

IDENTIFICATION OF THE ISOLATED COMPOUNDS FROM *Zingiber amaricans* BL. RHIZOME

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ABSTRACT

Five extracts were obtained from extraction of rhizomes of *Zingiber amaricans*. Hexane, dichloromethane and methanol extracts were obtained by maceration, while dichloromethane and acetone extracts the resulted of soxhlet extraction. By column chromatography technique 2,6,9-humulantrien-9-one (zerumbone) was isolated as the major constituent of the hexane, dichloromethane and methanol extracts. The minor constituents were phytosterol mixtures isolated from hexane and dichloromethane extracts. The mixtures consisted cholesterol, campesterol, stigmasterol and β -sitosterol. The structure elucidations of zerumbone was confirmed by spectroscopic method, whereas the phytosterol mixtures was identified by gas chromatography-mass.

Keywords: zerumbone, phytosterol, *zingiber amaricans*, spectroscopy

INTRODUCTION

Zingiber amaricans Bl.(Zingiberaceae) is locally known in Central Java as "Lempuyang emprit". It is distributed throughout Indonesia and resemble with *Zingiber zerumbet* which is locally named "Lempuyang Gajah". Both plants are traditionally used as "jamu" and its can substitute each other. The differences between them are the size each parts of the plant, *Zingiber amaricans* is slightly smaller than *Zingiber zerumbet* and the rhizomes of the former are harder and darker [1]

The juice of the fresh rhizome of *Zingiber amaricans* was used as an appetizer [1], and the juice was constituent of beverage "Cabepuyang". There was no other publication on *Zingiber amaricans* but *Zingiber zerumbet* continued to become the subject for further investigations.

In 1980, Matthes *et al.* isolated one new and five known compounds from *Zingiber zerumbet* rhizomes [2], all of which showed cytotoxic activity on neoplastic rat liver cells cultured strain. The molecular structure of the new compound was established by spectral data and chemical evidences as kaempferol-3- α -L-(3",4"-O-diacetyl) rhamnopyranoside or 3",4"-O-diacetyl afzelin. The known compounds were zerumbone and zerumbone epoxide, whereas diferuloylmethane, feruloyl-*p*-coumaroylmethane and di-*p*-coumaroylmethane have not previously been reported in *Zingiber zerumbet*, but are known in *Curcuma* species.

The aqueous extract of *Z. zerumbet* showed the bronchodilator effects on isolated tracheal and ileum of Guinea pig and antiinflammatory effect on mice experiment [3,4]. On the continuing study Asmawi *et al.* reported that the water extract of *Z. zerumbet* showed the antifertility on the female, Sprague-Dawley rats.

Masuda *et al.* also studied *Z. zerumbet* collected in Ryukyus, Japan [5]. Three new acetylated and one known kaempferol glycosides were isolated from the fresh rhizomes. The molecular structures of the aglycone were established as 3-O-(2-O-acetyl- α -L-rhamnopyranoside), 3-O-(3-O-acetyl- α -L-rhamnopyranoside), 3-O-(4-O-acetyl- α -L-rhamnopyranoside) and 3-O- α -L-rhamno-pyranoside .

Zerumbone was also isolated from rhizomes of *Z. zerumbet* collected from Thailand by Murakami *et al.* [6]. The methanol extract was partitioned with CHCl_3 , and the CHCl_3 soluble fraction was subjected to silica gel column chromatography and eluted with EtOAc/*n*-hexane mixtures.

This research aim to identify the constituents of *Z. amaricans* rhizome because of the compounds content hold the principal role on the foods or beverages safety if the plants are used as their constituents.

EXPERIMENTAL SECTION

Material

Zingiber amaricans was purchased from central market Beringharjo, Yogyakarta, identified in Pharmaceutical Biology Department, University of Gadjah Mada and the voucher specimen was deposited in the herbarium of that department.

Instruments

Melting points were determined on Kohfler melting points apparatus XSP-12 Model 500X equipped with microscope and were uncorrected. Ultraviolet spectra were recorded on Shimadzu UV-VIS 160 in absolute ethanol or methanol. The IR spectra were recorded using KBr mini disc on Perkin Elmer

FTIR spectrophotometer model 1725X. Mass spectra were recorded on an AE1-MS 12 spectrometer with ionisation induced by electron impact at 70 eV. Proton Nuclear Magnetic Resonance spectra were recorded on either a JEOL FTNMR (600 MHz) or a Bruker DRX-500 (500 MHz) spectrometer with tetramethylsilane (TMS) as an internal standard. ^{13}C -Nuclear Magnetic Resonance spectra with off resonance decoupling and DEPT experiments were determined with either a JEOL FTNMR or a Bruker DRX-500 spectrometer operating at 150 and 125 MHz, respectively. The solvent used was CDCl_3 unless otherwise specified. The ^1H - ^1H COSY, NOESY, HMQC and HMBC NMR spectra were obtained with the usual pulse sequences.

