# DPPH FREE RADICAL SCAVENGER ACTIVITY OF FLAVONOID FROM THE LEAVES OF FERN *Chingia sakayensis* (Zeiller) Holtt

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## ABSTRACT

*A flavonoid compound in flavonol type namely kaemferol was isolated from the ethyl acetate fraction of the methanol extract of the fern Chingia sakayensis (Zeiller) Holtt's leaves. The DPPH free radical scavenger activity of kaemferol was stronger than buthyl hyroxy toluene (BHT) but it was weaker than ascorbic acid (vitamin C) and $\alpha$-tocopherol (vitamin E).*

**Keywords:** *Chingia sakayensis, kaemferol, DPPH free radical scavenger activity*

## INTRODUCTION

One of the bioresources of Indonesia was the ferns (Pteridophytes) distributed throughout the Indonesian archipelago. Sastrapradja [1] estimated as many as 1,300 species of the ferns could be discovered in Indonesia. The ferns had been used for a long time as the ornamental plants, vegetables, traditional medicines, protector plants, fertilizer and building material [2].

*Chingia sakayensis* was one of the ferns in Thelypteridaceae family distributed in Thailand, Malaysia, Serawak, Sumatra and Java. It usually grew in the forest, often near streams, at altitude 150-1200 m. Because of the difference of environment condition, the specimens from Java and Sumatra were much ticker in texture, with very strongly raised veins and sinus membrane on the lower [3]. Until this time, the research of it's the secondary metabolites had been never reported. Therefore we were conducting the chemical investigation to it intensively and carrying out the bioactivity assays to its isolates.

In this paper, we reported the isolation and structure determination of a flavonoid isolated from the leaves of *C. sakayensis* as well as the evaluation of its DPPH free radical scavenger activity.

## EXPERIMENTAL SECTION

### General Experimental Procedures

Melting point was measured by Fisher John melting point apparatus and was uncorrected. The UV spectra were recorded on Shimadzu Pharmaspec UV-1700 spectrophotometer. The IR spectrum in KBr film was determined by JASCO FT/IR-5300 spectrophotometer. The $^{1}$H-NMR (400 MHz) , $^{13}$C-NMR (100.5 MHz) and 2D-NMR spectra were measured by JEOL JNM-ECP 400 spectrometer using tetra methyl silane (TMS) as the internal standart. The Mass spectrum (MS) was recorded on JEOL JMS-AX 500 spectrometer using ion mode FAB$^{+}$ and 3-nitro benzyl alcohol (m-NBA) as matrix. Kieselgel 60 GF-254 (Merck) and silica gel G 60 230-400 mesh (Merck) were used for vacuum liquid chromatography (VLC) and flash chromatography (FC), respectively. Precoated silica gel 60 GF-254 (Merck) was used for thin layer chromatography (TLC) and spots were detected by spraying with the sulphuric acid solution 5% (v/v) in ethanol followed by heating. DPPH (1,1-diphenyl-2-picrylhydrazil)(Sigma), ascorbic acid (Merck), $\alpha$-tocopherol (Merck) and BHT (Sigma) were used in assay of DPPH fee radical scavenger activity.

### Plant Material

The leaves of *C. sakayensis* was collected from Kletak forest, Nongkojajar, Pasuruan, East Java, Indonesia in January 2002. The sample was identified by Mr. Wardaya from the Purwodadi Botanical Garden, Indonesia and a voucher spesimen was deposited at the herbarium of the Purwodadi Botanical Garden, Indonesia.

### Isolation

The dried powdered leaves of *C. sakayensis* (1.5 kg) was exhaustively extracted successively with n-hexane (31.5 L), dichloromethane (25.5 L)

