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ORIGINAL PAPER

Comprehensive evaluation of antioxidant effects of Japanese Kampo medicines led to identification of Tsudosan formulation as a potent antioxidant agent

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Abstract
Oxidative stress due to the overproduction of reactive oxygen species plays an important role in the pathogenesis of various diseases. In the present study, we comprehensively evaluated the antioxidant activities of 147 oral formulations of Japanese traditional herbal medicines (Kampo medicines), representing the entire panel of oral Kampo medicines listed in the Japanese National Health Insurance Drug List, using in vitro radical scavenging assays, including the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity assay, the superoxide anion scavenging activity assay, and the oxygen radical absorption capacity assay. Three of the formulations tested, namely, Tsudosan, Daisaikoto, and Masiningan, showed the most potent in vitro antioxidant activities and were selected for further investigation of their intracellular and in vivo antioxidant effects. The results of the 2′,7′-dichlorodihydrofluorescin diacetate assay demonstrated that all three Kampo medicines significantly inhibited hydrogen peroxide-induced oxidative stress in human hepatocellular liver carcinoma HepG2 cells. In addition, Tsudosan significantly increased the serum biological antioxidant potential values when orally administrated to mice, indicating that it also had in vivo antioxidant activity. The potent antioxidant activity of Tsudosan may be one of the mechanisms closely correlated to its clinical usage against blood stasis.

Keywords Kampo · Antioxidant · Daisaikoto · Tsudosan · Masiningan

Introduction

Reactive oxygen species (ROS), including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and singlet oxygen ($^1O_2$), are generated during the cellular metabolism of molecular oxygen [1] and play important roles in cellular signaling and homeostasis [2, 3]. However, overproduction of ROS causes oxidative damage to lipids, proteins, and nucleic acids, potentially leading to the pathogenesis of various diseases, such as dyslipidemia, cerebral infarction, cancer, among others [4]. Consequently, the application of antioxidants as therapeutic agents to reduce oxidative stress by scavenging ROS has attracted the interest of researchers. Although various antioxidants have been adopted as drug candidates to date, the toxicity and safety of synthetic antioxidants are still open to question, as exemplified by butylhydroxyanisole (BHA) and butylhydroxytoluene, both of which have been reported to be possible carcinogens [5]. Thus, natural medicines, with their use recorded over long periods of medical practice, are considered to have the potential to be valuable sources of safe and effective antioxidants.

In Japan, 148 formulations of Kampo medicines (Japanese traditional medicines) are currently listed in the National Health Insurance System Drug List and applied under clinical conditions for the treatment of various diseases, including many oxidative stress-related diseases, such as hyperlipidemia, arteriosclerosis, and diabetic complications. Despite their extensive clinical usage, our knowledge of the antioxidant activities of Kampo medicines is still quite limited. A number of Kampo medicines, such as Saikokeishito [6] and Hangeshashinto [7], have been individually investigated for

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their antioxidant activity. However, to our knowledge, the antioxidant activity of these 147 formulations of Kampo medicines has only been comprehensively investigated in an in vitro oxygen radical absorption capacity (ORAC) assay [8]; additional further cellular and animal experiments have not been performed. In the study reported here, we evaluated the antioxidant activities of Kampo medicines using three different in vitro radical scavenging assays. The results indicated that these formulations have intracellular antioxidant activities against H_2O_2-induced oxidative stress, as well as in vivo antioxidant effects in mice. The Kampo formulation Tsudosan was the most potent antioxidant agent of the 147 formulations of oral Kampo medicines.

Materials and methods

Materials

In this investigation, we evaluated 147 formulations of Kampo medicines, representing the entire panel of oral Kampo medicines listed in the Japanese National Health Insurance Drug List. These formulations are manufactured by Tsumura & Co. (Tokyo, Japan), Kotaro Pharmaceutical Co., Ltd. (Osaka, Japan), Ohsugi Pharmaceutical Co., Ltd. (Osaka, Japan), Kracie Holdings, Ltd. (Tokyo, Japan), Sanwa Shoyaku Co., Ltd. (Tochigi, Japan), Taikoseido Pharmaceutical Co., Ltd. (Hyogo, Japan), or Toyo-Kampo Pharmaceutical Co., Ltd. (Osaka, Japan), respectively.

Chemicals

Trolox (purity > 98%) was purchased from Calbiochem (San Diego, CA, USA); 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (purity > 97%), BHA (purity > 98%), 2, and 2-diphenyl-1-picrylhydrazyl (DPPH) (purity > 95%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka Japan). Fluorescein sodium salt and all other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

In vitro antioxidant activity

Sample preparation of Kampo medicines We defined the amount of daily dose as 1 unit (U) for each Kampo medicine. The Kampo medicine under study was dissolved as 1/1000 of the daily dose in 1 mL of purified water (concentration 1 mU/mL) and extracted by sonication at room temperature for 15 min. The mixture was then centrifuged at 12,000 rpm for 15 min to remove insoluble matter affecting the measurement, and the supernatant or supernatant diluted in purified water was used as the sample solution for in vitro assays. The sample concentrations were set to a concentration that allowed data comparison among the different formulations.

DPPH free radical scavenging activity assay The DPPH assay was performed as previously reported by Dudonné et al. [9] with modifications. The DPPH radical in MeOH (0.15 mM, 90 µL) was mixed with 10 µL of aqueous solution of each Kampo medicine. The samples were then incubated for 30 min at room temperature, and the decrease in absorbance at 515 nm was measured. BHA was used as the positive control, and the half maximal (50%) effective concentration (EC_{50}) value was 26.7 ± 1.3 µM. The concentration–activity dependency relationship was examined at multiple concentrations ranging from 10 to 100 µU/mL. XY curves fitted with the non-linear model and the EC_{50} values were calculated by the logistic curve fitting method using GraphPad Prism® version 7 (GraphPad Software Inc., La Jolla, CA, USA).

