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Inhibition by *Rikko-san* and its Major Ingredients of LPS-stimulated Nitric Oxide Production by Mouse Macrophage-like Cells

NORIO HORIE[1,3], KAZUE SATOH[2,4], KEN HASHIMOTO[2], TETSUO SHIMOYAMA[3], TADAYOSHI KANEKO[1,5], KAORU KUSAMA[1] and HIROSHI SAKAGAMI[2]

[1]*Department of Oral Pathology and*
[2]*Department of Dental Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283;*
[3]*Department of Oral Surgery, Saitama Medical Center, Saitama Medical School, Kawagoe, Saitama 350-8550;*
[4]*School of Medicine, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142-8555;*
[5]*Department of Oral and Maxillofacial Surgery, Tokyo Medical University, Shinjyuku-ku, Tokyo, 160-0023, Japan*

Abstract. *Rikko-san and its ingredients were investigated for their activity to modify nitric oxide (NO) production by unstimulated and lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells. LPS significantly stimulated the NO production by Raw 264.7 cells, and Rikko-san effectively inhibited the stimulation effect of LPS even at non-cytotoxic concentrations. Among 5 Rikko-san ingredients, Kanzo showed a similar magnitude of inhibition of NO production. Shoma was also slightly inhibitory. On the other hand, Ryutan, Saishin and Bofu did not show such a clear-cut stimulation effect, due to the co-existence of both inhibitory and stimulatory substance(s) for NO production. Thus NO stimulators were present in Rikko-san and its four ingredients except for Kanzo. Western blot analysis demonstrated that LPS induced the production of inducible NO synthase (iNOS), and that non-cytotoxic concentrations of Rikko-san and Kanzo significantly inhibited the LPS-stimulated iNOS expression. ESR spectroscopy showed that Rikko-san, Kanzo, Shoma and Saishin, but not Ryutan and Bofu, produced radical(s) under alkaline condition. All samples scavenged superoxide (produced by hypoxanthine-xanthine oxidase reaction) and NO (produced by 1-hydroxy-2-oxo-3-(N-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7)), possibly by their general reducing activity. These data suggest that the inhibition of NO production by Chinese medicines investigated here may be the result of both the inhibition of iNOS expression and their radical scavenging activity.*

Correspondence to: Hiroshi Sakagami, Department of Dental Pharmacology Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: (+81) 49-279-2758, Fax: (+81) 49-285-5171, e-mail: Sakagami@dent.meikai.ac.jp, horien@saitama-med.ac.jp

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We have previously reported the diverse biological activity of poly-herbal extracts of Himalayan plants (1), Moxa extracts (2) and smoke (3), persimmon (*Diospyros Kaki*) peel extracts (4), fermented pine seed shell extract (5), kiwifruit extracts (6) and blended herbal extracts (7). Lignins stimulated mouse macrophage-like Raw 264.7 cells to produce nitric oxide (NO) and asparagine into the culture medium, to a comparable extent attained by lipopolysaccharide (LPS) (8). On the other hand, the precursor of lignin (phenylpropenoid monomers) (9), isoflavones (10), Himalayan plants (11), Barbados cherry extract (12) and dental materials (eugenol-related compounds) (13) inhibited the NO production by activated macrophages. These data suggested that plant extracts, including Chinese folklore medicines, might modify the function of macrophages, which play significant roles in immunological reactions. As an extension of these studies, we investigated whether *Rikko-san*, one of the Chinese medicines, and its five ingredients (*Kanzo, Shoma, Ryutan, Saishin* and *Bofu*) stimulate or inhibit the NO production by LPS-stimulated Raw 264.7 cells. The extracellular concentration of NO produced by activated macrophages is determined by the intracellular concentration of inducible NO synthase (iNOS), the enzyme activity of iNOS and the quenching effect of radical scavengers. Therefore, we also investigated the possible change of iNOS expression by Western blot analysis and the radical scavenging activity against superoxide anion (O_2^-) and NO radical, using ESR spectroscopy.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: *Rikko-san* (No. 2990110010), its five ingredients [*Kanzo* (No. 281013010), *Shoma* (No. 281081010), *Ryutan* (No. 281080010), *Saishin* (No. 2001026010), *Bofu* (No.