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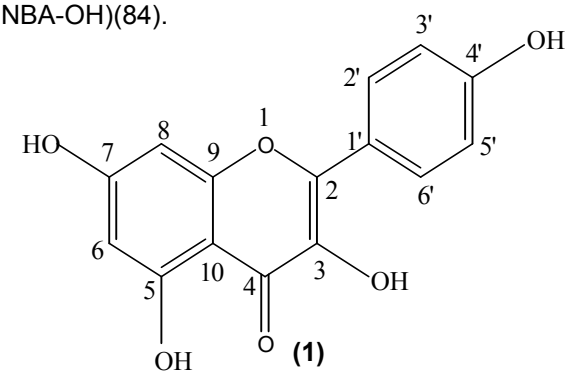
and methanol (24.0 L) at room temperature. The methanol extract was evaporated in vacuo to obtain the brown solid (203.5 g). Furthermore it was reextracted four times with 400 mL of ethyl acetate-water (1:1) mixture. Removal of the solvent under reduced pressure of the ethyl acetate fraction afforded the greenish brown solid (28.3 g). A part of it (8.34 g) was chromatographed by VLC and eluted with solvents of increasing polarity (n-hexane, n-hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH) yielded 200 fractions. Removal of the solvent under reduced pressure of the collection fractions 125-180 gave the brownish yellow solid (270.8 mg). A part of it (203.3 mg) was chromatographed by FC with $CHCl_3$-acetone (4:1) as eluen, obtained 25 fractions. The fractions 6-9 were collected, recrystalized in $CHCl_3$-acetone yielded compound 1 (28 mg). It showed a single spot by TLC on silica gel with Rf = 0.29 ($CHCl_3$ : acetone = 4 : 1), Rf = 0.57 ($CHCl_3$ : acetone = 1 : 1) and Rf = 0.71 ($CHCl_3$ : methanol = 3:1).

Compound 1 was obtained as yellow crystal ($CHCl_3$-acetone), mp. 271-273$^oC$, which gave positive test (green colour) with $FeCl_3$ and Shinoda test (Mg-HCl). UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) : 273 (2.87), 324 (sh) (2.63) and 375 (2.85) nm; (MeOH + NaOH): 285 (3.91) and 410 (3.69) nm; (MeOH+$AlCl_3$): 276 (3.00), 312 (sh) (2.49), 355 (sh) (2.53) and 432 (2.93) nm; (MeOH+$AlCl_3$+HCl): 276 (2.97), 311 (sh) (2.51), 355 (sh) (2.55) and 432 (2.89) nm; (MeOH+NaOAc): 282 (2.97) and 391 (2.81) nm; (MeOH+NaOAc+$H_3BO_3$): 274 (2.92) and 375 (2.87) nm. IR (KBr) $\nu_{max}$ : 3333 (OH), 1659 (chelated C=O), 1617, 1570, 1509 (aromatic C=C) $cm^{-1}$. $^1$H-NMR (400 MHz,$CD_3OH$) $\delta$ (ppm) : 6.18 (1H, *d*, *J* = 2 Hz, H-6); 6.39 (1H, *d*, *J* = 2 Hz, H-8); 6.90 (2H, *d*, *J* = 9 Hz, H-3' and H-5') and 8.08 (2H, *d*, *J* = 9 Hz, H-2' and H-6'). $^{13}$C-NMR (100.5 MHz, $CD_3OH$) $\delta$ (ppm) : 94.5 (C-8); 99.3 (C-6); 104.5 (C-10); 116.3 (C-3' and C-5'); 123.7 (C-1'); 130.7 (C-2' and C-6'); 137.1 (C-3); 148.1 (C-2); 158.3 (C-5); 160.5 (C-4'); 162.5 (C-9); 165.6 (C-7) and 177.4 (C-4). FABMS, m/z (rel.int.): 287 ($M+H^+$)(44), 176 (m-NBA + $Na^+$)(34), 154 (m-NBA + $H^+$)(100), 136 (m-NBA-OH)(84).




**(1)**

**Measurement of DPPH Free Radical Scavenger Activity**

In this research, DPPH free radical scavenger activity was measured by the method of Braca, et al [4]. This activity was expressed as an effective concentration at 50% ($EC_{50}$)(i.e. the concentration of the test solution required to give a 50% decrease in absorbance compared to that of a control solution). Compound **1** was dissolved in methanol to obtain the various concentrations (10,20,30,40,50,60,70 and 80 ppm). Each sample solution (300 $\mu$L) was added to 3 ml of 0.004% methanolic solution of DPPH, and then kept in the dark for 30 minutes. Absorbance was measured at $\lambda_{max}$ = 514 nm. A control was prepared by adding methanol instead of the sample solution. $EC_{50}$ value was calculated by the linier regression analysis. In the same way, it was also done the measurement of DPPH free radical scavenger activity of ascorbic acid, $\alpha$-tocopherol and BHT.

