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Antiinflammatory Activity of Heat-treated *Cassia alata* Leaf Extract and Its Flavonoid Glycoside

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Antiinflammatory activities of heat-treated *Cassia alata* leaf extract and kaempferol 3-*O*-gentiobioside (K3G) isolated from *C. alata* as an abundant flavonoid glycoside were studied by comparing their activities with the activities of sun-dried *C. alata* leaf extract. We observed strong inhibitory effects on Concanavalin A-induced histamine release from rat peritoneal exudate cells both in the extracts of heat-treated and sun-dried *C. alata* leaves. Furthermore, the heat-treated leaf extract exhibited stronger inhibitory effects than the effects of the sun-dried leaf extract at low concentrations in the studies of Concanavalin A-induced histamine release, 5-lipoxygenase inhibition, and also inhibition of cyclooxygenases (COX-1 and COX-2), whereas K3G showed weak inhibitory effects on Concanavalin A-induced histamine release, 5-lipoxygenase, and COX-1. No anti-hyaluronidase effect was detected in any of the materials tested.

Key words——*Cassia alata*; kaempferol gentiobioside; antiinflammatory activity; cyclooxygenase; 5-lipoxygenase; histamine release

INTRODUCTION

Cassia alata L. (Leguminosae) is a native of tropical America now grown in Indonesia, where it is often found in the wild. *C. alata* leaf extract has been reported to have various pharmacological activities, including antiinflammatory activities using carrageenan-induced edema methods,[1—3] the cotton implantation method,[1] and an assay for inhibitory effects on compound 48/80-induced histamine release[2]; analgesic activity,[4] laxative activity,[5,6] and antiplatelet-aggregating activity.[7]

Heat treatment of fresh *C. alata* leaf was effective in stabilizing kaempferol 3-*O*-gentiobioside (K3G), which was found abundantly in *C. alata* leaf, as previously reported.[8] Consequently, it is meaningful to study the biological significance, such as antiinflammatory activities, of K3G. Various flavonoid glycosides are known to have antiinflammatory activities.[9]

In the present study, we have investigated the extracts of *C. alata* leaves in addition to K3G isolated from *C. alata* leaf[8] as shown in Fig. 1 for further elaboration of their antiinflammatory effects, including the inhibitory effect on histamine release from rat peritoneal exudate cells induced by Concanavalin A and on enzymes such as cyclooxygenases (COX-1 and COX-2), 5-lipoxygenase, and hyaluronidase. Their effects were compared with those of the sun-dried *C. alata* leaf extract as a reference, of which the inhibitory effects were previously reported to be useful for evaluating antiinflammatory activities.[10—16]

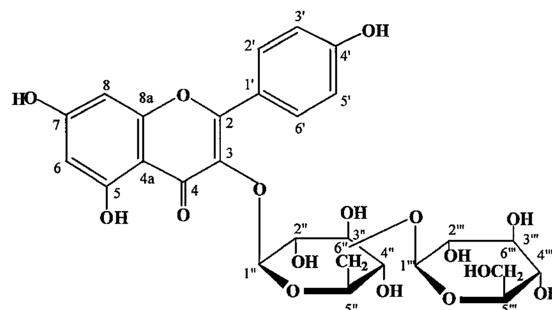

Fig. 1. Structure of Kaempferol 3-*O*-gentiobioside (K3G)

MATERIALS AND METHODS

Plant Materials All the plant materials (*C. alata* leaves) used in the experiments were obtained from PT Haldin Pacific Semesta (Jakarta, Indonesia). *C. alata* was grown in a region 80 km southeast of Jakarta. Leaflets with sizes ranging from 5 to 9 cm in width and from 10 to 16 cm in length were harvested in January 2000. They were stored in plastic bags at

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room temperature and were used for the present study within 3 months. Heat-treated and sun-dried leaves were prepared as previously described.[8)]

Preparation of Plant Extracts Twenty grams of each of pulverized heat-treated and sun-dried *C. alata* leaves were extracted with 1000 ml of distilled water in a water bath at 80℃ for 1 h. After filtration, the extracts were evaporated under reduced pressure and lyophilized. The yields were 18.6% and 19.9%, respectively.

