Anti-inflammatory Constituents of Sappan Lignum

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We performed an *in vitro* assay for seven compounds from methanolic extract of Sappan Lignum (CSE) that inhibit the chemical mediators of inflammation using the J774.1 cell line: brazilin (1), sappanchalcone (2), protosappanin A (3), protosappanin B (4), protosappanin C (5), protosappanin D (6), and protosappanin E (7). Those compounds were evaluated for their inhibitory effects on nitric oxide (NO) and prostaglandin E_2 (PGE_2) production and their suppressive effects on tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) mRNA expression. As a result, we clarified that 1 inhibited NO production, and almost no inhibition in PGE_2. On the contrary, 2, 6, and 7 inhibited both NO and PGE_2 production and suppressed TNF-α, IL-6, COX-2, and iNOS mRNA expression. An examination of carrageenin-induced mouse paw edema suggested that the CSE contained active compounds other than 1, the main constituent in CSE. It was thus revealed that several compounds and mechanisms contributed to the anti-inflammatory effect of CSE.

Key words *Caesalpinia sappan* L.; Sappan Lignum; anti-inflammatory effect; brazilin; carrageenin

Caesalpinia sappan L. (Leguminosae) is distributed in Southeast Asia, and its heartwood, Sappan Lignum, is famous as a red dyestuff. Sappan Lignum is also used as herbal medicine for inflammation or improvement for blood circulation.[1,2] In Japan, Sappan Lignum is newly listed in the 15th Japanese Pharmacopoeia.[3]

Aside from brazilin (**1**) and brazilein, the known constituents of Sappan Lignum, we have isolated sappanchalcone (**2**) and protosappanins A—E (**3**—**7**), which are dibenzoxocin derivatives, and elucidated their structures (Fig. 1).[4—9] Pharmacological studies of Sappan Lignum focusing on its vasorelaxation[10] or immunosuppressive effect[11] have concluded that **1** is the active compound. Compound **1** alone was reported to show anti-inflammatory effect,[12,13] lens-aldose reductase inhibitory effect,[14] and anti-hepatotoxic effect.[15] However, there are few pharmacological studies of other compounds except ours. We previously performed an *in vitro* assay for **1**, brazilein, **2**, **3**, **4**, and **5**, and clarified such properties as inhibition of nitric oxide (NO) production and inducible NO synthase gene expression, as well as free radical scavenging and antioxidant activity.[16] The results suggested that Sappan Lignum contained active compounds other than **1** that showed anti-inflammatory effect.

In this study, we performed an *in vitro* assay for **2**,[4] **3**,[5] **4**,[6] **5**,[7] **6** (isomeric mixtures),[8] and **7** (isomeric mixtures of E-1 and E-2)[9] to determine the mechanism underlying their inhibitory effects on NO and prostaglandin E_2 (PGE_2) production and their suppressive effects on the mRNA expression of typical chemical mediators of inflammation: tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and inducible NO synthase (iNOS). We then performed an *in vivo* assay for methanolic extract of Sappan Lignum (CSE) and **1** to evaluate their anti-inflammatory effect on carrageenin-induced mouse paw edema in order to estimate whether or not **1** was the only active constituent of CSE.

MATERIALS AND METHODS

Materials Brazilin (**1**, 5.1% from CSE), sappanchalcone (**2**, 0.38%), protosappanin A (**3**, 0.15%), protosappanin B (**4**, 0.80%), protosappanin C (**5**, 0.48%), protosappanin D (**6**, 0.15%), and protosappanin E (**7**, 0.32%) were isolated from Sappan Lignum purchased from Uchida Wakanyaku Co., Ltd. (Lot. No. 313116, December 27, 2006).

