Antioxidant Compound from the Rhizomes of *Kaempferia rotunda* L.

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**Abstract:** This research aimed to investigate the antioxidant effect from rhizomes of *K. rotunda* for finding the active compounds by DPPH free radical scavenging activity assay. The chloroform-soluble extract of the rhizomes of *K. rotunda* showed significant scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (IC$_{50}$ = 180 µg mL$^{-1}$). Two compounds of the chloroform-soluble extract were isolated and identified. Compound 1, 2'-hydroxy-4,4',6'-trimethoxy-chalcone was found as the active constituent (IC$_{50}$ = 142 µg mL$^{-1}$). Compound 2, (+)-cretopoxide, was inactive (IC$_{50}$ = 1316 µg mL$^{-1}$). The structures of compounds 1 and 2 were identified based on the basis of spectral evidence, Mass Spectrophotometry (MS) and 2D-NMR (2 dimension of Nuclear Magnetic Resonance) data including Heteromolecular Multiple Quantum Coherence (HMQC) and Heteromolecular Multiple Bond Correlation (HMBC) and comparison to published values.

**Key words:** *Kaempferia rotunda*, anticancer prevention, DPPH free radical scavenger, chalcone, cretopoxide

**INTRODUCTION**

*Kaempferia rotunda* L. is a very closely related to *Kaempferia angustifolia* Rosc. (syn. *K. roxburghiana* Schult.; *K. undulata* Teysm. and Binnend.; *K. gilbertii* W.Bull.) (Zingiberaceae). In Java these two plants have same local name as Kunci Pepet and Kunir Putih (Riswan and Setiyowati, 2000). This plant is indigenous to southeast Asia and cultivated in Indonesia for medicinal purposes (Woerdenbag et al., 2004). It is distributed from eastern Himalaya, Laos, Vietnam, Thailand and Java. *K. rotunda* is found in teak forest, low land rice and on calcareous marl up to 150 m altitude. In Java, this plant is cultivated and flowers from October to January. This small herb, with its small roots, tubers and rhizomes are fragrant and traditionally used as abdominal pain, dysentery, diarrhea, cold, obesity, astringent (cosmetic) and after childbirth (Ibrahim, 2003). Leaves and rhizomes are eaten fresh or cooked as vegetable, used in cosmetic powder and as a food flavoring spice. Recently, the dried powder of *K. rotunda* rhizomes is famous for traditional prevention and treatment for cancer diseases. The dried powder of the rhizomes is easily available and sold in Indonesian traditional medicine markets. This powder is eaten directly or made as a drink by adding sugar and hot water. It has been speculated that the active compounds are either its curcuminoinds or polysaccharides. Formulation containing the ethanol soluble extracts of the rhizomes of *K. rotunda* in combination with the extracts of *Boesenbergia pandurata*, *Allium tuberosum* and *Phylanthus niruri* possessed platelet activating factor. The combined extracts were used for the atopic dermatitis as skin external use agent for rough skin prevention which possessed improvement and preventive effect to various skin diseases of eczema (Maeda and Ota, 2001). A formulation for skin-lightening cosmetics comprised *K. rotunda* extracts containing melarin formation and tyrosinase inhibitors was reported to be safe and effective. Earlier phytochemical study from this plant and related species led to the isolation of (+)-apoxide and the 2'-zylerol-related substances, (+)-(1R,2S,3R,4S)-2-benzoyloxyethylcyclohex-5-ene-1,2,3,4-tetrol 1,4-dibenzoate and (1R,2S,3R,4S)-2-hydroxyethylcyclohex-5-ene-1,2,3,4-tetrol 1,4-dibenzoate, together with 2'-hydroxy-4,4',6'-trimethoxychalcone, cretopoxide, boesoxinb oxide and (+)-zeyleol (Pancharoen et al., 1996). In this study, we investigated the antioxidant effect from rhizomes of *K. rotunda* for finding the active compounds by DPPH free radical scavenging activity assay.

**MATERIALS AND METHODS**

This research was conducted at Natural Product and Pharmaceutical Laboratory, Research Centre for Chemistry, Indonesian Institute of Sciences (LIPI) on 2004-2005.

