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ANTIOXIDANT ACTION OF NATURAL HEALTH PRODUCTS AND CHINESE HERBS

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Abstract—The scavenging effects of reactive oxygen species (ROS) by two natural health products, antioxidant analogs (AOA), and green magma (GM), and 16 medical Chinese herbs were investigated in two in vitro ROS-generating systems, activated neutrophils and xanthine–xanthine oxidase. Native, unheated AOA and GM products significantly reduced ROS levels, while unheated Chinese herbs had a negligible effect on ROS levels. In contrast, heat-extracted Chinese herbs and AOA markedly, and GM mildly, suppressed the levels of ROS in both systems. The ROS scavenging activity of these native, unheated products was unaffected by dialysis, but that of heated products was markedly diminished by dialysis. Further, the incubation of these products with gastric juice obtained by a gastric tube from healthy volunteers revealed results comparable to those induced by heat treatment with or without dialysis. Although the antioxidant activity of these natural products appears to be partly due to enzymes such as superoxide dismutase (SOD), the predominant factor seems to be low-molecular-weight ROS scavengers that are liberated or activated by gastric juice digestion as observed after heat treatment.

INTRODUCTION

In recent years, many Chinese herbs traditionally used for medicinal purposes, as well as a variety of so-called natural medical foods or health foods, have become widely accepted in Japan, as well as in China, as mild, nontoxic medications. Although the active ingredients and mechanisms of action of these products are still largely obscure, it is not uncommon for such products to demonstrate apparent clinical efficacy in patients with a variety of disorders (1–5). In this respect, they resemble the flavonoids, naturally occurring plant products that have come into clinical use in Europe (1, 6, 7).

We have previously found that many of these products are potent scavengers of reactive oxygen species (ROS), and some of these natural products are

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promoted commercially as if predominant factors are enzymes such as SOD. However, it is unlikely that high-molecular-weight enzymes are intact in the stomach after digestion and that they still preserve antioxidant activity in vivo. These questions prompted us to elucidate what kinds of factors are predominantly responsible for quenching ROS in vivo. In an attempt to characterize the chemical nature of these scavenging activities, we noticed that the scavenging activities were (unchanged or) increased after heat treatment, suggesting that the active compounds were unlikely to be enzymes, which would be expected to be denatured by such treatment. However, dialysis of the native products usually had no effect on the scavenging activity, suggesting that low-molecular-weight compounds might not be involved. In this connection, we performed subsequent experiments and describe here recent laboratory data obtained that seem to provide a resolution of this mild paradox concerning the physicochemical nature of the ROS scavenging activity in plant medical products. We propose that the major clinical effect of these natural medical products is one of scavenging reactive oxygen species and that this effect is mediated largely by low-molecular-weight components.

MATERIALS AND METHODS

Among the commercially available natural products, antioxidant analogs (AOA) products that contain malt made from rice, green tea, eaves of "Daikon," soybean, germs of rice, and sesane (Antioxidants Inc., Osaka, Japan), Green magma (GM) products (Japan Pharmaceutical Development Co., L., Osaka, Japan) that are eluted from leaves of immature barley, and 16 Chinese herbs were selected for the present study. Therapeutic doses (expected serum concentration after administration of therapeutic doses, e.g., $\times 1$ = 1.6 mg/ml for AOA and 2.1 mg/ml for GM) of these agents were added to an in vitro ROS-generation systems (neutrophils and xanthine–xanthine oxidase) to observe the dose–response effects ($\times\frac{1}{10}$, $\times 1$, $\times 10$) of these agents on ROS levels. The effects of several specific scavengers (SOD for O_2^-, catalase for H_2O_2, benzoate for $OH\cdot$, xanthine for $OH\cdot$ and chemiluminescence) were also examined concurrently as standards for comparison with these natural products. Each natural product was used after ultrasonification. The effects of dialysis and heating were examined on the ROS-scavenging actions of the compounds studied. For dialysis, we used Immersible CX 10 TM membranes (No. PTGC 11K 25, Millipore, Massachusetts), which permit egress of molecules with molecular weights below 10,000. Heat treatment of the agents was carried out at 130°C for 30 min.

