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Anti-inflammatory constituents of *Zingiber zerumbet*

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Abstract

Zingiber zerumbet Smith has long been used as a botanical medicine for anti-inflammation in Southeast Asia. In this paper, zerumbone (**1**), 3-*O*-methyl kaempferol (**2**), kaempferol-3-*O*-(2, 4-di-*O*-acetyl-α-L-rhamnopyranoside) (**3**), and kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**4**) were isolated from the rhizome of *Z. zerumbet*. The inhibitory effects of these compounds on NO and PGE_2 production from lipopolysaccharide (LPS)-induced RAW 264.7 macrophages were measured. Among them, **1** and **2** demonstrated potent inhibition of NO production, with respective IC_{50} values of 4.37 and 24.35 μM, and also significantly suppressed iNOS expression in a dose-dependent manner. However, **1** and **2** could inhibit PGE_2 production only at high doses (20 and 40 μM, respectively), and COX-2 protein level was not affected. According to the *in vitro* study, **1** had greater anti-inflammatory effects than **2**. Therefore, mice were administered with **1** (10 mg/kg) 1 h before carrageenan injection, and the oedema was significantly attenuated compared to the vehicle control. Mature rhizomes were richer in **1** and lower in moisture. We suggest that the economic cultivation period of *Z. zerumbet* is the 5th month after seeding when its functions as food and anti-inflammatory are maximum, because **1** is dramatically increased at that time.

Keywords: *Zingiber zerumbet* Smith; Zingiberaceae; Zerumbone; 3-*O*-methyl kaempferol; iNOS; Paw oedema

1. Introduction

Numerous ginger spices (Zingiberaceae) are widely used in Southeast Asia, not only because of their unique flavour but also because of their medicinal properties. Some of the dietary ingredients have been identified and their biological activities elucidated (Aggarwal & Shishodia, 2006; Surh, 1999). *Zingiber zerumbet* Smith, also a spice in the ginger family, is used as an anti-inflammatory adjuvant for stomach ache, sprain, and fever in Taiwan. *Z. zerumbet* has important economic properties, as the rhizome can be used as both a spice and a traditional medicine (Chiu & Chang, 1986). Its "pine cones" are used as an ornamental in gardening, and the milky juice from its pine cones is used as a shampoo in Hawaii. Recently, several reports on the bioactivity of zerumbone, which was identified as a major compound of this plant, have been published, including anticarcinogenesis (Takada, Murakami, & Aggarwal, 2005) and anti-inflammation (Murakami et al., 2003), but other ingredients such as flavonoids of *Z. zerumbet* have barely been discussed. In this study, we explored the anti-inflammatory principal constituents of *Z. zerumbet* using *in vitro* and *in vivo* assay methods.

2. Materials and methods

2.1. Plant materials

The dried rhizomes of *Z. zerumbet* were provided by Wholesome Life Science Co. Taipei, Taiwan.

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2.2. Chemicals and reagents

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), methanol, chloroform, *n*-hexane, ethyl acetate and other chemicals were purchased from Sigma (St. Louis, MO). All other reagents and chemicals were of the highest purity grade available.

2.3. NMR instruments

^{1}H (500 MHz) and ^{13}C NMR (126 MHz) spectra were generated on a Bruker DRX 500 spectrometric system using acetone-d6 as solvent, and chemical shifts are given in δ (ppm) values. Two-dimension NMR spectra (^{1}H–^{1}H COSY, HMQC, HMBC, NOESY) were measured by Bruker DRX 500 using standard Bruker pulse sequences.

2.4. Extraction and isolation

Dried rhizomes of *Z. zerumbet* (600 g) were refluxed with methanol (6 l × 3 times) at 80 °C for 1 h. The combined filtrate was concentrated with a rotary evaporator to remove the methanol. The extract was coated onto a Celite 545 gel, and than subjected to silica gel column chromatography (10 cm i.d. × 40 cm) with chloroform and ethyl acetate. The chloroform- and ethyl acetate-eluted fractions contained zerumbone (**1**) and flavonoids, respectively. The ethyl acetate fraction was chromatographed on a silica gel column (7.0 cm i.d. × 75 cm) with a chloroform–methanol gradient (10:0→20:1→10:1). The chloroform–methanol (20:1) fraction was rechromatographed over a low-pressure silica gel column (2.5 cm i.d. × 300 mm), eluted with chloroform–methanol (20:1), and then purified by preparative HPLC to obtain 3-*O*-methylkaempferol (**2**, 77.0 mg). The chloroform–methanol (10:1) fraction was further chromatographed on a low-pressure silica gel column (1.5 cm i.d. × 200 mm), then purified by preparative HPLC (*n*-hexane:ethyl acetate = 1:1) to give kaempferol-3-*O*-(2,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**3**, 102.6 mg) and kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**4**, 138.6 mg).