J774.1 (Health Science Research Resources Bank), TRIzol reagent (Invitrogen), RevertAid H minus first strand cDNA synthesis kit (Fermentas), prostaglandin E_2 Correlate-EIA kit (Assay Designs), GoTaq (Promega), RT-PCR primers (Operon), carrageenin from seaweed (SIGMA), indomethacin

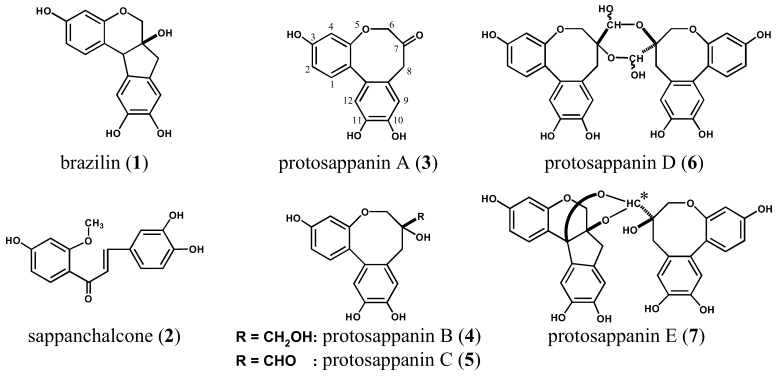

Fig. 1. Structures of Compounds

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(IM, SIGMA), sodium carboxymethyl cellulose (CMC, Kanto Chemical), fetal bovine serum (FBS, Biowest), were purchased from indicated sources. RPMI-1640, benzylpenicillin potassium, streptomycin sulfate, and lipopolysaccharide (LPS) were from Wako Pure Chemical Co., Ltd.

Cell Culture Mouse macrophage-like J774.1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, benzylpenicillin potassium (100 U/ml) and streptomycin sulfate (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

Measurement of NO and PGE_2 Concentration J774.1 cells were incubated in medium containing LPS (1.0 μg/ml) with or without the test compound (3, 10, 30, 100 μM). After 24 h, the concentrations of NO and PGE_2 in the medium were measured. NO concentration was measured using our previous method.[16] The LPS concentration of the present study was 1.0 μg/ml, although 10 μg/ml was used previously. PGE_2 concentration was measured using the prostaglandin E_2 correlate-EIA kit according to the manufacturer's instructions. Cell viability was measured with 0.4% trypan blue and NO and PGE_2 concentration values were corrected by the viability.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from the J774.1 cell pellet using TRIzol reagent after incubation for 24 h in the medium containing LPS (1.0 μg/ml) with or without the test compound, and was reverse-transcribed into cDNA using RevertAid H minus first strand cDNA synthesis kit according to the manufacturer's instructions. The generated cDNA was subjected to PCR using GoTaq and the primers of murine β-actin, TNF-α, IL-6, COX-2, and iNOS.[17] The cycling program of PCR (TaKaRa Dice) was as follows: 94 °C for 1 min, followed by 19 cycles (β-actin, TNF-α, and IL-6), 20 cycles (COX-2), or 24 cycles (iNOS) of 94 °C for 15 s, 60 °C for 60 s, and 72 °C for 60 s, and finally one cycle of 72 °C for 10 min. The PCR products were resolved by electrophoresis on 1% agarose gel. Relative mRNA expression of TNF-α, IL-6, COX-2, and iNOS was measured by Scion Image (NIH). PCR products of the primers for β-actin were used as control.

Animals Six-week-old male ddY mice were purchased from Tokyo Experimental Animals Co. (Tokyo, Japan). This study was carried out in accordance with the guide for the committee on the care and use of laboratory animals of Hoshi University.

Carrageenin-Induced Mouse Paw Edema Edema was induced on the right hind paw by intraplantar injection of carrageenin (2% in physiological saline solution, 30 μl). CSE, **1**, vehicle (0.1% CMC) or indomethacin (IM) was orally administered 30 min prior to the carrageenin injection. Edema values were calculated by subtracting the thickness of the left paw injected with physiological saline alone (30 μl) from that of the right paw. Paw thickness was measured with Dial Thickness Gauge G-1A (Peacock). Percentage inhibition of edema was expressed as the percentage thickness increase compared to right paw thickness before carrageenin injection.

