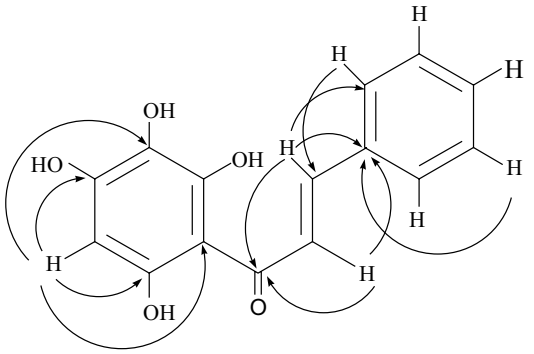


Fig. 2. Major HMBC correlations of 2′,3′,4′,6′-tetrahydroxychalcone.

dihydroflavokawain B (Itokawa *et al.*, 1981). Apparently, 2′,3′,4′,6′-tetrahydroxychalcone has never been isolated from natural source before, although few chalcones with tetrasubstitution in ring B have been reported which include 2′,3′,4′,6′-tetramethoxychalcone, 2′-hydroxy-3′,4′,6′-trimethoxychalcone and 2′,4-dihydroxy-3′,4′,6′-trimethoxychalcone, from *Popowia cauliflora* (Panichpol and Waterman, 1978).

From the TLC autographic assay, the spots belonging to **5**, **6** and **7** reduced the purple coloration of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, while those belonging to **1**, **2**, **3**, and **4** were completely inactive. Thus the $IC_{50}$ values of **5**, **6** and **7** were determined using a colorimetric analysis. The activity of 2′,3′,4′,6′-tetrahydroxychalcone ($IC_{50}$ = 55 $\mu$M) was much higher than that of vitamin C ($IC_{50}$ = 91 $\mu$M) and $\alpha$-tocopherol ($IC_{50}$ = 96 $\mu$M), but weaker than that of quercetin ($IC_{50}$ = 15.8 $\mu$M); **5** and **6** were found to be inactive (Table I). 2′,3′,4′,6′-Tetrahydroxychalcone (**7**) showed significant DPPH free radical scavenging

Table I. $IC_{50}$ values of cardamonin (**5**), (−)-pinostrobin (**6**) and 2′,3′,4′,6′-tetrahydroxychalcone (**7**) compared to those of quercetin, $\alpha$-tocopherol and vitamin C.

| Compound | $IC_{50}$ [μM] |
|---|---|
| Cardamonin | nd |
| (−)-Pinostrobin | nd |
| 2′,3′,4′,6′-Tetrahydroxychalcone | 55.0 ± 1.1 |
| Quercetin | 15.8 ± 0.6 |
| $\alpha$-Tocopherol | 96.0 ± 0.4 |
| Vitamin C | 91.0 ± 0.2 |

nd = $IC_{50}$ exceeds the concentration range which could not be determined. The absorbance of control (DPPH in MeOH) is considered as 0% inhibition.

activity, whereas flavokawin B (**2**), cardamonin (**5**) and (–)-pinostrobin (**6**) did not show any significant activity. Our results provide clear evidence that the chalcone can act as a free radical scavenger not only if its molecule has a catechol group in ring B, but also if it has a catechol group in ring A as in the structure of 2′,3′,4′,6′-tetrahydroxychalcone (**7**). Moreover, ineffectiveness of flavokawin B and cardamonin as radical scavengers was possibly due to the inability of ring A to form a co-planar conformation with the carbonyl after the radical formation at the 2′-hydroxyl, since they lack the additional free hydroxyl group to form intramolecular hydrogen bonds with the carbonyl (Mathiesen *et al*., 1997). In the case of (–)-pinostrobin, the absence of the catechol form and a double bond at C2–C3 renders it to be inactive as a free radical scavenger.

## Experimental

### *General experimental procedures*

Melting points were determined on a Kofler hot-stage apparatus and were uncorrected. The UV and IR spectra were recorded on a Shimadzu UV-VIS 160 and a Perkin-Elmer 1650 FTIR spectrometer, respectively. $^1$H and $^{13}$C NMR spectra were recorded on a Varian Unity 500 (Varian Inc., Palo Alto, CA) measured at 500 and 125 MHz, respectively, and the spectra were interpreted by the aid of field gradient correlation spectroscopy (FGCOSY), field gradient heteronuclear multiple bond correlation (FGHMBC) as well as field gradient heteronuclear quantum correlation (FGHMQC) experiments. Mass spectra were recorded on a Hitachi M2000 mass spectrometer for EIMS and Finnigan MAT95XL-T for HREIMS. The ionization voltage was 70 eV. Column chromatography utilized silica gel Merck 7734 and Merck 9385 while analytical TLC utilized Merck silica gel DC-Plastikfolien 60 $F_{254}$. Preparative TLC used in the purification of compound utilized PSC-Fertigplatten Kieselgel 60 $F_{254}$ (2.0 mm thickness, 20 × 20 cm; Merck).

