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ORIGINAL PAPER

Quantitative analysis of anti-inflammatory activity of orengedokuto: importance of combination of flavonoids in inhibition of PGE_2 production in mouse macrophage-like cell line J774.1

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Abstract Orengedokuto is a Kampo formula which has been used for removing "heat" and "poison" to treat inflammation, hypertension, gastrointestinal disorders, and liver and cerebrovascular diseases. In this report, we quantitatively analyzed the anti-inflammatory effect of the component crude drugs of orengedokuto and their constituents, using inhibition of prostaglandin E_2 (PGE_2) production in the murine macrophage-like cell line J774.1. First, we compared PGE_2 production inhibitory activities of extracts of combinations of the component crude drugs, which showed that the activity could be ascribed to Scutellaria Root. Next, as baicalin (**1**), one of the major constituents of Scutellaria Root, did not show any activity, and baicalein (**2**), the aglycon of **1**, showed only weak activity (IC_{50} 92 μM), a hot-water extract of Scutellaria Root was fractionated under the guidance of the activity to give wogonin (**3**) (IC_{50} 28 μM), 6-methoxywogonin (**4**) (IC_{50} 7.2 μM) and oroxylin A (**5**) (IC_{50} 45 μM) from the most active fraction. However, the activities of these compounds at concentrations equivalent to those in the extract were weaker than that of the extract, and none of these compounds alone could explain the activity of the extract. Therefore, we examined the activity of combinations of compounds **2**–**5**. Comparison of all combinations of the four compounds in a ratio which is the same as in the extract revealed that wogonin (**3**) had an essential role in the activity, and a combination of baicalein (**2**) and wogonin (**3**), together with 6-methoxywogonin (**4**) or oroxylin A (**5**), was necessary to show activity equivalent to that of the extract.

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Introduction

Orengedokuto (黄連解毒湯) is a Kampo formula consisting of Scutellaria Root (**S**, root of *Scutellaria baicalensis*), Phellodendron Bark (**P**, bark of *Phellodendron amurense* or *P. chinense*), Coptis Rhizome (**C**, rhizome of *Coptis japonica*, *C. chinense*, *C. deltoidea*, or *C. teeta*) and Gardenia Fruit (**G**, fruit of *Gardenia jasminoides*) [1]. It has been used for removing "heat" and "poison" to treat inflammation, hypertension, gastrointestinal disorders, and liver and cerebrovascular diseases [2]. There have been many reports on the anti-inflammatory activities of the component crude drugs of orengedokuto. A water extract of Scutellaria Root inhibited production of nitric oxide (NO), interleukin (IL)-3, IL-6, IL-10, IL-12p40 and IL-17, interferon-inducible protein (IP)-10 and vascular endothelial growth factor (VEGF) in RAW264.7 macrophages [3]. Baicalein and wogonin from Scutellaria Root inhibited production of pro-inflammatory cytokines [tumor necrosis factor (TNF)-α, IL-6, IL-10 and NO] [4–6]. Methanolic extracts of Phellodendron Bark ameliorated acute airway inflammation in mouse [7], and inhibited inducible nitric oxide synthase (iNOS) and TNF-α gene expression in BV2 cells [8]. Berberine, the main constituent of Coptis Rhizome and Phellodendron Bark, inhibited IL-6 mRNA expression in YES-2 cells [9], and inhibited IL-1β and TNF-α production through inhibition of inhibitor kappa B (IκB)-α degradation in human lung cells [10]. It also inhibited cyclooxygenase (COX)-2 transcription in human colon cancer cells [11], and intercellular adhesion molecule

(ICAM)-1, transforming growth factor (TGF)-β1, iNOS and fibronectin in rat mesangial cells [12]. An extract of Gardenia Fruit showed anti-inflammatory effect on acute hepatopathy in rats [13], and its constituents, crocin [14] and iridoid glycosides [15–18], showed anti-inflammatory effects.

In spite of these reports on the effects of the individual crude drugs and their constituents, studies on the anti-inflammatory effect of orengedokuto itself in connection with the constituents taking part in the activity are limited. Zeng et al. [19] reported an analysis of the anti-inflammatory effect of orengedokuto, and revealed that a mixture of baicalein from Scutellaria Root and coptisine from Coptis Rhizome inhibited production of leukotriene B_4 (LTB_4) through inhibition of the lipoxygenase pathway. Lu et al. [20] reported that a baicalin-containing fraction of orengedokuto extract mainly inhibited the production of pro-inflammatory mediators [malondialdehyde (MDA), NO, superoxide dismutase (SOD), PGE_2, TNF-α, IL-6 and IL-10]. However, the quantitative relationship between the activity and the amount of the constituents in the extract is not described in these reports.