Neutrophils were isolated from the peripheral blood of healthy volunteers by a previously described technique (8). For generation of ROS, the neutrophils were suspended in Krebs-Ringer phosphate buffer (KRP) containing 5 mM glucose and gelatin (1 mg/ml). KRP containing only glucose was used for $OH\cdot$ generation assay; Hanks' solution (Nissui Pharmaceutical Co., Tokyo, Japan) containing only gelatin was used for the determination of chemiluminescence. Each ROS was assessed by methods we have previously described (8–10). In brief, formation of O_2^- was determined by ferricytochrome *c* (type III, Sigma Chemical Co., St. Louis, Missouri) reduction by O_2^- produced from 4×10^6 neutrophils stimulated with 1 mg/ml opsonized zymosan (Sigma); absorbance was measured at 550 nm.

Generation of H_2O_2 was determined by using 2.5×10^6 PMNs stimulated with 1 mg/ml

opsonized zymosan, 0.1 ml of 50 mmol/liter scopoletin (Sigma) in KRP buffer, and 0.1 ml of 1 mg/ml horseradish peroxidase (type II, Sigma). The rate of decrease in fluorescence intensity of the scopoletin within 30 min was quantitated in a fluorescence spectrometer (Hitachi Co., Ltd., Tokyo) set at 370 nm for excitation and 460 nm for emission.

Hydroxyl radical (OH·) was quantitated by the amount of ethylene gas formed from the reaction of α-keto-methiol butylic acid (Sigma) with the PMN-generated OH·; 2×10^6 PMNs stimulated with 1 mg/ml opsonized zymosan and 1 mmol α-keto-methiol butylic acid were used, and the total amount of ethylene gas formed at 10, 20, and 30 min was determined on a gas-chromatograph (Hitachi).

Chemiluminescence was measured in a scintillation spectrometer (Packard, Downers Grove, Illinois). Five million PMNs in 3 ml Hanks' solution containing 1 mg/ml opsonized zymosan, but no luminol, were incubated in the dark. Chemiluminescence was monitored on the spectrometer, which was operated in the out-of-coincidence summation mode.

ROS generation in the xanthine–xanthine oxidase system was performed as follows: Instead of adding neutrophils and opsonized zymosan, 0.1 ml of 13.5 mg hypoxanthine in physiological saline plus 0.05 ml of 50 mM EDTA were diluted in 2 ml of KRP (pH 7.2–7.4). Thereafter, 0.1 ml of 0.1 unit/ml dialyzed xanthine oxidase was added to generate O_2^-.

In order to determine the effect of oral administration of these natural products on antioxidant action, the products were added to ROS-generating system after being treated with gastric juice as follows: samples were dissolved in fasting gastric juice which was obtained from healthy volunteers by a gastric tube. Thereafter, they were incubated at 37°C for 2.5 h and centrifuged at 2000 rpm for 10 min. Then, the supernates were obtained and neutralized with appropriate amounts of NaOH and introduced to ROS-generating systems.

The effect of gastric juice alone neutralized with NaOH on ROS levels was assessed as controls both in neutrophil and xanthine–xanthine oxidase systems.

We also assessed the activity of 2-nitropropane dioxygenase by the method of Kido et al., (11), because AOA products consisted mainly of rice-derived malt, which is related to the yeasts in which Kido et al. identified the activity of an oxidizing enzyme (dioxygenase) (11). The standard reaction mixture, consisting of 50 μmol 2-nitropropane, 1-nitropropane, or nitroethane and 100 μmol potassium phosphate (pH 7.0–8.0), was incubated aerobically for 20 min at 37°C in the presence of modest amounts of rice-derived malt. The reaction was stopped by the addition of 0.1 ml glacial acetic acid; after centrifugation, 0.1-ml aliquots of the supernatant were used for the determination of nitrite and acetone. Nitrite was determined by the method of Little (12). Acetone was determined spectrophotometrically by the method of Paz et al. (13). One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 nmol of nitrite or acetone per min.

The generation of chemiluminescence is negligible in the xanthine–xanthine oxidase system, and hence its measurement was omitted from these experiments (9). Regarding the results of 16 Chinese herbs on ROS generation, comparable data were obtained from each. Therefore, a mean value of triplicate determinations in each drug was assessed, and thereafter the mean of 16 Chinese agents was determined and expressed in the figures and table as a single point.