**RESULT AND DISCUSSION**

Compound 1 was isolated from ethyl acetate fraction of methanol extract of the *C. sakayensis*'s leaves. It showed the characteristic colour reaction of a flavonoid with $FeCl_3$ test (green) and Shinoda-test (orange). The presence of two peaks at $\lambda$ = 273 nm (band II) and 375 nm (band I) in the UV spectrum supported that compound 1 was a flavonol with a free 3-hydroxyl group [5]. The batochromic shift of band I on adding NaOH reagent (35 nm) and $AlCl_3$ + HCl reagent (57 nm) showed the presence of a hydroxyl group at C-4' and C-5, respectively. The presence of a hydroxyl group at C-7 was exhibited by batochromic shift of band II (9 nm) on adding NaOAc reagent. No batochromic shift on adding NaOAc + $H_3BO_3$ reagent supported that compound 1 did not have ortho-di hydroxyl group. The IR spectrum of 1 clearly disclosed absorbtion bands for OH group (3333 $cm^{-1}$), chelated carbonyl group (1659 $cm^{-1}$) and aromatic ring (1617, 1570 and 1509 $cm^{-1}$ ).

The $^1$H-NMR spectrum of 1 exhibited four doublet proton signals at $\delta_H$ : 6.18; 6.39; 6.90 and 8.08 ppm. Two doublet proton signals at $\delta_H$ : 6.18 and 6.39 ppm with *J* = 2 Hz (meta position) due to H-6 and H-8, respectively, supported the presence of a hydroxyl group at C-5 and C-7 in compound 1. While two doublet proton signals at $\delta_H$ : 6.90 and 8.08 ppm with *J* = 9 Hz (ortho position) due to H-3',5' and H-2',6', respectively, confirmed the presence of a hydroxyl group at C-4'. The $^{13}$C-NMR spectrum exhibited 15 carbon signals which corresponded to compound 1, containing five oxy aryl carbons [$\delta_C$ = 148.1 (C-2); 158.3 (C-5); 160.5 (C-4'); 162.5 (C-9) and 165.6 (C-7)], one oxy olefine

carbon [$\delta_C$ =137.1 (C-3)] and one carbonyl carbon [$\delta_C$ = 177.4 (C-4)]. The FABMS spectrum showed quasi molecular ion peak at m/z = 287 [M + $H^+$] which corresponded to compound 1 with molecular formula $C_{15}H_{10}O_6$.

The correlation spectroscopy homonuclear (COSY $^1$H-$^1$H) and heteronuclear (HSQC and HMBC) (Table 1, Figure 1 and Figure 2) supported that compound 1 was kaemferol (3,5,7,4'-tetrahydroxy flavone).

Futher supporting evidence of structure 1 for kaemferol came from comparison of the $^1$H-NMR and $^{13}$C-NMR spectral data with those of reported data in literature [6,7,8]. From the above results, compound 1 was proposed for the structure of kaemferol.

The measurement of DPPH free radical scavenger activity showed that $EC_{50}$ value of kaemferol, ascorbic acid, $\alpha$-tocopherol and BHT were 58.305 ; 38.176 ; 49.813 and 58. 869 ppm. Thus DPPH free radical scavenger activity of kaemferol was stronger than BHT but was weaker than ascorbic acid and $\alpha$-tocopherol. Therefore kaemferol denoted the natural antioxidant which could be considered in developing the new food suplemen and food preservation. Free radical scavenger activity of kaemferol was due to the presence of hydroxyl fenolic group at C-5, C-7 and C-4' which could be oxidized easily to quinone. Moreover the presence of a double bond at C-2,3 conjugated with 4-oxo (carbonyl) group and a hydroxyl group at C-3 could enhance the free radical scavenger activity (antioxidant) of kaemferol [9].