In this report, we quantitatively analyzed the role of the component crude drugs of orengedokuto and their constituents in the anti-inflammatory effect of orengedokuto, using inhibition of PGE_2 production in murine macrophage-like cells J774.1, and revealed the importance of the combination of flavonoids from Scutellaria Root.

Experimental

General procedure

HPLC analyses were performed on an LC-10A HPLC system (Shimadzu, Japan) consisting of a column compartment (CTO-10A), a degasser (DGU-12A), a pump (LC-10AD*vp*) and a detector (SPD-10A). A LC-9201 HPLC system (Japan Analytical Industry Co., Ltd., Japan) equipped with a JAIGEL-2H column was used for recycling preparative HPLC. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL FT-NMR ECP-600 spectrometer, and chemical shifts were expressed in δ (ppm) with TMS as an internal standard. MALDI-TOF MS were recorded on a Voyager RP spectrometer.

Materials

Crude drugs used for preparation of extracts, Scutellaria Root (Lot 004609007), Phellodendron Bark (Lot 001310001), Coptis Rhizome (Lot 001210002) and Gardenia Fruit (Lot 001110001), were purchased from Tochimoto Co., Japan. Baicalin (Lot STL2939) and baicalein (Lot PKF2197) were purchased from Wako Pure Chemical Industries, Ltd., Japan.

Preparation of extracts of each crude drug and combined formula

Cut crude drug materials [single crude drug or a combination of multiple crude drugs (combined formula), 20 g] were heated in distilled water (400 mL) by a decoction maker (UIH-650N, Uchida Wakanyaku Ltd., Japan) until the volume was reduced to a half in 30 min. The extract was filtered through gauze and centrifuged at 1000*g* at 25 °C for 10 min. The supernatant was separated, its volume measured, and freeze-dried to give an extract. The procedure was repeated three times, and average weight and standard error were calculated. The composition ratio of the component crude drugs of orengedokuto is **S**:**P**:**C**:**G** = 3:1.5:1.5:2 [1].

Preparation of blended formula

The extracts of the component crude drugs prepared as above were mixed to prepare blended formulae corresponding to the combinations of entries 5–15 in Table 1 in the ratio **S**:**P**:**C**:**G** = 4.7:1:1.3:2.2. The ratio was calculated from the product of the yield of the extract and composition

Table 1 The amount of each extract prepared from 20 g of crude drug materials

Entry	**S**[a]	**P**[a]	**C**[a]	**G**[a]	Weight (g)[b]
1	○				6.34 ± 0.17
2		○			2.72 ± 0.07
3			○		3.44 ± 0.12
4				○	4.55 ± 0.20
5	○	○			4.98 ± 0.14
6	○		○		4.98 ± 0.29
7	○			○	6.44 ± 0.54
8		○	○		2.88 ± 0.05
9		○		○	3.65 ± 0.47
10			○	○	4.27 ± 0.13
11	○	○	○		3.69 ± 0.13
12	○	○		○	5.24 ± 0.16
13	○		○	○	5.43 ± 0.83
14		○	○	○	3.65 ± 0.36
15	○	○	○	○	4.22 ± 0.55

Crude drugs were combined in a ratio of **S**:**P**:**C**:**G** = 3:1.5:1.5:2

[a] **S**: Scutellaria Root, **P**: Phellondendron Bark, **C**: Coptis Rhizome, **G**: Gardenia Fruit

[b] Average of three experiments ± SE

ratio in orengedokuto of each crude drug: **S**:**P**:**C**:**G** = (6.34 × 3):(2.72 × 1.5):(3.44 × 1.5):(4.55 × 2).