RESULTS

Effect of Agents on Levels of ROS Before and After Heat Treatment, Figure 1A and 1B show the results of experiments measuring the scavenging effects of the natural products AOA, GM, and 16 Chinese herbs on the levels of superoxide anion, hydrogen peroxide, hydroxyl radical, and chemi-

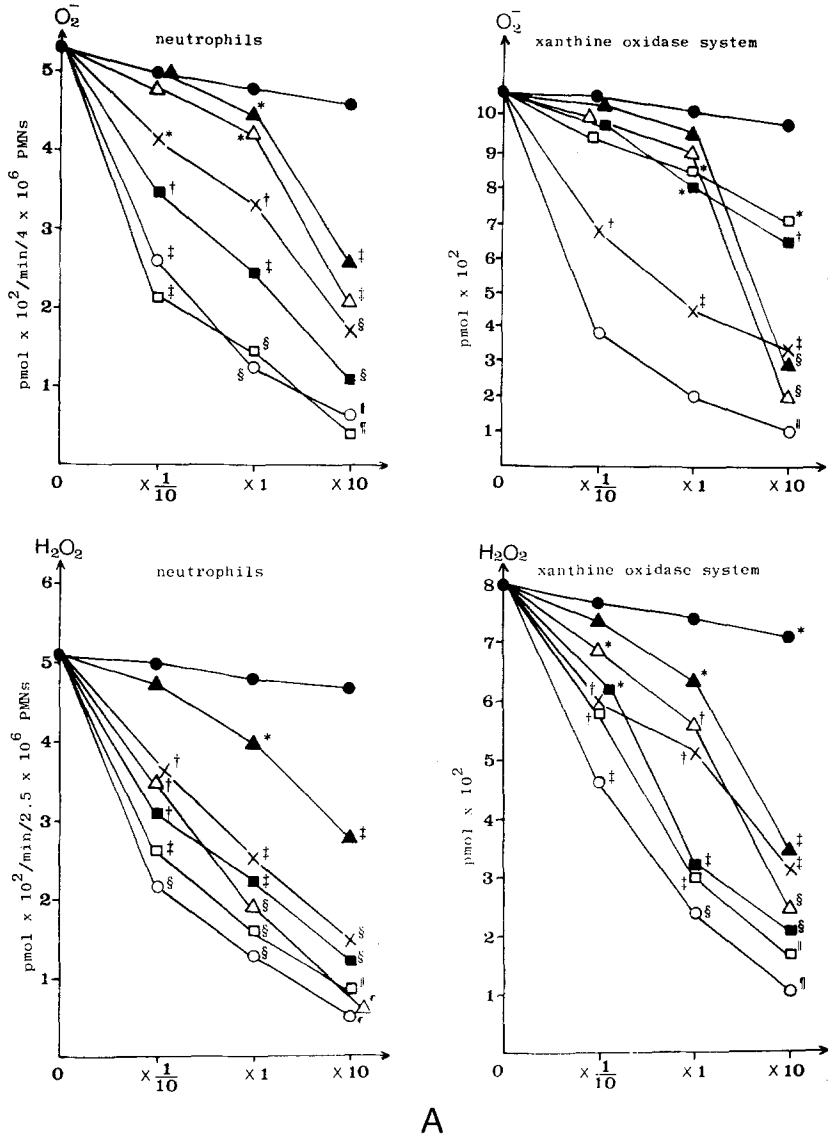
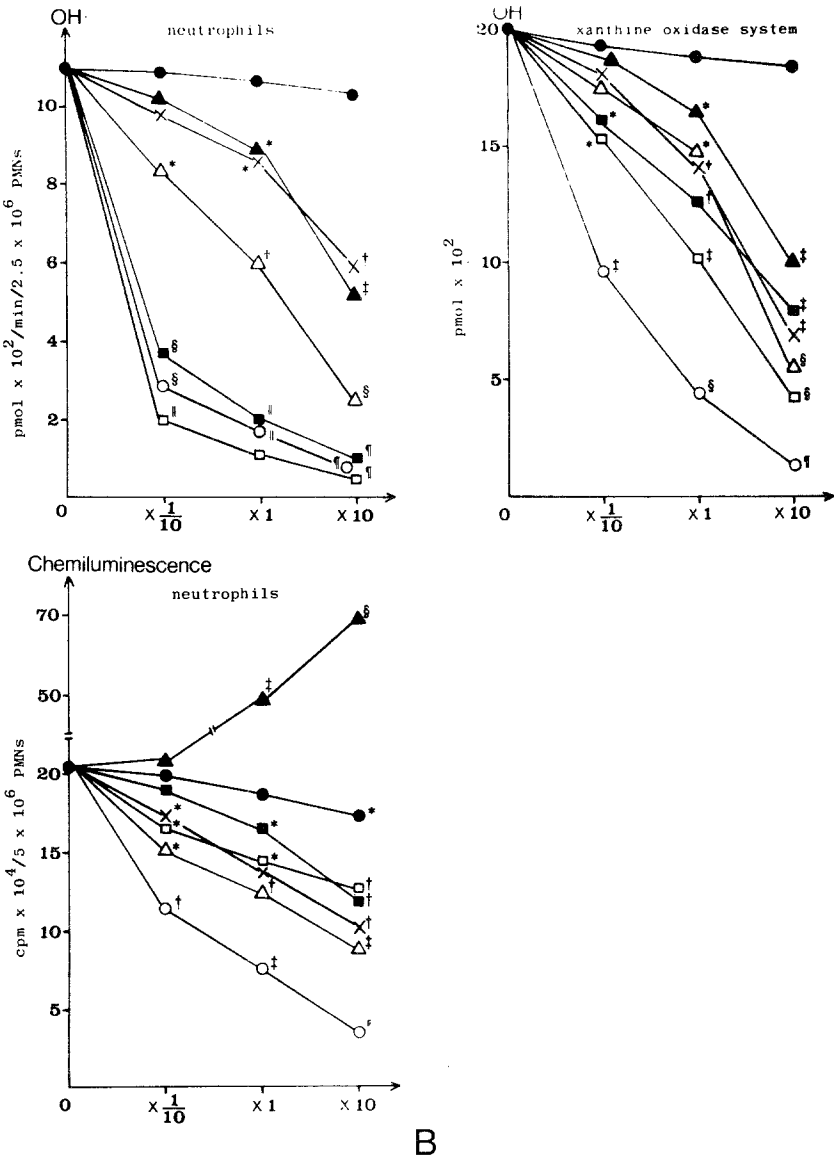