Superoxide anion scavenging activity assay Superoxide anion scavenging activity was determined by the WST [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2*H*-tetrazolium, monosodium salt] reduction method, using a superoxide dismutase (SOD) assay kit–WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. BHA was used as the positive control, and the EC_{50} value was 17.0 ± 1.0 µM. The concentration–activity dependency relationship was measured at concentrations ranging from 0.42 to 83.3 µU/mL. XY curves fitted with the non-linear model and the EC_{50} values were calculated by the logistic curve fitting method (GraphPad Prism® version 7; GraphPad Software Inc.).

ORAC assay The ORAC assay is based on the scavenging of peroxyl radicals generated by AAPH and was performed as reported previously by Dudonné et al. [9] and Prior et al. [10] with modifications. Namely, 35 µL of the blank solution, trolox standard solutions (25.0, 12.5, and 6.25 µM), or the sample solutions in 75 mM potassium phosphate buffer (pH 7.0) was added to each well of a 96-well polypropylene plate, followed by the addition of 115 µL of 94.4 nM fluorescein solution to each well. The plate was then covered with a lid and incubated in the preheated (37 °C) fluorescence reader (EnSpire Multimode Plate Reader; Perkin Elmer, Yokohama, Japan) for 10 min, with shaking for 3 of the 10 min. The fluorescence (excitation 485 nm, emission 520 nm) was measured every 2 min for up to 90 min after the addition of the AAPH solution (31.7 mM, 75 µL) to each well of the plate. The ORAC values were expressed as micromole trolox equivalents per unit of Kampo medicine.

Intracellular antioxidant activities

Cells and cell culture Human hepatocellular carcinoma HepG2 cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank/National Institutes of Biomedical Innovation, Health and Nutrition (Tokyo, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum (JRC Biosciences, Lenexa, KS, USA), under a humidified atmosphere containing 5% CO_2 at 37 °C.

Analysis of intracellular ROS level Intracellular ROS level was determined by 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) staining according to Higai et al. [11]. The sample concentrations were set to a concentration that does not affect cell growth. HepG2 cells (2×10^5 cells/mL) on 35-mm well plates (1 mL/well) were incubated with the aqueous solution of the respective Kampo medicine (10 μU/mL) for 24 h. After the culture medium was removed, the cells were incubated with 100 μL of DCFH-DA solution (50 μM in DMEM medium) for 15 min at 37 °C, following which H_2O_2 was added to a final concentration of 1 mM. The ROS level in the cells was estimated by scanning the fluorescence of 2′,7′-dichlorofluorescein (DCF) using a model LSMS 510 Meta confocal laser scan microscope (Carl Zeiss, Oberkochen, Germany), and the time-dependent change of fluorescence intensity was calculated at 0, 10, 20, 30, 40, 50 min using the image processing software ImageJ.

WST-1 cell proliferation assay HepG2 cells were seeded on 96-well culture plates (100 μL per well, 2×10^5 cell/mL) and incubated for 6 h at 37 °C. The cells were then exposed to different concentrations of sample solutions for 48 h. The number of living cells was measured using a Premix WST-1 Cell Proliferation Assay System (TaKaRa, Shiga, Japan), according to the manufacturer's instructions.

In vivo antioxidant effect

Animals Four-week-old male ICR mice were purchased from Clea Japan (Tokyo, Japan). Prior to experimentation, the mice were acclimatized for 1 week to a temperature of 25 ± 2 °C, humidity of 50 ± 10%, and a 12/12-h light/dark cycle. They were fed standard pellet chow and water ad libitum. All behavioral observations were conducted between 1000 hours and 1500 hours. The measurement of biological antioxidant potential was performed with nine mice in each group. Experiments were performed in accordance with the Toho University guidelines for animal care, handling, and termination, which are in line with the international and Japanese guidelines for animal care and welfare (Approval no. 17-51-357).

Evaluation of serum biological antioxidant potential values in mice The biological antioxidant potential (BAP) value of an ion represents the reducing ability of that ion against active oxygen, which is used as an index of antioxidant activity. In this study, we used the BAP test to examine the concentration of antioxidants in the serum of mice that reduced iron from the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form [12]. The experimental animals were randomly divided into six experimental groups. The mice in the control group were orally administered purified water. Vitamin C (purity 99.6%, Wako Pure Chemical Industries, Ltd.), as a positive control, was administered to mice orally at a dose of 2.5 g/kg body weight twice a day for 2 days. The Kampo medicines were administered to mice orally at a dose of 2.5 g/kg body weight twice a day for 2 days, i.e., the same dose as the positive control. Thirty minutes after the final administration, the whole blood of each mouse was collected from the jugular vein with the mice under dormicum (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan)/butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) mixed anesthesia and ether anesthesia. The whole blood was centrifuged at 3000 rpm for 15 min at 4 °C to separate serum. The BAP levels were measured using a free radical analyzer (FREE Carrio Duo; Diacron Int. s.r.l., Grosseto, Italy) according to the manufacturer's instructions.

Statistical analysis

All results were expressed as the mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance followed by Dunnett's test with significance set at $p < 0.05$ or $p < 0.01$ versus the vehicle or the Newman–Keuls method with significance set at $p < 0.05$. All statistical analyses were performed using GraphPad Prism® version 7 (GraphPad Software Inc.).