Extraction and isolation

Scutellaria Root (1.5 kg) (Lot P031010201) was extracted with hot water (4.5 L) for 40 min, and the extraction was repeated four times. The combined extracts were applied to a column of Diaion HP-20 (12 × 10 cm), and eluted with H_2O (8 L) followed by MeOH (40 L) to afford water elute [fr. DW, 492 g (IC_{50} > 50 μg/mL)] and MeOH elute [fr. DM, 59 g (IC_{50} 40.0 μg/mL)]. A portion of fr. DM (19 g) was chromatographed on a column of silica gel (5 × 30 cm) with $CHCl_3$–MeOH to afford seven fractions [fr. DM-1: $CHCl_3$ (2.2 L), 1.21 g (IC_{50} 1.87 μg/mL); fr. DM-2: $CHCl_3$:MeOH = 9:1 (2 L), 3.88 g (IC_{50} > 50 μg/mL); fr. DM-3: $CHCl_3$:MeOH = 9:1 (0.5 L), 425 mg (IC_{50} > 50 μg/mL); fr. DM-4: $CHCl_3$:MeOH = 4 : 1 (5 L), 2.33 g (IC_{50} > 50 μg/mL); fr. DM-5: $CHCl_3$:MeOH = 3:2 (3 L), 1.63 g (IC_{50} > 50 μg/mL); fr. DM-6: $CHCl_3$:MeOH = 1:4 (11 L), 6.20 g (IC_{50} > 50 μg/mL); fr. DM-7: MeOH (14 L), 1.89 g (IC_{50} > 50 μg/mL)]. A portion of fr. DM-1 (1.21 g) was crystallized from $CHCl_3$ to give wogonin (**3**) [21] (646 mg). A part of the mother liquor (81 mg) was subjected to preparative recycling-HPLC ($CHCl_3$) to give 6-methoxywogonin (**4**) [22] (8.0 mg) and oroxylin A (**5**) [23] (12.5 mg). The structures of the isolated compounds were confirmed by comparisons of their spectral data with those reported.

Quantitative analysis of flavonoids

Quantitative analyses of flavonoids were carried out by published methods with appropriate modifications.

Baicalin [24]: Chromatographic analysis was carried out on a Capcell Pak C18 AQ type (4.6 × 250 mm i.d.) (Shiseido Fine Chemical Co., Japan), with 0.1 % trifluoroacetic acid (TFA) in water (A)-MeCN (B) using the following gradient: 0–4 min (10 % MeCN) → 15 min (26 % MeCN) → 27 min (28 % MeCN) → 35 min (70 % MeCN) → 55–60 min (90 % MeCN) at a flow rate of 1.0 mL/min. The column was maintained at 40 °C and 280 nm was used for the detection of baicalin.

Baicalein, wogonin [25]: Chromatographic analysis was carried out on a BDS Hypersil C18 (4.6 × 250 mm i.d.) (Thermo Scientific Co., Japan), with water–MeCN mixture (68:32) containing 5 mM tetra-*n*-amylammonium bromide (TAA), whose pH was adjusted to 4 by H_3PO_4. The column was kept at 40 °C with a flow rate of 1.0 mL/min, and 280 nm was used for the detection of the compounds.

6-Methoxywogonin, oroxylin A [20]: Chromatographic analysis was carried out on a Capcell Pak C18 AQ type (4.6 × 250 mm i.d.) (Shiseido Fine Chemical Co., Japan), with 0.06 % H_3PO_4 in water (A)–MeCN (B) using the following gradient: 0–5 min (20 % MeCN) → 30 min (30 % MeCN) → 55 min (55 % MeCN) → 60 min (90 % MeCN) at a flow rate of 1.0 mL/min. The column was maintained at 30 °C and 280 nm was used for the detection of the compounds.

Inhibition of PGE_2 production

A murine macrophage-like cell line J774.1 was grown in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies Japan Ltd.) supplemented with 10 % FBS and 1 % penicillin/streptomycin/glutamine at 37 °C under humidified 5 % CO_2. The cells were seeded in wells of a 96-well culture plate (Falcon) at a density of 5.0 × 10^5 cells/mL in 200 μL of the medium, and allowed to adhere to the plate for 24 h. The medium was then replaced with a fresh medium containing lipopolysaccharide (LPS, 1 μg/mL) from *E. coli.* (Sigma) and a test compound, and incubated for 24 h. The concentration of PGE_2 in the culture medium was measured by a PGE_2 ELISA kit (Cayman Chemical Co., USA) according to the manufacturer's instructions [26]. Absorbance at 405 nm was measured with a Microplate Reader (model no. MTP-810 lab, Corona Co., Japan). The percentage inhibition was calculated as follows: % of control = [As/Ac] × 100, where Ac and As are absorbance of control treated with LPS alone and that treated with LPS and a sample, respectively. Indomethacin (Ind, IC_{50} 3.9 μM), a COX inhibitor, was used as a positive control [27].