Statistical Analysis Means of data were presented together with S.E.M. Statistical comparisons were made by Bonferroni test and $p<0.05$ was considered significant.

RESULTS

Measurement of NO and PGE_2 Concentration Compounds **1** and **6** showed inhibition of NO production by J774.1 with the IC_{50} values of 3.7 μM and 9.6 μM, respectively, and then **2** and **7** showed inhibition with 11.2 μM and 25.6 μM. In the case of **3**, **4**, and **5**, the IC_{50} value of them was more than 100 μM (Table 1). On the other hand, **1** minimally inhibited PGE_2 production; it showed inhibition with the IC_{50} value of more than 100 μM. As opposed to **1**, **2** and **6** showed inhibition of PGE_2 production by J774.1 with the IC_{50} values of 7.7 μM and 7.8 μM, respectively, and then **5** and **7** showed inhibition with 22.6 μM and 22.9 μM. It was revealed that **2**, **6** and **7** inhibited both NO and PGE_2 production. No measurements were conducted for **1** and **6** at 100 μM because these compounds were cytotoxic at that concentration. Note that the reported cytotoxicity of **1** at 40 μM[13] reinforces our finding of the cytotoxic effects of **1** and **6** at 100 μM; the cell viability was found to be more than 95% for the rest.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The ability to suppress TNF-α, IL-6, COX-2, and iNOS mRNA expression in J774.1 was measured after incubation for 24 h in the presence of LPS and the seven compounds (Table 1). Regarding TNF-α mRNA expression, **3**, **7** and **6** showed inhibition with the IC_{50} values of 12.3 μM, 12.6 μM and 14.2 μM, respectively. Regarding IL-6 mRNA expression, **6** and **2** showed inhibition with the IC_{50} values of 3.0 μM and 17.4 μM, respectively. Regarding COX-2 mRNA expression, **6** showed inhibition with the IC_{50} value of 21.4 μM. Regarding iNOS mRNA expression, **1**, **6**, and **2** showed inhibition with the IC_{50} values of 3.6 μM, 13.2 μM and 16.6 μM, respectively. Clearly, **1** showed significant inhibition of only iNOS mRNA expression. No measurements were conducted for **1** and **6** at 100 μM because these compounds were cytotoxic at that concentration.

Table 1. IC_{50} Value of Seven Compounds in *in Vitro* Assay

Compound	NO production (μM)	PGE_2 production (μM)	mRNA expression of TNF-α (μM)	mRNA expression of IL-6 (μM)	mRNA expression of COX-2 (μM)	mRNA expression of iNOS (μM)
1	3.7	100<	100<	100<	100<	3.6
2	11.2	7.7	47.8	17.4	75.1	16.6
3	100<	64.3	12.3	43.1	100<	100<
4	100<	100<	100<	100<	100<	100<
5	100<	22.6	100<	100<	52.4	100<
6	9.6	7.8	14.2	3.0	21.4	13.2
7	25.6	22.9	12.6	51.9	48.0	53.3

