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Anti-inflammatory kavalactones from *Alpinia zerumbet*

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ABSTRACT

Alpinia zerumbet (Pers.) B.L.Burtt & R.M.Sm. (Zingiberaceae) is a perennial plant native to the East Indies and is widely distributed in South America, Oceania, and Asia. The mature fruits of the plant have been used in traditional medicine in China. In this study, we compared the chemical constituents in the methanol extracts of the leaves, the placenta, the pericarps, and the seeds obtained from the same plant using LC-MS, and we examined the NO inhibitory activities of the respective extracts and the isolated compounds. As a result of LC-MS analyses, kavalactone derivatives (**1**–**6**) were detected in the methanol extracts of the leaves, placenta, and pericarps. Of these, compound **6** was identified as a new asymmetrical cyclobutane dimer of 5,6-dehydrokawain. Quantitative analysis showed that the total amounts of kavalactone derivatives were highest in the methanol extract of the pericarps. Moreover, the results of measurements of the anti-inflammatory activity revealed that the pericarps extract showed the strongest activity. The compounds responsible for the anti-inflammatory activity of the extracts from *A. zerumbet* were identified. Of these, five were known kavalactone derivatives and one was a new kavalactone derivative (aniba dimer C). The results showed that the pericarps of *A. zerumbet* are a rich source of kavalactone derivatives, and that the pericarps of *A. zerumbet* can be utilized as an important medicinal resource.

1. Introduction

Alpinia zerumbet (Pers.) B.L.Burtt & R.M.Sm. (Zingiberaceae) is a perennial plant native to the East Indies and is widely distributed in South America, Oceania, and Asia [1,2]. In Okinawa, Japan, the leaves of *A. zerumbet* have been used to prepare a traditional food, mu-chi, and it is widely believed that it can prevent the common cold [3]. In addition, its seeds have been used occasionally as a substitute for the crude drug Amomum Seed [4,5]. In Japan, only its seeds are considered to be possibly medicinal, whereas in China, the mature fruits (the seeds, the pericarps, and the placenta) have been used for the treatment of cardiovascular diseases by the Miao people in the Guizhou province [6]. However, the chemical constituents and biological activities of the placenta and the pericarps of *A. zerumbet* have not been well investigated.

In carrying out phytochemical studies of the plant, flavonoids, kava pyrones, phenolic acids, phenylpropanoids, sterols, and terpenoids have been isolated [7]. Among these, kava pyrones (also known as kavalactones), dihydro-5,6-dehydrokawain (DDK) and 5,6-dehydrokawain (DK), are major compounds in *A. zerumbet* [8], and it is reported that these compounds have various biological activities, such as anti-obesity [9], anti-oxidant [10], anti-glycation [11], anti-platelet [12], osteogenic [13], and neuraminidase inhibitory [14] activities. Most *in vitro* and *in vivo* studies on the anti-inflammatory activities of the extracts of *A. zerumbet* [6,15–17] have focused on the activities of the essential oils or flavonoids, and there have been few reports on the pharmacological effects of kavalactone derivatives including DDK and DK.

With regard to inflammation, nitric oxide (NO) is an important mediator, which is produced by hepatocytes (the major cell type) and Kupffer cells (resident macrophages) in the liver in response to inflammatory stimulation [18]. In primary cultured rat hepatocytes, NO is synthesized by inducible nitric oxide synthase (iNOS), and the proinflammatory cytokinin interleukin (IL) -1β induces expression of the iNOS gene. Because the induction of iNOS mimics an inflammatory response, the suppression of NO production is correlated with anti-inflammatory activity. Therefore, we have used NO as a marker to estimate the anti-inflammatory activity of medicinal plants [19,20].

In this study, we prepared the methanol extracts of different parts of *A. zerumbet* obtained from the same plant and quantitatively compared the amounts of kavalactone derivatives in the extracts using LC-MS. In addition, the NO-suppressing effects as an index of the anti-inflammatory activity of the extracts and isolated kavalactone derivatives

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Table 1
The chromatographic and mass spectrometric data of compounds in the methanol extracts of *A. zerumbet*.

