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Antitumor Potential of Three Herbal Extracts against Human Oral Squamous Cell Lines

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Abstract. *Three Chinese herbal extracts of Drynaria baronii, Angelica sinensis and Cornus officinalis Sieb. et Zucc (referred to as DB, AS, CO, respectively) were investigated for their antitumor potential. These extracts showed very weak cytotoxicity against all nine cultured human cells (normal and tumor cells), but with some tumor-specific cytotoxicity displayed by DB and CO. These extracts showed little or no growth stimulation effects at lower concentrations (so-called 'hormetic effect'). Human oral squamous cell carcinoma cell lines (HSC-2, NA) were relatively resistant to committing apoptosis, as compared with human promyelocytic leukemia HL-60 cells. Electron-spin resonance spectroscopy shows that DB and CO scavenged superoxide anion (generated by hypoxanthine and xanthine oxidase reaction) and hydroxyl radical (generated by Fenton reaction) more efficiently than AS. DB and CO, but not AS, produced broad radical peak(s) and enhanced the superoxide scavenging activity of vitamin C. However, none of the extracts clearly enhanced the cytotoxicity of mitoxantrone, an anthracycline antitumor antibiotic. DB, but not CO and AS, showed weak anti-HIV activity. These data demonstrate several unique antitumor properties of DB.*

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Chinese herbal extracts, such as those from *Drynaria baronii*, *Angelica sinensis* and *Cornus officinalis* Sieb. et Zucc (referred to as DB, AS, CO, respectively), have been reported to display diverse biological activities: the osteogenic (1) and bone resorption inhibitory (2) activities of DB; the radioprotective (3), antioxidative (4) and hematopoietic (3) activities of AS; the antibacterial (5), anti-diabetic (6), anti-inflammatory (7), antiarrhythmic (8) and antioxidant (9, 10) activities of CO; and the antitumor activity of AS and CO or some compounds extracted from them (11, 12).

However, there is little direct evidence of any antitumor potential of these extracts. We have recently established an *in vitro* system for the assay of the antitumor potential of natural and synthetic compounds (13). Several anthracycline antibiotics such as doxorubicin, nocobactins, mitomycin and some cyclic α,β-unsaturated ketones were found to induce the highest tumor-selective toxicity, whereas hundreds of low molecular weight flavonoids and tannins were much less tumor specific (13, 14). This assay system was applied to measure the antitumor potential of DB, CO and AS, using three normal oral cells (human gingival fibroblast HGF, human pulp cell HPC, human periodontal ligament fibroblast HPLF) and seven human tumor cell lines (five oral squamous cell carcinoma (OSCC) HSC-2, HSC-3, HSC-4, Ca9-22, NA; one glioblastoma T98G and promyelocytic leukemia HL-60) as target cells.

It has been reported that many toxic substances, environmental hormones, inorganic compounds and even irradiation modulate the growth of cultured cells in a bi-phasic fashion, stimulating or inhibiting the growth at lower and higher concentrations, respectively. This growth stimulating effect at lower concentrations is known as hormesis (15, 16). We investigated whether these extracts exert such hormetic effects on various normal and tumor cells.

We have previously reported that (i) ascorbate derivatives that produce radicals were cytotoxic, whereas that do not produce radicals were not cytotoxic; (ii) lignin enhanced both the cytotoxicity and radical intensity of sodium ascorbate, whereas tannins and sodium ascorbate counteracted each other (17). Therefore, we also investigated whether these three herbal extracts stimulate the cytotoxicity of sodium ascorbate and mitoxantrone, an antitumor antibiotic (18).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); mitoxantrone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD) (Sigma Chemical Ind., St. Louis, MO, USA); sodium ascorbate (vitamin C) (Tokyo Kasei Kogyo Co., Ltd., Tokyo); diethylenetriamine-pentaacetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Dojin, Kumamoto, Japan).

Preparation of herb extracts. *D. baronii, A. sinensis* and *C. officinalis* Sieb. et Zucc were supplied by the Department of Pharmacology, School of Stomatology, The Fourth Military Medical University, Xi'an, China, and extracted for 2 hours twice with 12 volumes of water at 100˚C. The supernatants, obtained after centrifugation at 3,000 rpm for 10 minutes, were lyophilized to a dried powder (referred to as DB, AS and CO, respectively). Lyophilization and measurement of the dry weight of DB, AS and CO showed that 1 g of dried powder was obtained from 3.3 g (DB), 2.2 g (AS) and 2.15 g (CO) solid raw material, respectively. Dried powder was dissolved in sterile distilled water at the concentration of 100 mg/ml, and stored at -30°C until use.