Cell viability

Cell viability was determined by the mitochondrial respiration-dependent 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction method [28]. Briefly, after incubation for 24 h as stated above in 50 μL of the medium, the cells were added to 5 mg/mL MTT solution (Dojindo, Japan) (5 μL), and incubated for 3 h at 37 °C under humidified 5 % CO_2. The supernatant was then removed and violet crystals of formazan in viable cells were dissolved in 50 μL of DMSO, and absorbance at 595 nm was measured.

Statistics

All data are presented as mean ± SE of three independent experiments. The differences between the activity of LPS control and those of the samples were tested by a one-tailed Student's *t* test using StatPlus 2009 for Mac OS (AnalystSoft Inc.). Dunnett's multiple comparison test was used for multigroup comparisons.

Results and discussion

The component crude drugs of orengedokuto, Scutellaria Root (**S**), Phellodendron Bark (**P**), Coptis Rhizome (**C**) and Gardenia Fruit (**G**), together with all combinations of them, were separately extracted in the manner of preparing a Kampo decoction, and the extracts, after removal of precipitates, were freeze-dried. For the combinations, a component crude drug ratio of **S**:**P**:**C**:**G** = 3:1.5:1.5:2 as listed in the Japanese Pharmacopoeia [1] was used. Table 1 shows the amounts of the extracts prepared from 20 g of the crude drug materials. Of the single crude drugs (entries 1–4), Scutellaria Root (entry 1) gave the largest amount of the extract, followed by Gardenia Fruit (entry 4), Coptis Rhizome (entry 3) and Phellodendron Bark (entry 2).

There is a possibility that constituents of component crude drugs interact with each other during the decoction process to cause chemical changes in the extract. Therefore, in addition to the extracts of mixed crude drugs (combined formulae, Table 1, entries 5–15), we prepared mixtures of each extract of the component crude drugs (blended formulae) in the ratio corresponding to the ratio of the amount of each crude drug extract, and compared their activity.

As a preliminary test, we measured PGE_2 production inhibitory activity of orengedokuto extract on LPS-treated J774.1 macrophages at various concentrations (25 μg/mL, 9.7 %; 50 μg/mL, 35.8 %; 100 μg/mL, 65.6 %) and selected 50 μg/mL for comparison of the various combinations of crude drugs. Figure 1 shows the inhibitory activities of single crude drugs, combined formulae and blended formulae at 50 μg/mL. Not all the samples showed cytotoxicity, indicating that the inhibition of PGE_2 production was not caused by a cytotoxic effect. Of the single crude drugs (**S**–**G**), only Scutellaria Root (**S**) showed significant inhibitory activity. Among the combinations (**SP**–**SPCG**), **S**-containing combinations also showed significant activity. As the extract of **S** showed more potent PGE_2 production inhibitory activity than those of orengedokuto (**SPCG**) and any other combination, the activity can be ascribed to Scutellaria Root. No significant difference was observed between the activities of the combined and blended formulae, indicating that no change which influences the activity occurred in the extracts during the decoction process. Thus, the constituents originally contained in Scutellaria Root are responsible for the PGE_2 production inhibitory activity of orengedokuto.

Baicalin (**1**), one of the major constituents of Scutellaria Root, has been reported to inhibit COX-2 and its gene expression, resulting in inhibition of PGE_2 production [29]. However, **1** showed no activity up to 100 μM (data not shown) in our assay. Quantitative analysis of **1** revealed that the concentration of **1** in 100 μg/mL solution of **S** extract was 57.8 μM (Table 2). Thus, **1** does not contribute to the activity of Scutellaria Root.