Procedure

Chromatography analysis

Vacuum column chromatography was performed using Silica gel 60 PF₂₅₄ MERCK 7749 as absorbent. Length and diameter of column are 40 cm and 8 cm, respectively. The ratio of sample to the stationary phase was 1 part sample to 20 parts of stationary phase. The solvents or the mobile phases used were combination of hexane, petroleum ether, chloroform, dichloromethane, ethyl acetate, acetone, and methanol. Water pump was applied during the elution to absorb the pressure in the receiver flask. The gravity column chromatography was also used to further separate the fractions obtained from vacuum column chromatography. The column was prepared using silica gel 60 (70-230 mesh ASTM), MERCK 7734. The diameter and length of the applied column depend on the weight of sample. Generally, the ratio of sample to silica gel was 1 part of sample to 40 part of silica gel. On the special cases, if the amounts of samples are minimum, silica gel (230-400 mesh ASTM) MERCK 9385 was used.

Thin Layer chromatography (TLC) was used to detect the various components present in crude extract and fractions collected during elution or to examine the impurities of the isolated compounds. TLC plastic sheets pre-coated with Silica gel 60 F₂₅₄ (0.2 mm thickness, 20x20 cm) were used. The samples were spotted onto the proper width of TLC plate using glass capillary tubes and then developed in the chromatographic tank containing saturated solution of the mobile phase. The spots on the plates were examined under UV lamp at λ 254 and 366 nm or kept in iodine tank. Analytical gas chromatography (GC) were carried out on a Shimadzu model GC-16A gas-chromatograph fitted with CBP-1 capillary column of dimension 30 m x 0.25 mm x 0.25 μm and Helium was used as carrier gas. The GC oven temperature was programmed from 80-265°C at 3°C/minute with an initial hold 2 minute and final hold 10 minute. GC-MS analyses were performed on the gas chromatograph (Shimadzu GC-17A) coupled to a

Shimadzu GCMS-QP5050 mass spectrometer. A fused silica capillary column BPX-5 (30 m x 0.25 mm x 0.25 mm), ionisation induced by electron impact at 70 eV and ion source temperature of 170°C were applied.

Extraction and Fractionation of Extract of Rhizomes of *Zingiber amaricans*

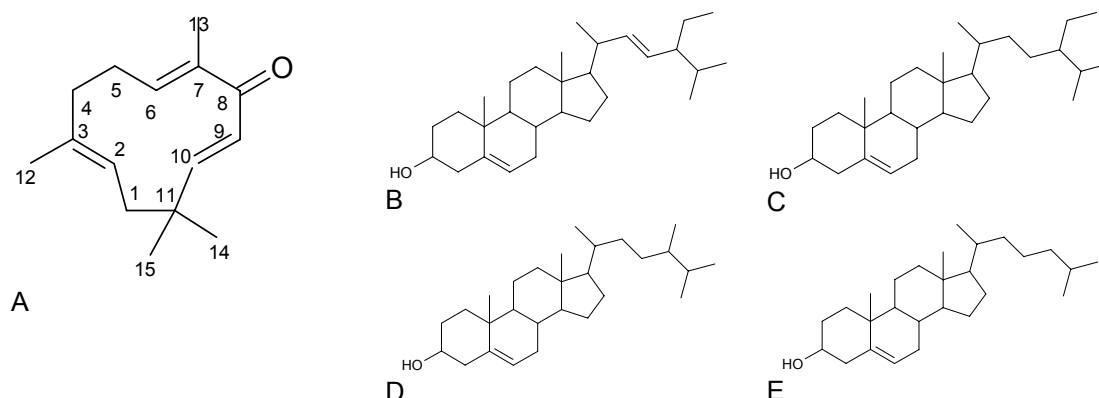
The fresh rhizomes was sliced thinly, dried at the room temperature and ground into powdered form. The sample (1.5 kg) was subsequently extracted with hexane, dichloromethane and methanol. Follow-up procedures gave corresponding hexane extract (33.1 g), dichloromethane extract (32.4 g) and methanol extract (43.3 g). Another portion of the sample (1.1 kg) was extracted using Soxhlet apparatus. The solvent used was dichloromethane, which was followed by acetone. The extractions produced 64.8 g and 6.3 g of extracts, respectively.