Results and discussion

The first step in our study was to comprehensively evaluate the antioxidant activities of the 147 Kampo medicines under study, which covered the entire panel of oral Kampo medicines listed in Japanese National Health Insurance Drug List, using three of the most common in vitro radical scavenging assays, namely, the DPPH, SOD, and ORAC assays (Table 1). The DPPH assay is one of the simplest of antioxidant assay methods, and its results represent the ability to scavenge the stable free radical generated by DPPH [13]. The SOD assay was carried out using a WST-1 based SOD inhibition method to measure the scavenging ability of superoxide anion radicals, one species of ROS [14]. The ORAC assay is a well-established method to measure the scavenging ability of peroxyl free radicals thermally generated from

Table 1 In vitro antioxidant activity of the 147 formulations of oral Kampo medicines tested in this study

Number	Name of oral Kampo formulation	DPPH (%)		SOD (%)		ORAC (μM TE/U)
		100 μU/mL	50 μU/mL	83.3 μU/mL	41.6 μUnit/mL	
1	Anchusan[c]	30.4 ± 1.7		70.7 ± 8.3		1384 ± 13
2	Bakumondoto[c]	35.7 ± 1.8		37.2 ± 10.3		11 ± 72
3	Bofutsushosan[a]	92.8 ± 0.3	82.1 ± 0.5	98.8 ± 0.7	88.7 ± 1.3	8306 ± 29
4	Boiogito[a]	52.7 ± 1.3		84.0 ± 2.1		1419 ± 47
5	Bukuryoin[c]	34.3 ± 0.5		57.3 ± 7.0		2545 ± 75
6	Bukuryoingohangekobokuto[f]	70.1 ± 2.2		83.7 ± 2.8		2759 ± 135
7	Bushirichuto[d]	52.6 ± 0.6		52.2 ± 3.3		1931 ± 252
8	Byakkokaninjinto[c]	23.8 ± 11.0		67.3 ± 10.6		1310 ± 63
9	Chikujountanto[f]	33.5 ± 0.7		45.7 ± 7.0		3444 ± 178
10	Choijokito[f]	91.2 ± 0.3	83.0 ± 0.1	98.3 ± 1.8	89.1 ± 0.3	4314 ± 98
11	Choreito[c]	7.4 ± 1.7		56.8 ± 5.3		0 ± 0.0
12	Choreitogoshimotsuto[f]	44.4 ± 0.8		76.2 ± 2.3		774 ± 69
13	Chotosan[f]	47.2 ± 0.7		79.4 ± 5.8		2505 ± 83
14	Choyoto[c]	62.1 ± 0.3		87.1 ± 1.4		278 ± 46
15	Daibofuto[f]	58.8 ± 1.4		77.8 ± 4.5		3759 ± 213
16	Daijokito[a]	91.0 ± 0.2	71.2 ± 0.4	96.3 ± 1.4	79.3 ± 1.3	9498 ± 158
17	Daikenchuto[c]	35.2 ± 1.8		45.2 ± 1.6		741 ± 35
18	Daiobotampito[c]	90.8 ± 0.8	63.8 ± 0.5	91.8 ± 4.3	78.4 ± 0.4	3563 ± 291
19	Daiokanzoto[a]	92.6 ± 0.2	69.6 ± 0.5	94.9 ± 1.7	84.7 ± 1.5	5426 ± 105
20	Daisaikoto[c]	90.3 ± 2.1	92.4 ± 0.5	96.0 ± 4.3	96.9 ± 1.9	11567 ± 61
21	Daisaikotokyodaio[c]	94.1 ± 0.9	82.9 ± 0.5	100.0 ± 1.4	91.1 ± 0.9	5769 ± 121
22	Eppikajutsuto[c]	79.9 ± 1.4		91.3 ± 0.5	76.4 ± 2.4	2579 ± 120
23	Gokoto[b]	45.0 ± 0.9		84.1 ± 0.2		1942 ± 271
24	Goreisan[c]	21.6 ± 1.7		66.9 ± 6.9		1006 ± 27
25	Gorinsan[f]	92.8 ± 0.3	53.4 ± 0.9	93.5 ± 0.8	78.5 ± 1.8	5943 ± 265
26	Goshajinkigan[f]	63.2 ± 1.4		82.7 ± 2.0		1929 ± 177
27	Goshakusan[c]	68.5 ± 2.4		80.5 ± 1.7		3497 ± 26
28	Goshuyuto[c]	87.3 ± 1.5		82.6 ± 1.7		2079 ± 123
29	Hachimijiogan[a]	85.4 ± 2.2		88.9 ± 3.1		1450 ± 124
30	Hainosankyuto[a]	62.6 ± 1.9		81.4 ± 2.2		3314 ± 93
31	Hangebyakujutsutemmato[c]	37.1 ± 2.3		25.6 ± 0.4		2022 ± 186
32	Hangekobokuto[c]	91.6 ± 0.9	53.0 ± 0.8	81.5 ± 3.1		3770 ± 22
33	Hangeshashinto[c]	66.6 ± 0.6		80.0 ± 3.0		3000 ± 25
34	Heiisan[a]	65.3 ± 2.1		69.2 ± 1.5		2472 ± 197
35	Hochuekkito[c]	52.2 ± 2.5		72.1 ± 2.2		3059 ± 60
36	Inchingoreisan[f]	71.5 ± 1.0		89.5 ± 1.4		3512 ± 125
37	Inchinkoto[a]	90.3 ± 0.7	59.1 ± 1.0	87.6 ± 3.6		4277 ± 277
38	Ireito[f]	63.4 ± 1.6		78.6 ± 1.8		2864 ± 131
39	Jidabokuippo[f]	92.9 ± 0.3	89.7 ± 0.8	97.9 ± 1.6	92.7 ± 1.7	6853 ± 38
40	Jiinkokato[f]	49.5 ± 2.6		70.2 ± 1.5		1662 ± 167
41	Jiinshihoto[f]	76.4 ± 3.0		74.5 ± 2.1		2962 ± 117
42	Jinsoin[f]	41.8 ± 1.0		70.2 ± 2.4		2124 ± 73
43	Jizusoippo[f]	86.2 ± 1.4		95.2 ± 1.1	77.1 ± 1.2	5680 ± 413
44	Jumihaidokuto[a]	60.0 ± 0.2		76.7 ± 18.7		2314 ± 19
45	Junchoto[f]	94.2 ± 0.7	81.0 ± 1.4	94.0 ± 2.0	87.9 ± 2.6	4674 ± 229
46	Juzentaihoto[c]	82.1 ± 2.4		79.8 ± 4.4		2813 ± 122
47	Kakkonkajutsubuto[d]	93.2 ± 1.6	61.4 ± 0.3	89.1 ± 0.3		5713 ± 242
48	Kakkonto[c]	91.4 ± 0.1	61.1 ± 0.8	93.1 ± 7.0	87.3 ± 0.3	6596 ± 288