Peak No.	t_R (min)	*m/z*	Abundant ions	Formula	Compounds
1	19.13	231.1013	$[M + H]^+$	$C_{14}H_{14}O_3$	7,8-Dihydro-5,6-dehydrokawain
2	19.98	229.0851	$[M + H]^+$	$C_{14}H_{12}O_3$	5,6-Dehydrokawain
3	20.63	457.1645	$[M + H]^+$	$C_{28}H_{24}O_6$	6,6′-((1*α*,2*α*,3*β*,4*β*)-2,4-Diphenylcyclobutane-1,3-diyl)bis(4-methoxy-2*H*-pyran-2-one)
4	21.92	457.1654	$[M + H]^+$	$C_{28}H_{24}O_6$	*rel*-6,6′-((1*R*,2*S*,3*R*,4*S*)-3,4-Diphenylcyclobutane-1,2-diyl)bis(4-methoxy-2*H*-pyran-2-one)
5	22.42	474.1897	$[M + NH_4]^+$	$C_{28}H_{24}O_6$	Aniba dimer A
6	22.93	474.1904	$[M + NH_4]^+$	$C_{28}H_{24}O_6$	Aniba dimer C

were examined using primary cultured rat hepatocytes.

2. Experimental

2.1. Plant materials and reagents

Alpinia zerumbet was obtained from Okinawa Chosei Yakusou Headquarters (Okinawa, Japan). All the specimens were deposited in the Museum of Materia Medica, College of Pharmaceutical Sciences, Ritsumeikan University (RIN). Analytical grade chemicals and LC-MS grades of chromatographic solvents and reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2.2. Analytical instruments

The LC-MS analyses were performed using a Shimadzu LC-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an ESI interface. The ESI parameters were as follows: source voltage, +4.5 kV (positive ion mode); capillary temperature, 200 °C; nebulizer gas flow rate, 1.5 L/min. The mass spectrometer was operated in the positive ion mode, scanning from *m/z* 150 to 1500. A Waters Atlantis T3 column (i.d. 2.1 mm × 150 mm, 5 μm) was used and the column temperature was maintained at 40 °C. The mobile phase was a binary eluent of (A) 5 mM $(NH_4)OAc$ solution and (B) CH_3CN under the following gradient conditions: 0–30 min, linear gradient from 10% to 100% B, 30–40 min, isocratic at 100% B. The flow rate was 0.2 mL/min. Semi-preparative HPLC was performed using a Shimadzu Prominence HPLC System with a SPD-20A Prominence UV/Vis Detector (Shimadzu, Kyoto, Japan). Analyses were carried out using a COSMOSIL Cholester Packed Column (i.d. 10 mm × 250 mm, 5 μm, Nacalai Tesque, Inc., Kyoto, Japan). The chromatograms were obtained by monitoring UV absorption at a wavelength of 254 nm. NMR spectra were measured in $CDCl_3$ using a JNM-ECS400 NMR spectrometer (JEOL Ltd., Tokyo, Japan) with tetramethylsilane as an internal standard. The optical rotation was measured on a DIP-370 polarimeter (JASCO Corporation, Tokyo, Japan).

2.3. Sample preparation

The leaves (2.8 g), placenta (0.9 g), pericarps (6.5 g), and seeds (8.3 g) were extracted using an Extraction System B-811 LSV (BUCHI, Flawil, Switzerland) and gave the methanol extracts of 532 mg, 144 mg, 927 mg, and 1460 mg, respectively. Samples were individually dissolved in methanol at a concentration of 10 mg/mL. Each solution was filtered through a 0.45 μm Millipore filter unit (Advantec, Tokyo, Japan), and the filtrate samples (1 μL) were injected into the LC–MS system for analysis.