Cell culture. Human oral normal cells (HGF, HPC, HPLF) were prepared from periodontal tissues according to the guideline of the Intramural Board of Ethic Committee (No. 0206, No. A0808), after obtaining informed consent from the patients. Since these normal cells have a limited lifespan of about 20 population doubling level (PDL) due to *in vitro* senescence (19), cells at the 8-12 PDL were used for the present study. These normal cells, human OSCC cell lines (HSC-2, HSC-3, HSC-4, Ca9-22, NA) (kindly supplied by Prof. Nagumo, Showa University) and human glioblastoma cell line (T98G) (kindly supplied by Dr. Iida, Showa University) were cultured in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Human promyelocytic leukemic cell lines HL-60 (supplied by Professor Nakaya, Showa University) and human T-cell leukemia virus I (HTLV-I)-bearing CD4-positive human T-cell line, MT-4 (supplied by Professor Yamamoto, Tokyo Medical Dental University) were cultured in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, as described elsewhere (20).

Assay for cytotoxic activity. Near-confluent cells were treated for 48 hours with different concentrations of test substances, and the viable adherent cell number was then determined by the MTT method, as described elsewhere (21). The viability of HL-60 cells was determined by hemocytometer after staining with trypan blue. The 50% cytotoxic concentration (CC_{50}) was determined from the dose response curve. The tumor-specificity index (TS) was measured by the following equation: TS=(CC_{50}HGF + CC_{50}HPC + CC_{50}HPLF) / (CC_{50}HSC-2 + CC_{50}HSC-3 + CC_{50}HSC-4 + CC_{50}Ca9-22 + CC_{50}NA + CC_{50}T98G)× (6/3) (Table I).

Assay for DNA fragmentation. HSC-2 or NA cells (1.2×10^5) were inoculated onto a 6-well plate (9.6 cm^2) and incubated for 48 hours to complete adherence to the plate. Adherent HSC-2 and NA cells or freshly prepared HL-60 cells (5×10^5) were cultured for 6, 24 or 48 hours (HL-60 cells: 6 hours) in fresh culture medium (3 ml) without (control) or with ×1, ×2, ×4 CC_{50} of ascorbate sodium and mitoxantrone. Cells were then harvested, washed once with PBS(–) and lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 1-2 hours at 50°C, and then mixed with 50 μl NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and then 200 μl of ethanol. After centrifugation for 20 minutes at 20,000 × g, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Sample (10-20 μL) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m^2/min, 1 min) were run in parallel as positive controls (22). After staining with ethidium bromide, DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc-It, UVP, Inc., Upland, CA, USA) (21).

Assay for caspase activation. HSC-2 cells (6×10^5) were inoculated onto a 85-mm dish and incubated for 48 hours to allow complete adherence. The adherent HSC-2 cells or freshly prepared HL-60 cells (3×10^6 in 6-well plate) were further incubated for 6, 24 or 48 hours in fresh medium without (control), or with sodium ascorbate or mitoxantrone. Cells were washed with PBS(–) and lysed in the lysis solution [50 mM Tris-HCl (pH 7.5), 0.3% NP-40, 1 mM DTT]. After standing for 10 minutes on ice and centrifugation for 5 minutes at 21,000 × g, the supernatant was collected. Lysate (50 μl, equivalent to 200 μg protein) was mixed with 50 μl lysis solution containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore pNA was measured by microplate reader (21).

Assay for anti-HIV activity. MT-4 cells were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock-infected (control) MT-4 cells were incubated for 5 days with different concentrations of test substances, and the relative viable cell number was determined by MTT assay. The 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) were determined from the dose-response curve with mock-infected or HIV-infected cells, respectively (20). All the data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated by the following equation: SI=CC_{50}/EC_{50}.

Radical-scavenging activity. The radical intensity was determined at 25˚C in 0.1 M phosphate buffer (pH 7.4), 0.1 M $Na_2CO_3/NaHCO_3$ (pH 10.0) or 0.1 M KOH (pH 13.0), using electron-spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band; 100 kHz modulation frequency) (23). The instrument settings were: center field,

Table I. *Cytotoxic activity of herb extracts against cultured human normal and tumor cells.*

	CC_{50} (μg/ml)								
	DB			AS			CO		
Cell	H	I	L	H	I	L	H	I	L
HGF	>500	>500	>500	>500	481	479	>500	>500	>500
HPC	>500	>500	>500	>500	440	>500	>500	>500	>500
HPLF	>500	>500	>500	>500	463	482	>500	>500	>500
HSC-2	>500	434	391	>500	455	477	454	411	458
HSC-3	>500	>500	500	>500	>500	471	>500	>500	467
HSC-4	>500	>500	>500	>500	439	384	479	429	732
Ca9-22	>500	471	463	398	449	460	389	481	472
NA	>500	>500	399	>500	>500	>500	>500	>500	>500
T98G	>500	>500	>500	>500	>500	>500	>500	>500	>500
TS	>1.00<	>1.03<	>1.09<	>1.04<	>1.06<	>1.05<	>1.06<	>1.10<	>1.06<