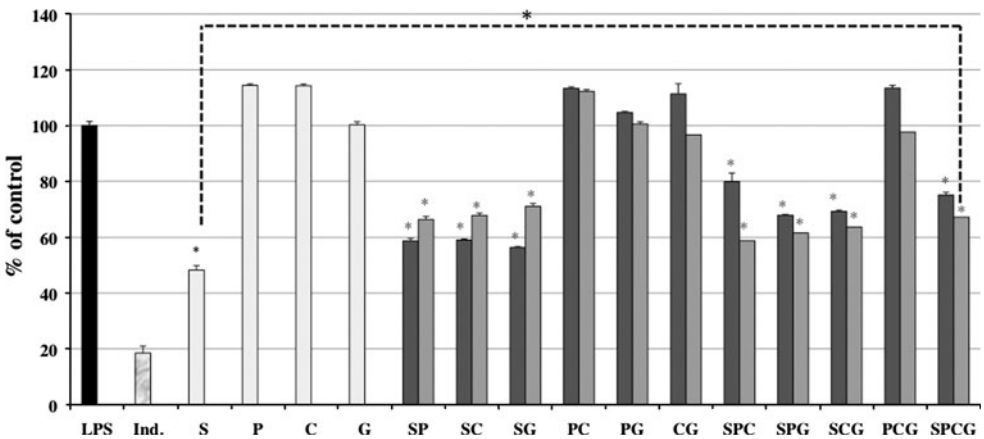

Fig. 1 PGE_2 production inhibitory activity of the extracts (50 μg/mL). *LPS* lipopolysaccharide, 1 μg/mL, *Ind* indomethacin, 5 μM. *Single asterisk* indicates a significant difference ($p < 0.05$). *Light grey* single crude drug, *black* blended extract, *thick grey* combined extract

Table 2 Quantitative analysis of compounds **1**–**5** in the extract of Scutellaria Root

	Baicalin (**1**)	Baicalein (**2**)	Wogonin (**3**)	6-Methoxywogonin (**4**)	Oroxylin A (**5**)
Amount *a* (mg)[a]	1640	124	47.9	1.01	2.74
Content *b* (%) in the extract[b]	25.9	1.96	0.756	0.016	0.043
Concentration (μM) in 100 μg/mL of the extract[c]	57.8	7.26	2.66	0.051	0.152

[a] The amount (mg) contained in the extract prepared from 20 g of Scutellaria Root

[b] *a*/6340 × 100

[c] 100 (μg/mL) × *b*/100 × 1000/MW

Next, we examined the activity of baicalein (**2**), the aglycon of **1**. Baicalein (**2**) showed activity with an IC_{50} value of 92 μM. However, the concentration of **2** in the extract solution of **S** (100 μg/mL) was 7.3 μM (Table 2), and the activity of **2** at 10 μM (about 80 % of the control) was much lower than that of the corresponding extract solution (100 μg/mL, 20 % of the control). Thus, **2** also does not contribute to the activity of the extract (Fig. 2).

In order to identify the active principles in Scutellaria Root, its hot-water extract was fractionated under the guidance of PGE_2 production inhibitory activity, resulting in the isolation of three flavonoids, wogonin (**3**), 6-methoxywogonin (**4**) and oroxylin A (**5**), from the most potent fraction (Fig. 3). These compounds showed appreciable activity with IC_{50} values of 28 μM (**3**), 7.2 μM (**4**) and 45 μM (**5**). However, quantitative analyses of **3**–**5** in the extract (100 μg/mL) revealed that the concentrations of these compounds were 2.66 μM, 0.051 μM and 0.152 μM, respectively (Table 2), indicating that **3**–**5** also do not individually contribute to the activity of the extract (Fig. 4).

Therefore, the activities of a mixture of compounds **2**–**5** were examined. Compounds **2**–**5** were mixed in the ratio equivalent to that in the extract (**2**:**3**:**4**:**5** = 120:47:1:2.7) (Table 2), and the activity of the mixture was determined (Fig. 5). The combination of all of the four compounds greatly increased the activity. The mixture showed significant inhibitory activity at 1 μg/mL, and the activity at 2.5 μg/mL was comparable to that of the extract (100 μg/mL). The total concentration of compounds **2**–**5** in the 100 μg/mL solution of the extract of Scutellaria Root was 2.78 μg/mL. Therefore, the PGE_2 production inhibitory activity of Scutellaria Root extract, and orengedokuto, can be ascribed to the combination of these four compounds.