2.4. Isolation of the compounds

The pericarps of *A. zerumbet* (55 g) were pulverized and extracted with methanol under reflux for 1 h, then concentrated to yield the methanol extract (4.3 g). This was suspended in water and extracted with ethyl acetate (EtOAc) and water to give an EtOAc-soluble fraction (2.5 g) and a water-soluble fraction (1.8 g). The EtOAc-soluble fraction was subjected to an ODS column (2 L UNIVERSAL COLUMN Packed with High Performance ODS, Yamazen Corporation, Osaka, Japan) with a solvent of MeOH–H_2O (8:2 → 1:0) to yield seven fractions. Fraction 2 (141 mg) was further purified by semi-preparative HPLC using isocratic elution with 55% aq. CH_3CN (flow rate; 5 mL/min) to yield compound **3** (1.9 mg). Fraction 4 (93 mg) was further purified by semi-preparative HPLC with the same condition to yield compounds **1** (16.8 mg), **2** (6.0 mg) and **5** (1.9 mg). Fraction 5 (115 mg) was further purified by semi-preparative HPLC with the same condition to give compound **4** (2.2 mg) and a new compound **6** (aniba dimer C, 2.3 mg). The structures of these isolated compounds were revealed by spectroscopic analysis and by comparing them with those reported in the literature [13,21,22]. Assignment of the NMR signals of aniba dimer A (**5**) have not as yet been fixed [21,23,24]. Therefore, we carefully assigned the NMR signals of aniba dimer A (**5**) based on the COSY, HMQC and HMBC spectral data.

Aniba dimer C (**6**): Colorless needles; $[\alpha]_D^{25}$ 0 (*c* 0.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): shown in Table 2; HRESIMS: shown in Table 1.

2.5. Quantitative analysis

The isolated compounds (**1**–**6**) were dissolved in methanol to prepare stock solutions. These stock solutions were serially diluted to obtain calibration standard solutions. Calibration was performed in the range of 1–500 μg/mL using dilutions of the respective stock solutions, and calibration graphs were prepared by plotting the respective peak area *vs* the concentration (μg/mL). Each calibration curve was prepared from three or four different concentrations.

2.6. Preparation of primary cultured rat hepatocytes

All animal care and experimental procedures were performed in accordance with the laws and guidelines of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus. Male Wistar rats (5–6 weeks old; Charles River Laboratories Japan, Inc., Yokohama, Japan) were housed at 21–23 °C under a 12 h light-dark cycle and fed with a CRF-1 diet (Charles River Laboratories Japan, Kanagawa, Japan) and had water available *ad libitum*. The animals were acclimatized to their housing. Hepatocytes were isolated from the rat liver by collagenase perfusion, according to a previously published method [25]. Briefly, pentobarbital sodium was intraperitoneally administered to the rat, and the liver was perfused with collagenase. The dispersed cells were centrifuged, resuspended, and seeded at 1.2×10^6 cells per 35-mm diameter dish. The cells were then incubated at 37 °C for 2 h. After that the medium was replaced, and the cells were incubated at 37 °C overnight until the NO assay.

2.7. Measurement of NO production and LDH activity

On Day 1, each extract or compound was added to the medium with 1 nM interleukin (IL)-1β (PeproTech, Rockey Hill, NJ), and the hepatocytes were incubated for 8 h. The nitrite (a stable metabolite of NO) in the medium was measured using the Griess method [26]. The half-maximal inhibitory concentration (IC_{50}) values against nitrite