**Table 1** $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$H COSY, HSQC and HMBC spectral data of **1**

| Position of Atom C | $^1$H-NMR ($\delta$ ppm, mult.,*J*) | $^{13}$C-NMR ($\delta$ ppm) | $^1$H-$^1$H COSY | $^1$H-$^{13}$C HSQC | $^1$H-$^{13}$C HMBC |
|---|---|---|---|---|---|
| 1 | - | - | - | - | - |
| 2 | - | 148.1 | - | - | - |
| 3 | - | 137.1 | - | - | - |
| 4 | - | 177.3 | - | - | - |
| 5 | - | 158.3 | - | - | - |
| 6 | 6.18 (*d, J* =2 Hz) | 99.3 | H-8 | C-6 | C-7,C-8,C-9,C-10 |
| 7 | | 165.6 | - | - | - |
| 8 | 6.39(*d, J* =2 Hz) | 94.5 | H-6 | C-8 | C-4,C-5,C-6,C-7,C-10 |
| 9 | - | 162.5 | - | - | - |
| 10 | - | 104.5 | - | - | - |
| 1' | - | 123.7 | - | - | - |
| 2' | 8.08 (*d, J* = 9 Hz) | 130.7 | H-3' | C-2' | C-2,C-3',C-4',C-5',C-6' |
| 3' | 6.90 (*d, J* = 9 Hz) | 116.3 | H-2' | C-3' | C-1',C-4',C-5' |
| 4' | | 160.5 | - | - | - |
| 5' | 6.90 (*d, J* = 9 Hz) | 116.3 | H-6' | C-5' | C-1',C-3',C-4' |
| 6' | 8.08 (*d, J* = 9 Hz) | 130.7 | H-5' | C-6' | C-2,C-2',C-3',C-4',C-5' |

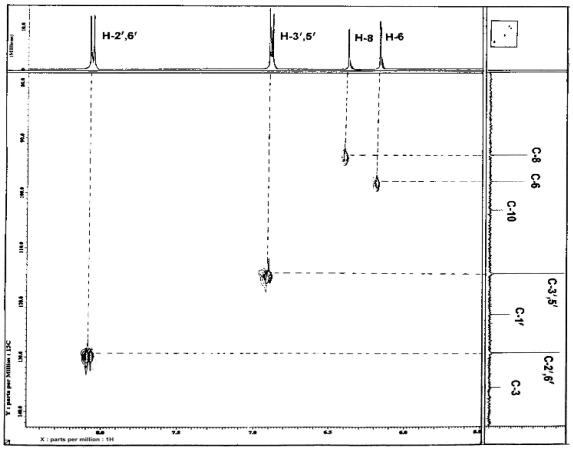



**Figure 1** HSQC spectrum of 1

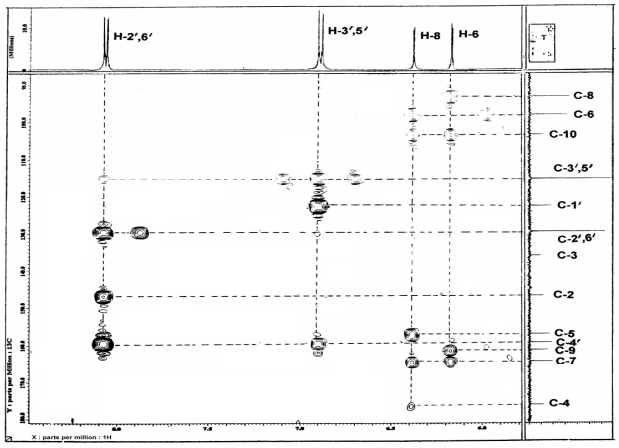



**Figure 2** HMBC spectrum of 1

## CONCLUSION

Kaemferol (3,5,7,4'-tetrahydroxy flavon) had been isolated from the ethyl acetate fraction of the methanol extract of the *Chingia sakayensis*'s leaves. DPPH free radical scavenger activity of kaemferol was stronger than BHT but was weaker than ascorbic acid and $\alpha$-tocopherol. Kaemferol denoted a natural antioxidant which could be considered in developing the new food suplemen and food preservation.

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