Isolation of Kaempferol 3-*O*-Gentiobioside Isolation of K3G was achieved using a preparatory HPLC column as described previously.[8)] Purity was confirmed to be 99% with HPLC.

Chemicals Concanavalin A and phosphatidyl-L-serine were purchased from Sigma Chemical (St. Louis, MO, USA), [1-^{14}C] arachidonic acid was from Amersham American Biosciences Corp. (Piscataway, NJ, USA), and trichloroacetic acid was obtained from Wako Pure Chemical Industry (Japan). RPMI-1640 was obtained from Dainippon Pharmaceutical Co., Ltd. (Japan). HEPES was purchased from Dojindo Laboratories (Japan). COX-1 and COX-2 were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Unless otherwise specified, all other reagents were of analytical grade.

Animals Male Wistar rats (300—500 g) were raised as described previously.[12)]

Isolation of Rat Peritoneal Mast Cells Male Wistar rats were exsanguinated and injected intraperitoneally with 15 ml of physiological salt solution (PSS) consisting of 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.6 mM $CaCl_2$, 10 mM HEPES, 0.05% gelatin, and 0.41 mM NaH_2PO_4 in distilled water at pH 7.4. The abdominal region was gently massaged for 1.5 min and then the peritoneal exudate cells were collected in a polystyrene tube. The cell suspensions were centrifuged at 265×g for 15 min at 4℃ and washed several times with the physiological suspension.

Assay of Histamine Release from Rat Peritoneal Exudate Cells Induced by Concanavalin A A 0.05 ml aliquot of varying concentrations of the extracts dissolved in dimethylsulfoxide (DMSO) was mixed with 1.75 ml of the aforementioned physiological solution to which 5.6 mM glucose and phosphatidyl-L-serine 30 μg/ml (PSS+glucose) were added. The mixed solution was preincubated at 37℃ for 5 min, 0.05 ml of peritoneal exudate cell suspension was added, and the mixture was incubated at 37℃ for 15 min. The test sample was replaced with PSS+glucose as a control. The preincubated peritoneal cell suspension was mixed with 0.2 ml of Concanavalin A (4.0×10^{-4} g/ml) solution and incubated at 37℃ for 10 min. The physiological solution was added in place of Concanavalin A solution as a blank. The mixture was cooled to 4℃ and centrifuged at 300×g for 10 min at this temperature. Histamine in the supernatant and residue was measured according to a previously reported method.[13,17)] The percent inhibition was expressed as follows:

$$\text{Histamine release (\%)} = \frac{Ps}{Ps+Pr}\times 100 = A$$

where Ps is histamine in supernatant and Pr is residue histamine in cells.

$$\text{Inhibition (\%)} = \left(1-\frac{S-B}{C-B}\right)\times 100$$

where S is A obtained from the test sample, C is A obtained from the control, and B is A obtained from the blank.

Assay for COX-1 and COX-2 Inhibition A modification of the method of Futaki *et al*[10)] was used. COX-1 and COX-2 enzymes were isolated and purified from ram seminal vesicles and sheep placenta, respectively. A mixture of Tris-HCl buffer 10 μl (100 mM), hematin 10 μl (1 μM), phenol 10 μl (2 mM), and 50 μl of distilled water was added to 10 μl of test materials dissolved in 1% DMSO. Then 200 U of COX-1 or COX-2 was added to the mixture. The reaction medium was preincubated with each of the extract samples for 2 min at 37℃, and then 2 μl of 51.4 μM [1-^{14}C] arachidonic acid was added and incubated for 2 min at 37℃. After terminating the reaction in an ice bath, 0.4 ml of *n*-hexane/ethyl acetate (2 : 1, v/v) was added to the reaction mixtures and the mixtures were centrifuged at 2000×g for 1 min at room temperature. The aqueous phase was kept and the organic solvent phase was discarded. The extraction procedure was repeated twice and then 50 μl of ethanol was added to the aqueous phase and the mixtures were centrifuged at 2000×g for 1 min at room temperature. The amount of radioactivity in the supernatant was measured using a scintillation counter.