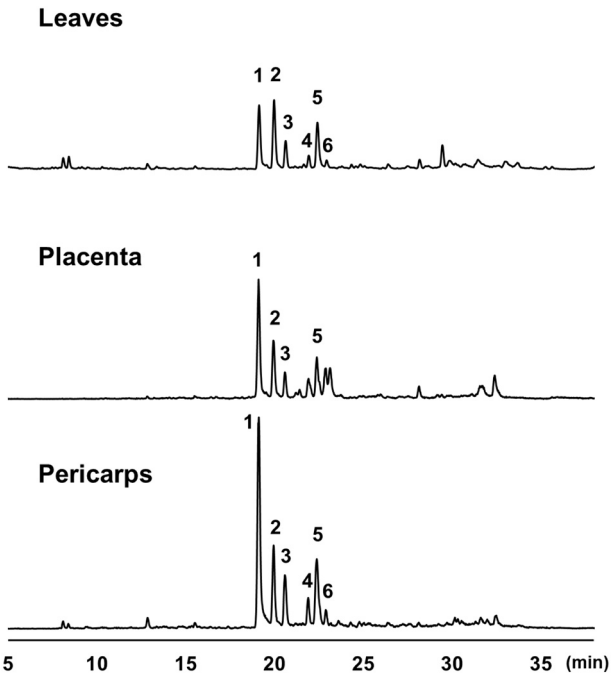

Fig. 1. Total ion chromatograms (TICs) of the methanol extracts. The peak numbers in the figure indicate the compounds in Table 1 and Fig. 2.

production were determined in triplicate for at least three different concentrations. When the extract or compound did not show cytotoxicity, the IC_{50} value was calculated to evaluate its potency to suppress NO production. Using LDH Cytotoxicity Detection Kits, the LDH activity in the medium was measured as an indicator of cytotoxicity (Takara Bio Inc., Otsu, Japan).

2.8. Statistical analysis

The results using hepatocytes are representative of at least three independent experiments that yielded similar findings. The values are presented as the mean ± standard deviation (SD). The differences were analyzed using Student's *t*-test. The significance was set at $P < .05$ and $P < .01$.

3. Results and discussion

3.1. LC-MS profiling and quantitative analysis

Fig. 1 shows LC-MS total ion chromatograms (TIC) of the methanol extracts of the leaves, placenta and pericarps of *A. zerumbet* analyzed in the positive ion mode. By comparison of the retention times and mass spectral data with those of isolated compounds, five known compounds, 7,8-dihydro-5,6-dehydrokawain (DDK, **1**) [13], 5,6-dehydrokawain (DK, **2**) [13], 6,6′-((1*α*,2*α*,3*β*,4*β*)-2,4-diphenylcyclobutane-1,3-diyl)bis (4-methoxy-2*H*-pyran-2-one) (**3**) [21], *rel*-6,6′-((1*R*,2*S*,3*R*,4*S*)-3,4-diphenylcyclobutane-1,2-diyl)bis(4-methoxy-2*H*-pyran-2-one) (**4**) [22], and aniba dimer A (**5**) [21], and one new compound (aniba dimer C, **6**) were detected as shown in Table 1 and Fig. 2. We also analyzed the methanol extract of the seeds of *A. zerumbet* by LC-MS. However, the amounts of compounds **1–6** were very low and peaks for the compounds were not detected in the TICs. Therefore, the methanol extracts from the seeds of *A. zerumbet* were not used in further studies, and the TICs of it are not shown.

The amounts of kavalactone derivatives (**1–6**) in the leaves, placenta, and pericarps of *A. zerumbet* are shown in Table 2. Of the three different parts of the plant, the pericarps contained the highest amount of DDK, and the leaves contained the highest quantity of DK. In addition, the total amount of kavalactone derivatives in pericarps was the highest, followed by the placenta and the leaves. In previous reports, of the different parts of *A. zerumbet*, the rhizomes and leaves have been reported as being rich sources of DDK and DK [8,11,27]. In this study, we show that the pericarps are also a rich source of kavalactone derivatives (**1–6**), more so than the leaves. Furthermore, it was clarified that the total amount of kavalactone dimers (**3–6**) in the pericarps was more than that in the placenta. It is considered that UV radiation could affect the production of kavalactone dimers (**3–6**) by [2 + 2] photocycloaddition [21,28,29] leading to the characteristic accumulation of kavalactone dimers (**3–6**) in the pericarps. As far as we know, this is the first report comparing the amounts of kavalactone dimers in different parts of *A. zerumbet*.