Fig. 1. (A) Comparison of the effect among natural products (heat-treated or untreated) and each specific scavenger on O_2^- and H_2O_2 levels generated by stimulated neutrophils and xanthine–xanthine oxidase system. (B) Comparison of the effect among natural products (heat-treated or untreated) and each specific scavenger on OH· and chemiluminescence levels generated by stimulated neutrophils and xanthine–xanthine oxidase system. ○—○, "herb" heated; ●—●, "herb" unheated; △—△, GM heated; ▲—▲, GM unheated; □—□, AOA heated; ■—■, AOA unheated; x—x, each specific scavenger; each specific scavenger: SOD (×1 = 120 unit/ml) for O_2^-, catalase (×1 = 120 unit/ml) for H_2O_2, benzoate (×1 = 1.0 mM) for OH·, and xanthine



B

(×1 = 0.8 mg/ml) for chemiluminescence. ×1 for herb, GM, or AOA indicates the therapeutic dose of each drug, e.g., 1.6 mg/ml for AOA and 2.1 mg/ml for GM. *SEM* (standard error of mean) of each value plotted in all figures (in both Figures 1 and 2) was within 12%. *SEM* was not described in each value to avoid complicating the figures. The effects of 16 Chinese herbs on ROS generation were comparable. Therefore, the mean of 16 Chinese agents was determined and indicated as a single symbol in the figures and table after a mean value of triplicate determinations in each drug was assessed. *$0.01 < P < 0.05$ compared with control medium containing no natural product, † $P < 0.01$, ‡ $P < 0.001$, § $P < 0.0001$; ‖ $P < 0.00001$; ¶ $P < 0.000001$.

luminescence generated by zymosan-stimulated neutrophils and by the xanthine–xanthine oxidase system. Optimal concentrations of specific scavengers for each of the ROS were included as positive controls (superoxide dismutase, catalase, benzoate, and xanthine, respectively).