2.5. Cell cultures

The RAW 264.7 macrophage cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 10% heat-inactivated foetal bovine serum (FBS) from Gibco BRL (Grand Island, NY), and then incubated at 37 °C in a humidified incubator containing 5% CO_2.

2.6. Measurement of NO production

This assay was performed as previously described (Wang, Huang, Chen, Lee, & Yang, 2002). Briefly, cells generated NO in the medium after 24 h of incubation with or without samples and/or lipopolysaccharide (LPS; 500 ng/ml). NO production was measured spectrophotometrically at 530 nm after the Griess reaction. The inhibition ratio (NO inhibition%) was calculated based on the following equation:

$$\text{NO inhibition } (\%) = [1 - (T/C)] \times 100\%,$$

where T and C represent the mean optical density of LPS-stimulated-RAW 264.7 cells with or without samples, respectively.

2.7. Cell viability

Cell viability was determined, as previously described, by the mitochondrial-dependent reduction of MTT to formazan (Wang et al., 2002). Briefly, after the indicated time of treatment, cells were incubated with MTT for 4 h, and then solubilised in isopropanol containing 0.04 N HCl. The amount of reduction was measured using an MRX microplate reader (Dynex Technologies, Guernsey, Channel Islands, UK) at 600 nm.

2.8. Prostaglandin E_2 assays

Sample treatment conditions were similar to those previously described. The culture medium was collected after 24 h incubation with a sample, and PGE_2 concentrations were determined with an enzyme-linked immunosorbant assay (ELISA) kit (Amersham Pharmacia Biotech, Amersham, UK) as previously described (Tseng, Lee, Chen, Wu, & Wang, 2006).

2.9. Carrageenan-induced paw oedema in mice

Male ICR mice were housed in a controlled environment and provided sufficient chow and water. Oedema in the left hind paw of mice was induced by an intraperitoneal injection of 50 μl of 1% (w/v) carrageenan from Sigma in saline. Mice were divided randomly and consisted of six animals in each group. The paw volume was measured with a plethysmometer (Ugo Basile, Comerio VA, Italy) before the injection and after 1, 2, 3, 4, 5, and 6 h. Zerumbone (10 mg/kg) or indomethacin (100 mg/kg, as a reference substance) was given 1 h orally before the injection.

2.10. Western blot analysis

The RAW 264.7 cell line was seeded at an initial density of 4×10^5 cells/well in 6-well tissue culture plates overnight. Cells were exposed to LPS (500 ng) and treated with or without a sample at the proper concentration. Non-treated cells were used as the controls. Triptolide (0.1 μM), an

iNOS and COX-2 protein inhibitor, was used as the positive control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was used to monitor that equal amounts of protein were in each lane. Protein samples were collected and prepared as described previously (Wang et al., 2002), and the iNOS and COX-2 expression levels were investigated using Western blot analysis. Briefly, proteins (25 μg) were separated by 10% SDS-PAGE and than transferred to a nitrocellulose membrane. Membranes were probed using antibodies specific to COX-2, iNOS, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) and visualised using a BCIP/NBT kit (Gibco BRL).