Tumor cells were inoculated at 1.5, 3 or 6×10^3 cells (referred to as low (L), intermediate (I) or high (H) cell density) in each well of 96-microplates. Confluent normal cells were trypsinized and inoculated at the density of 1:8 (L), 1:4 (I) or 1:2 (H) of confluency. After incubation for 48 hours, the media were replaced with fresh media containing various concentrations of DB, AS or CO, and the viable cell number was determined by MTT method after 48 hours. Each value represents the mean from 3 independent experiments. S.D. <20% . TS: Tumor-specificity.

Table II. *Very low level of hormetic effect of DB, AS and CO against both human normal and tumor cells.*

	Maximun response (% increase in the cell number at the maximum)											
	DB				AS				CO			
	L	I	H	mean±S.D.	L	I	H	mean±S.D.	L	I	H	mean±S.D.
HGF	4.4	6.8	0.0	3.7±3.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HPC	0.0	0.0	6.3	2.1±3.7	8.2	4.0	0.0	4.1±4.1	8.5	0.0	0.0	2.8±4.9
HPLF	7.4	2.3	5.1	5.0±2.6	1.9	1.8	17.0	6.9±8.7	3.8	3.7	1.3	2.9±1.4
HSC-2	10.0	0.0	8.7	6.2±5.4	23.0	12.7	30.0	22.0±8.7	6.8	4.0	12.8	7.9±4.5
HSC-3	14.8	10.7	2.9	9.4±6.0	12.0	17.0	19.5	16.2±3.8	5.1	21.4	0.0	8.9±11.2
HSC-4	25.7	21.4	20.8	22.6±2.7	2.9	0.0	3.3	2.1±1.8	4.3	9.2	4.0	5.8±2.9
Ca922	8.6	6.5	17.0	10.7±5.6	15.8	15.4	29.2	20.1±7.8	71.0	17.6	23.0	37.2±29.4
NA	0.0	33.4	4.8	12.8±18.1	11.4	21.5	11.7	14.9±5.8	11.6	8.5	18.1	12.7±4.9
T98G	40.4	6.7	10.7	19.3±18.4	39.5	18.0	6.3	21.3±16.9	27.5	17.2	2.7	16.0±12.5

The cells were inoculated and treated with herbal extracts as described in Table I. Maximum response (% of increase of cell number vs control) was determined as previously reported (15).

335.5±5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT: gain, 630, time constant, 0.03 s; scanning time, 2 min.

The superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μl) (2 mM HX in 0.1 M phosphate buffer [pH 7.4] [PB] 50 μl, 1 mM DETAPAC 10 μl, 10% DMPO 30 μl, sample 40 μl, H_2O 40 μl, XOD [1 U/ml in PB] 30 μl) was determined, using the same instrument settings described above (23).

For the determination of hydroxyl radical (in the form of DMPO-OH) produced by Fenton reaction (200 μl) [1 mM $FeSO_4$ (containing 0.2 mM DETAPAC) 50 μl, 0.1 M phosphate buffer (pH 7.4) 50 μl, 92 mM DMPO 20 μl, sample (in H_2O) 50 μl, 1 mM H_2O_2, 30 μl], the gain was changed to 400 (23).

Results

Cytotoxic activity. These herbs extracts (DB, AS, CO) showed very weak cytotoxicity against both human oral normal cells (HGF, HPC, HPLF) (mean CC_{50}>500, >461, >500 μg/ml) and human cancer cell lines (OSCC lines, HSC-2, HSC-3, HSC-4, Ca9-22 and NA: mean CC_{50}>481, >469, >464 μg/ml, respectively; human glioblastoma T98G: mean CC_{50}>500 μg/ml), making it difficult to accurately determine the tumor-specificity index (TS=>1.03<, >1.06< and >1.1<, respectively) (Table I). It should, however, be