Table I. *Major components of five ingredients in Rikko-san.*

Ingredients	Major components
Kanzo	glycyrrhizin, glabric acid, liquiritin, licoricone licoflavone
Shoma	cimigenol, dahurinol,acerinol, β-sitosterol, cimicifugoside
Ryutan	gentiopicroside, trifloraside, swertiamarine, gentisin
Saishin	β-pinen, eucarvoe, 1,8-cineol, *l*-sarinin, higenamine
Bofu	deltoin, bergapten, psoralen, hamaudol, cimifugin

2991031010)] (Tsumura Corp., Tokyo, Japan) [42.5 g *Rikko-san* contained *Kanzo* (1.5 g), *Shoma* (2.0 g), *Ryutan* (1.0 g), *Saishin* (2.0 g) and *Bofu* (2.0 g). Major components of five ingredients in *Rikko-san* are listed in Table I]; Dulbecco's modified Eagle medium (DMEM), phenol red-free DMEM (Invitrogen Corp, Carlsbad, CA, USA), fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA); LPS from *Escherichia coli* (Serotype 0111:B4), 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenylte-trazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepentaacetic acid (DETAPAC) (Sigma Chem. Ind., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2-(4-carboxyphenyl)-4,4,5, 5-tetramethyli- midazoline-1-oxyl-3-oxide (carboxy-PTIO) (a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) (NO generator), superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan).

Cell culture. Raw 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere.

Assay for cytotoxic activity. Near confluent Raw 264.7 cells were treated for 24 hours with various concentrations of each test sample. Cells were washed once and incubated for 4 hours at 37˚C with 0.1 mL of fresh medium containing 0.2 mg/mL MTT. After removal of the medium, the cells were lysed with 0.1 mL of DMSO, and the absorbance at 540 nm (A_{540}) of the cell lysate, which reflects the relative viable cell number, was then determined. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (1).

Assay for NO production. Near confluent Raw 264.7 cells were incubated for 24 hours with various concentrations of *Rikko-san* or its ingredients in phenol red-free DMEM supplemented with 10% FBS. The extracellular concentration of NO was quantified with Griess reagent, using the standard curve of NO_2^- (11). The net NO production was obtained by subtracting the value (measured without cells) from that (measured with the cells). The concentration of each sample to inhibit the LPS-induced NO production by 50% (IC_{50}) was determined from the dose-response curve. The inhibitory effect of NO production was evaluated by the selectivity index (SI), which was calculated by the following equation:

$$SI=CC_{50}/IC_{50}$$

Assay for iNOS protein. The cell pellets were suspended in PBS and mixed with an equal volume of 2x sodium dodecyle sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol), boiled for 10 minutes. The protein in the cell lysate was determined by Protein Assay Kit (Bio Rad, Hercules, CA, USA) and the aliquots equivalent to 20 μg protein were applied to the 15% SDS polyacrylamide gel electrophoresis, and then transferred to PVDF membrane (Immobilon-P, Millipore Corp, Bedford, MA, USA). The membranes were then blocked with 5% skim milk in Tris-HCl-buffered saline plus 0.05% Tween 20 overnight at 4˚C and incubated with antibodies against iNOS (BD Biosciences, Pharmingen, San Diego, CA, USA) (1:1000) for 90 minutes at room temperature, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 60 minutes at room temperature. Immunoblots were developed with a Western Lightning^{TM} Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) and analyzed on a Macintosh (Power Macintosh 7600/120) computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy. nimh. nih. gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, USA, part number PB95-500195GEI).

Assay for radical intensity. The radical intensity of the test sample was determined at 25˚C in 0.1 M Tris-HCl buffer (pH 7.4), 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9, 10) or in 0.1 M KOH (pH 12.5), using ESR spectrometer (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 seconds; scanning time, 2 minutes. The radical intensity was defined as the ratio of the peak height of these radicals to that of MnO.

To determine O_2^-, produced by HX-XOD reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, PB 20 μL, sample (in H_2O) 50 μL, XOD (0.5 U/mL in PB) 30 μL], the gain, time constant and scanning time were changed to 500, 0.1 second and 1 minute after mixing. The O_2^- scavenging activity was expressed as SOD unit/mg sample, by calibration with the standard curve of SOD (1).

For the determination of NO radical, sample was added to the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7 in 0.06 M phosphate buffer, pH 7.4. The gain and scanning time were changed to 250 and 2 minutes, respectively. The NO radical intensity was defined as the ratio of peak height of the 1st peak of carboxy-PTI (indicated by arrows in Figure 5) (14), which was produced by the reaction of NO (derived from NOC-7) with carboxy-PTIO, to that of MnO.