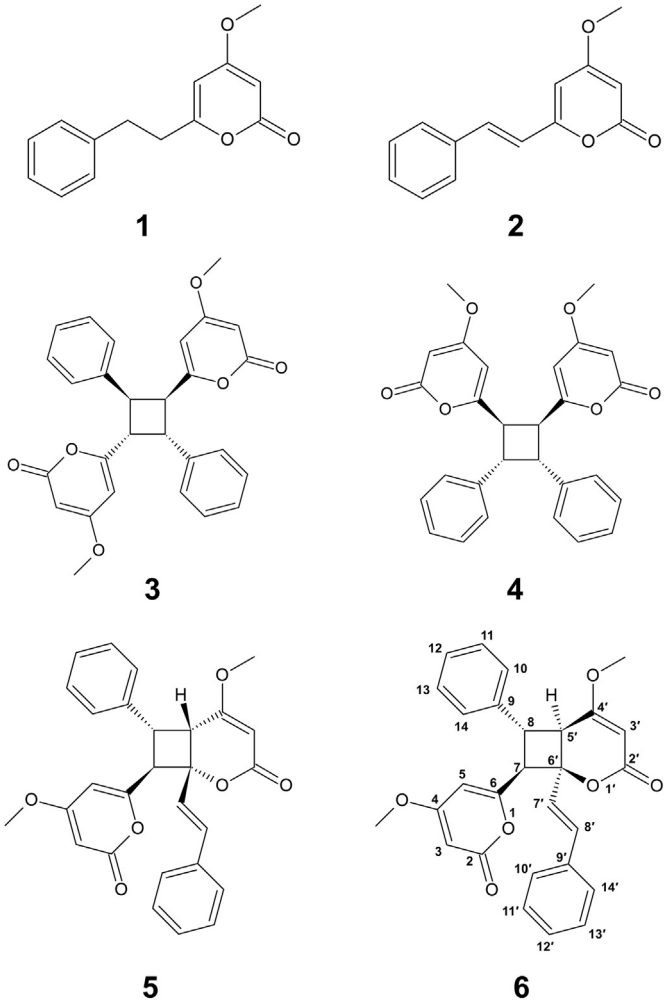

Fig. 2. Structures of compounds **1–6**. The structure of compound **5** with the relative configuration (5′*R*, 6′*S*, 7*R*, 8*R*) is shown. The structure of compound **6** with the relative configuration (5′*S*, 6′*R*, 7*R*, 8*R*) is shown.

3.2. Structure elucidation of the new compound 6

Compound **6** exhibited an $[M + NH_4]^+$ peak at *m/z* 474.1904 corresponding to the molecular formula $C_{28}H_{24}O_6$, which is the same molecular formula as that of aniba dimer A (**5**). The ^1H NMR and ^{13}C NMR data (Table 3) of **6** were very similar to those of aniba dimer A (**5**), which is an asymmetrical cyclobutane dimer of 5,6-dehydrokawain (**2**). However, there are slight differences in the chemical shifts between compounds **6** and **5** in the ^1H and ^{13}C NMR data at positions 5′, 6′, 7′, 7, 8, and 9. This suggests the two compounds have different configurations at the cyclobutane ring. The relative configuration of **6** was

Table 2
Comparison of six main compounds in the leaves, placenta, and pericarps of *A. zerumbet*.

Parts	Extraction yield (%)	kavalactone contents						Total kavalactones
		1	2	3	4	5	6	
Leaves	19.2	2145.98	2043.91	261.10	56.84	187.14	78.11	4773.08
Placenta	16.6	4874.34	1321.77	104.62	29.20	132.53	29.39	6491.85
Pericarps	14.2	5524.10	1561.70	521.78	162.15	361.42	127.15	8258.29

Values are expressed in μg / g of dried each parts of *A. zerumbet*.

