ANTIOXIDANT COMPOUND FROM THE RHIZOMES OF *KAEMPFERIA ANGUSTIFOLIA* COLLECTED FROM PURWOREJO, CENTRAL JAVA SECONDARY FOREST

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Abstract

Kaempferia angustifolia *Rosc. (Zingiberaceae) dan* ***K. rotunda*** *L. di Jawa dikenal sebagai Kunci pepet dan Kunir putih. Kedua macam tumbuhan tersebut biasanya tumbuh di daerah hutan sekunder di Jawa. Rhizoma dari* ***K. angustifolia*** *berbentuk serbuk kering atau tunbuhan segar biasa dijual di pasar tradisional di Indonesia, dan dikenal sebagai pencegah dan pengobatan kanker secara tradisional. Ekstrak khloroform dari rhyzoma* ***K. Angustifolia*** *menunjukkan efek antioksidan (scavenging) yang signifikan terhadap radikal bebas 1,1-difenil-2 pikril hidrasil (DPPH). (IC50 = 370 ug/mL). Dua macam senyawa dari ekstrak khloroform telah diisolasi dan diidentifikasi. Senyawa 1, suatu khalkon yang telah dikenal, yaitu, 2'-hidroksi-4', 4', 6'-trimetoksi khalkon adalah senyawa yang aktif (IC_{50} = 97 ug/mL). Senyawa 2, suatu sikloheksan epoksid yang telah dikenal, (+)-krotepoksida, tidak aktif (IC_{50} = 690 ug/mL) Struktur senyawa 1 dan 2 diidentifikasi berdasarkan data spektroskopik, MS dan 2D-NMR (HMQC dan HMBC) dan dibandingkan dengan data yang telah dipublikasikan.*

Kata kunci:
Kaempferia angustifolia *Rosc, Zingiberaceae, antikanker, DPPH radikal bebas "scavenger," khalkon, (+) krotepoksida.*

Introduction

Kaempferia angustifolia Roscoe (syn. *K. roxburghiana* Schult.; *K. undulata* Teysm. & Binnend.; *K. gilbertii* W. Bull.) (Zingiberaceae) is very closely related to *K. rotunda* L. (syn. *K. longa* Jacq.). In Java these two plants have the same local names as Kunci Pepet and Kunir Putih (1). This plant is distributed from eastern Himalaya, Laos, Vietnam, Thailand, and Java. *K. angustifolia* is found in teak forest, low land rice, and on calcerous marsh up to 150 m altitude. In Java, this plant is cultivated and flowers from October to January. *K. angustifolia* is a small herb, with its small roots, tubers and rhizomes are fragrant and is traditionally used for abdominal pain, dysentery, diarrhea, cold, obesity, astringent of (cosmetic) and tonic for women after childbirth (2). Leaves and rhizomes are eaten fresh or cooked as vegetable, used in cosmetic powder and as a food flavouring spice. Recently, the dried powder of *K. angustifolia* rhizomes becomes famous for traditional prevention and treatment for caneer diseases in Indonesia. The dried powder of the rhizomes is easily available and sold in Indonesian traditional medicine markets. This powder is taken directly or made as a syrup (solution) by adding sugar and hot water. It has been speculated that the active compounds are either its curcuminoids or polysaccharides (1).

Formulation containing the ethanol soluble extracts of the rhizomes of *K. angustifolia* in combination with the extracts of *Boesenbergia pandurata*, *Allium tuberosum* and *Phyllanthus niruri* possessed platelet activating factor. The combined extracts were used for atopic dermatitis as skin external use agent for rough skin prevention which possessed improvement and preventive effect to various skin diseases or eczema (3). A formulation for skin lightening cosmetics comprised *K. angustifolia* extracts containing melanin formation and tyrosinase inhibitors was reported to be safe and effective. (4). Previous phytochemical study from this plant and related species led to the isolation of (-)-pipoxide and the 2 (+)-zeylenol-related substances, (-)-(1R,2S,3R,4S)-2-benzoyl oxymethylcyclohex-5-ene-1,2,3,4-tetrol 1, 4-dibenzoate, and (1R,2S,3R, 4S) – 2 -hydroxymethylcyclohex- 5 – ene-1,2,3,4-tetrol 1,4-dibenzoate, together with 2'-hydroxy-4,4',6'-trimethoxychalcone,crotepoxide, boesenboxide and (+)-zeylenol (5).