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**General experimental procedures:** Melting points were measured on a Buchi B-540 apparatus and uncorrected. UV and IR spectra were measured on Hitachi U-2000 spectrometer in MeOH and on Perkin Elmer FT-IR spectrometer in KBr, respectively. 1H- and 13C-NMR spectra were measured on a Varian VXR-500 instrument at 500 MHz using TMS as internal standard. EIMS and HR-EIMS, CI-MS (iso-butane for a gas) were measured on a Hitachi M-4100 instrument. TLC was performed using Silica gel 60 F254, 0.25 mm (Merck), with detection provided by UV light (254 nm) and by spraying 10% H2SO4 solution, followed by heating, or 5% FeCl3 reagent. Gravity column chromatography was performed using silica gel for column chromatography (Merck).

**Plant material:** The fresh rhizomes of *K. rotunda* (5 kg) were collected in Purworejo Central Java, the plant was identified by Dr. S. Riswan and the voucher specimen was deposited at Herbarium Bogoriense, Bogor.

**Extraction and isolation:** The dried powdered rhizomes (1.2 kg) were defatted with n-hexane (1x2 L) to yield 18 g (IC_{50}=1000 µg mL\(^{-1}\)) of hexane extract. The defatted materials then was extracted with methanol (3x2.5 L) and evaporated to give the MeOH extract (85 g; IC_{50} = 398 µg mL\(^{-1}\)). The methanol extract was partitioned in chloroform and water, 1 L each and evaporated to obtain chloroform soluble extract (35 g; IC_{50}=180 µg mL\(^{-1}\)) and water soluble part extract. The water-soluble part was partitioned further with ethyl acetate (1:1, 1 L each), to obtain ethyl acetate soluble extract (9 g; IC_{50}=192 µg mL\(^{-1}\)) and water part. The water part was partitioned with n-butanol (1 L), then both extract were evaporated to obtain n-butanol-soluble extract (8 g; IC_{50} = 251 µg mL\(^{-1}\)) and water-soluble extract (32 g; IC_{50}>1000 µg mL\(^{-1}\)). The chloroform, ethyl acetate and n-butanol extracts were the active extracts. The ethyl acetate and n-butanol extracts were not studied further. The chloroform extract was subsequently passed through a silica gel column chromatography with chloroform as the solvent system to give 9 main fractions. Fraction 5 (IC_{50} = 160 µg mL\(^{-1}\)) was found to be the active fraction. Further chromatographic elution of the Fraction 5 with a mixture of chloroform and methanol (99:1) then chloroform and methanol (97:3) as the solvent systems, compounds 1 and 2 were isolated.

**Assay for DPPH free radical scavenger activity:** The antioxidant assay was performed on scavenging effect of stable free radicals 1,1-diphenyl-2-pircryl-hydrazyl (DPPH) (Sigma). An extract of 4 mg was dissolved in 4 mL Dimethyl Sulfoxide (DMSO) to obtain 1000 µg mL\(^{-1}\) as mother solution of test sample. This test samples were diluted with ethanol to concentrations of 10, 40, 200 and 1000 µg mL\(^{-1}\) for extracts and 10, 20, 50, 100 and 200 µg mL\(^{-1}\), for pure compounds, respectively. The test samples were mixed with the ethanol solution of 300 µM DPPH in 90-Well micro-titer plate and incubated at 37°C for 30 min. The absorption was measured at 515 nm. Inhibition percentage of the test samples was compared to that of control (DMSO), as shown in Table 1. The positive control test were use solutions of t-Butyl Hydroxy Anisole (BHA), t-Butyl Hydroxy Toluene (BHT) and ascorbic acid (Vit C). The IC_{50} value is the ability of the test sample to scavenge 50% of free radicals, DPPH (Yen and Chen, 1995).