Table 3
^{13}C NMR (100 MHz) and ^{1}H NMR (400 MHz) data of compound **5** and **6** in $CDCl_3$ (δ in ppm).

position	δ_C		δ_H, mult (*J* in Hz)	
	5	**6**	**5**	**6**
2	163.9	165.3		
3	88.7	89.0	5.36 d (2.1)	5.48 d (2.1)
4	170.5	170.6		
5	102.7	102.2	5.92 d (2.1)	6.01 d (2.1)
6	158.6	158.6		
7	54.5	54.1	4.17 d (11.0)	3.71 d (10.4)
8	39.1	46.0	4.37 dd (9.8, 11.0)	4.11 dd (9.4, 10.4)
9	135.6	139.3		
10/14	127.5	127.0	7.20–7.45[a]	7.28-7.43[a]
11/13	128.8	128.9	7.20–7.45[a]	7.28-7.43[a]
12	128.3	128.6	7.20–7.45[a]	7.28-7.43[a]
4-OMe	55.9	55.9	3.72 s	3.79 s
2′	164.6	164.2		
3′	91.8	89.4	5.30 s	5.23 s
4′	169.9	171.3		
5′	45.7	43.7	3.60 d (9.8)	3.27 d (9.4)
6′	79.4	82.4		
7′	124.4	127.3	6.61 d (15.9)	6.32 d (16.0)
8′	131.5	132.0	6.95 d (15.9)	6.71 d (16.0)
9′	135.9	135.2		
10′/14′	126.9	126.3	7.20–7.45[a]	7.28-7.43[a]
11′/13′	128.5	128.7	7.20–7.45[a]	7.28-7.43[a]
12′	127.9	127.6	7.20–7.45[a]	7.28-7.43[a]
4′-OMe	55.4	56.3	3.28 s	3.80 s

[a] overlapped signals.

determined from the coupling constants and NOESY correlations. The relatively large coupling constants of H-7/H-8 and H-8/H-5′ were determined to be 10.4 and 9.4 Hz, respectively, which are similar to those of velutinindimer C, which is a 12,12′-dimethoxy derivative with relative configuration (5′*S*, 6′*R*, 7*R*, 8*R*) [30]. The NOESY correlations between H-5/H-8, H-7′/H-7, and H-5′/H-7′ indicate the relative configuration of **6** (Fig. 3). It was clarified by X-ray crystallography that aniba dimer A and velutinindimer C are actually a racemic mixture [30,31]. Compound **6** showed no optical activity, similar to aniba dimer A and velutinindimer C. These results indicate that compound **6** is also a racemate of 5′*S*, 6′*R*, 7*R*, 8*R* and 5′*R*, 6′*S*, 7*S*, 8*S*. Thus, the structure of **6** was determined to be another new asymmetrical cyclobutane dimer of 5,6-dehydrokawain (**2**), and it was named aniba dimer C. There is a hypothesis that the formation of these cyclobutanes takes place through [2 + 2] photocycloaddition, and it has been reported to occur in some plants [32,33]. Therefore, compound **6** was presumably formed by a [2 + 2] photocycloaddition reaction between two molecules of 5,6-dehydrokawain (**2**). Considering the absolute configuration, a [2 + 2] photocycloaddition reaction between two molecules of monomeric 5,6-dehydrokawain (**2**) in the trans–trans form can generate four possible asymmetric dimers as two racemates (Fig. 4). Of these, one of the racemates has been determined to be aniba dimer A (**5**), but the other racemate has not been isolated. In this study, we isolated and identified the aniba dimer C (**6**) as the other racemate.

3.3. Anti-inflammatory activity

Because iNOS expression and NO production are induced in rat hepatocytes by the proinflammatory cytokine, IL-1β [18], we added the methanol extracts to estimate the NO levels. As shown in (Fig. 5), the anti-inflammatory activity differed depending on the part of the plant from which the extract had been obtained. The LDH activity of the medium indicated that none of the extracts produced cytotoxicity at the concentrations indicated (data not shown). Of the three different parts, the pericarps of *A. zerumbet* markedly suppressed NO production with