Materials and Methods

General

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. ^{1}H- and ^{13}C-NMR spectra were measured on a Varian VXR-500 instrument at 500 MHz using TMS as internal standard. EIMS and HR-EIMS, CIMS (iso-butane for a gas) were measured on a Hitachi M-4100 instrument. TLC was performed using Silica gel 60 F_{254}, 0.25 mm (Merck), with detection provided by UV light (254 nm) and by spraying 10% H_2SO_4 solution, followed by heating, or 5% $FeCl_3$ reagent. Gravity column chromatography was performed using silica gel for column chromatography (Merck).

Extraction and isolation

- *Plant material*: The fresh rhizomes of *K. angustifolia* (5 kg) were collected in Purworejo, Central Java, in August 1999. 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 (1x2l) to yield 18 g (IC_{50}>1000 µg/mL) of hexane extract. The defatted materials then was extracted with methanol (3 x 2.5 liters) and evaporated to give the MeOH extract (85 g; IC_{50} = 262 µg/mL). The methanol extract was partitioned in Chloroform and water, one liter each, and evaporated to obtain chloroform soluble extract (35 g; IC_{50}= 370 µg/mL) and water soluble part extract. The water-soluble part was partitioned further with ethyl acetate (1:1, 1 liter each), to obtain ethyl acetate soluble extract (9 g; IC_{50}=160 µg/mL) and water part. The water part was partitioned withn-butanol (1 liter), then both extract were evaporated to obtain n–butanol-soluble extract (8 g; IC_{50} = 701 µg/mL) and water - soluble extract (32 g; IC_{50}>1000 µg/mL). 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) 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.
- *Compound 1*: Yellow amorphous; mp 151-153 °C; UV (MeOH): λ_{max} (log ε) = 258 (4.20), 368 (3.50); IR (KBr): ε_{max} = 3432 (OH), 1710 (CO), 1686 (ArH), 1615 cm^{-1} (Ar); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 14.37 (1H, 2'-OH), 7.81 (2H, *s*, H_ and **H_**), 7.57 (2H, *d*,

J=10 Hz, H-2 and H-6), 6.94 (2H, *d*, J=10 Hz, H-3 and H-5), 6.12 (1H, *d*, J=2.8 Hz, H-3'), 5.98 (1H, *d*=2.8 Hz, H-5'), 3.93 (3H, *s*, 6'-OCH_3), 3.87 (3H, *s*, 4-OCH_3) and 3.85 (3H, *s*, 4'-OCH_3), ^{13}C-NMR (125 Hz, $CDCl_3$): δ 192.7 (C=O), 168.4 (C-2'), 166.1 (C-4'), 162.5 (6'), 161.4 (C-4), 142.5 (C-β), 130.1 (C-2 and C-6), 128.4 (C-1), 125.2 (C-α), 114.4 (C-3 and C-5), 106.5 (C-1'), 93.9 (C-3'), 91.3 (C-5'), 55.6 (4'-O<u>C</u>H_3), 55.5 (4-O<u>C</u>H_3) and 55.4 (2'-O<u>C</u>H_3); HR-EIMS: *m/z* (rel. int.) = 315.1057 $[M+H]^+$ (20), 314.1127 $[M]^+$ (100), 313.0906 $[M-H]^+$(65), 297.1076 $[M-OH]^+$(10), 286.0604 $[M-C_2H_4]^+$ (15), 207.0656 $[M- C_2H_4 - C_5H_3O]^+$ (35), 180.0381 $[M-C_9H_{10}O]^+$ (34), 161.0576 $[M-C_2H_4 - C_6H_5O_3]^+$ (9), 152.0436 $[M-C_9H_{10}O - CO]^+$ (10), 137.0110 $[M- C_9H_{10}O - CO - CH_3]^+$ (9), 134.0658 $[M-C_9H_8O_4]^+$ (38), 121.0678 $[M-C_9H_8O_4-CH]^+$ (38). HR-EIMS: *m/z* = 314.1127 $[M]^+$ (Calcd for $C_{18}H_{18}O_5$, 314.1405).