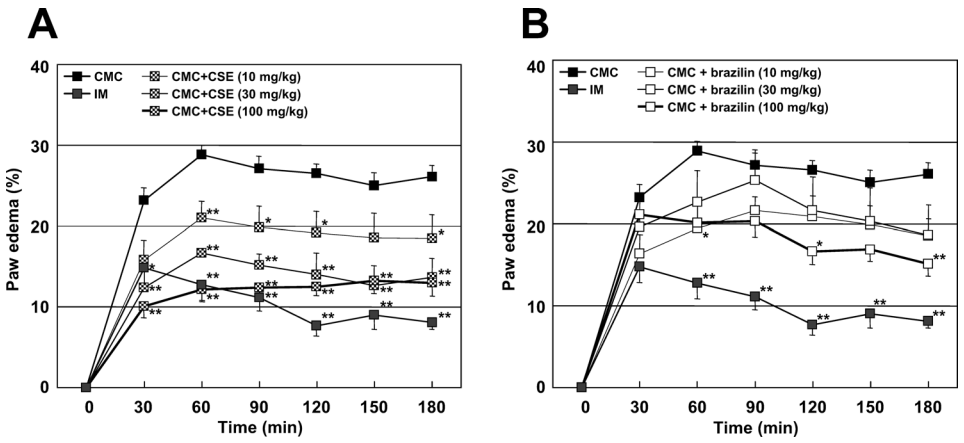

Fig. 2. Effects of CSE and Brazilin (**1**) on Mouse Paw Edema Induced by Carrageenin

Time course of percentage inhibition of CSE (A) and brazilin (**1**) (B) up to 180 min. Sample was administered orally 30 min before carrageenin injection. The results are shown as means±S.E. of 5—8 data. $*p<0.05$, $**p<0.01$ when compared to CMC groups. Abbreviations are as follows: CMC, carboxymethyl cellulose; IM, indomethacin; CSE, methanol extract of Sappan Lignum.

Carrageenin-Induced Mouse Paw Edema Orally administered CSE showed a concentration-dependent inhibitory effect on carrageenin-induced mouse paw edema (Fig. 2A). CSE at 100 mg/kg showed a similar effect to IM at 10 mg/kg. The accumulation under curve (AUC) evaluation demonstrated that CSE showed 27.8% (10 mg/kg), 45.9% (30 mg/kg), and 53.4% inhibition (100 mg/kg), while IM showed 58.7% inhibition. In contrast, orally administered **1** (10, 30, 100 mg/kg) was less effective than CSE and its effect was not concentration-dependent (Fig. 2B). The AUC evaluation demonstrated that **1** showed 25.2% (10 mg/kg), 17.3% (30 mg/kg), and 28.5% inhibition (100 mg/kg). At 100 mg/kg, the effect of CSE was superior to that of **1**.

DISCUSSION

It is known that the large amount of NO synthesized through iNOS gene induces tissue injury and that PGE_2 synthesized through COX-2 gene accelerates inflammation. Thus, compounds that suppress iNOS gene or COX-2 gene expression are expected to show anti-inflammatory effect by decreasing NO or PGE_2 production. TNF-α, also an early-stage inflammation mediator, induces the synthesis of IL-6 or serotonin, resulting in the activation of T cells and inflammation-related cells. Thus, compounds that suppress TNF-α and IL-6 mRNA expression are also expected to show anti-inflammatory effect.

We investigated the inhibitory effects of the seven compounds on the chemical mediators of inflammation and compared their activities and mechanisms. Using J774.1, we examined the inhibitory effects on NO and PGE_2 production, and the suppressive effects on TNF-α, IL-6, COX-2, and iNOS mRNA expression. Compound **1** most effectively suppressed NO production, followed by **6**, **2**, and **7**. In contrast, **1** had almost no effect on PGE_2 production while **2**, **6**, **5**, and **7** were effective.

It was reported that CSE and **1** were responsible for the anti-inflammatory effect of Sappan Lignum. Hong *et al.* reported that 10 μg/ml CSE inhibited NO production by 71% and PGE_2 production by 40% in LPS-induced mouse RAW264.7.[18] Bae *et al.* reported that **1** suppressed iNOS protein and mRNA expression in RAW264.7.[13] Hikino *et al.* showed through *in vivo* experiments that **1** inhibited carrageenin-induced rat paw edema.[12] Thus, the anti-inflammatory effects of CSE and **1** were well established; however, it has not been clear if the effects of CSE were contributed by only **1**, because no comparative study of CSE and **1** has been conducted. Only a few studies were conducted of compounds other than **1**, such as the anti-complementary activity[19] of **7** and the xanthine oxidase inhibitory activity[20] of **2**. Our previous study revealed that **2** showed similar anti-oxidant activity to **1**,[16] suggesting that the anti-inflammatory effect of CSE is due to not only **1**.