Isolation and Spectroscopic Analysis of Compounds from Hexane Extract

The hexane extract (5 g) was fractionated using vacuum column chromatography from which collected 35 fractions. Fractions 10-12 were combined to give residue which then afforded compound **A** (2.5 g) while fractions 26-27 give phytosterol mixtures (65 mg). Follow-up procedure on dichloromethane and methanol extract also resulted compound **A**, together with some oily mixtures and unidentified in organic compound. Similar fractionation on dichloromethane fraction obtained from Soxhlet apparatus extraction afforded only compound **A** and phytosterol mixtures. In contrast, further work on acetone extract did not lead to identification of any compounds.

RESULT AND DISCUSSION

Compound **A** appeared as colourless crystals with melting point 60-62°C. Infrared spectrum showed peak at 3025 cm^{-1} indicating the olefinic C-H stretching, and peaks at 2964, 2924 and 2864 cm^{-1} correspond to aliphatic C-H. A strong peak at 1653 cm^{-1} attributed to C=O group, while weak peaks at 1456 and 1264 cm^{-1} were signal of C=C group and C-O bond, respectively. The mass spectrum showed molecular ion at m/z 218 which corresponds to the molecular formula of $\text{C}_{15}\text{H}_{22}\text{O}$. ^1H -NMR spectrum showed four singlets each integrated to three protons at δ 1.07, 1.20, 1.54 and δ 1.80 attributed to the four methyl groups, of which two groups attached to C-3 and C-7, while other two groups attached to C-11. The two doublets ($J = 16.4$ Hz) observed at δ 1.90 and 2.35 were due to two nonequivalent protons of H-1, while methylene protons of H-4 and H-5 exhibited multiplets around δ 2.19-2.36. A doublet of doublet ($J=16.4, 4.0$ Hz) at δ 5.25 was due to the signal of H-2, which coupled to nonequivalent

**Table 1.** NMR spectroscopic data of compound A

C number	¹ H-NMR	¹³ C-NMR (ppm)	DEPT	HMQC	HMBC
1	1.90, <i>d.</i> (<i>J</i> =13.2Hz) 2.35, <i>d.</i> (<i>J</i> =13.2Hz)	42.4	CH	H-1	H-14, H-15, H-10
2	5.25, <i>dd.</i> (<i>J</i> =16.4,4.0Hz)	125.0	CH	H-2	H-1, H-4, H-12
3	-	136.3	C	-	H-4, H-5, H-12
4	2.19-2.36, <i>m</i>	39.5	CH ₂	H-4	H-2, H-12
5	2.26, <i>m</i>	24.4	CH ₂	H-5	H-4
6	6.02, <i>t.</i> (<i>J</i> =13.2Hz)	148.8	CH	H-6	H-13
7	-	137.9	C	-	H-6, H-13
8	-	204.4	C	-	H-9, H-10, H-13
9	5.97, <i>d.</i> (<i>J</i> =16.4Hz)	127.2	CH	H-9	H-10
10	5.86, <i>d.</i> (<i>J</i> =16.4Hz)	160.8	CH	H-10	H-9, H-14, H-15
11	-	37.9	C	-	H-9, H-14, H-15
12	1.54, <i>s.</i>	15.2	CH ₃	H-12	H-2, H-4
13	1.80, <i>s.</i>	11.8	CH ₃	H-13	-
14*	1.20, <i>s.</i>	24.2	CH ₃	H-14	H-15
15*	1.07, <i>s.</i>	29.4	CH ₃	H-15	H-14

Table 2. Chemical constituents of phytosterol mixtures

Number peaks	Retention Time (min)	Molecular Weight	Molecular Formula	Composition (%)	Compound
1	23.74	386	C ₂₇ H ₄₆ O	1.12	Cholesterol (E)
2	25.64	400	C ₂₈ H ₄₈ O	12.38	Campesterol (D)
3	26.47	412	C ₂₉ H ₄₈ O	30.16	Stigmasterol (B)
4	27.98	414	C ₂₉ H ₅₀ O	56.28	β-Sitosterol (C)

two protons of H-1. The triplet (*J*=13.2 Hz) observed at δ 6.02 was due to H-6 which overlapped with doublet (*J*=16.4 Hz) at δ 5.97 attributed to H-9 and the later correlated with H-10 to give another doublet (*J*=16.4 Hz) at δ 5.87.

