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A Potential Cytotoxic Principle of *Zingiber cassumunar*

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Abstract – A bioassay-guided fractionation of the active chloroform extracts of the rhizomes of *Zingiber cassumunar* Roxb. led to the isolation of a potential cytotoxic principle, curcumin (**1**), along with two inactive compounds, (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (**2**) and (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (**3**). Curcumin (**1**) showed a significant cytotoxicity against several human cancer cell lines (Col2; 2.30, A549; 12.30, SNU638; IC_{50} 18.80 μg/ml)

Keywords – *Zingiber cassumunar*, Zingiberaceae, rhizome, curcumin, (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol, (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate, cytotoxicity.

Introduction

One of the Indonesian plants, *Zingiber cassumunar* Roxb., belongs to the family Zingiberaceae. The Zingiberaceae is a well-known plant family in Southeast Asia. Numerous species have been used as traditional medicines for the treatments of many diseases. For example, in Malaysia, various ginger rhizomes are widely used in the treatments of stomach problems, nausea, vomiting, epilepsy, sore throat, cough, bruises, wounds, childbirth, eyewash, sore eyes, liver complaints, rheumatism, muscular pains, ringworm, asthma, fever, malignancies, swelling and several other disorders (Burkill, 1966). Several compounds including phenybutenoids (Masuda and Jitoe, 1995; Tuntiwachwuttikul *et al.*, 1981; Jitoe *et al.*, 1993), cassumunins (Masuda *et al.*, 1993), and cassumunarins (Jitoe *et al.*, 1994) have been reported previously as the constituents of *Z. cassumunar*. This plant was found to be potential antioxidants (Masuda *et al.*, 1993; Jitoe *et al.*, 1994) and was reported to have anti-inflammatory effect (Ozaki *et al.*, 1991).

During our screening program to find anticancer potentials from terrestrial plants, the chloroform extracts of the rhizomes of *Z. cassumunar* showed significant cytotoxic activity. Herein we report a cytotoxic principle along with two inactive compounds derived from this plant.

Experimental

General – Melting points were measured on a J-923 (Jisico, Korea) and are uncorrected. Optical rotations were measured on a P-1010 digital polarimeter (JASCO, Japan) at 25°C. UV spectrum was obtained using a U-3000 spectrophotometer (Hitachi, Japan) and IR spectrum was recorded on a FTS-135 FT-IR spectrometer (Bio-Rad, USA). NMR experiments were run on a Unity INOVA 400 MHz FT-NMR (Varian, CA), and TMS was used as an internal standard. Mass data were obtained using a JMS-700 Mstation HRMS spectrometer (JEOL, Japan). Flash column chromatography was carried out on Si gel 60 (70-230 mesh, Merck, Darmstadt, Germany). Column chromatography was monitored by TLC (Si gel 60 F_{254} plates, 0.25 mm thickness) with visualization under UV light (254 and 365 nm) and 1% sulfuric acid in EtOH.

Plant materials – The rhizomes of *Zingiber cassumunar* Roxb. (Zingiberaceae) were collected in Surabaya, Indonesia, in 2001, and was identified by Prof. Tri Windono (University of Surabaya, Indonesia). The voucher specimen has been deposited at University of Surabaya.

Extraction and isolation – The dried rhizomes of *Z. cassumunar* (500 g) were ground and extracted with MeOH by percolation. The filtered MeOH extracts were evaporated under vacuum. The aqueous MeOH extract was partitioned with hexanes, chloroform, and *n*-butanol, subsequently. The $CHCl_3$ extracts (13 g) were subjected to a silica gel flash column chromatography using CH_2Cl_2-MeOH (gradient, 100:0 → 0:100) as a solvent system, to produce 13 fractions (I-XIII). Fractions III-VI eluted with CH_2Cl_2-MeOH (100:0 → 99.75:0.25) from the first column chromatography were combined due to their overlapping TLC pattern. The combined fractions (III-VI) were subjected to a Si gel CC using hexanes-acetone (100:0 → 0:100), as

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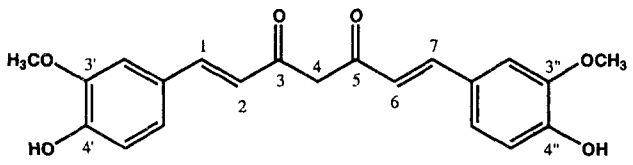

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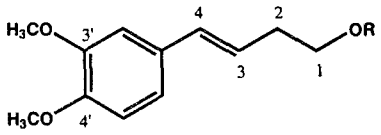

2: R = H, 3: R = Ac

a solvent system. Precipitation of compound **1** was obtained from the fraction III-10 eluted with hexanes-acetone (5:1). On the other hand, the combined factions (III-3~III-5) eluted with hexanes-acetone (20:1) were chromatographed on Si gel with hexanes-EtOAc (8:1), affording compound **2**. Fraction I eluted with CH_2Cl_2-MeOH (100:0) from the first column chromatography was subjected to a Si gel CC using hexanes-EtOAc (100:0 → 0:100), as a solvent system. Compound **3** was obtained from fraction I-7 eluted with hexanes-EtOAc (50:1).