**RESULTS AND DISCUSSION**

**DPPH free radical scavenging activity:** Scavenging effect values of extracts and isolates were various. The active compounds were presumed in the chloroform, ethyl acetate- and n-butanol-soluble extracts. Table 1 showed the scavenging effects of n-hexane-, chloroform-, ethyl acetate-, n-butanol- and water-soluble extracts. This fact indicated that more than one compound were active. In the chloroform-soluble extract, compound 1 and 2 were isolated. Only compound 1 showed DPPH free radical scavenging effect activity. However, compound 1 was less active to standard controls BHA, BHT and ascorbic acid, used in this experiment. This result at least gave indication that the traditional used as cancer prevention and anticancer traditional medicines of the rhizomes of *K. rotunda* having correlation to the active of compound 1 (as antioxidant) and compound 2 that has been known as potential anticancer compound (Kupchan et al., 1968; Slung and Tam, 1998). The speculated that probably the active compounds were its curcumainoids or polysaccharides as claimed in traditional application has not been known.

**Isolation of the antioxidant:** The methanol extract of the *K. rotunda* rhizomes showed weak activity as DPPH scavenger. However, when this extract was partitioned
Compound 1 was identified as 2'-hydroxy-4,4',6'-trimethoxy-chalcone, based on the physical and spectroscopic data of UV, IR, low and high resolution mass, 1H- and 13C-NMR (gHSQC and gHMBC) and comparison to published values (Pancharoen et al., 1989). This compound was recently isolated from K. angustifolia and K. rotunda rhizomes (Pancharoen et al., 1989; Sirat et al., 2001). The stereoisomer of the double bond at C-α and C-β was identified as a trans, judging from the fact that the β-protons of 2'-hydroxy-4,4',6'-trimethoxy chalcone did not split to a doublet (Kurosawa et al., 1978; Song-San et al., 1989; Lien et al., 2000). The gHSQC spectrum showed that the 1H-resonance at δ 7.81 (s) is crossed related to δ 145.5 (C-β) and 125.2 (C-α) supported that the H-α and H-β did not split. The gHMBC spectrum showed that the singlet peak at δ 7.81 were crossed related to 13C-NMR at δ 192.7, 130.1 and 128.4. The 1H-NMR resonance at δ 7.57 (H-2 and H-6) showed crossed related peaks to 13C-NMR at δ 114.4, 128.4, 142.5 and 161.4. The 1H-NMR resonance at δ 7.94 (H-3 and H-5) showed crossed related peaks to 13C-NMR at δ 182.4 (C-1), 130.1 (C-2 and C-6) and 161.4. The 1H-NMR resonance at δ 6.12 (H-3') showed crossed related peaks to 13C-NMR at δ 186.4 (C-2'), 1661 (C-4'), 106.5 (C-1') and 91.3 (C-3'). The 1H-NMR resonance at δ 5.98 (H-5') showed crossed related peaks to 13C-NMR at δ 1661 (C-4'), 162.5 (C-6, C-1') and 93.9 (C-3'). The methoxy peaks at δ 3.93, 3.87 and 3.85 showed crossed peaks at δ 162.5 (C-6'), 161.4 (C-4) and 1661 (C-4'), respectively. The hydroxy peak (δ 14.37, 1H, s) showed crossed related peak to δ 168.4 (C-2').