Table 1 (continued)

Number	Name of oral Kampo formulation	DPPH (%)		SOD (%)		ORAC (μM TE/U)
		100 μU/mL	50 μU/mL	83.3 μU/mL	41.6 μUnit/mL	
49	Kakkontokasenkyushin'i[a]	92.2 ± 0.5	56.3 ± 0.3	96.3 ± 3.5	92.9 ± 0.9	5594 ± 82
50	Kambakutaisoto[c]	31.6 ± 1.7		66.0 ± 4.9		1792 ± 41
51	Kamikihito[a]	48.7 ± 1.2		50.0 ± 4.7		2165 ± 149
52	Kamishoyosan[c]	91.4 ± 0.1	54.3 ± 0.5	78.0 ± 1.3		1429 ± 24
53	Kanzoto[b]	15.7 ± 2.0		25.5 ± 5.8		654 ± 119
54	Keigairengyoto[a]	91.3 ± 0.3	59.8 ± 1.2	88.6 ± 2.2		5622 ± 99
55	Keihito[f]	35.7 ± 2.9		11.4 ± 3.5		481 ± 144
56	Keimakakuhanto[g]	93.5 ± 0.4	53.8 ± 1.2	99.0 ± 3.6	88.6 ± 1.4	2154 ± 139
57	Keishakuchimoto[d]	93.6 ± 2.7	61.6 ± 1.9	96.1 ± 3.4	85.1 ± 1.6	3691 ± 111
58	Keishibukuryogan[c]	92.0 ± 0.9	76.4 ± 1.5	94.2 ± 2.2	92.3 ± 1.2	887 ± 102
59	Keishibukuryogankayokuinin[f]	90.6 ± 1.1	47.7 ± 4.4	92.8 ± 2.7	70.2 ± 2.8	926 ± 238
60	Keishikajutsubuto[c]	90.6 ± 0.8	52.6 ± 0.6	87.0 ± 7.7		2981 ± 489
61	Keishikakakkonto[g]	77.0 ± 0.9		100.0 ± 7.0	61.4 ± 4.6	2676 ± 99
62	Keishikakobokukyoninto[g]	92.5 ± 0.7	51.8 ± 0.8	99.7 ± 8.6	77.7 ± 0.8	1803 ± 12
63	Keishikaogito[g]	77.0 ± 2.7		91.3 ± 1.5	66.6 ± 0.7	1285 ± 51
64	Keishikaryojutsubuto[a]	91.4 ± 0.5	50.5 ± 1.2	98.3 ± 2.1	84.8 ± 1.0	3285 ± 9
65	Keishikaryukotsuboreito[c]	76.6 ± 1.2		84.0 ± 2.2		0 ± 0
66	Keishikashakuyakudaioto[f]	93.0 ± 0.9	91.7 ± 0.1	86.0 ± 0.5		3573 ± 167
67	Keishikashakuyakuto[c]	91.1 ± 0.0	51.4 ± 0.0	94.6 ± 1.3	79.6 ± 1.3	4842 ± 588
68	Keishininjinto[f]	33.3 ± 0.7		45.8 ± 12.3		912 ± 126
69	Keishito[c]	91.7 ± 0.6	52.4 ± 1.2	94.0 ± 0.8	71.9 ± 3.6	3080 ± 253
70	Kihito[f]	22.4 ± 0.7		46.5 ± 5.5		837 ± 34
71	Kikyosekko[c]	4.1 ± 1.4		0.0 ± 0		0 ± 0
72	Kikyoto[f]	6.5 ± 1.5		0.0 ± 0		745 ± 200
73	Kososan[c]	53.7 ± 0.8		71.0 ± 2.8		3179 ± 207
74	Kumibinroto[c]	92.1 ± 0.5	90.1 ± 0.6	100.0 ± 1.7	93.5 ± 3.2	7560 ± 153
75	Kyukichoketsuin[e]	66.7 ± 1.0		71.5 ± 3.4		2578 ± 4
76	Kyukikyogaito[c]	98.1 ± 0.4	74.7 ± 1.5	94.9 ± 0.8	90.4 ± 1.7	4622 ± 2
77	Makyokansekito[c]	45.3 ± 1.5		87.9 ± 2.9		4150 ± 333
78	Makyoyokukanto[c]	32.4 ± 0.2		55.3 ± 5.4		399 ± 1
79	Maobushisaishinto[a]	40.6 ± 1.3		94.8 ± 4.3	44.7 ± 4.9	797 ± 1202
80	Maoto[c]	69.2 ± 0.7		81.0 ± 1.0		478 ± 79
81	Masiningan[a]	92.6 ± 0.3	92.5 ± 0.6	48.8 ± 2.6		13409 ± 244
82	Mokuboito[c]	51.9 ± 1.5		85.7 ± 2.9		1758 ± 306
83	Nichinto[f]	14.1 ± 0.7		43.9 ± 7.3		1237 ± 112
84	Nijutsuto[f]	85.4 ± 1.7		89.1 ± 1.3		3485 ± 104
85	Ninjinto[f]	22.0 ± 1.4		3.2 ± 1.4		222 ± 12
86	Ninjin'yoeito[c]	81.7 ± 1.9		84.3 ± 2.9		2895 ± 77
87	Nyoshinsan[f]	92.9 ± 1.3	62.1 ± 0.4	98.1 ± 2.9	84.8 ± 0.6	3339 ± 16
88	Ogikenchuto[f]	91.1 ± 0.6	54.4 ± 0.7	87.3 ± 1.6		2965 ± 20
89	Ogonto[d]	94.3 ± 0.6	66.9 ± 0.9	94.8 ± 2.5	72.0 ± 1.8	2353 ± 97
90	Orengedokuto[a]	46.4 ± 1.6		73.1 ± 3.7		4034 ± 130
91	Orento[a]	39.8 ± 1.1		71.2 ± 2.7		1069 ± 75
92	Otsujito[c]	91.6 ± 1.4	73.3 ± 0.8	95.7 ± 2.0	90.2 ± 0.8	6255 ± 79
93	Rikkosan[f]	65.6 ± 1.0		78.7 ± 1.8		3090 ± 2
94	Rikkunshito[c]	26.3 ± 0.4		69.4 ± 6.4		2258 ± 117
95	Rokumigan[f]	64.5 ± 0.6		80.3 ± 2.1		309 ± 77
96	Ryokankyomishingeninto[a]	32.9 ± 0.7		69.8 ± 4.6		1899 ± 1