¹³C-NMR spectrum (BCM) exhibited fifteen signals for each of fifteen carbons present in the molecule. The DEPT spectrum showed that four methine carbons appeared at 124.9, 127.2, 148.8 ppm and 160.7 ppm. HMQC spectrum confirmed the assignments of ¹H and ¹³C-NMR data. The spectrum revealed that resonances for H-1 protons at δ 2.35 and 1.90 correlated with carbon signal for C-1 (δ 42.4). The signal of methylene protons of H-4 and H-5 (δ 2.20-2.50) showed correlation with

carbon signals at δ 39.5 (C-4) and 24.4 (C-5). The crosspeaks were also shown by signals of methyl protons of C-13, C-12, C-14 and C-15 with their respective carbon signals.

Some of the long-range C-H correlations of the compound are displayed by HMBC spectrum. The positions of the methyl groups were confirmed by correlations of methyl proton signal at δ 1.54 (C-12) with carbon signal at δ 136.3 (C-3), methyl proton signal at δ 1.80 (C-13) with carbon signal at δ 137.9 (C-7), and methyl proton signal at δ 1.20/1.07 (C-14/C-15) with carbon signal at δ 37.9 (C-11). The presence of carbonyl group was also confirmed by correlation of carbon peak at δ 204.4 (C-8) with proton signals at δ

5.97 (H-9) and 1.80 (13-CH₃). The protons signal at around δ 2.19-2.36 (H-4) showed crosspeaks with carbon signals at δ 24.4 (C-5), δ 136.3 (C-3), δ 15.2 (C-12), and δ 125.0 (C-2), while proton signal at δ 2.35 (H-1) showed correlation with carbon signals at δ 125.0 (C-2) and δ 136.3 (C-3). Other HMBC correlations and other NMR data are summarized in Table 1.

Based on the spectroscopic data obtained and comparison to the data reported previously [2], compound **A** was elucidated as 2,6,9-humulatrien-8-one (zerumbone). The mass fragmentation pattern of the compound supported the assignment of the sesquiterpene.

The phytosterol mixtures was washed with acetone and followed by recrystallization using hexane to give needles shape crystals with melting point of 138-140 °C. GC – MS analysis showed that the substance consisted four component. The major components were stigmasterol (**B**)(30.16%), β -sitosterol (**C**) (56.28%), campesterol (**D**) (12.38%) and cholesterol(**E**) (1.12%). Their retention time were obtained from the chromatogram and their structures were identified based on the similarities of their mass spectra patterns with the library data in the GC-MS system. The results of GC-MS analysis are listed in Table 2. β -sitosterol (**C**); mass spectrum m/z (% intensity) : 412(M⁺, 81), 400(25), 396(20), 369(18), 351(20), 314(28), 300(45), 271(60), 255(65), 231(18), 213(36), 163(31), 159(51), 145(55), 107(58), 81(80), 55(100). Stigmasterol (**B**); mass spectrum m/z (% intensity): 412(M⁺, 81), 400(25), 396(20), 369(18), 351(20), 314(28), 300(45), 271(60), 255(65), 231(18), 213(36), 163(31), 159(51), 145(55), 107(58), 81(80), 55(100). Campesterol (**D**); mass spectrum m/z (% intensity) : 400(M⁺,53), 382 (26), 367 (23), 315 (47), 289 (48), 273 (27), 255 (33), 231 (31), 213 (60), 173 (33), 159 (63),145 (84), 133 (53), 119 (64), 105 (98), 91 (86), 81 (82), 55(100). Cholesterol (**E**); mass spectrum m/z (% intensity) : 386 (M⁺,47), 368 (23), 353 (22) , 301 (46), 275 (59), 255 (29), 231 (23), 213 (47), 159 (59), 145 (81), 119 (64) , 105 (97), 91 (91), 55 (100).

CONCLUSION

The major constituent of *Z. amaricans* rhizome was 2,6,9-humulatrien-8-one (zerumbone), and the minor constituent was a mixture of phytosterol consisted β -sitosterol, cholesterol, campesterol and stigmasterol.

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