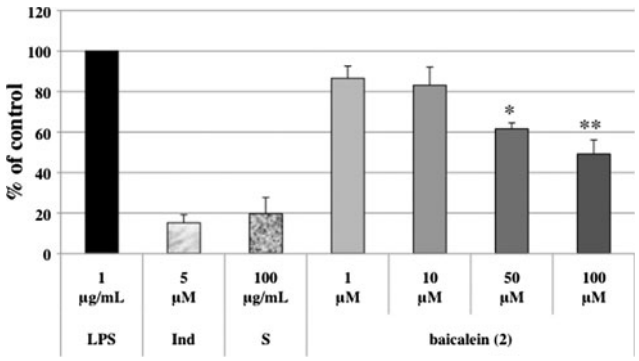

Fig. 2 PGE_2 production inhibitory activity of baicalein (**2**). *LPS* lipopolysaccharide, *Ind* indomethacin, **S**: extract of Scutellaria Root. *Single asterisk* indicates a significant difference ($p < 0.05$). *Double asterisk* indicates a very significant difference ($p < 0.01$). The concentration of **2** in the extract (**S**) was 7.26 μM

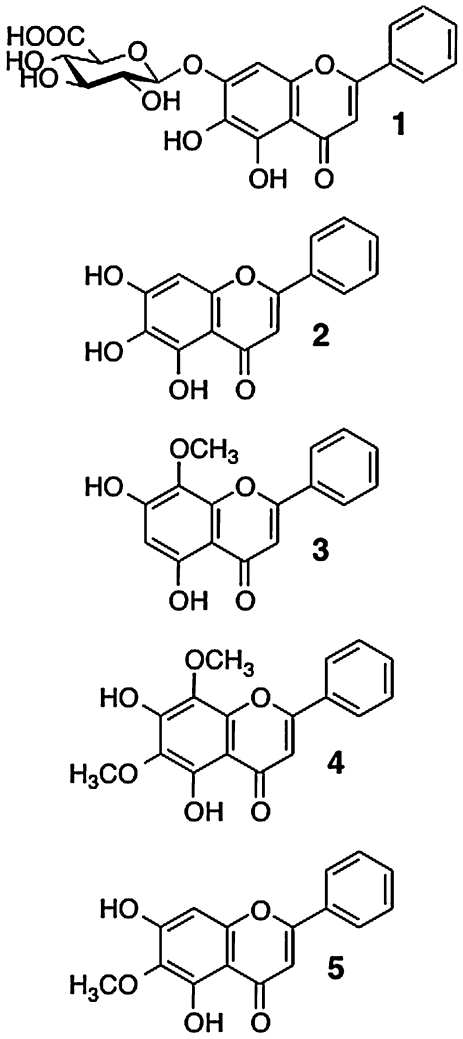

Fig. 3 Structures of the flavonoids of Scutellaria Root

Next, we examined the activity of all combinations of these four compounds in ratios equivalent to those in the extract of Scutellaria Root (Table 3, Fig. 6). Table 3 shows the IC_{50} values of the combinations. The mixture of compounds **2**–**5** (**2**:**3**:**4**:**5** = 120:47:1:2.7) showed significantly stronger activity (IC_{50} 0.67 μg/mL) than individual compounds (baicalein (**2**), IC_{50} 24.8 μg/mL; wogonin (**3**), IC_{50} 7.81 μg/mL; 6-methoxywogonin (**4**), IC_{50} 2.26 μg/mL; oroxylin A (**5**), IC_{50} 12.8 μg/mL). Among the mixtures consisting of three of the four compounds (entries C2–C5), the mixture without **3** (entry C3) showed significantly less activity compared to the full mixture (C1). In addition, among the mixtures of two compounds (entries C6–C11), those containing **3** (entry C6, C7, C9) showed stronger activity than those without **3** (entry C8, C10, C11). Thus, although the IC_{50} value of wogonin (**3**) is larger than that of oroxylin A (**5**), **3** seems to play an important role in the inhibition of PGE_2 production.