Curcumin (1): orange solid; m.p.: 175-178°C; IR ν_{max} (KBr) cm^{-1}: 3418, 2943, 2841, 2352, 1626, 1588, 1515, 1221, 1036; UV λ_{max} log ε ($CHCl_3$): 421(4.1); EIMS *m/z* (%): 368 (25) $[M]^+$, 350 (25), 208 (100), 207 (35), 177 (30), 145 (15), 51 (10); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.59 (2H, d, *J* = 15.8 Hz, H-1 and 7), 7.12 (2H, dd, *J* = 8.0, 2.0 Hz, H-6' and 6"), 7.05 (2H, d *J* = 2.0 Hz, H-2' and 2"), 6.93 (2H, d, *J* = 8.0 Hz, H-5' and 5"), 6.48 (2H, d, *J* = 15.8 Hz, H-2 and 6) 5.80 (1H, s, H-4), 3.95 (6H, s, OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz) : 183.3 (C-3 and 5), 147.9 (C-4' and 4"), 146.8 (C-3' and 3"), 140.6 (C-1 and 7), 127.7 (C-1' and 1"), 122.9 (C-6' and 6"), 121.8 (C-2 and 6), 114.8 (C-5' and 5"), 109.6 (C-2' and 2"), 101.2 (C-4), 56.0 ($O\underline{C}H_3$); ^{1}H-^{13}C HMBC correlations ($CDCl_3$, 400 MHz): H-1, H-7/C-2', C-2", C-6', C-6", C-3, C-5; H-6', H-6"/C-2', C-2", C-5', C-5", C-1, C-7, C-4', C-4"; H-2', H-2"/C-6', C-6", C-1, C-7, C-4', C-4"; H-5', H-5"/C-1', C-1", C-3', C-3"; H-2, H-6/C-4, C-1', C-1", C-3, C-5; H-4/C-2, C-6, C-3, C-5; OCH_3/C-3', C-3".

(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (2): a pale yellow oil; IR ν_{max} (liquid film) cm^{-1}; 3400, 2935, 1599, 1460, 1263, 1139, 1025; UV λ_{max} log ε ($CHCl_3$): 209.5 (4.1); FABMS *m/z*: 208.2478[M] $^+$ calcd. 208.2595 for $C_{12}H_{16}O_3$; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 6.92 (1H, d, *J* = 1.8 Hz, H-2'), 6.89 (1H, dd, *J* = 8.2, 1.8 Hz, H-6'), 6.81 (1H, d, *J* = 8.2 Hz, H-5'), 6.43 (1H, d, *J* = 15.6 Hz, H-4), 6.07 (1H, dt, *J* = 15.6, 7.2 Hz, H-3), 3.90 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 3.75 (1H, t, *J* = 6.4 Hz, H-1), 2.47 (2H, dt, *J* = 7.2, 6.4 Hz, H-2); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 149.0 (C-3'), 148.6 (C-4'), 132.5 (C-4), 130.4 (C-1'), 124.4 (C-3), 119.1 (C-6'), 111.2 (C-5'), 108.6 (C-2'), 62.1 (C-1), 55.9 (OCH_3), 55.8 (OCH_3), 36.4 (C-2); ^{1}H-^{13}C HMBC correlations ($CDCl_3$, 400 MHz): H-2'/C-6', C-1', C-4', C-4; H-6'/C-2'; H-5'/C-1', C-3'; H-4/C-1', C-6', C-2, C-2'; H-3/C-2, C-1, C-1'; OCH_3/C-3'; OCH_3/C-4'; H-1/C-2, C-3'; H-2/C-1, C-3, C-4.