Table 1 (continued)

Number	Name of oral Kampo formulation	DPPH (%)		SOD (%)		ORAC (μM TE/U)
		100 μU/mL	50 μU/mL	83.3 μU/mL	41.6 μUnit/mL	
97	Ryokeijutsukanto[a]	45.1 ± 0.2		79.7 ± 3.7		465 ± 58
98	Ryokyojutsukanto[a]	18.9 ± 1.4		15.8 ± 11.1		1776 ± 116
99	Ryutanshakanto[c]	90.8 ± 1.6	64.3 ± 0.6	92.0 ± 0.4	89.8 ± 0.3	6466 ± 33
100	Saibokuto[f]	95.0 ± 1.1	59.7 ± 1.4	92.0 ± 1.0	80.8 ± 3.0	4246 ± 90
101	Saikanto[c]	78.2 ± 3.8		97.0 ± 1.6	72.8 ± 3.7	5577 ± 183
102	Saikokaryukotsuboreito[f]	62.6 ± 0.4		86.3 ± 0.8		3113 ± 108
103	Saikokeishikankyoto[c]	91.4 ± 0.4	52.2 ± 0.4	86.8 ± 3.2		5335 ± 484
104	Saikokeishito[c]	91.7 ± 0.2	44.4 ± 0.4	84.4 ± 2.9		2926 ± 184
105	Saikoseikanto[c]	91.1 ± 1.8	61.2 ± 0.9	93.8 ± 1.0	87.7 ± 1.8	8117 ± 80
106	Saireito[f]	85.0 ± 1.5		91.7 ± 1.7	75.1 ± 2.9	3629 ± 40
107	Sammotsuogonto[f)]	86.4 ± 0.0		91.1 ± 1.8	79.2 ± 1.8	2403 ± 133
108	San'oshashinto[c]	43.1 ± 1.4		83.1 ± 3.3		2286 ± 302
109	Sansoninto[a]	36.8 ± 0.7		10.9 ± 1.6		905 ± 37
110	Seihaito[f]	64.5 ± 0.7		74.9 ± 2.0		3741 ± 124
111	Seijobofuto[a]	92.4 ± 0.4	53.6 ± 0.3	96.0 ± 1.6	77.0 ± 1.4	4995 ± 168
112	Seishinrenshiin[f]	93.0 ± 0.7	46.4 ± 1.0	85.7 ± 3.0		4483 ± 259
113	Seishoekkito[f]	25.5 ± 1.4		46.6 ± 1.2		1396 ± 195
114	Senkyuchachosan[f]	90.7 ± 0.5	63.3 ± 0.8	95.9 ± 1.7	85.5 ± 1.9	1972 ± 241
115	Shakanzoto[c]	59.7 ± 1.2		78.6 ± 0.2		2262 ± 33
116	Shakuyakukanzobushito[d]	91.5 ± 0.1	49.2 ± 1.0	94.0 ± 3.5	86.4 ± 1.6	1966 ± 188
117	Shakuyakukanzoto[c]	88.0 ± 1.0		94.1 ± 1.5	79.0 ± 1.1	1044 ± 84
118	Shichimotsukokato[a]	75.0 ± 0.1		93.0 ± 0.4	71.1 ± 6.0	3896 ± 32
119	Shigyakusan[f]	37.1 ± 0.9		52.5 ± 14.5		1109 ± 70
120	Shikunshito[f]	13.5 ± 0.9		58.3 ± 4.9		1040 ± 42
121	Shimbuto[c]	44.7 ± 0.6		63.0 ± 1.0		0 ± 0
122	Shimotsuto[c]	54.1 ± 0.5		87.9 ± 0.3		1329 ± 101
123	Shimpito[c]	88.5 ± 1.8		92.4 ± 0.2	82.1 ± 2.5	5237 ± 77
124	Shin'iseihaito[a]	94.3 ± 1.3	74.2 ± 0.2	88.9 ± 2.3		5119 ± 1
125	Shireito[a]	6.7 ± 1.0		10.1 ± 10.2		498 ± 103
126	Shishihakuhito[c]	34.1 ± 0.5		48.5 ± 8.4		1091 ± 52
127	Shofusan[c]	71.2 ± 1.4		91.3 ± 5.2	67.4 ± 1.0	2214 ± 48
128	Shohangekabukuryoto[c]	4.6 ± 1.8		57.5 ± 0.75		829 ± 19
129	Shokenchuto[c]	91.5 ± 1.2	49.7 ± 2.8	87.7 ± 3.0		1607 ± 20
130	Shomakakkonto[f]	80.6 ± 3.4		86.2 ± 0.3		5295 ± 95
131	Shosaikoto[c]	87.0 ± 1.3		80.4 ± 1.0		4646 ± 55
132	Shosaikotokakikyosekko[f]	62.6 ± 2.0		86.5 ± 1.8		2699 ± 207
133	Shoseiryuto[c]	87.9 ± 1.9		86.2 ± 2.2		3229 ± 100
134	Sokeikakketsuto[f]	43.4 ± 2.1		67.4 ± 12.7		2602 ± 57
135	Tokakujokito[c]	91.8 ± 1.0	89.1 ± 0.2	99.0 ± 1.8	95.4 ± 2.4	6678 ± 261
136	Tokiinshi[f]	54.5 ± 1.2		76.2 ± 1.4		2589 ± 174
137	Tokikenchuto[f]	65.0 ± 1.9		86.8 ± 1.1		2367 ± 46
138	Tokishakuyakusan[c]	90.8 ± 0.4	39.2 ± 0.6	84.7 ± 6.5		211 ± 167
139	Tokishakuyakusankabushi[a]	94.4 ± 1.1	55.7 ± 1.0	89.3 ± 0.8		1890 ± 20
140	Tokishigyakukagoshuyushokyoto[f]	91.0 ± 0.9	48.9 ± 0.2	92.6 ± 1.0	79.4 ± 0.8	2882 ± 34
141	Tokito[f]	80.2 ± 1.7		84.1 ± 0.2		2354 ± 54
142	Tsudosan[c]	90.2 ± 2.2	92.0 ± 1.2	100.0 ± 3.8	92.6 ± 0.7	28660 ± 355
143	Unkeito[c]	90.3 ± 2.6	39.6 ± 3.1	92.1 ± 1.0	81.1 ± 3.3	4247 ± 71
144	Unseiin[c]	92.6 ± 1.6	76.8 ± 0.8	97.5 ± 3.8	83.9 ± 8.0	6991 ± 119