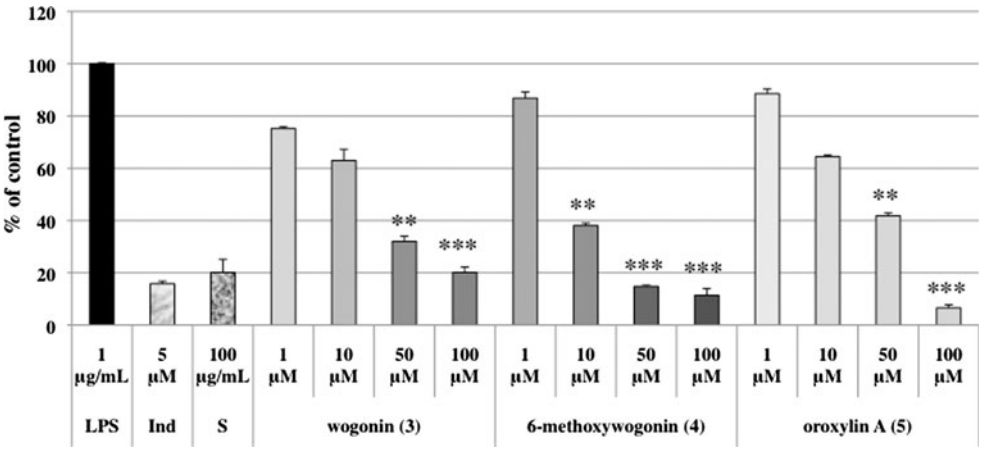

Fig. 4 PGE_2 production inhibitory activity of wogonin (**3**), 6-methoxywogonin (**4**) and oroxylin A (**5**). *LPS* lipopolysaccharide, *Ind* indomethacin, **S**: extract of Scutellaria Root. *Double asterisk* indicates a very significant difference ($p < 0.01$). *Triple asterisk* indicates an extremely significant difference ($p < 0.001$). The concentrations of **3**, **4** and **5** in the extract (**S**) were 2.66 μM, 0.051 μM and 0.152 μM, respectively

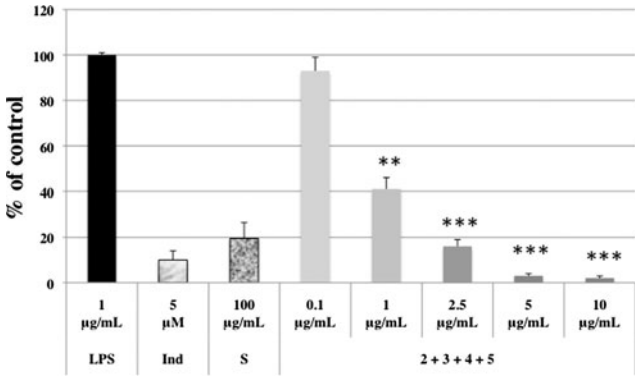

Fig. 5 PGE_2 production inhibitory activity of the combination of four active constituents. *LPS* lipopolysaccharide, *Ind* indomethacin, **S**: extract of Scutellaria Root. *Double asterisk* indicates a very significant difference ($p < 0.01$). *Triple asterisk* indicates an extremely significant difference ($p < 0.001$). Total content of **2**–**5** in **S** was 2.78 μg/mL

Table 3 IC_{50} values of each combination of compounds **2**–**5**

Combination	**2**	**3**	**4**	**5**	IC_{50} (μg/mL) ± SE[a]
C1	○	○	○	○	0.67 ± 0.01
C2		○	○	○	0.45 ± 0.09
C3	○		○	○	3.56 ± 0.30
C4	○	○		○	1.02 ± 0.15
C5	○	○	○		1.25 ± 0.23
C6		○	○		0.49 ± 0.16
C7		○		○	2.40 ± 0.66
C8			○	○	1.01 ± 0.02
C9	○	○			4.27 ± 0.33
C10	○		○		2.71 ± 0.97
C11	○			○	3.84 ± 0.95

The compounds were mixed in the ratio of **2**:**3**:**4**:**5** = 120:47:1:2.7

[a] Average of at least 3 experiments ± SE

Figure 6 shows the activity of each combination at the concentration equivalent to that in the hot-water extract of Scuterallia Root (100 μg/mL). The mixture of the four compounds (C1, **2** + **3** + **4** + **5**) showed slightly stronger activity than that of the extract, and combinations C4 (**2** + **3** + **5**) and C5 (**2** + **3** + **4**) showed comparable activity to the extract. However, the activity of the combination C9, containing only **2** and **3**, was significantly lower than those of C1, C4 and C5. Therefore, it may be concluded that the combination of at least three constituents (**2** + **3**, and **4** or **5**) is required for the activity of the extract. Although the contents of **4** and **5** are very small compared to those of **2** and **3** (Table 2), **4** and **5** also have important roles in the activity of the extract.