Results

NO production. Rikko-san showed very weak cytotoxic activity against Raw 264.7 cells with or without LPS (100 ng/mL) stimulation [CC_{50}>4000μg/ml (-LPS), CC_{50}=3915 μg/mL (+LPS)] (Figure 1A, Table II). Unstimulated Raw 264.7 cells produced a lower amount of NO (0.5 μM) into the culture medium. LPS (100 ng/mL) enhanced the NO production by Raw 264.7 cells (Figure 1A) to a higher level (16 μM). *Rikko-san* slightly enhanced the NO production at

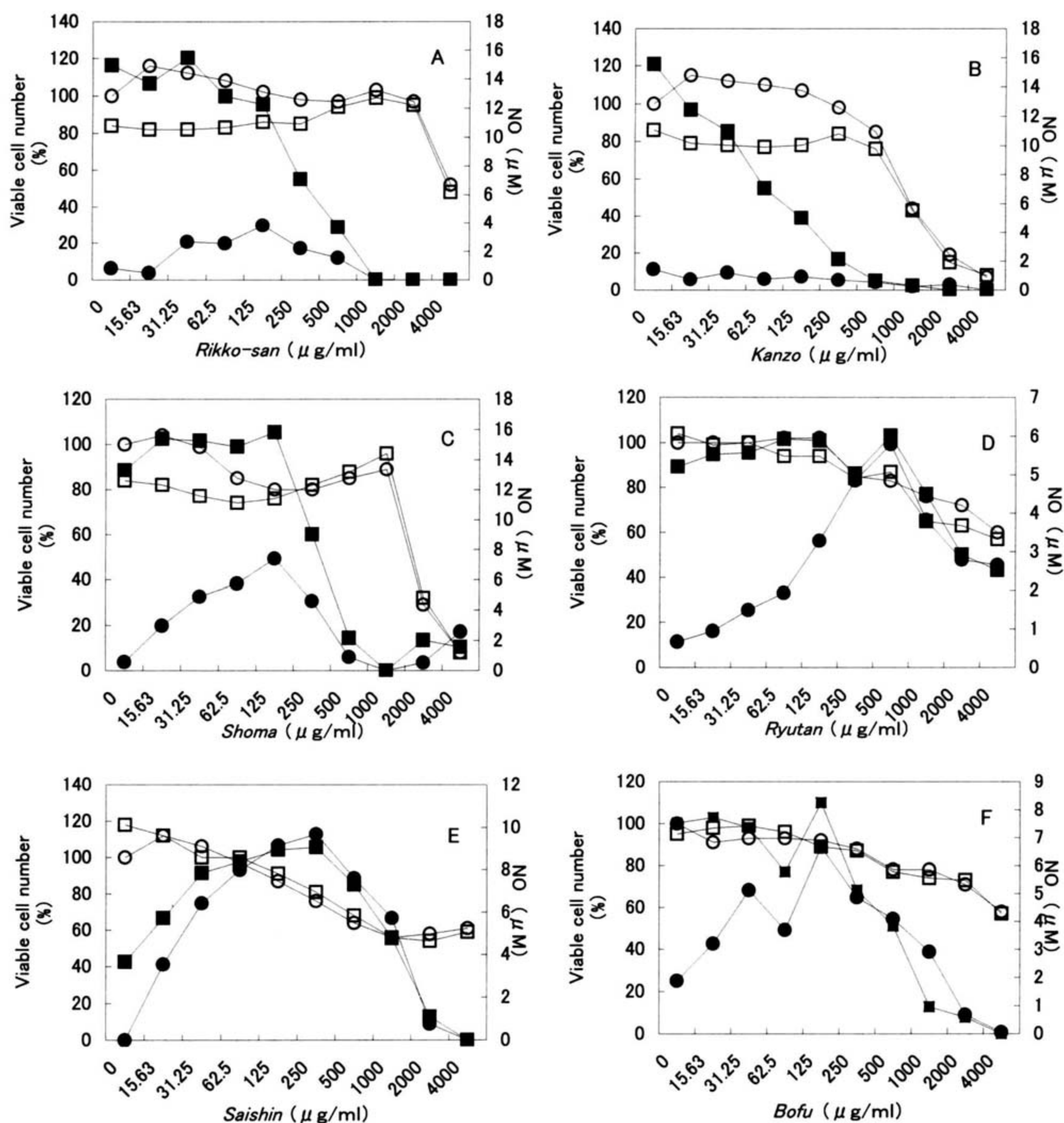

Figure 1. *Effect of Rikko-san and its ingredients on the viable cell number and NO production by unstimulated or LPS-stimulated Raw 264.7 cells. Near confluent 264.7 cells were incubated for 24 hours with 0.1 mL of fresh phenol red-free DMEM supplemented with 10% FBS containing the indicated concentrations of Rikko-san (A), or its ingredients such as Kanzo (B), Shoma (C), Ryutan (D), Saishin (E) or Bofu (F) in the presence (□, ■) or absence (○, ●) of 100 ng/mL LPS. The viable cell number (% of control) (○, □) and extracellular NO concentration (●, ■) were then determined. Each symbol represents the mean of 4 determinations.*

micromolar concentration (31-250 μg/mL), but completely inhibited the LPS-stimulated NO production at higher concentration (1000 μg/mL) (Figure 1A), suggesting the presence of both inhibitory and stimulatory substances in *Rikko-san*. It should be noted that the inhibitory effect of *Rikko-san* was observed at its non-cytotoxic concentration, thus yielding a higher selectivity index of inhibition of NO production (SI=16.4) (Table II).