When added in the untreated state, none of the Chinese herbs showed any significant scavenging of ROS generated by either system ($P > 0.05$). In contrast, both unheated and heated AOA and GM, as well as heated Chinese herbs, showed potent ROS scavenging activity. Of particular interest was the observation that heated products unexpectedly showed no decrease but generally greater scavenging effects than the unheated products. Levels of superoxide in the neutrophil generating system were highly significantly reduced by therapeutic concentrations of heated AOA ($P < 0.0001$), unheated AOA ($P < 0.001$), and heat-extracted Chinese herbs ($P < 0.0001$) while heated and unheated GM agents reduced O_2^- slightly ($0.01 < P < 0.05$ for each). Heated Chinese herbs and heated and unheated AOA more effectively reduced O_2^- levels than SOD, which is a specific scavenger of O_2^-, at concentrations usually used in our laboratory. The scavenging of hydrogen peroxide by natural products was generally comparable to that of superoxide anion, indicating that not only SOD alone but also other scavengers such as catalase, peroxidase, and other undefined antioxidants are contained in these natural drugs because H_2O_2 levels are not reduced when only SOD is present (8, 9). Thus, heat treatment produced a significant enhancement of the scavenging effect of the Chinese herbs and of the GM products, but not of the AOA products (except for O_2^- generated in the neutrophil system).

In Figure 1B, unheated GM products are shown to enhance chemiluminescence, while heated GM products show the opposite effect. We interpret this to indicate that one or more pigmented compounds contained in the dense green GM product is responsible for the increased chemiluminescence and that this effect is inactivated by heating.

Effect of Dialysis on ROS Values. In Table 1, the effects of dialysis upon the scavenging of neutrophil-generated ROS by unheated and heated natural products are shown. Dialysis had no effect on the scavenging capacity of any of the unheated products. These results suggest that scavenging substances in unheated products are retained inside the dialysis membrane and thus have molecular weights above 10,000. In contrast, dialysis of the heated agents resulted in a significant loss in ROS scavenging capacity, suggesting that potent scavenging compounds are newly produced by heat treatment and thus have molecular weights below 10,000. Parallel experiments with the xanthine–xanthine oxidase system gave results similar to those of the neutrophil system (data not shown).

Effect of Gastric Juice on ROS Levels. Comparable results were obtained both after gastric juice treatment and after heat treatment with or without dialysis. Therapeutic concentrations of gastric juice-treated natural products (ex-

Table 1. Effect of Treatment with Heat or Gastric Juice (GJ) after Dialysis on Oxygen Radical Levels Generated by Neutrophils

	Dialysis	O_2^- (pmol × 10^2/min/4 × 10^6 PMNs)	H_2O_2 (pmol × 10^2/min/2.5 × 10^6 PMNs)	OH (pmol × 10^2/min/2.5 × 10^6 PMNs)	Chemiluminescence (cpm × 10^4/5 × 10^6 PMNs)
GM (×I)					
Unheated	before	4.52 ± 0.42	4.06 ± 0.43	9.02 ± 1.1	45.6 ± 5.2
	after	4.45 ± 0.38	4.13 ± 0.48	8.93 ± 0.9	47.8 ± 6.1
Heated	before	4.32 ± 0.49	1.82 ± 0.24	6.09 ± 0.62	10.7 ± 1.9
	after	5.21 ± 0.43[a]	4.23 ± 0.56[b]	8.78 ± 1.1[a]	18.1 ± 1.6[b]
GJ-treated	before	3.45 ± 0.41	3.58 ± 0.43	1.80 ± 0.22	69.5 ± 7.4
	after	3.80 ± 0.45	4.12 ± 0.49[a]	2.21 ± 0.31	57.8 ± 6.3
AOF (×I)					
Unheated	before	2.56 ± 0.31	2.04 ± 0.28	2.12 ± 0.20	15.4 ± 1.9
	after	2.67 ± 0.29	1.97 ± 0.22	2.01 ± 0.23	14.7 ± 2.0
Heated	before	1.53 ± 0.09	1.75 ± 0.14	1.07 ± 0.09	10.8 ± 2.1
	after	4.33 ± 0.49[c]	4.28 ± 0.55[c]	8.72 ± 0.92[e]	16.4 ± 2.0[b]
GJ-treated	before	1.18 ± 0.21	1.22 ± 0.18	0.56 ± 0.07	20.5 ± 2.7
	after	3.62 ± 0.43[c]	4.01 ± 0.51[d]	2.08 ± 0.26[d]	30.2 ± 4.1[b]
Herb (×I)					
Unheated	before	4.81 ± 0.53	4.72 ± 0.61	10.85 ± 2.1	15.8 ± 2.2
	after	4.84 ± 0.50	4.69 ± 0.60	10.92 ± 1.8	15.4 ± 2.4
Heated	before	1.32 ± 0.11	1.35 ± 0.21	1.82 ± 0.08	5.7 ± 0.7
	after	4.28 ± 0.56[c]	4.32 ± 0.51[c]	9.13 ± 1.1[e]	17.5 ± 2.3[c]
GJ-treated	before	1.75 ± 0.22	1.02 ± 0.14	0.75 ± 0.01	10.7 ± 1.6
	after	2.41 ± 0.32[a]	1.93 ± 0.24[a]	1.33 ± 0.21[a]	15.6 ± 2.2[a]