The inhibitory rate (%) of COX activities was expressed as follows:

$$\text{Inhibition rate (\%)} = \left(1-\frac{\text{RA (sample)}-\text{RA (blank)}}{\text{RA (control)}-\text{RA (blank)}}\right)\times 100$$

where RA is radioactivity (cpm).

Assay for 5-Lipoxygenase Inhibition The previously reported method was used[11,16] with slight modifications. Briefly, rat basophilic leukemia-1 (RBL-1) cells were grown in RPMI-1640 medium containing 10% heat-inactivated newborn calf serum (NCS), penicillin 100 units/ml, and streptomycin 100 mg/ml. Cells were cultured at 37°C in 5% CO_2/air. Cells in the growth phase were collected by centrifuging at 100×g for 5 min and suspended at a density of 3×10^7 cells/ml in 50 mM phosphate buffer (0.25 M sucrose, 1 mM EDTA, 2 mM glutathione, pH 7.4). The RBL-1 cells containing 5-lipoxygenase were stored at −80°C until use. The assay system (0.5 ml) consisted of 50 mM of the aforementioned phosphate buffer, varying concentrations of the samples in 1% DMSO, 2 mM $CaCl_2$, 0.2 mg/ml of arachidonic acid (10 mg/ml MeOH, 10 μl), and ultrasonic pressed RBL-1 cell (1×10^7 cells/ml) in a final volume of 0.5 ml. Reaction mixtures were incubated at 37°C for 3 min, and the reaction was terminated with the addition of methanol (0.5 ml). The mixtures were centrifuged and then 5-HETE in the supernatant was analyzed by HPLC as performed previously.[11]

Assay for Anti-Hyaluronidase Activity Hyaluronidase activity was determined according to the method described previously.[15]

Statistical Analysis Statistical comparisons were performed by Bonferroni's multiple *t*-test. A *p* value$<$0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The heat-treated and sun-dried *C. alata* leaf extracts and K3G isolated from *C. alata* leaf were found to have various antiinflammatory activities. Among them, the inhibitory effects on the histamine release induced by Concanavalin A were notable in the extracts of heat-treated and sun-dried *C. alata* leaves, exhibiting a concentration dependence as shown in Table 1. K3G also inhibited the histamine release in a concentration-dependent manner, although the extent of the inhibition was less than that by the leaf extracts. The result using sun-dried *C. alata* leaf extract was in agreement with that of a previous study[2]; however, the extent of inhibition was more potent than the extract of the previously investigated plant material[2] even when using low concentrations of our leaf extracts. Although reason for the difference in the results is not clear, it may have been due to the use of a different histamine releaser in the experiment. Compound 48/80 and Concanavalin A as histamine releasers were reported to give a difference in the inhibitory rate.[12]

Table 1. Inhibitory Effects on Concanavalin A-Induced Histamine Release from Rat Peritoneal Exudate Cells

Material	Conc. (mg/ml)	Inhibition (%)
CA	0.01	0.5±0.8
	0.10	71.0±3.7
	1.00	96.7±0.9
HCA	0.01	3.4±0.2
	0.10	95.0±1.4$^{b)}$
	1.00	98.4±0.5
K3G	0.01	−0.4±2.9$^{a)}$
	0.10	8.1±1.3$^{b)}$
	1.00	32.8±4.2$^{c)}$

CA: sun-dried *C. alata* leaf, HCA: heat-treated *C. alata* leaf, K3G: kaempferol 3-*O*-gentiobioside. $n=3$, ±SE.
a), *b*), *c*) Statistical significance at $p<0.05$ when compared with CA.