2.11. Phytochemicals and moisture analysis

The harvested samples were carefully cleaned and stored at −20 °C until analysis. For the HPLC analysis, samples were cut into pieces and extracted with methanol by ultrasound, for 30 min at 40 °C. HPLC was performed with a system composed of a Shimadzu (Kyoto, Japan) LC-10ATvp liquid chromatograph equipped with a DGU-14A degasser, an FCV-10ALvp low-pressure gradient flow control valve, an SIL-10ADvp auto injector, an SPD-M10Avp diode array detector, and an SCL-10Avp system controller. Peak areas were calculated with Shimadzu Class-VP software (Version 6.12 sp5). A Purospher STAR RP-18e reversed-phase column (250 × 4 mm i.d.) with a Purospher STAR RP-18e guard column (4 × 4 mm i.d.) (both Merck, Darmstadt, Germany); flow rate, 1.0 ml/min; mobile phase, composed of 0.05% trifluoroacetic acid–acetonitrile (v/v) with gradient elution (0–10 min, 65: 35; 10.01–20 min, 35: 65; 20.01–40 min, 0: 100; 50 min, 0: 100). The retention times of 3-*O*-methylkaempferol (**2**) and zerumbone (**1**) were 9.00 and 29.24 min, respectively. The linearity of the peak area (y) versus concentration (x, μg/ml) curve for zerumbone (**1**) was used to calculate the contents of the biomarker substances in *Z. zerumbet*. For the moisture analysis, samples were cut into pieces to fit into the moisture analyser (AND MX-50, Tokyo, Japan). The accuracy of the weight change was set to <0.01%.

2.12. Statistical analysis

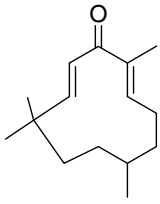
Results are presented as the mean ± standard deviation (SD) from three independent experiments. Data were analyzed using Student's t-test, and results were considered statistically significant when $p < 0.05$.

3. Results

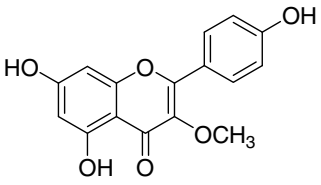
In this work, we obtained four phytochemicals: one sesquiterpene (**1**), one flavone (**2**) and two flavonoid glycosides (**3** and **4**) from the rhizome of *Z. zerumbet* (Fig. 1). Each compound was identified by direct comparison of its spectroscopic data with authentic samples (Damodaran & Dev, 1968; Masuda, Jitoe, Kato, & Nakatani, 1991; Nakatani, Jitoe, Masuda, & Yonemori, 1991). Purity tests of those four compounds were shown to be greater than 95% by HPLC. The anti-inflammatory effects of the four compounds were evaluated by the following experiments.

3.1. Effects of the four compounds from Z. zerumbet on LPS-induced RAW 264.7 cells

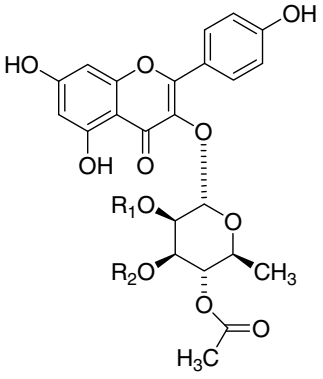
After activation with LPS (500 ng/ml) for 24 h, RAW 264.7 cells exhibited enhanced production of pro-inflam-



zerumbone (**1**)



3-O-methyl kaempferol (**2**)



	R_1	R_2
kaempferol-3-O-(2,4-di-O-acetyl-α-L-rhamnopyranoside) (**3**)	Ac	H
kaempferol-3-O-(3,4-di-O-acetyl-α-L-rhamnopyranoside) (**4**)	H	Ac

Fig. 1. Chemical structures of the four components isolated from *Zingiber zerumbet*.

matory factors which were released into the culture medium, such as NO and PGE_2. Primarily, the inhibitory effects of these compounds (**1**, **2**, **3** and **4**) were detected by NO and PGE_2 production from LPS-induced RAW 264.7 cells. To ensure that these compounds did not interfere with the survival of the macrophages, it was determined that none of the concentrations used in the experiment was cytotoxic (data not shown). As shown in Table 1, treatment with **1** or **2** can significantly suppress LPS-induced NO production in a dose-dependent manner, with respective IC_{50} values of 4.37 and 24.35 μM, while **3** and **4** showed only slight inhibitory effects. Among these compounds, **1** exhibited the most potent inhibitory effect on NO production under inflammatory stimulation. PGE_2, another critical mediator converted from arachidonic acid by COX-2, was also significantly induced by LPS in macrophages. The generation of PGE_2 was suppressed by **1** and **2** only at the respective high doses of 20 and 40 μM (Table 1).