Table II. *Inhibition by Rikko-san and its ingredients of LPS-induced stimulated NO production.*

	CC_{50} (μg/mL)		IC_{50}(μg/mL)	
	LPS(-)	LPS(+)	LPS(+)	SI=CC_{50}/ IC_{50}
Rikko-san	>4000	3915	239	16.4
Kanzo	927	894	56	16
Shoma	1650	1719	337	5.1*
Ryutan	>4000	>4000	3500	>1.1*
Saishin	>4000	>4000	1800	>2.2*
Bofu	>4000	>4000	513	>7.8*

*The precise calculation of CC_{50} was difficult, due to the co-existence of both inhibitory and stimulatory substances for NO production.

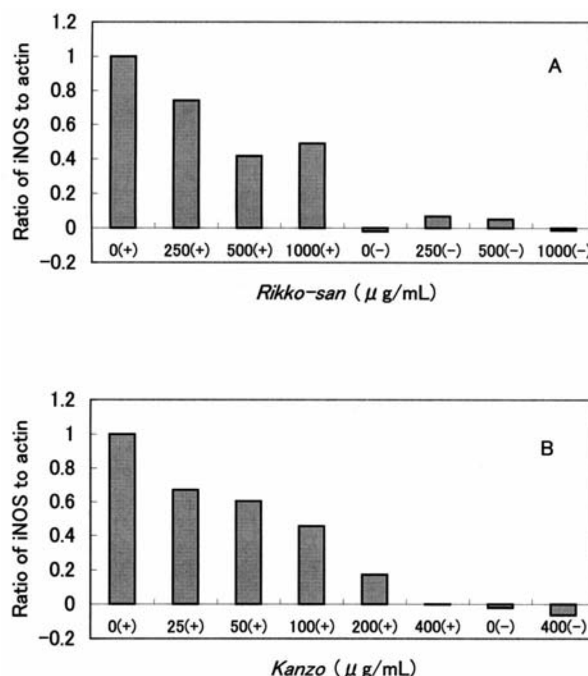

Figure 2. *Effect of Rikko-san (A) and Kanzo (B) on iNOS protein expression in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 hours without (-) or with (+) 100 ng/mL LPS in the presence of the indicated concentrations of Rikko-san (A) and Kanzo (B), and iNOS protein was quantified by Western blot analysis.*

Among 5 major *Rikko-san* ingredients, *Kanzo* showed the highest cytotoxity (CC_{50}=927 μg/mL) (Figure 1B), followed by *Shoma* (CC_{50}=1650μg/ml) (Figure 1C), *Ryutan* (Figure 1D), *Saishin* (Figure 1E) and *Bofu* (Figure 1F) (CC_{50}>4000 μg/mL) (Table II). *Saishin* (Figure 1E) and *Ryutan* (Figure 1D) stimulated the NO production up to a level comparable with that attained by LPS. *Shoma* (Figure 1C) and *Bofu* (Figure 1F) also stimulated the NO production, but to lesser extents. *Kanzo* showed no such stimulation effect (Figure 1B). *Kanzo* significantly reduced the LPS-stimulated NO production (IC_{50}=56 μg/mL; SI=16). Other ingredients also showed similar inhibitory activity, but the precise calculation of the IC_{50} value was difficult, due to the co-existence of both inhibitory and stimulatory substances for NO production.

Western blot analysis showed that LPS stimulated the expression of iNOS protein, and that *Rikko-san* inhibited the LPS-stimulated expression of iNOS protein, with a slight increase of iNOS expression at 1000 μg/mL (Figure 2A). *Kanzo* effectively inhibited the LPS-stimulated iNOS expression, without any apparent shoulder (Figure 2B).

Radical generation and scavenging activity. Rikko-san and all five ingredients did not produce any detectable ESR signal at neutral pH (pH 7.4) (Figure 3). At higher pH (pH 9～12.5), *Rikko-san* (A), *Kanzo* (B), *Shoma* (C) and *Saishin* (E) generated detectable amounts of radical, whereas no radical production was observed in *Ryutan* (D) and *Bofu* (F).

Shoma (C) most effectively scavenged O_2^-, generated by HX-XOD reaction (41.5 SOD unit/mg), followed by *Saishin* (19.7 SOD unit/mg) (E) > *Rikko-san* (18.4 SOD unit/mg) (A) > *Kanzo* (9.4 SOD unit/mg) (B) > *Bofu* (9.0 SOD unit/mg) (F) > *Ryutan* (5.9 SOD unit/mg) (D) (Figure 4). These data indicate some connection between the radical generation and O_2^- scavenging activity (Table III).