[a] $0.01 < P < 0.05$ compared with before dialysis.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P < 0.0001$.
[e] $P < 0.00001$.

cept for GM products) markedly reduced ROS levels generated in the neutrophil generating system (Chinese herb: $P < 0.001$ for O_2^- and OH·, $P < 0.0001$ for H_2O_2 and chemiluminescence; AOA: $0.01 < P < 0.05$ for chemiluminescence, $P < 0.0001$ for O_2^-, H_2O_2, and OH·; GM: $0.01 < P < 0.05$ for OH·) (Figure 2). Dialysis of the gastric juice-treated agents also significantly decreased ROS scavenging activity (Table 1). Similar results were obtained in xanthine–xanthine oxidase system (not shown). These results also suggest that low-molecular-weight substances that effectively quench ROS are produced in stomach.

However, in case of GM products, as shown in Table 1, little difference of ROS values was observed before and after dialysis since only the highest concentration (×10) significantly reduced ROS levels (Figure 1 and 2) and only the product of ×1 concentration was compared before and after dialysis (Table 1).

2-Nitropropane Dioxygenase Activity in AOA Agents. The malt contained in the AOA products was found to contain no detectible *n*-nitropropane dioxygenase activity (not shown). Thus, it is unlikely that the antioxidant action of the AOA agents is due to the action of a dioxygenase enzyme such as that found in the yeast *Hansenula mrakii* by Kido et al. (11).

DISCUSSION

The present experiments demonstrated that AOA, GM, and Chinese herbs markedly reduce the ROS levels generated by both stimulated neutrophils and the xanthine–xanthine oxidase systems; these products thus appear to have potent ROS scavenging activity. Although enzymes such as SOD, catalase, and peroxidase are considered to constitute part of the active principles of these natural medical products, it is unlikely that the potent properties in these products to exert antioxidant activities consist exclusively of enzymes since the products are biochemically and pharmacologically active when taken orally and the active principles are likely to be largely resistance to acid and proteolysis. The results of our experiments using heat treatment, dialysis, and gastric juice suggest that heat- and gastric juice-resistant, low-molecular-weight compounds perform the bulk of the ROS scavenging associated with these natural products.

From these findings, we speculate that the ROS scavenging compounds are, for the most part, low-molecular-weight substances that in the native products either are bound to high-molecular-weight compounds or constitute part of the repeating subunits of high-molecular-weight polymers. Furthermore, gastric juice, as speculated from our heat-treatment experiments, would be expected to liberate the active low-molecular-weight compounds by cleaving either covalent, noncovalent bonds, or both in stomach. Thus the antioxidant properties of

the native products would be augmented by oral administration. The modest ROS scavenging activity of the native products, which we have demonstrated in these experiments, might be due either to the presence of scavenging enzymes such as SOD or to low, but detectable, activity exerted by the bound forms of the scavengers that are liberated by (heating or) gastric juice treatment, or to both. In fact, clinically, the natural medical products (e.g., AOA) (5) whose ROS scavenging activity following the dialysis after gastric juice treatment was greater (Table 1) revealed clinically more effective in the patients related to an increase in ROS (8, 9, 14–22). This seems to support our hypothesis concerning the anti-oxidant properties of these native natural products.

If low-molecular-weight ROS scavengers play a major role in mediating the pharmacologic effects of many natural products, chemical identification of these compounds might be highly informative. Flavonoids (1, 6, 7, 23–26), alpha-tocopherol, vitamin C, and carotinoid are possible candidates. Most of the medical Chinese herbs contain ginseng or ren shen (*Panax ginthenz*), gan cao (*Glycyrrhiza uralensis*), and huang-chin (*Radix scutellariae*); these plants contain high concentration of flavonoids (27–29). We speculate that it is the flavonoids that exert a major part of the pharmacologic action of these natural medicinal products, since these substances have high and various chemical reactivities (1, 6, 7,).