Table 1 (continued)

Number	Name of oral Kampo formulation	DPPH (%)		SOD (%)		ORAC (μM TE/U)
		100 μU/mL	50 μU/mL	83.3 μU/mL	41.6 μUnit/mL	
145	Yokuininto[f]	90.6 ± 0.6	50.6 ± 0.7	89.3 ± 3.3		5398 ± 165
146	Yokukansan[a]	75.8 ± 4.6		79.2 ± 7.8		3381 ± 326
147	Yokukansankachimpihange[c]	93.0 ± 3.4	46.7 ± 0.9	86.6 ± 0.3		3559 ± 103

Data are presented as the mean of triplicate measurements for each group ± standard deviation (SD)

DPPH 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity rate, *ORAC* oxygen radical absorbance capacity, *SOD* (sodium dioxide dimutase) superoxide anion scavenging activity rate, *TE* trolox equivalents, *μU* microunit

[a]Ohsugi Pharmaceutical Co., Ltd. (Osaka, Japan)

[b]Kracie Holdings, Ltd. (Tokyo, Japan)

[c]Kotaro Pharmaceutical Co., Ltd. (Osaka, Japan)

[d]Sanwa Shoyaku Co., Ltd. (Tochigi, Japan)

[e]Taikoseido Pharmaceutical Co., Ltd. (Hyogo, Japan)

[f]Tsumura & Co. (Tokyo, Japan)

[g]Toyo-Kampo Pharmaceutical Co., Ltd. (Osaka, Japan)

AAPH [15, 16]. We found that at a final concentration of 0.5 mU/mL, five formulations of the 147 Kampo medicines tested, namely, Masiningan, Tsudosan, Daisaikoto, Keishikashakuyakudaioto, and Kumibinroto, completely scavenged the DPPH free radical (inhibition ratio > 90%). At a final concentrations of 41.6 μU/mL, ten Kampo medicines, namely, Daisaikoto, Jidabokuippo, Tsudosan, Kumibinroto, Tokakujokito, Kyukikyogaito, Daisaikotokyodaio, Otsujito, Keishibukuryogan, and Kakkontokasenkyusini, had completely scavenged this superoxide anion radical (inhibition ratio > 90%). The concentration dependence of the top five formulations of Kampo medicines was then evaluated in the DPPH assay, at concentrations ranging from 10 to 100 μU/mL (Fig. 1a), and in the SOD assay, at concentrations ranging from 0.42 to 83.3 μU/mL (Fig. 1b). The EC_{50} and ORAC values of the five formulations of Kampo medicines showing the most potent activities in each assay are shown in Table 2.

A common ingredient of Kampo medicines showing high in vitro antioxidant intensity is Rhei Rhizoma. Rhei rhizome, one of the widely used crude drugs found in Kampo medicines, has been reported to have various biological effects, such as purgative, antipyretic, anti-inflammatory, antiangiogenic, and antineoplasmic activities [17]. Zhong et al. demonstrated that anthraquinones have a protective effect against oxidative stress-related endothelial cell injury [18]. Bupleuri Radix and Scutellariae Radix, which are components of the Kampo formulation Daisaikoto, have been reported to have a potential to protect hepatic cells from free radicals in endotoxemia [19]. In other studies, the Kampo formulation Shosaikoto was found to scavenge superoxide anions, hydroxyl radicals, DPPH radicals in a dose-dependent manner [20], and the Daisaikoto formulation limited the development of arteriosclerosis in the aorta of KHC rabbits and showed an antioxidant capacity for low-density lipoprotein

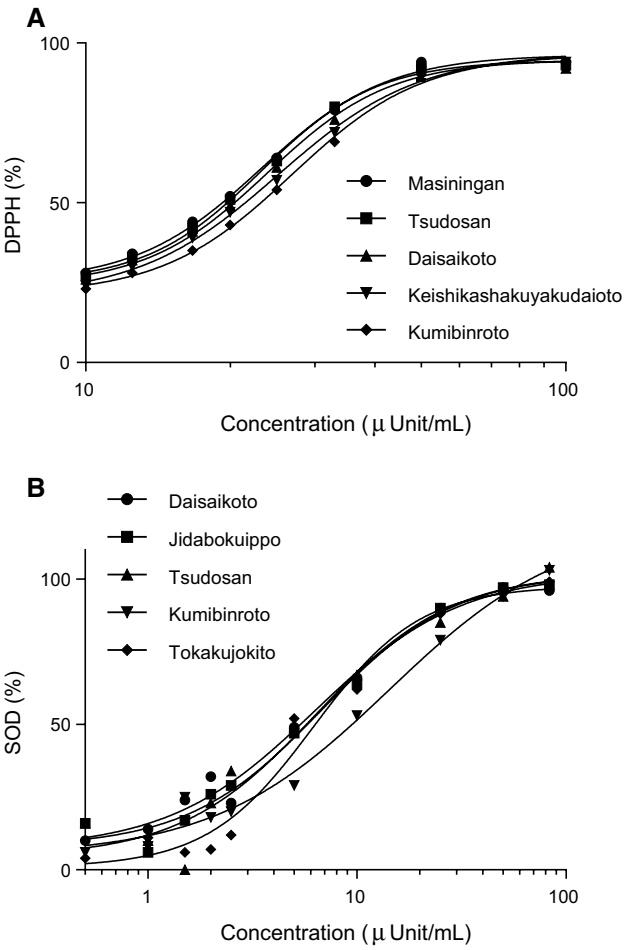

Fig. 1 Dose–response curve of the five formulations of Kampo medicines showing the most potent activities in the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assay (**a**) and in the superoxide anion scavaging activity (*SOD* sodium oxide dimutase) assay (**b**)