### *Plant material*

The ripe fruits of *Alpinia rafflesiana* were harvested from the Medicinal Plant Garden, Laboratory of Natural Products (LNP), University Putra Malaysia (UPM) from May 2000 to February 2001. The samples were dried and kept in the refrigerator prior to use. A voucher specimen (No. 10264) was deposited at the Herbarium of LNP, UPM.

### *Extraction and isolation*

The dried material (0.5 kg) was ground and extracted five times each with 1 l MeOH. The extract was evaporated under reduced pressure to give 60 g of the methanolic crude extract. 50 g of the MeOH extract were subjected to column chromatography on silica gel (5 × 40 cm) and eluted with petroleum ether/$CHCl_3$ (1:4 v/v), followed by gradient polarity elution with EtOAc/$CHCl_3$ (1:9–1:1 v/v), to give nine combined fractions, A–I. Flavokawin B (**2**) (83 mg) was isolated from fraction B after column chromatography on silica gel eluted with 30% $CHCl_3$ in hexane, followed by multiple developed (4×) using preparative TLC ($CHCl_3$/hexane, 3:7 v/v). 5,6-Dehydrokawain (**1**) (2.6 g) was isolated as a major compound after repeated recrystallisation of fraction C from diethyl ether. 1,7-Diphenyl-5-hydroxy-6-hepten-3-one (**3**) (10 mg) was isolated after column chromatography of supernatant (diethyl ether soluble) of fraction C, eluted with EtOAc/petroleum ether (1:9 v/v) followed by recrystallisation from hexane/EtOAc. (–)-Pinocembrin (**4**) (80 mg) and cardamonin (**5**) (200 mg) were isolated as pure compounds after recrystallisation from hexane/EtOAc from fractions D and F, respectively. (–)-Pinostrobin (**6**) (100 mg) was isolated as white crystals from fraction H after washing with diethyl ether and repeated recrystallisation from $CHCl_3$/MeOH. Fraction I was triturated with EtOAc to separate the yellow amorphous solid from the dark brown solution. 2′,3′,4′,6′-Tetrahydroxychalcone (**7**) (43 mg) was isolated after column chromatography on silica gel of the yellow solid eluted with EtOAc/hexane (3:7, v/v), followed by recrystallisation from hexane/diethyl ether.

### *2′,3′,4′,6′-Tetrahydroxychalcone* (**7**)

Yellow-orange amorphous solid, m.p. 182–184 °C. – UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) = 339 nm (4.33). – IR (KBr): $\nu_{max}$ = 3520 (OH), 1614 and 1580 cm$^{-1}$. – $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ = 5.92 (1H, s, H-5′), 8.23 (1H, d, $J$ = 15.6 Hz, H-$\alpha$), 7.74 (1H, d, $J$ = 15.6 Hz, H-$\beta$), 7.63 (2H, m, H-2, H-6), 7.42 (3H, m, H-3, H-4, H-5). – $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ = 104.8 (C-1′), 164.9* (C-2′,6′), 165.0* (C-3′), 165.4* (C-4′), 128.1 (C-2,6), 128.8 (C-3,5), 192.9 (C=O), 94.9 (C-5′),

165.5* (C-6′), 135.9 (C-1), 129.9 (C-4), 127.8 (C-$\alpha$), 142.8 (C-$\beta$); *values maybe interchangeable. – MS (EI, 70 eV): *m/z* (rel. int.) = 272 (1), 270 (35), 193 (20), 166 (100), 138 (45), 77 (12), 69 (21). – HREIMS: *m/z*: 272.0680 $[M]^+$, calcd. for $C_{15}H_{12}O_5$ 272.0685

*DPPH free radical scavenging assay*

The radical scavenging assay was conducted by the modified method described previously (Cavin *et al*., 1998). Briefly, the TLC plate was spotted with the crude extract or fraction and then developed and dried. The TLC was sprayed with 0.2% 1,1-diphenyl-2-picrylhydrazyl (DPPH) methanolic solution and the plate was stored in the dark. The TLC was observed on ordinary light after 30 min. The active components appeared as yellow spots against a purple background. This method was used to locate the active DPPH free radical scavenger components from *Alpinia rafflesiana*. To determine the $IC_{50}$ value of the active component, the technique using 96-well microplates was employed (Lee *et al*., 1998). A solution of DPPH was prepared by dissolving 5 mg DPPH in 2 ml of methanol, and the solution was kept in the dark at 4 °C. A stock solution of the compounds was prepared at 1 mg/ml in DMSO. The stock solution was diluted to varying concentrations in 96-well microplates. Then, 5 $\mu$l of methanolic DPPH solution (final concentration 300 $\mu$M) was added to each well. The plate was shaken to ensure thorough mixing before being wrapped with aluminium foil and placed into the dark. After 30 min, the optical density (OD) of the solution was read using an ELISA Reader (EL340 Biokinetic reader, Bio-Tek Instrumentation, USA) at the wavelength 517 nm. A methanolic solution of DPPH served as a control. Percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [1 - \text{OD(DPPH + sample)}/\text{OD(DPPH)}] \times 100\%$$

A dose response curve was plotted to determine the $IC_{50}$ values. $IC_{50}$ is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged. The standards used in this assay were vitamin C, quercetin and $\alpha$-tocopherol (Sigma, USA).

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