Addition of Chinese medicine (200 μg/mL) except *Ryutan* significantly attenuated the intensity of NO radical, produced by NOC-7 in the presence of carboxy-PTIO (Figure 5, Table III). *Shoma* most potently attenuated the NO radical intensity (76.8% attenuation), followed by *Saishin* (32.9%) > *Rikko-san* (28.6%) > *Kanzo* (26.8%) > *Bofu* (15.7%) (Table III). However, these samples directly attenuated the radical intensity of carboxy-PTI (parentheses in Table III). It was therefore concluded that the apparent NO radical scavenging activity of these samples was mainly due to their reducing activity.

Discussion

We found that Chinese medicine significantly reduced the extracellular NO concentration, and is therefore expected to modify the biological action of the NO radical. NO is produced from L-arginine by NOS in the presence of NADPH, and displayed diverse biological activities such as vasodilation, inhibition of endothelial leukocyte adhesion and regulation of energy metabolism (15). We have previously reported that LPS stimulated Raw 264.7 cells to produce tumor necrosis factor (TNF), NO, citrulline and asparagines (8, 10). We found that the Chinese medicine *Rikko-san* and its ingredient *Kanzo* reduced the extracellular NO concentration by LPS-activated macrophages. This was mostly due to the decreased expression of iNOS protein and radical

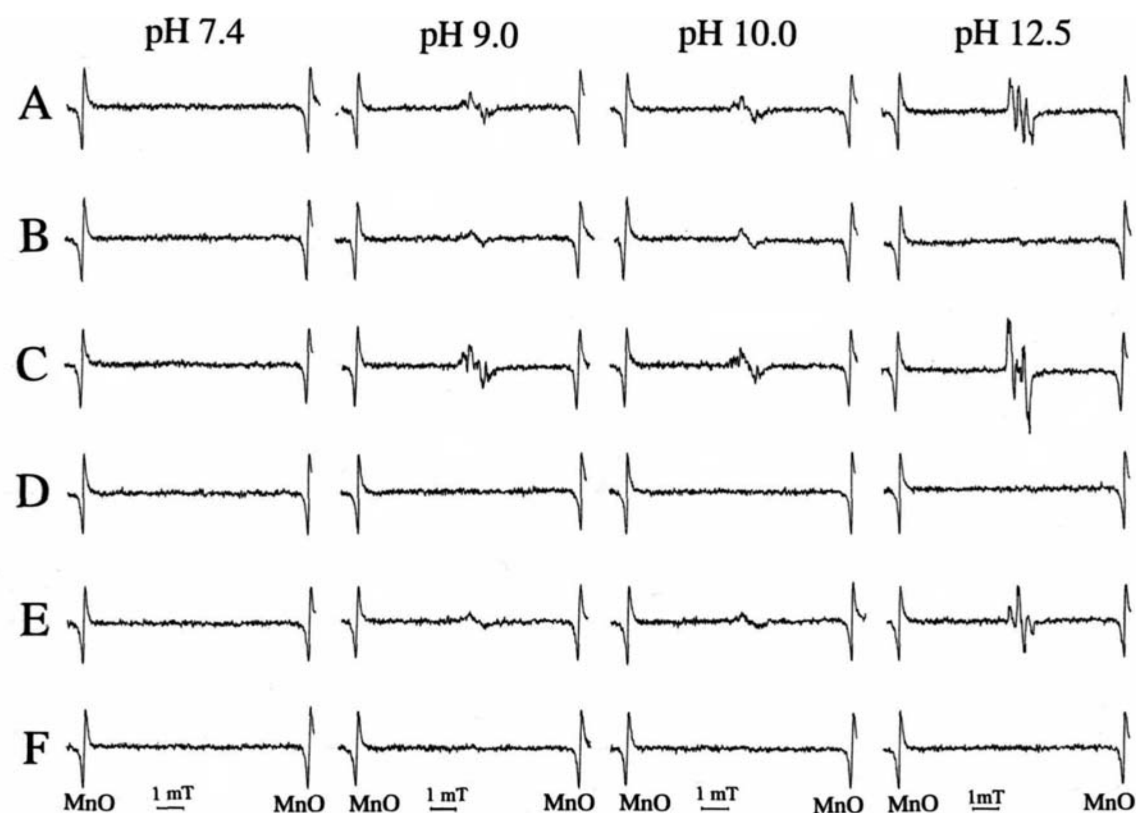

Figure 3. *ESR spectra of Rikko-san (A) and its ingredients Kanzo (B), Shoma (C), Ryutan (D), Saishin (E) or Bofu (F) (5 mg/mL) in 0.1 M Tris-HCl, pH 7.4, 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 9 or 10) or in 0.1 M KOH (pH 12.5).*