- *Compound 2*: White needle crystals; mp 148-150 °C; $[\alpha]_D$= + 72° (*c* 0.5, $CHCl_3$), UV (MeOH): λ_{max}(log ε) = 259 (4.12), 296 (3.50), 312 (3.86); IR (KBr): ν_{max}= 1756 (CO), 1720 (CO), 1686 (ArH), 1276, 1068 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.02 (2H, dd, *J* = 7.5; 1.5 Hz, H-2'and 6'), 7.58 (1H, dd, *J* = 7.5; 1.5 Hz, H-4'), 7.45 (2H, dd, *J*=7.5; 7.5 Hz, H-3'and 5'), 5.69(1H, *d*, J=9 Hz, H-4), 4.99 (1H, *dd*, *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, *dd*, *J*=2.7, 3.9 Hz, H-1), 3.09 (1H, *dd*, *J*=1.6, 3.9 Hz, H-2), 2.11 (3H, *s*, C<u>H</u>$_3$CO at C-4) and 2.02 (3H, *s*, C<u>H</u>$_3$CO at C-3). Its ^{13}C-NMR spectrum showed resonance at δ170.21 (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 (<u>C</u>H_3- acetyl at C-4) and 20.79 (<u>C</u>H_3- acetyl at C-3); EIMS: *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-CH-O-benzyl]$^+$ (3.4), 227 [M- benzyl- $COCH_3$]$^+$ (13.5), 104 $[M-CH_2$ O-benzyl – 2 acetyl]$^+$ (100); CI-MS (positive mode, isobutane) : 365 $[M]^+$+1. HR-EIMS: *m/z* = 364.1118 $[M]^+$ (Calcd for $C_{18}H_{20}O_8$, 364.1546).

Assay for DPPH free radical scavenger activity

The antioxidant assay was performed on scavenging effect of stable free radicals 1,1-diphenyl-2-picryl-hydrazyl (DPPH)(Sigma). An extract of 4 mg was dissolved in 4 mL DMSO to obtain 1000 µg/mL as mother solution of test sample. This test samples were diluted with ethanol to concentrations of 10, 40, 200 and 1000 µg/mL for extracts and 10, 20, 50, 100, and 200 µg/mL, 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 minutes. 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 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 (6).

Results and Discussion

Isolation of the antioxidant

The methanol extract of the *K. angustifolia* rhyizomes showed weak activity as DPPH scavenger. However, when this extract was partitioned between chloroform and water, ethyl acetate and water then n-butanol and water, the chloroform-soluble extract showed significant activity. The fraction of silica gel column showed that the significant active fraction was fraction 5. Compound **1** and **2** were isolated from this fraction.

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, ^{1}H- and ^{13}C-NMR (*gHSQC* and *gHMBC*) and comparison to published values (5). This compound was recently isolated from *K. angustifolia* and *K. rotunda* rhizomes (5,7,8). The stereochemistry of the double bond at C-α and C-β was identified as a *trans*, judging from the fact that the α , β-protons of 2'-hydroxy-4,4',6'- trimethoxy chalcone did not split to a doublet (9,10,11). The *gHSQC* spectrum showed that the two protons at δ 7.81 (s) is crossed related to δ 142.5 (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 was crossed related to the carbons at δ 192.7, 130.1 (X-6) and 128.4 (C-1). The proton at δ 7.57 (H-2 and H-6) showed crossed related peaks to the carbons at δ 114.4 (C-3), 128.4 (C-1), 142.5 (C-α) and 161.4 (C-4). The proton at δ 6.94 (H-3 and H-5) showed crossed related peaks to the carbons at δ 128.4 (C-1),130.1 (C-2 and C-6) and 161.4. The proton at δ 6.12 (H-3') showed crossed related the carbons at δ 168.4 (C-2'), 166.1 (C-4'), 106.5 (C-1'), and 91.3 (C-5'). The proton at δ 5.98 (H-5') showed crossed peaks to the carbons at δ 166.1 (C-4'), 162.5 (C-6'), 106.5 (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 166.1 (C-4'), respectively. The hydroxy peak (δ 14.37, -OH), showed crossed related peak to δ 168.4 (C-2').