Table 2. Inhibitory Effects on COX-1 and COX-2

Material	Conc. (mg/ml)	Inhibition (%)	
		COX-1	COX-2
CA	0.03	5.7±5.2	−6.1±1.5
	0.10	30.0±9.5	−0.4±2.1
	0.30	86.6±1.7	28.0±4.0
	1.00	100.5±0.7	72.9±1.3
HCA	0.03	9.8±6.0	15.5±4.3$^{a)}$
	0.10	42.2±6.2	32.5±18.0$^{b)}$
	0.30	92.5±1.1$^{c)}$	57.4±5.8$^{c)}$
	1.00	96.6±2.0$^{d)}$	84.6±3.5$^{d)}$
K3G	0.03	5.2±4.0	4.7±3.4$^{a)}$
	0.10	−0.2±7.6$^{b)}$	7.0±3.7
	0.30	13.5±3.0$^{c)}$	12.9±2.6$^{c)}$
	1.00	37.3±0.7$^{d)}$	14.0±2.6$^{d)}$

CA: sun-dried *C. alata* leaf, HCA: heat-treated *C. alata* leaf, K3G: kaempferol 3-*O*-gentiobioside. $n=3$, ±SE.
a), *b*), *c*), *d*) Statistical significance of difference at $p<0.05$ when compared with CA.

Table 2 shows the results of the inhibitory effects on COX-1 and COX-2 activities. Extracts of both heat-treated and sun-dried *C. alata* leaves demonstrated strong inhibitory effects, whereas K3G exhibited weak inhibitory effects on COX-1 and had little or no effect on COX-2. When the inhibitory effect of heat-treated leaf extract was compared with that of sun-dried leaf extract, the heat-treated leaf extract showed stronger inhibitory effects than the sun-dried leaf extract, with

Table 3. Inhibitory Effects on 5-Lipoxygenase

Material	Conc. (mg/ml)	Inhibition (%)
CA	0.01	−3.2±8.9
	0.10	64.9±1.0
	1.00	98.0±0.1
HCA	0.01	26.9±3.1[a]
	0.10	84.1±0.8[b]
	1.00	99.2±0.1
K3G	0.01	3.9±2.6
	0.10	3.6±2.6[b]
	1.00	10.2±1.0[c]

CA: sun-dried *C. alata* leaf, HCA: heat-treated *C. alata* leaf, K3G: kaempferol 3-*O*-gentiobioside. $n=3$, ±SE.
a),*b*),*c*) Statistical significance of difference at $p<0.05$ when compared with CA.

significant differences in COX-1 at 0.30 mg/ml and in COX-2 at all concentrations tested.

COX-1 plays an important role in prostaglandins and thromboxane A_2 synthesis. The inhibitory effect of *C. alata* leaf extract on the enzyme explains the result of our previous study on the antiplatelet aggregating activity of the leaf extract using the same plant, with strong inhibitory effects on the platelet aggregation induced by ADP or collagen as an aggregating agent.[7] Consequently, the extract inhibited the development of small-sized platelet aggregates into large-sized platelet aggregates.

The inhibitory effects on 5-lipoxygenase are shown in Table 3. We confirmed that the extracts of heat-treated and sun-dried *C. alata* leaves have strong inhibitory effects, which were particularly significant for the heat-treated leaf extract at low concentrations (0.01 and 0.10 mg/ml). We also observed that K3G had little inhibitory effect on 5-lipoxygenase activity.