**Compound 2:** White needle crystals; mp 148-150 °C; [α]D20 = +72° (c 0.5, CHCl3). UV (MeOH): 194 (4.21), 296 (3.50), 312 (3.86), IR (KBr): νmax = 1756 (CO), 1720 (CO), 1686 (ArH), 1266 (OH); 1HNMR (500 MHz, CDCl3): δ 8.02 (2H, d, J = 7.5, 15 Hz, H-2 and H-6'), 7.18 (1H, d, J = 7.5, 15 Hz, H-5'), 7.54 (1H, d, J = 7.5, 1.5 Hz, H-4'), 7.45 (2H, d, J = 7.5, 1.5 Hz, H-3' and 5'), 5.69 (1H, d, J = 9 Hz, H-4), 4.99 (1H, d, J = 1.5, 9 Hz, H-3), 4.57 (1H, d, J = 12 Hz, H-6), 4.23 (1H, d, J = 12 Hz, H-6), 3.65 (1H, d, J = 2.7 Hz, H-7), 3.44 (1H, d, J = 2.7, 3.9 Hz, H-1), 3.04 (1H, d, J = 1.6, 3.9 Hz, H-2), 2.11 (3H, s, CH3OH at C-4) and 2.02 (3H, s, CH3OH at C-3). Its 13C-NMR spectrum showed resonance at δ 170.21 (C = O acetyl at C-4), 169.89 (C = O acetyl at C-3), 165.98 (C = O benzyl), 133.72 (C-4'), 130.01 (C-2 and 6'), 129.40 (C-1'), 128.76 (C-3' and 5'), 70.61 (C-4), 69.72 (C-3), 62.69 (C-6), 59.60 (C-5), 54.01 (C-7), 52.80 (C-2), 48.25 (C-1), 20.83 (CH2 acetyl at C-4) and 20.79 (CH2 acetyl at C-3). EI-MS: m/z (rel.int.) = 364 [M]+ (0.1), 363 [M+H]+ (0.5), 304 [M+acetyl]+ (0.1), 303 [M-H-acetyl]+ (0.4), 260 [M-benzyl]+ (0.4), 231 [M-CH3O-benzyl]+ (3.4), 227 [M-benzylCOCH]+ (1.3), 104 [M-CH3O-benzyl-2-acetyl]+ (100); CI-MS (positive mode, isobutane): 364 [M]+, HR-EI-MS: m/z = 364.1118 [M]+ (Caled for C16H15O6S, 364.1158).
Compound 2 was identified as (+)-crotopexide based on the physical (mp, a$_D$) and spectroscopic data of UV, IR, low and high resolution mass, H- and 13C-NMR (COSY, DEPT, gHSQC and gHMBC) and to published values (Shing and Tam, 1998). This compound was first discovered from the fruits of Croton macrostachys (Kupchan et al., 1968), Piper futokadzura (Takahashi, 1969) and has been shown to display significant tumor-inhibitory activity against Lewis lung carcinoma in mice and Walker intramuscular carcinosarcoma in rats. Recently this compound was also isolated from the rhizomes of K. angustifolia and K. rotunda (Pai et al., 1970; Pancharoen et al., 1996; Sirat et al., 2001) and was synthesized (Shing and Tam, 1998). In HMQC spectrum, proton peaks at δ 8.02, 7.58, 7.45 had crossed related peaks with 13C-resonances at δ 136.01, 133.72 and 128.76, respectively and were assigned for H- and 13C-of aromatic carbons, of 2’ and 6’, 4’ and 3’ and 5’. Other proton peaks δ 5.69, 4.99, 3.65, 3.44 and 3.09 had crossed related peaks with 13C-resonances δ 70.61, 69.72, 54.01, 48.25 and 52.80, were assigned for H- and 13C-resonances for C-4, 3, 7, 1 and 2, respectively. The 13C-resonance peak at δ 62.63 had crossed peaks with δ3-aromone at δ 4.57 and 4.23 was assigned as C-6 and H-6, respectively. In HMBC spectrum aromatic proton of H-2’ and 6’ (δ 7.58) had crossed peaks with δ 165.98 and 133.72, assigned for C = O benzyl and C-4’. The H-3’ and 5’ (δ 7.45) has crossed peaks with 129.40 and 133.72 assigned as C-1’ and 4’. The resonance proton of H-6 (δ 4.23 and 4.57) had crossed peaks with δ 165.98, 70.61 (C-4) and 54.01 (C-7). The H-4 (δ 5.69) had crossed peaks with 13C-NMR at δ 54.01 (C-7), 52.80 (C-2) and 170.21 (C = O, acetyl). The H-3 (δ 4.98) had crossed peaks with 13C-NMR at δ 59.60 (C-5), 48.25 (C-1) and 169.89 (C = O, acetyl). The H-2 (δ 3.09) had crossed peaks with 13C-NMR at δ 70.61 (C-4) and 54.01 (C-7).

ACKNOWLEDGMENT

The authors wish to thank to Dr. Soedarsono Riswan, Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, for plant identification.

REFERENCES