Compound 2 was identified as (+)-crotepoxide based on the physical (mp, α_D) and spectroscopic data of UV, IR, low and high resolution mass, ^{1}H- and ^{13}C-NMR (COSY, DEPT, *gHSQC* and *gHMBC*) and compared to published values (12). This compound was first discovered from the fruits of *Croton macrostachys* (13), *Piper futokadzura* (14), 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 rhyzomes of *K. angustifolia* and *K. rotunda* (7,8,15) and was synthesized (12).

In HMQC spectrum, the proton peaks at δ 8.02, 7.58, 7.45 had crossed related peaks with the carbons at δ 130.01, 133.72 and 128.76, respectively, and were assigned for protons and carbons of 2'and 6', 4' and 3'and 5' on the aromatic ring. Other proton peaks δ 5.69, 4.99, 3.65, 3.44 and 3.09 had crossed related peaks with the carbons at δ 70.61, 69.72, 54.01, 48.25, and 52.80, were assigned for protons and carbons for C-4, 3, 7, 1 and 2, respectively. The carbon at δ 62.63 had crossed peaks with protons 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 benzoyloxy 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 the carbons 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 the carbons 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 the carbons at δ 70.61 (C-4), and 54.01 (C-7).

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. Figure 2, 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. angustifolia* having correlation to the active of compound **1** (as antioxidant), and compound **2** that has been known as potentia l anticancer compound (12,13). The speculation that probably the active compounds were its curcuminoids or polysaccharides has not been known.

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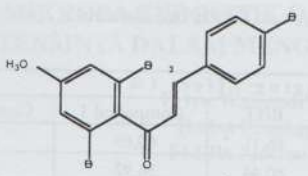
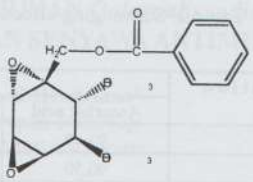


Compound 1: 2'-hydroxy-4,4',6'- trimethoxy chalcone



Compound 2 : (+)-crotepoxide

Table 1. DPPH free radical scavenging effect of extracts* of the rhizomes of *K. angustifolia*

Concs (µg/ml)	Scavenging Effect (%)								
	Ascorbic acid	BHA	BHT	Methanol Extract	n-Hexane Extract	Chloroform Extract	Etil Asetat Extract	n-Buthanol Extract	Water Extract
500	93,71	91,97	88,02	65,60	7,45	20,09	86,85	32,285	30,039
300	93,71	89,89	86,22	48,52	4,49	19,96	74,005	18,485	17,908
200	92,49	88,12	86,11	36,26	3,72	14,06	56,226	18,118	14,634
100	90,50	86,09	80,44	19,90	2,38	14,12	32,67	13,03	10,141
50	84,60	81,45	52,89	2,50	0,39	2,82	12,709	9,371	9,949
20	55,42	53,76	29,56	4,37	3,85	7,51	7,702	8,023	9,05
10	41,24	38,34	25,41	5,01	4,24	2,63	4,685	7,445	6,418
IC_{50} (µg/ml)	**10,82**	**15,26**	**49,69**	**262,72**	**1517,50**	**370,01**	**160,87**	**701,94**	**1329,65**