(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (3): a pale yellow oil; IR ν_{max} (liquid film) cm^{-1}: 2923, 2853, 1738, 1602, 1515, 1461, 1265, 1236, 1028; UV λ_{max} log ε ($CHCl_3$): 268.5 (4.1), 239(3.8); EIMS: *m/z* (%) = 250 (20) $[M]^+$, 190 (35), 159 (25), 117 (15), 85 (100), 83 (100), 55 (10); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 6.91 (1H, d, *J* = 1.8 Hz, H-2'), 6.88 (1H,dd, *J* = 8.0, 1.8 Hz, H-6'), 6.81 (1H, d, *J* = 8.0 Hz, H-5'), 6.41 (1H, d, *J* = 16.0 Hz, H-4), 6.03 (1H, dt, *J* = 16.0, 6.8 Hz, H-3), 4.18 (2H, t, *J* = 6.8 Hz, H-1), 3.90 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 2.53 (2H, qd, *J* = 6.8, 1.2 Hz, H-2), 2.06 (3H, s, $OCOCH_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 171.3 ($O\underline{C}OCH_3$), 149.3 (C-3'), 148.8 (C-4'), 132.3 (C-4), 130.6 (C-1'), 123.8 (C-3), 119.3 (C-6'), 111.4 (C-5'), 108.8 (C-2'), 64.1 (C-1), 56.2 (OCH_3), 56.0 (OCH_3), 32.5 (C-2), 21.2 ($OCO\underline{C}H_3$); ^{1}H-^{13}C HMBC correlations ($CDCl_3$, 400 MHz): H-2'/C-4', C-6', C-4; H-6'/C-2', C-4, C-4', H-5'/C-3', C-1', H-4/C-2', C-6', C-2; H-3/C-1', C-2; H-1/C-3, C-2, $O\underline{C}OCH_3$; OCH_3/C-3'; OCH_3/C-4'; H-2/C-4, C-3, C-1; $OCOCH_3$/$O\underline{C}OCH_3$.

Chemicals – All chemicals and reagents used were of highest purity. Trichloroacetic acid (TCA), and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Minimal essential medium with Earles salt (MEME), fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100X), trypsin-EDTA solution (1X) and antibiotic-antimycotic solution (PSF) were from GIBCO-BRL (Grand Island, NY, USA).

***In vitro* Cytotoxicity assay** – Cytotoxic potential was determined as described previously (Lee *et al.*, 1998). Briefly, cells (in log growth phase) were counted, diluted to 5×10^4 cells/ml with fresh medium, and added to 96-well microtiter plates (190 μl/well) containing test materials (10 μl in 10% aqueous DMSO). Test plates were incubated for 3 days at 37°C in CO_2 incubator. For zero day controls, the cells were incubated for 30 min at 37°C in CO_2 incubator. All treatments were performed in triplicate. After the incubation periods, the cells were fixed by the addition of 50 μl of cold 50% aqueous trichloroacetic acid (4°C for 30 min), washed 4-5 times with trap water, and air-dried. The fixed cells were stained with sulforhodamin B (SRB) (0.4% w/v SRB in 1% aqueous acetic acid) for 30 min. Free SRB

solution was then removed by rinsing with 1% acetic acid. The plates were then air-dried, the bound dye was solubilized with 200 μl of 10 mM tris-base (pH 10.0), and absorbance was determined at 515 nm using an ELISA plate reader. Finally, the absorbance values obtained with each of the treatment procedures were averaged, and the averaged value obtained with the zero day control was subtracted. These results were expressed as a percentage, relative to solvent-treated control incubations, and IC_{50} values were calculated using non-liner regression analyses (percent survival versus concentration).

Results and Discussion

The extracts of the rhizomes of *Z. cassumunar* exhibited significant cytotoxic activity during our screening program to find cytotoxic plant extracts. To the best of our knowledge, the cytotoxic activity of *Z. cassumunar* has never been reported previously. Therefore, the $CHCl_3$ extracts of the rhizomes of *Z. cassumunar* were subjected to the bioassay-guided fractionation, leading to the isolation of compound **1** as an active principle along with the inactive compounds **2** and **3.** Compound **1** showed a significant cytotoxic activity with an IC_{50} value of 2.30 μg/ml in the Col2 which is a colon human cancer cell line. In addition, compound **1** also showed a moderate cytotoxicity against the A549 (lung carcinoma) and SNU-638 (stomach cancer) cell lines with IC_{50} values of 12.30 μg/ml and 18.80 μg/ml, respectively.

However, compounds **2** and **3** did not exhibit significant cytotoxic activity in the tested cell lines in the present study. These compounds **1**, **2**, and **3** were identified as curcumin (**1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (**2**), and (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (**3**), respectively, by analysis of the 1D and 2D NMR data including DEPT, HSQC, and HMBC NMR techniques as well as by comparison of their spectral data with the published values (Syu *et al.*, 1998; Masuda and Jitoe, 1995).

In conclusion, three compounds, curcumin (**1**), (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol (**2**), and (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-yl acetate (**3**), were isolated from the Indonesian plant extracts of the rhizome of *Z. cassumunar* by the bioassay-guided fractionation, and curcumin (**1**) was considered as a potential cytotoxic principle of this cytotoxic plant extracts.

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