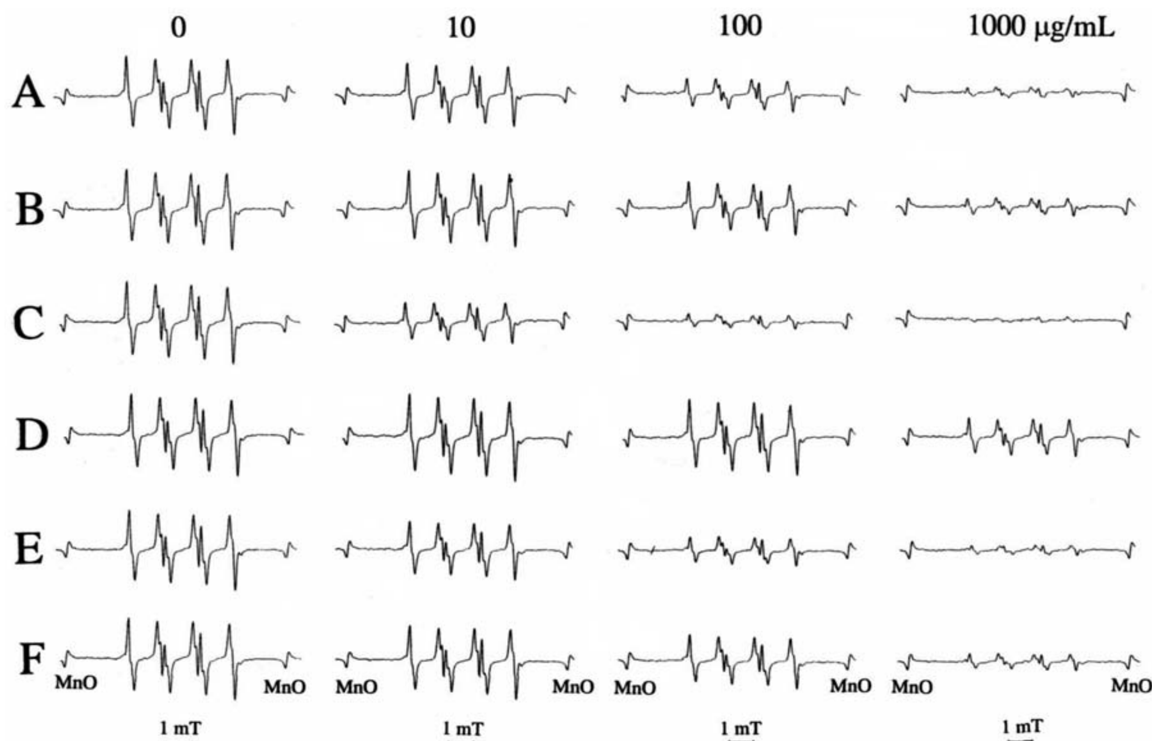

Figure 4. *ESR spectra of DMPO-OOH adduct produced in HX-XOD reaction mixture with the indicated concentrations of Rikko-san (A) and its ingredients such as Kanzo (B), Shoma (C), Ryutan (D), Saishin (E) or Bofu (F).*

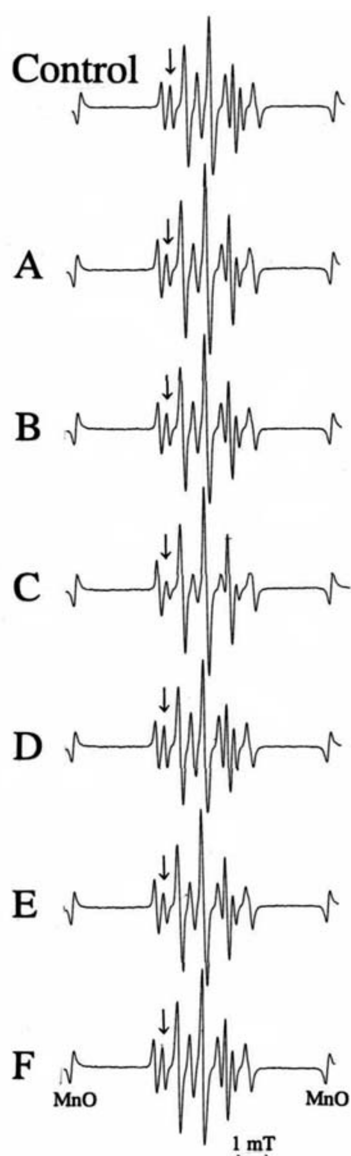

Figure 5. *NO radical scavenging activity of Rikko-san and its ingredients. The reaction mixture contained 20 μM carboxy-PTIO + 50 μM NOC-7 without (control) or with 200 μg/mL of Rikko-san (A), Kanzo (B), Shoma (C), Ryutan (D), Saishin (E) or Bofu (F). The mixture was subjected to ESR spectroscopy 3 minutes later. The arrows indicate the first peak of carboxy-PTI, produced by NOC-7 and carboxy-PTIO, which was used for the measurement of NO radical intensity.*