Flavonoids of *Scutellaria baicalensis* have been reported to inhibit LPS-induced production of PGE_2 in mouse macrophage cells [5, 30–32]. Chi et al. [5] reported that baicalein (**2**) and wogonin (**3**) inhibited LPS-induced expression of COX-2 and iNOS and **3** also showed direct inhibition of COX-2, resulting in inhibition of PGE_2 production, and similar results were reported by Han et al. [30]. On the contrary, Kaneko et al. [31] reported that these compounds inhibited LPS-induced PGE_2 production, but did not inhibit induction of COX-2, and Chen et al. [32] reported that LPS-induced expression of iNOS was inhibited by **2** and **3**, but only **3** inhibited expression of COX-2 without affecting its enzyme activity. Oroxylin A (**5**) was also reported to inhibit gene expressions of iNOS and COX-2 [32]. As it is reported that nitric oxide activated COX and a combination of COX inhibitor and iNOS inhibitor effectively reduced PG production [33], the increase in the activity of the flavonoid mixtures revealed in this work can be explained, in part, as a total effect of inhibition of expression of COX-2, direct inhibition of COX activity and inhibition of COX activation by NO through inhibition of iNOS expression. Further investigation is necessary to clarify the underlying mechanism of the increased activity of the mixture.

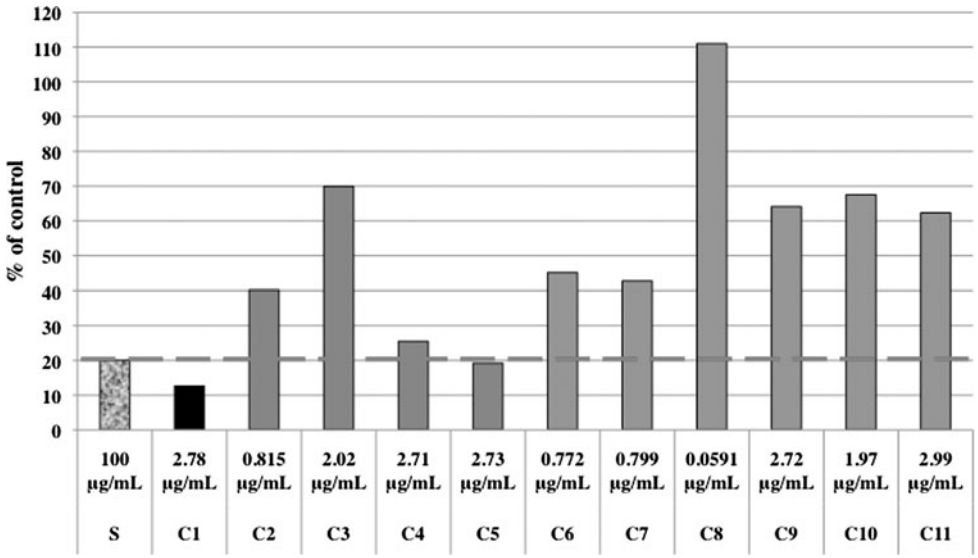

Fig. 6 PGE_2 production inhibitory activity of each combination of compounds **2**–**5** at the concentration in the extract of Scutellaria Root (100 μg/mL). **S**: extract of Scutellaria Root; **C1**–**C11**: see Table 3

In this paper, we investigated PGE_2 production inhibitory activity of a Kampo formula, orengedokuto. The activity was ascribed to one of the component crude drugs, Scutellaria Root, and the active constituents were revealed to be a mixture of baicalein (**2**), wogonin (**3**), 6-methoxywogonin (**4**) and oroxylin A (**5**), among which **3** plays an important role in the activity of the extract. We used a mouse macrophage-like cell line J774.1 of ascites origin as a model system of inflammation, and the response of this cell line to the flavonoid mixture might be different from that of human macrophages. However, as a human response to a Kampo formula is very complex, a simplified in-vitro system such as cultured cells is a versatile tool for analyzing such a complex response. Further experiments to clarify the response of human macrophages to the flavonoid mixture will be necessary as the next step in connecting this result to the mechanism of action of orengedokuto.

In the search for active constituents of crude drugs, it is often found that the activity decreases or disappears in the course of separation of the constituents. In this work, based on quantitative analyses of the constituents, we clearly showed that a combination of constituents plays an essential role in the activity of the extract. This result demonstrates the importance of quantitative analysis of the relationship between a biological activity and combinations of constituents. It is generally believed that the effectiveness of Kampo formulae relies on effects of multiple constituents of component crude drugs. Our strategy will contribute to clarifying the scientific basis of the effectiveness of Kampo formulae.

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