Our previous study revealed that heat treatment played an important role in stabilizing K3G, detecting more K3G in heat-treated leaf (about 1.0%, w/w dry wt.) than in sun-dried leaf (about 0.6%, w/w dry wt.).[8] We assumed that the difference in the K3G concentrations between the leaves might be attributable to the presence of an enzyme(s) such as glucosidase on K3G. It is therefore possible that a similar stabilization or change may have occurred in other constituents in heat-treated leaf, thereby resulting in the stronger inhibitory effects of the heat-treated leaf extract on the Concanavalin A-induced histamine release, COX-1, COX-2, and 5-lipoxygenase than sun-dried leaf. Furthermore, it is apparent the stronger inhibitory effects of heat-treated leaf extract than those of sun-dried leaf extract may not be attributed to K3G, but to some other constituents stabilized by heat treatment because K3G was found to have weak inhibitory effects on all the antiinflammatory activities studied.

In conclusion, we found that the extracts of heat-treated and sun-dried *C. alata* leaves have strong inhibitory effects on concanavalin A-induced histamine release, COX-1 and COX-2, and 5-lipoxygenase activities, whereas K3G as a major flavonoid glycoside in *C. alata* leaf showed weak or little inhibitory effects on the aforementioned activities. Therefore, K3G contributes little to the inhibitory effects of the heat-treated and sun-dried leaf extracts. Consequently, because heat-treated leaf extract exhibited more potent inhibitory effects in some of the activities at lower concentrations than the sun-dried extract, it is assumed that the heat-treated extract may contain other constituents responsible for the stronger inhibitory effects. Furthermore, none of the tested materials showed inhibitory effects on hyaluronidase activity.

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REFERENCES

1) Palanichamy S., Nagarajan S., *Fitoterapia*, **61**, 44–47 (1990).
2) Ohsumi K., Ito M., Ohmori Y., Kishi M., Katada T., Riswan S., Konishi H., *Nat. Med.*, **49**, 472–474 (1995).
3) Abatan M.O., *Fitoterapia*, **61**, 336–338 (1990).
4) Palanichamy S., Nagarajan S., *J. Ethnopharmacol.*, **29**, 73–78 (1990).
5) Elujoba A. A., Ajulo O.O., Iweibo G.O., *J. Pharm. Biomed. Anal.*, **7**, 1453–1457 (1989).
6) Ogunti E.O., Elujoba A. A., *Fitoterapia*, **64**, 437–439 (1993).
7) Moriyama H., Iizuka T., Nagai M., Terazono M., Hoshi K., *Nat. Med.*, **56**, 178–183 (2002).
8) Moriyama H., Iizuka T., Nagai M., *Yakugaku Zasshi*, **121**, 817–820 (2001).
9) ''The Flavonoids: Advances in Research since 1986,'' ed. by Harborne J. B., Chapman & Hall, London, 1994, pp. 624–635.

10) Futaki N., Takahashi S., Yokoyama M., Arai S., Higuchi S., Otomo S., *Prostaglandins*, **47**, 55–59 (1994).

11) Sogawa S., Nihro Y., Ueda A., Miki T., Matsumoto H., Satoh T., *J. Med. Chem.*, **36**, 3904–3909 (1993).

12) Miyataka H., Nishiki M., Matsumoto H., Fujimoto T., Matsuka M., Isobe A., Satoh T., *Biol. Pharm. Bull.*, **21**, 723–729 (1998).

13) Kakegawa H., Matsumoto H., Satoh T., *Chem. Pharm. Bull.*, **40**, 1439–1442 (1992).

14) Kakegawa H., Matsumoto H., Sato T., *Planta Med.*, **54**, 385–389 (1988).

15) Miyataka H., Nishiki M., Matsumoto H., Fujimoto T., Matsuka M., Satoh T., *Biol. Pharm. Bull.*, **20**, 496–501 (1997).

16) Blackham A., Griffiths R. J., Hallman C., Mann J., Mitchell P. D., Norris A. A., Simpson W. T., *Agents Actions*, **30**, 432–442 (1990).

17) Shore P. A., Barkhalter A., Cohn V. H., *J. Pharmacol. Exp. Ther.*, **127**, 182–186 (1959).