Table III. *Radical scavenging activity of Chinese medicine.*

	Radical intensity at pH 12.5	O_2^- scavenging activity (SOD unit/mg)	NO scavenging activity at 200μg/mL, expressed as % inhibition of NO intensity[a)]
Rikko-san	0.84	18.4	28.6 (17.0)[b)]
Kanzo	0.11	9.4	26.8 (19.5)
Shoma	1.51	41.5	76.8 (30.1)
Ryutan	<0.05	5.9	0 (0)
Saishin	0.90	19.7	32.9 (24.7)
Bofu	<0.05	9.0	15.7 (14.6)

*Reduction (%) of the peak height of carboxy-PTI.
Representative data are shown in Figure 5.
[a)] Carboxy-PTIO, NOC-7 and sample were mixed. Three minutes later, the radical intensity of the first peak of carboxy-PTI was determined by ESR spectroscopy and expressed as % of inhibition.
[b)] Carboxy-PTIO and NOC-7 were incubated for 10 minutes to achieve the complete conversion to carboxy-PTI. Sample was then added and further incubated for 3 minutes to determine the radical intensity, which was expressed as % of inhibition.

scavenging activity of these extracts. It seems likely that this is due to the decline of NO production by these Chinese medicines, as has been reported in dietary photo chemicals (16), since they reduced the intracellular concentration of iNOS proteins almost at the same concentration that reduced the extracellular production of NO. It remains to be investigated whether these Chinese medicines inhibit LPS-activation of NF-Î B expression (17). These substances efficiently scavenged O_2^-, but also showed potent reducing activity, which might produce apparent NO scavenging activity. This radical scavenging activity may further diminish the effective concentration of NO in the culture medium.

The extracellular NO concentration may decline through instantaneous capture by thiol compounds or serum proteins to form the *S*-nitroso adduct (18). The nitrosothiol will be decomposed in the presence of reducing agents such as ascorbic acid (19, 20) or copper (21), so as to again release NO. Another scavenging substance for NO is dioxygen, which significantly reduces the biological activity of NO (22). NO reacts with superoxide to form peroxynitrite, which has potent oxidizing activity (23). It is unclear whether peroxynitrite is produced at concentrations sufficient to damage the tissue *in vivo*.

We also found that lower concentrations of *Rikko-san* stimulated NO production (as indicated by closed circle in Figure 1A) and this was due to the increase of the intracellular concentration of iNOS (small shoulder in Figure 2A). This suggests that *Rikko-san* also contains macrophage-stimulating substances. This stimulator remains to be identified, and whether it acts *via* receptors similar to that of LPS (24) needs elucidation. We found that other *Rikko-san* ingredients, such as *Shoma*, *Ryutan*, *Saishin* and *Bofu*, showed much higher stimulation activity of NO production by Raw 264.7 cells. Such stimulating activity may be distributed among many other Chinese medicines, though the principles of this stimulating activity remains to be identified.

In conclusion, the present study demonstrated, for the first time, that *Rikko-san* and its 4 major ingredients have

both inhibitors and stimulators for NO production by macrophage-like cells. Further study is required to identify the physiological significance of these substances.