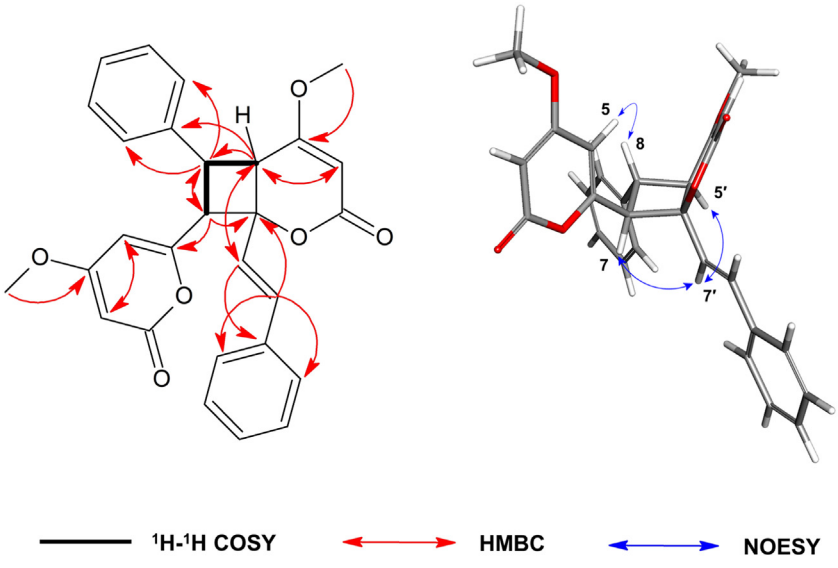

Fig. 3. Key 2D NMR correlations of aniba dimer C (**6**).

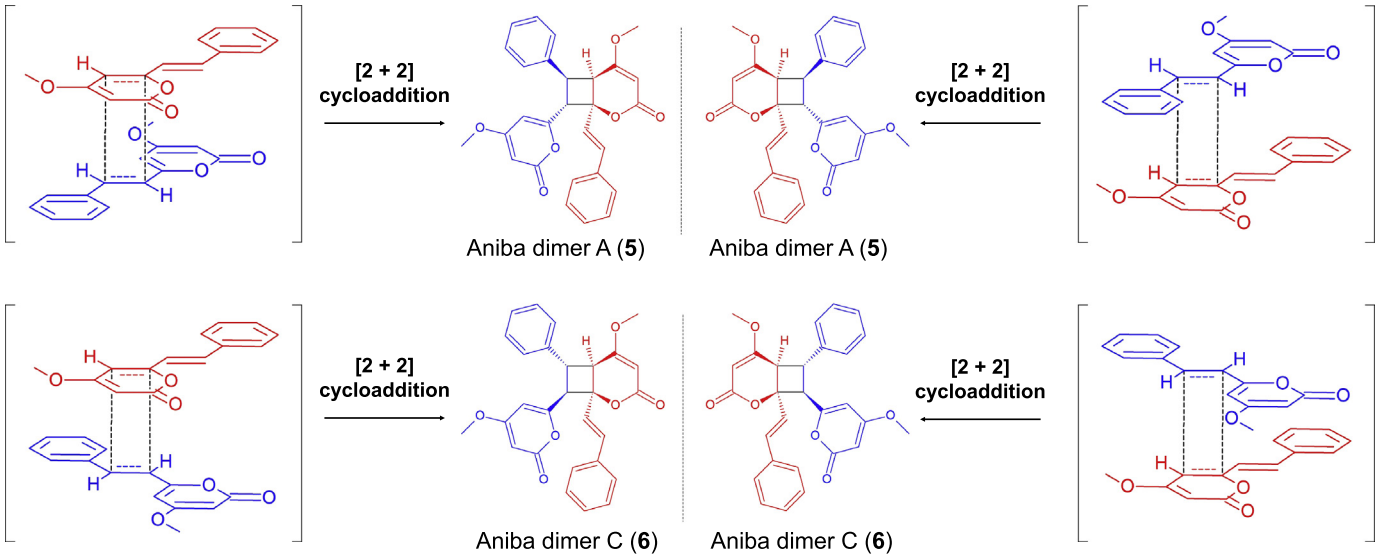

Fig. 4. Putative biosynthetic pathways toward the formation of compounds **5** and **6**.