To further delineate the possible mechanism of these bioactive compounds as potential anti-inflammatory compounds, we examined their inhibitory effects on the protein expressions of COX-2 and iNOS in activated RAW 264.7 cells, by a Western blot analysis. Moreover, **1** (2.5–20 μM) and **2** (2.5–40 μM) inhibit LPS-induced iNOS protein expression in a dose-dependent manner. These results are in agreement with the inhibitory effect on NO production (Fig. 2). Thus, the results of NO inhibition may be directly mediated by downregulation of iNOS expression.

When the macrophages were incubated with LPS for 24 h, COX-2, an enzyme which converts arachidonic acid to produce PGE_2, was significantly expressed. However, **1** did not seem to affect COX-2 protein expression even at a high concentration (Fig. 2). Triptolide, a natural compound isolated from *Triperidium wilfodii*, and a COX-2 and iNOS inhibitor, was used as a positive control in this experiment (Brinker, Ma, Lipsky, & Raskin, 2007). In contrast, the inhibitory effects on COX-2 expression by triptolide (0.1 μM) were more significant than that of compound **1** in LPS-stimulated RAW 264.7 cells (Fig. 2). In conclusion, **1** showed greater anti-inflammatory effects than the other compounds in the *in vitro* study, followed by **2**.

3.2. Effects of *1* on carrageenan-induced paw oedema in mice

Since **1** demonstrated an active component *in vitro*, carrageenan-induced paw oedema in ICR mice was used to further evaluate its anti-inflammatory effect *in vivo*. The time course of paw oedema is shown in Table 2. When carrageenan was intraplantar-injected into the hind paw, significant swelling was observed in 1 h by as much as 2.5-fold that of untreated mice. The oral administration of **1** (10 mg/kg) or indomethacin (100 mg/kg) produced a significant ($p < 0.05$) reduction in paw oedema, compared with non-treated controls.

Subsequently, the *in vivo* study also indicated that **1** could effectively attenuate the carrageenan-induced paw oedema in mice. Pretreatment with **1** suppressed the oedematous response 1 h after the carrageenan injection and this effect continued until 6 h. The potency of **1** on paw oedema attenuation was comparable to that of indomethacin, and the inhibitory effect lasted longer. These results are

Table 1
Effects of the natural components from *Zingiber zerumbet* on LPS-stimulated RAW 264.7 cells for 24 h

Compounds	Dose (μM)	NO inhibition (%)	PGE_2 inhibition (%)
1	10	84.1 ± 2.31	51.0 ± 12.7
2	40	47.1 ± 2.97	68.6 ± 15.9
3	40	14.1 ± 5.59	12.7 ± 19.3
4	40	14.8 ± 9.74	3.89 ± 17.5