**Extracts were considered active when IC_{50} < 400 µg/ml.*

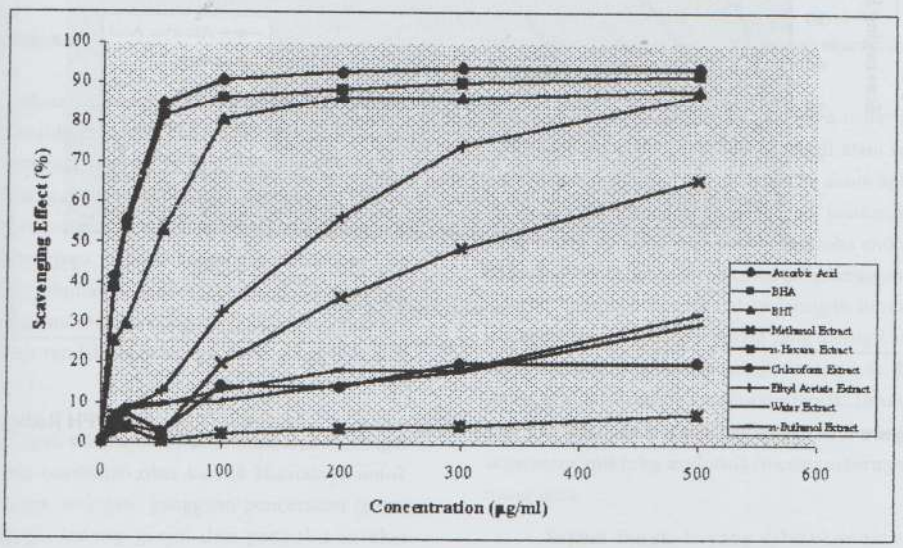


Figure 1. Scavenging Effect of *K. angustifolia* Rosc. extracts on DPPH free radicals

Table 2: Scavenging effects of the isolates* on DPPH free radicals.

Concentration (µg/ml)	Scavenging Effect (%)				
	Ascorbic acid	BHA	BHT	Compound 1	Compound 2
200	92,49	88,12	86,11	60,69	0,94
100	90,50	86,09	80,44	56,92	1,41
50	84,60	81,45	52,89	11,05	2,55
20	55,42	53,76	29,56	9,65	4,76
10	41,24	38,34	25,41	8,44	7,9
IC_{50} (µg/ml)	**10,82**	**15,26**	**49,69**	**97,68**	**690,88**

**Pure compounds were considered active when IC_{50} < 200 µg/ml.*

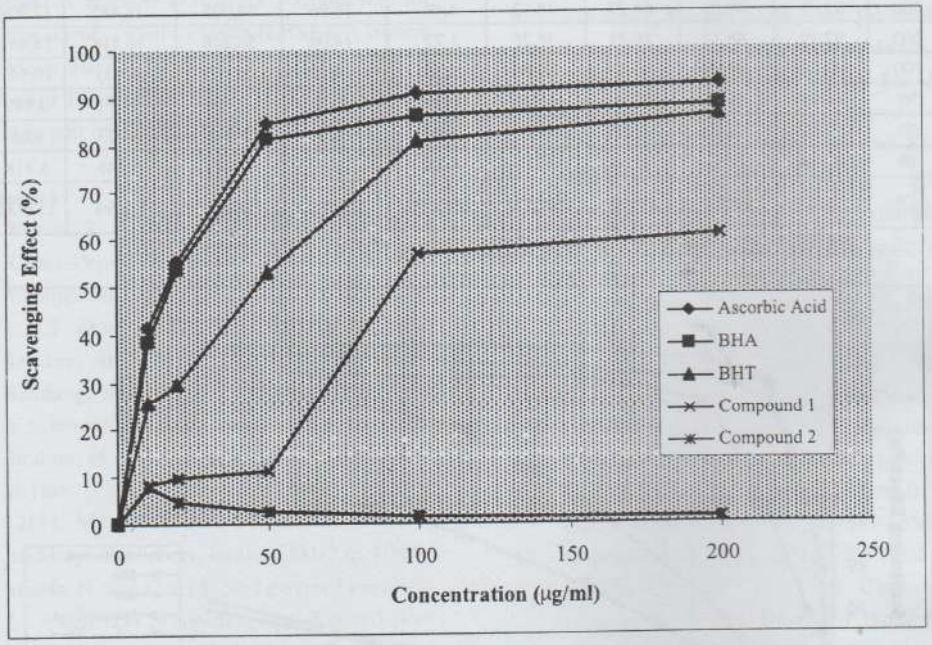


Figure 2. Scavenging Effect of Compound **1** and **2** isolated from *K. angustifolia* Rosc. on DPPH Radicals