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References

1 Miyamoto M, Sakagami H, Minagawa K, Kikuchi H, Nishikawa H, Satoh K, Komatsu N, Fujimaki M, Nakashima H, Gupta M, Sarma DNK and Mitra SK: Tumor-specificity and radical scavenging activity of poly-herbal formula. Anticancer Res *22*: 1217-1224, 2002.
2 Hatsukari I, Hitosugi N, Ohno R, Nakamura S, Mizukami S, Nagasaka H, Matsumoto I, Kikuchi H, Nishikawa H, Niitsu M, Kawase M, Negoro T, Satoh K, Nakashima H and Sakagami H: Partial purification of cytotoxic substance from Moxa extract. Anticancer Res *22*: 2777-2782, 2002.
3 Hitosugi N, Ohno R, Hatsukari I, Mizukami S, Nagasaka H, Matsumoto I, Komatsu N, Fujimaki M, Nakashima H, Satoh K and Sakagami H: Diverse biological activity of Moxa extract and smoke. In Vivo *15*: 249-254, 2001.
4 Kawase M, Motohashi N, Satoh K, Sakagami H, Nakashima H, Tani S, Shirataki Y, Kurihara T, Wolfard K and Molnar J: Biological activity of persimmon *(Diospyros Kaki)* peel extracts. Phytotherapy Res *17*: 495-500, 2003.
5 Mihara S, Unten S, Kakuta H, Satoh K, Negoro T, Nakashima H, Komatsu N, Fujimaki M, Kikuchi H, Nishikawa H, Minagawa K, Suzuki F, Satoh T and Sakagami H: Diverse biological activities of fermented pine seed shell extract. Anticancer Res *22*: 1569-1574, 2002.
6 Motohashi N, Shirataki Y, Kawase M, Tani S, Sakagami H, Satoh K, Kurihara T, Nakashima H, Musci Ilona, Varga A and Molnar J: Cancer prevention and therapy with Kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. J Ethno Pharmacol *81*: 357-364, 2002.
7 Nemoto Y, Toriizuka K, Satoh K, Tobe T, Sakagami H, Nakashima H and Ida Y: Cytotoxic and radical scavenging activity of blended herbal extracts. In Vivo *16*: 327-332, 2002.
8 Suzuki F, Okayasu H, Tashiro M, Hashimoto K, Yokote Y, Akahane K, Hongo S and Sakagami H: Effect of lignins and their precursors on nitric oxide, citrulline and asparagine production by mouse macrophage-like cell Raw 264.7. Anticancer Res *22*: 2719-2724, 2002.
9 Ogiwara T, Satoh K, Negoro T, Okayasu H, Sakagami H and Fujisawa S: Inhibition of NO production by activated macrophages by phenolcarboxylic acid monomers and polymers with radical scavenging activity. Anticancer Res *23*: 1317-1324, 2003.
10 Tashiro M, Suzuki F, Shirataki Y, Yokote Y, Akahane K, Motohashi N, Ishihara M, Satoh K and Sakagami H: Effects of isoflavones from *Sophora* species on growth and activation of mouse macrophage-like cell line. Anticancer Res *22*: 2185-2192, 2002.
11 Miyamoto M, Hashimoto K, Minagawa K, Satoh K, Komatsu N, Fujimaki M, Nakashima H, Yokote Y, Akahane K, Gupta M, Sarma DNK, Mitra SK and Sakagami H: Effect of poly-herbal formula on NO production by LPS-stimulated mouse macrophage-like cells. Anticancer Res *22*: 3293-3302, 2002.
12 Wakabayashi H, Fukushima H, Yamada T, Kawase M, Shirataki Y, Satoh K, Tobe T, Hashimoto K, Kurihara T, Motohashi N and Sakagami H: Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by Barbados cherry, a fruit of *Malpighia emarginata* DC. Anticancer Res *23*: 3237-3242, 2003.
13 Fujisawa S, Atsumi T, Kadoma Y and Sakagami H: Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. Forum "phenolic compounds: free radical mechanisms of toxicity, catalysis, and protection". Toxicology *177*: 39-54, 2002.
14 Noda Y, Mori A and Packer L: Gliclazide scavenges hydroxyl, superoxide and nitric oxide radicals. An ESR study. Res Commun Mol Pathol Pharmacol *96*: 115-124, 1997.
15 Moncada S, Palmer PM and Higgs EA: Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev *43*: 109-142, 1991.
16 Chan MMY, Ho CT and Huang HI: Effect of three dietary phytochemicals from tea, rosemary and turmeric on inflammation-induced nitrite production. Cancer Lett *96*: 23-29, 1995.
17 Lin YL and Lin JK: (-) Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down regulating lipopolysaccharide-induced activation of transcription factor nuclear factor-Î B. Mol Pharm *52*: 465-472, 1997.
18 Butler AR and Rhodes P: Chemistry, analysis, and biological roles of *S*-nitrosothiols. Anal Biochem *249*: 1-9, 1997.
19 Kashiba-Iwatsuki M, Yamaguchi M and Inoue M: Role of ascorbic acid in the metabolism of *S*-nitroso-glutathione. FEBS Lett *389*: 149-152, 1996.
20 Scorza G, Pietraforte D and Minetti M: Role of ascorbate and protein thiols in the release of nitric oxide from *S*-nitroso-albumin and *S*-nitroso-gluthathione in human plasma. Free Rad Biol Med *22*: 633-642, 1997.
21 Gorren AC, Schrammel A, Schmidt K and Mayer B: Decomposition of *S*-nitrosoglutathione in the presence of copper ions and glutathione. Arch Biochem Biophys *330*: 219-228, 1996.
22 Goldstein S and Czapski G: Kinetics of nitric oxide autoxidation in aqueous solution in the absence and presence of various reductants. The nature of oxidizing intermediates. J Am Chem Soc *117*: 12078-12084, 1995.
23 Hurie RE and Padmaja S: The reaction of NO with superoxide. Free Rad Res Commun *18*: 195-199, 1993.
24 Nishijima H: Intracellular signaling pathway induced by LPS (in Japanese). Mol Med *36*: 499-503, 1999.

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