The flavonoids are a large family of benzo-gamma-pyrone derivatives (1). Structurally, they somewhat resemble nucleotides and folic acid; this similarity is thought to be the main basis for their physiologic and pharmacologic action. They are highly reactive chemically, showing strong binding affinity for biological polymers (1, 6, 7) and heavy metal ions (1, 23), active catalysis of electron transport (1, 24), and scavenging of free radicals (25, 26). Although several reports have recently appeared in the Western medical literature of the pharmacologic potency and potential clinical use of the flavonoids (1, 6, 7), the clinical applications to date have still been empirical and only equivocally successful (1).

This is consistent with two empiric observations regarding natural medical products that are often cited in traditional Chinese medicine. These may be roughly translated as, "One drug can be effective in various diseases with different pathogeneses," and "One drug may be effective in only some of the patients with the same disease." This former saying may be due to the various and different clinical reactivities, and the latter to the different binding affinity for biochemical polymers among the patients to whom natural medical products were administered, as explained above. Regarding the mechanism of flavonoids in removing ROS (in Chinese Herbs), we have recently reported that ROS appears to play an important role in the pathogenesis of Behçet's disease (18); successful treatment of Behçet's disease with Chinese herbs has been reported

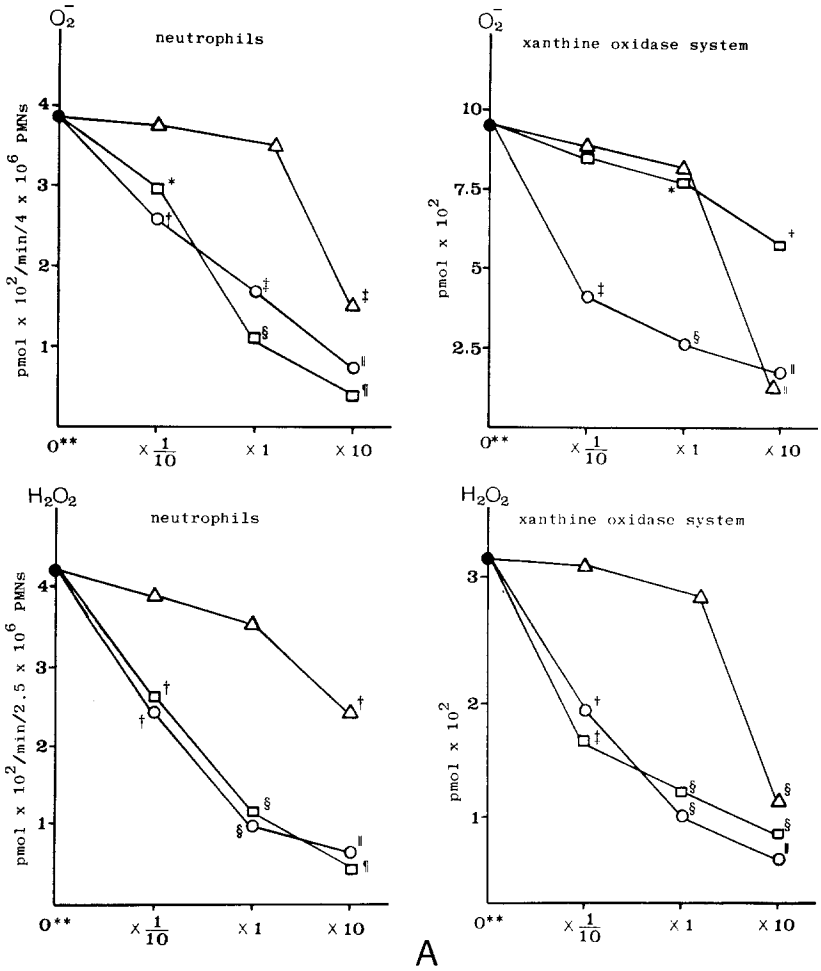


Fig. 2. (A) Effect of gastric juice-treated natural products on O_2^- and H_2O_2 levels generated by neutrophils and xanthine–xanthine oxidase system. (B) Effect of gastric juice-treated natural products on OH· and chemiluminescence levels generated by neutrophils and xanthine–xanthine oxidase system. ○——○, "herb"; △——△, GM; □——□, AOA. After these (unheated) products were dissolved in gastric juice, the medium was neutralized by NaOH. Thereafter, the medium containing the products and gastric juice was introduced to the ROS generating systems and assessed for ROS levels. **Control contains no natural products but gastric juice alone neutralized by NaOH. *$0.01 < P < 0.05$ compared with control. Other symbols: see legend for Figure 1.