Table 2 In vitro antioxidant activity of the five formulations of Kampo medicines with the highest activity

DPPH		SOD		ORAC	
Kampo formulation	EC_{50} (μU/mL)	Kampo formulation	EC_{50} (μU/mL)	Kampo formulation	ORAC (μM TE/U)
Masiningan	23.2 ± 0.9	Daisaikoto	6.2 ± 1.1	Tsudosan	28660 ± 355
Tsudosan	23.2 ± 0.6	Tsudosan	6.3 ± 1.9	Masiningan	13409 ± 244
Daisaikoto	23.9 ± 1.1	Tokakujokito	6.3 ± 1.1	Daisaikoto	11567 ± 61
Keishikashakuyakudaioto	25.2 ± 1.1	Jidabokuippo	6.8 ± 1.1	Daijokito	9498 ± 158
Kumibinroto	26.9 ± 0.9	Kumibinroto	14.3 ± 4.5	Bofutsushosan	8306 ± 29

Data represent the mean of triplicate measurements of each group ± SD

EC_{50} Half maximal (50%) effective concentration

[21]. Magnoliae Cortex is present in Kampo formulations Tsudosan and Masiningan. Son et al. reported that two major compounds of Magnoliae Cortex, namely, magnolol and honokiol, inhibited the production of inducible nitric oxide synthase and tumor necrosis factor alpha in their experiment system using RAW264.7 cells [22], and Chin et al. reported that these two compounds also eliminated hydroxyl radicals and inhibited lipid hyperoxidation in rats, resulting in prevention of liver damage by the peroxide radical [23]. Our assay results show that the Tsudosan, Daisaikoto, and

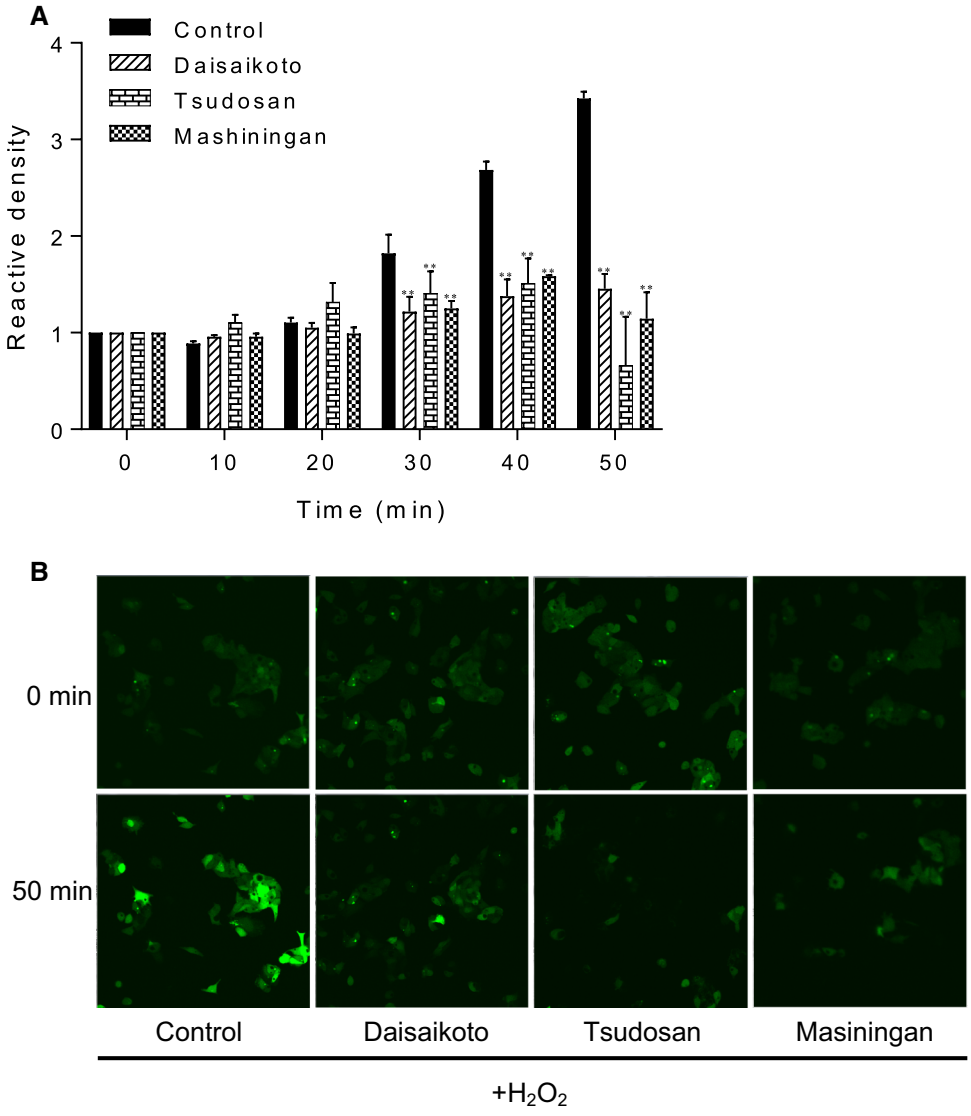

Fig. 2 Intracellular antioxidant effect of the Kampo formulations Tsudosan, Daisaikoto, and Masiningan on human hepatocellular carcinoma HepG2 cells. **a** The level of reactive oxygen species (ROS) in H_2O_2-treated HepG2 cells over time, **b** determination of ROS in the HepG2 cells 50 min following the addition of H_2O_2, by fluorescent microscopic study. Data are presented as the mean ± standard deviation (SD) of three measurements ($n = 3$). Statistical significance was determined with Dunnett's test. Double asterisks indicate significance vs. the control group at $p < 0.01$

Masiningan formulations were the most potent in vitro antioxidants of the Kampo formulations tested; we therefore selected these for further investigation of their intracellular and in vivo antioxidant activities.