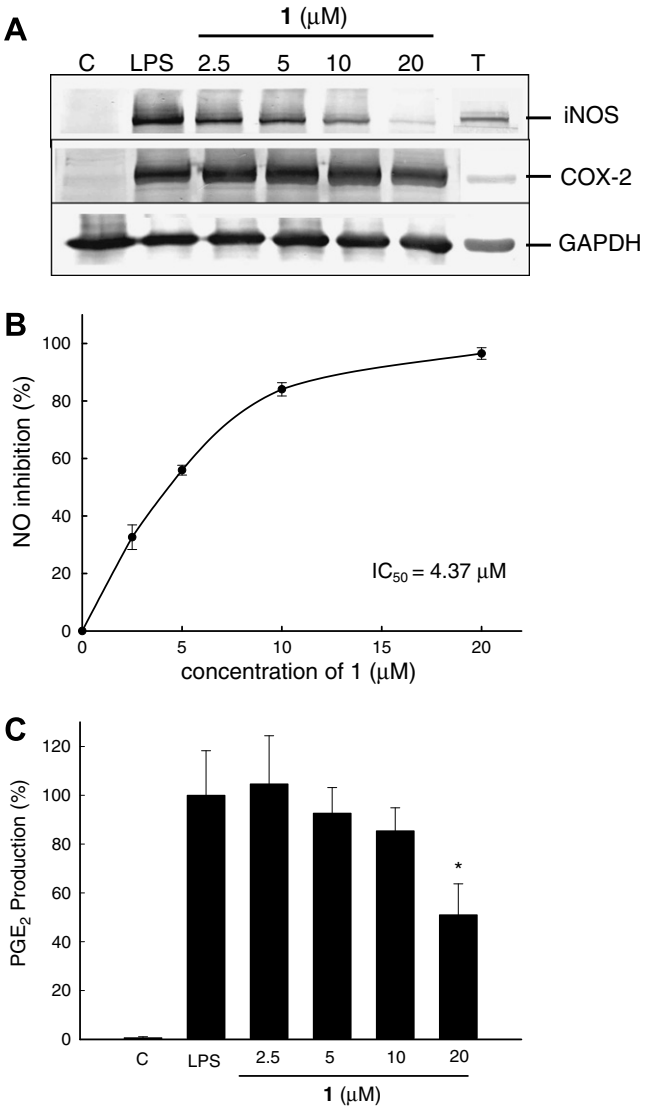


Fig. 2. Effects of zerumbone (**1**) on LPS (500 ng/ml)-stimulated RAW 264.7 cells after treatment for 24 h. (A) Detection of iNOS and COX-2 protein expressions by Western blotting. T is triptolide (0.1 μM) (B) NO inhibition percentage of **1** in a series of concentrations (2.5–20 μM). (C) PGE_2 inhibition percentage of **1** in a series concentration (2.5–20 μM). * $p < 0.01$ compared to the LPS.

Table 2
Anti-inflammatory effects of zerumbone (**1**) on carrageenan-induced mouse paw oedema, compared to indomethacin

Time (h)	Increase in paw volume (%)		
	Control	Indomethacin	**1**
0	0.00 ± 24.0	0.00 ± 21.1	0.00 ± 12.0
1	151 ± 19.7	71.4 ± 27.9*	63.3 ± 12.7*
2	133 ± 22.8	53.1 ± 12.8*	55.1 ± 6.32*
3	105 ± 22.8	44.9 ± 12.0*	32.7 ± 12.0*
4	86.1 ± 7.21	34.7 ± 15.5*	36.7 ± 16.3*
5	90.7 ± 22.8	36.7 ± 14.3	40.8 ± 29.7
6	105 ± 39.1	51.0 ± 16.7	38.8 ± 16.7*

Results are presented as the mean ± SD from six mice.
* $p < 0.01$ compared to the control at the same time.

in agreement with a previous report, which showed that enhanced NO production was highly associated with carrageenan-induced paw oedema formation in mouse.

3.3. The active components and water content of Z. zerumbet in different growth periods

According to the above results, **1** is not only the major compound but also the most-bioactive component of *Z. zerumbet* in terms of the plant's anti-inflammatory activity. Therefore, the amount of **1** in *Z. zerumbet* was measured by an HPLC system, and the results demonstrated that **1** slightly increased until the 5th month except in the 1-month-old rhizome, which may have been caused by sampling of the original part not new growth buds (Fig. 3). During the 5th month the amount of **1** increased dramatically and was quantified at 1393 μg/g which is far greater than in the 4-month-old rhizome at 378 μg/g. The maximum content of **1** was found in 8-month-old rhizomes (Table 3). In addition, **2** also significantly increased after the 5th month. The results suggest that the more mature rhizome has greater amounts of both **1** and **2**.

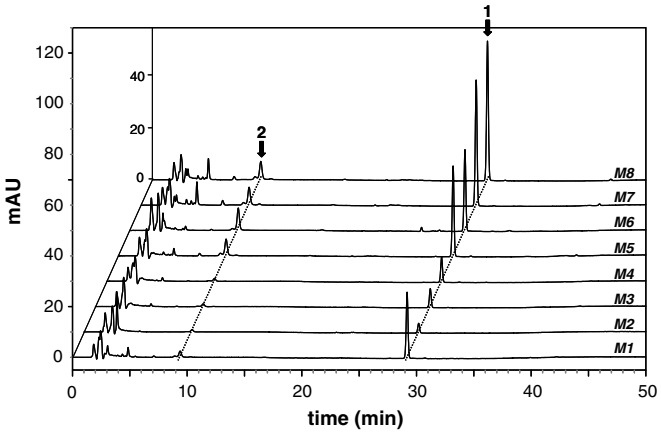


Fig. 3. Chromatograms at 254 nm of the methanol extract of the rhizome of *Zingiber zerumbet* harvested at different growth periods.M1–M8 represent cultivation periods: 1 month (M1) to 8 months (M8).