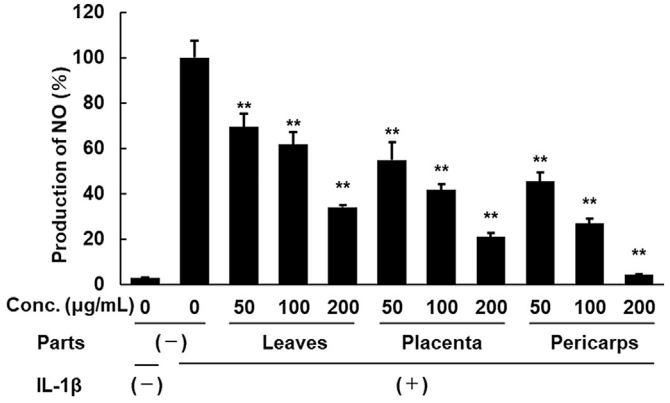

Fig. 5. Effects of the methanol extracts from each part of the plant on nitric oxide (NO) suppression in IL-1β-treated hepatocytes. The hepatocytes were treated with IL-1β and/or each extract for 8 h. The significance level was set at $^{**}P < .01$ *versus* IL-1β alone.

Table 4
Effects of the compounds of *Alpinia zerumbet* on nitric oxide (NO) suppression in IL-1β-treated hepatocytes.

Compounds	IC_{50} (μM)
1	27.5 ± 8.4
2	27.0 ± 5.8
3	33.8 ± 13.3
4	25.5 ± 10.6
5	34.2 ± 14.7
6	25.3 ± 16.1

an IC_{50} value of 63.9 ± 15.6 μg/mL. Next, to investigate whether the kavalactone derivatives (**1–6**) isolated from *A. zerumbet* suppress the production of the pro-inflammatory mediator NO, we added each compound to rat hepatocyte primary cell cultures in the presence of IL-1β. As shown in Table 4, all the compounds showed similar NO-suppressing activity, and their IC_{50} values were calculated. The LDH activities showed that none of the compounds showed cytotoxicity at the concentrations indicated (data not shown). The IC_{50} values of the major compounds were similar, and the ability of each extract to inhibit NO production depended on the total amounts of the major compounds. Therefore, we considered that high amounts of the kavalactone derivatives (**1–6**) might contribute to the potent anti-inflammatory activity of the pericarps compared to the other parts of the plant. Primary cultured rat hepatocytes have been used to evaluate the simple NO-suppressing activity of the compounds [34]. The IC_{50} values of compounds (**1–6**) in this study were compared with those of other chemical constituents, which were determined using the same system [19,20,34]. Comparing the activity of these compounds, kavalactone derivatives (**1–6**), coumarins, and chromons suppressed NO production with similar IC_{50} values. Coumarins and chromons are major active constituents of the root and rhizome of *Glehnia littoralis* and *Saposhnikovia divaricate*, respectively, and these medicinal plants have been used for their expected anti-inflammatory effects in Japanese traditional medicine [19]. From these results, it is suggested that the pericarps of *A. zerumbet* which contain high amounts of the kavalactone derivatives (**1–6**) may possibly be utilized as a new resource having anti-inflammatory activity.

4. Conclusion

In this study we investigated the amounts of kavalactone derivatives in the leaves, placenta, pericarps, and seeds of *A. zerumbet* for the first time. The results revealed that six kavalactone derivatives including one new compound (**6**) are contained in the leaves, placenta, and pericarps of *A. zerumbet*, and that the pericarps are a rich source of kavalactone derivatives. In addition, comparison of the anti-inflammatory activities of three extracts from different parts of the plant showed that the pericarps had the strongest activity of the three. The six kavalactone derivatives showed NO-suppressing activity, and it was considered that the anti-inflammatory activities of the extracts may be due to these compounds. It is well known that the plant is also a rich source of other phytochemicals such as essential oils. Therefore, further studies of the chemical constituents and biological activities are needed to precisely evaluate the medicinal values of the placenta and the pericarps of *A. zerumbet*. However, our results indicate that the pericarps and the placenta may affect the biological activity of the mature fruits of *A. zerumbet*, and that these parts can also be regarded as medicinal parts of *A. zerumbet*.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgment

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