We evaluated the intracellular antioxidant activities of these three Kampo medicines against H_2O_2-induced oxidative stress in human hepatocellular liver carcinoma HepG2 cells using the DCFH-DA assay. DCFH-DA is the most widely used cell-permeable probe for detecting intracellular H_2O_2 and oxidative stress. In this assay, DCFH-DA is hydrolyzed intracellularly to the DCFH carboxylate anion and subsequently oxidized to fluorescent DCF. Each of the three Kampo medicines was pre-incubated with the HepG2 cells, and then removed from the extracellular component. We then measured the time-course of the reductive effect of each intercellular Kampo medicine on H_2O_2-induced DCF fluorescence intensity. As shown in Fig. 2a, the addition of H_2O_2 to the HepG2 cells resulted in a notable induction of DCF fluorescence intensity after 30 min, but this intensity was significantly reduced in those cells pre-incubated with the selected Kampo formulations at the final concentration of 10 μU/mL. At the time-point of 50 min, there were fewer cells stained with DCF among the HepG2 cells treated with the Tsudosan formulation than among the (untreated) controls (Fig. 2a, b). It should be noted that cytotoxicity was not observed when the cells were pre-incubated with the selected Kampo formulations. These results reveal that the Tsudosan, Daisaikoto, and Masiningan formulations effectively inhibited H_2O_2-induced oxidative stress intracellularly.

Since all three Kampo formulations exhibited intracellular antioxidant activities, their antioxidant effects were subsequently evaluated in vivo in mice using the BAP test, which is based on the ability of a plasma blood sample to reduce ferric ions to ferrous ions as a FRAP assay (ferric reducing ability of plasma). BAP values indicate systemic antioxidative properties and reflect the reduction potential of the serum. In simple terms, non-enzymatic antioxidants, such as vitamin C, can be described as reductants, and inactivation of oxidants by reductants can be described as redox reactions. The oxidative stress index is an arbitrary parameter derived from BAP values to represent an individual compound's antioxidant potential against the condition of oxidative stress [24]. The Tsudosan, Daisaikoto, and Masiningan formulations and vitamin C were orally administrated to mice at a dose of 2.5 g/kg body weight, twice a day for 2 days. The serum BAP values were measured. In comparison to the control group, the serum BAP values were significantly increased in those groups of mice orally administrated the Tsudosan formulation and vitamin C, respectively, whereas the groups of mice administrated the Daisaikoto and Masiningan formulations tended to show an increased serum BAP value (Fig. 3).

Although our knowledge of the change in serum BAP values after the administration of Kampo medicines in animal experiments is still quite limited, it has been reported that the administration of the Kampo formulations Tokakujokito and Keisibukuryogan increased rat serum BAP values and improved blood stasis [25]. The Kampo Tsudosan formulation consists of ten crude drugs: glycyrrhiza (Glycyrrhizae Radix), immature orange (Aurantii Fructus Immaturus), safflower (Carthami Flos), magnolia bark (Magnoliae Cortex), sappan wood (Sappan Lignum), rhubarb (Rhei Rhizoma), citrus unshiu peel (Citri Unshiu Pericarpium), Japanese angelica root (Angelicae Acutilobae Radix), anhydrous sodium sulfate (Sal Mirabilis Anhydricus), and akebia stem (Akebia Caulis). This formulation is considered to be one of strongest Kampo medicines against blood stasis and is used clinically to treat chronic inflammatory diseases. The increase in mice serum BAP values following treatment with the Kampo Tsudosan formulation in our study may be closely related to its blood stasis effect.

In summary, comprehensive evaluation of the in vitro antioxidant activities of 147 formulations of oral Kampo medicines resulted in the identification of formulations Tsudosan, Daisaikoto and Masiningan having the most potent antioxidant activity. Further investigation of the intercellular and in vivo antioxidant activities of these three Kampo medicines led to the identification of the Tsudosan formulation as a potent antioxidant agent. The results of our study suggest that the clinical usage of the

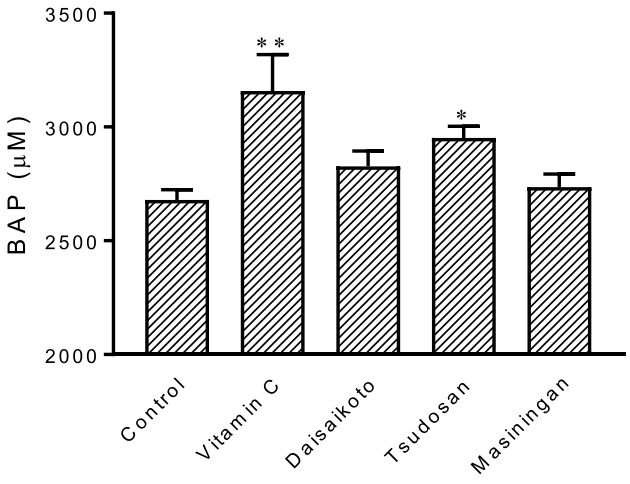

Fig. 3 Values of the biological antioxidant potential (*BAP*) following the administration of selected Kampo formulations (Tsudosan, Daisaikoto, Masiningan) and the non-enzymatic antioxidant vitamin C. Data are presented as the mean ± SD of nine measurements ($n = 9$). Statistical significance was determined with Dunnett's test. Single and double asterisks indicate significance vs. the control group at $^*p < 0.05$ and $^{**}p < 0.01$, respectively

Tsudosan formulation may be closely correlated to its potent antioxidant effects.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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