Table 3
Moisture and zerumbone (**1**) contents of the rhizome of *Zingiber zerumbet* harvested after different growth periods

Cultivation period (month)	Moisture content (%)	Content of **1** (μg/g)
1	91.1	1009 ± 17.2
2	92.2	141 ± 1.73
3	93.5	280 ± 5.32
4	91.2	378 ± 8.27
5	86.9	1393 ± 18.3
6	86.8	1254 ± 7.34
7	79.2	1931 ± 28.7
8	73.9	2158 ± 20.5

Variations in the moisture content of rhizomes of *Z. zerumbet* are important. The percentage of water content in rhizomes was almost 92% after cultivation for 4 months. When cultivated for 5 months, the percentage of water rapidly decreased to 86.9% and then steadily decreased to 73.9% when harvested in the 8th month (Table 3).

4. Discussion

RAW 264.7 cells stimulated by LPS produce a variety of pro-inflammatory mediators, including interleukin, cytokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2). Thus, agents that down-regulate these pro-inflammatory mediators would be beneficial in the treatment of inflammation (Chen et al., 2001; Wang & Mazza, 2002). Furthermore, carrageenan inducing mice paw oedema is a well-known animal model to examine the effect of agents against acute inflammation (Gepdiremen, Mshvildadze, Suleyman, & Elias, 2004). The mechanism involved in carrageenan-induced oedema formation has also been reported to be associated with COX-2 and iNOS overexpression (Nishikori, Irie, Suganuma, Ozaki, & Yoshioka, 2002).

In our previous study, we isolated and identified **1** from *Z. zerumbet* as the major natural product, which can induce cell cycle arrest in HL-60 leukaemia cells and prolong the lifespan of P-388D_1-bearing CDF_1 mice (Huang, Chien, Chen, & Wang, 2005). In the present study, we further modified the chromatographic procedure to obtain more flavonoids and improve the yield of **1,** the major bioactive component. Although **2** exhibited similar anti-inflammatory activity, **1** remains the most potent. Therefore, we believe that **1** is still the dominant bioactive compound in *Z. zerumbet*.

In addition, results of the *in vivo* assay indicated that **1** showed anti-inflammatory activity against carrageenan-induced mouse paw oedema and was as efficacious as indomethacin. The anti-inflammatory mechanisms of indomethacin are thought to suppress COX-2 expression. However, it appears that **1** is an inhibitor of iNOS rather than COX-2. The reason for the decrease in PGE_2 not affecting COX-2 expression was not examined in the present study. It is possible that the inhibitory effect may be mediated through inhibition of COX-2 enzymatic activity, similar

to that of a specific COX-2 inhibitor, NS-398 (Lin et al., 2004). In addition, a previous study showed that low NO concentrations promoted COX-2 expression in murine macrophages (Babcock et al., 2002). Through molecular cross-talk, the low level of NO in **1**-treated LPS-stimulated RAW 264.7 cells might have also led to enhanced COX-2 expression. Beside, NO also plays an important role in carrageenan-induced paw oedema. It is known that inhibition of iNOS activity and expression produces an anti-inflammatory effect on rat paw oedema (Rioja et al., 2000). Moreover, *N*-nitro-L-arginine-methyl ester (L-NAME) attenuated mouse paw oedema through inhibition of NO production (Tan-No et al., 2006). The present results are in agreement with a previous report (Murakami et al., 2002) that **1** potently inhibits NO production and iNOS expression; thus the anti-oedematogenic mechanism of **1** may also be related to the inhibition of NO.

Because **1** is responsible for the biological activity as determined in this and other scientific reports (Murakami, Miyamoto, & Ohigashi, 2004), developing an analysis system is necessary for controlling the quality of this plant. The advantage of the analysis system we developed is that it is quick. It takes only about 1.5 h from extraction to the chromatograph report. According to the results of chromatography, rhizome maturation had a great influence on the content of the natural constituents. The concentration of **1** was three times greater in mature 5-month-old rhizomes, compared to young rhizomes. On the other hand, the moisture also dropped significantly after 5 months. Both secondary metabolites and the moisture level of *Z. zerumbet* were affected by the stage of maturity, and dramatic changes occurred at 5 months of growth. It is suggested that the economic cultivating period of *Z. zerumbet* is 5 months, which is sufficient to produce a functional food.

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