In this study, we performed a comparative study of the effects of CSE and **1** at the same concentration on carrageenin-induced mouse paw edema and found that CSE showed stronger activity than **1** at the same concentration (Fig. 2), indicating that active constituents other than **1** exist in CSE.

As it has been reported that the edema induced by carrageenin involved PGE_2 and NO production,[21,22] it is suggested that the inhibitory effect of CSE in Fig. 2 was due to **2**, **6**, and **7** as well as **1**. Unfortunately, we could not conduct *in vivo* experiments of **2**, **6** or **7** because we had limited amounts of the compounds.

Regarding the activity of protosappanin derivatives, **3** suppressed TNF-α and IL-6 mRNA expression. Compound **4**, in contrast, had almost no effect. Compound **5** inhibited PGE_2 production and suppressed COX-2 mRNA expression more effectively than **3** and **4**. Compounds **3**, **4**, and **5** have different functional groups on dibenzoxocin skeleton at C-7 position: a carbonyl group for **3** and an aldehyde group for **5**, and those groups seemed to contribute to their respective activities.

Compound **6** had almost the same suppressive effect as **2** on both NO and PGE_2 production. Moreover, **6** showed the strongest inhibition in the TNF-α, IL-6, COX-2, and iNOS mRNA expression with the IC_{50} values from 3.0 to 21.4 μM. Additionally, **7** inhibited both NO and PGE_2, as well as TNF-α, IL-6, COX-2, and iNOS mRNA expression. Compound **6** was the most effective against COX-2 among the seven compounds. Protosappanins A—E (**3**—**7**) are all dibenzoxocin derivatives, and our data suggest that the dibenzoxocin skele-

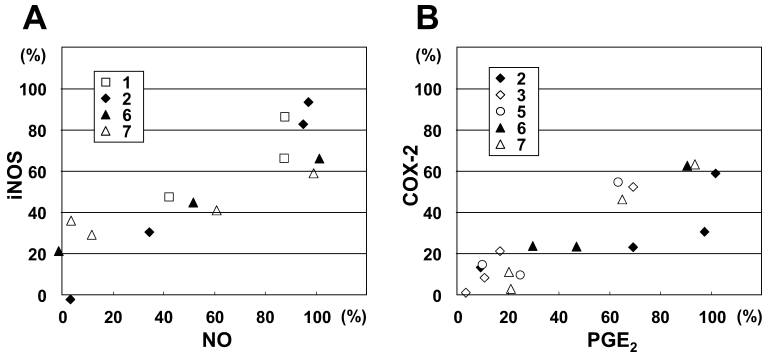

Fig. 3. Correlation Diagrams of (A) NO–iNOS Percentage Inhibition and (B) PGE_2–COX-2 Percentage Inhibition

ton and the functional groups at C-7 position would be important for the activity.

We also estimated the mechanisms underlying the inhibition of NO and PGE_2 production by the compounds. We found that NO–iNOS inhibition by **1**, **2**, **6**, and **7** showed a direct correlation (Fig. 3A). The results indicated that those compounds inhibited NO production by suppressing iNOS mRNA expression. In a similar way, PGE_2–COX-2 inhibition by **3**, **5**, **6**, and **7** showed a direct correlation (Fig. 3B), suggesting that those compounds inhibited PGE_2 production by suppressing COX-2 mRNA expression. In contrast, **2** showed not a direct correlation but a declinate curve, implying that **2** inhibited not only COX-2 mRNA expression but also COX-2 or PGE_2 synthase activity.

In this study, we clarified that **2**, **6**, and **7** inhibited both NO and PGE_2 production and suppressed the mRNA expression of TNF-α, IL-6, COX-2, and iNOS, which are inflammation mediators, while that **1** inhibited NO production and suppressed iNOS expression. Those compounds are also thought to possess anti-inflammatory activity.

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