(2, 3), lending support to our hypothesis that scavenging of ROS by the Chinese herbs is an important basis for their apparent clinical efficacy.

In addition to inflammatory diseases induced by an increase in ROS generation (8, 9, 14–22), there is mounting evidence that industrial pollution leads

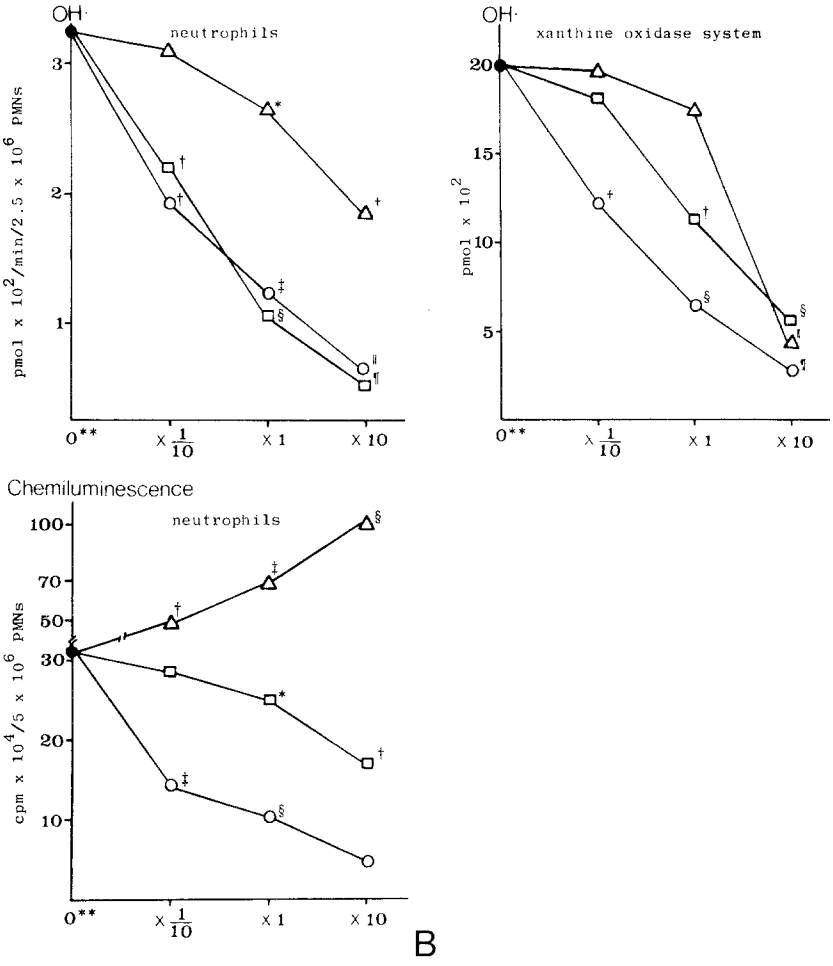


Fig. 2. (Continued).

to generally higher levels of ROS production in man, with resultant cytotoxic effects (30–32). Aging and carcinogenesis (33–35) are also thought to be accelerated by ROS. By virtue of their ROS scavenging action, the natural products we have investigated may exert favorable effects not only in these disease states (14–22), but also in retarding the aging process and in protection against carcinogenesis.

Many natural health products are promoted commercially in Japan, Europe, and the United States, either as SOD itself or as consisting primarily of enzymes such as SOD (e.g., orgotein in the United States and, green magma in Japan). Our results suggest that, although these products contain ROS-scav-

enging enzymes, their main pharmacologic actions are more likely due to low-molecular-weight components. The kind of chemical substances of low-molecular-weight responsible for the ROS quenching activity in these natural products is under investigation.

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