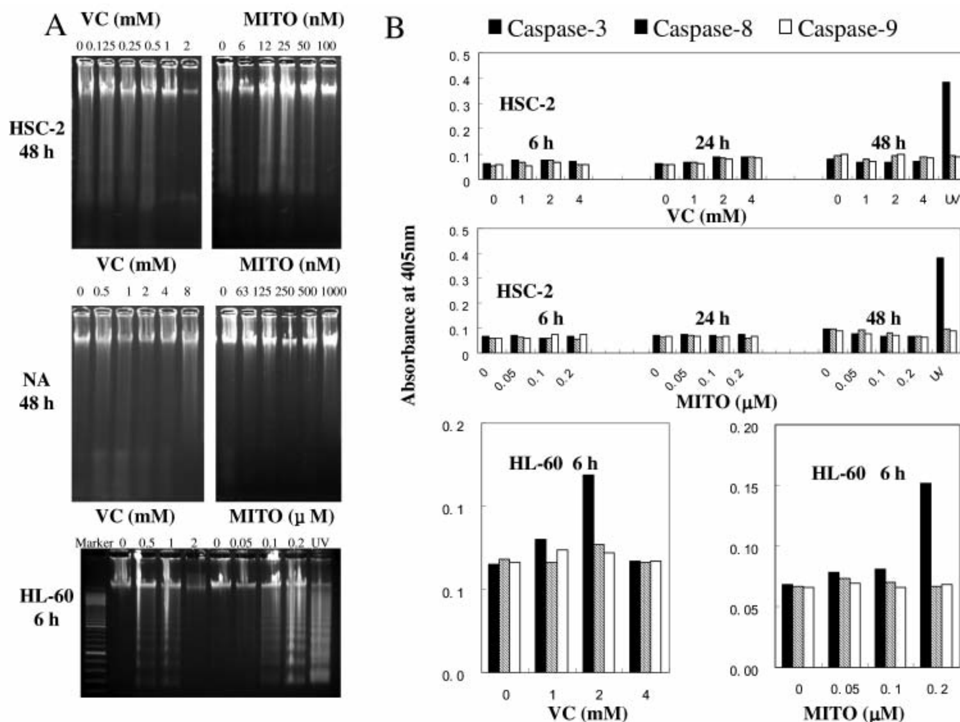

Figure 1. *Effect of sodium ascorbate and mitoxantrone on DNA fragmentation of human OSCC (HSC-2, NA) and promyelocytic leukemia HL-60 cell lines. Cells were incubated for 6, 24 or 48 hours with the indicated concentrations of sodium ascorbate (VC) or mitoxantrone (MITO), and processed for DNA fragmentation (A) and caspase activation (B).*

noted that DB and CO showed only slightly higher cytotoxicity against OSCC cell lines, as compared with normal oral cells.

In contrast to previous reports (15, 16), the three herbal extracts showed little or no hormetic growth stimulation (0-37.2% of the maximum response) at wide ranges of lower concentrations (Table II).

Induction of DNA fragmentation by sodium ascorbate and mitoxantrone. We first investigated OSCCs and HL-60 cell lines for their response to sodium ascorbate (vitamin C) or mitoxantrone, by measuring the induction of several apoptosis markers. We have selected HSC-2 and HL-60 cells due to their higher sensitivity to many apoptosis-inducing agents (24). Both vitamin C and mitoxantrone induced internucleosomal DNA fragmentation (Figure 1A) and caspase activation (Figure 1B) in HL-60 cells after 6 hours incubation, confirming previous reports (17, 18). However, vitamin C and mitoxantrone induced these apoptotic markers very weakly in HSC-2 cells, and rather induced a smear pattern of DNA fragmentation without activating the caspases in NA cells, even after 48 hours incubation (Figure 1). Vitamin C and mitoxantrone also failed to induce DNA fragmentation and caspase activation at early stages (6 and 24 hours after treatment) (data not shown).

Combination effect with vitamin C and mitoxantrone. We have investigated the combination effect of herbal extracts and vitamin C or mitoxantrone, using MTT method (that is more quantitative) rather than DNA fragmentation assay (that is more qualitative). DB and CO, but not AS, only slightly enhanced the cytotoxicity of vitamin C (only at 1 mM) (Figure 2). Combination of mitoxantrone with any of the three extracts produced complicated patterns of viability (Figure 3), suggesting the presence of both stimulators and inhibitors in the extracts. It remains to be investigated the interaction of herbal extracts and vitamin C or mitoxantrone in other cell lines.

Synergistic radical-scavenging activity with vitamin C. ESR spectroscopy showed that DB and CO produced a broad radical peak under alkaline conditions, and its radical intensity increased with increasing pH, whereas AS did not produce any detectable radical intensity (Figure 4).

When the superoxide anion (generated by HX-XOD reaction) was reacted with DMPO, four radical peaks of the spin adduct (DMPO-OOH) were generated. The height of the superoxide anion peak was diminished in the presence of increasing concentrations of CO (Figure 5). CO showed the greatest superoxide anion scavenging activity (IC_{50}=36 μg/ml), followed by DB (IC_{50}=40 μg/ml) and AS (IC_{50}=206 μg/ml)

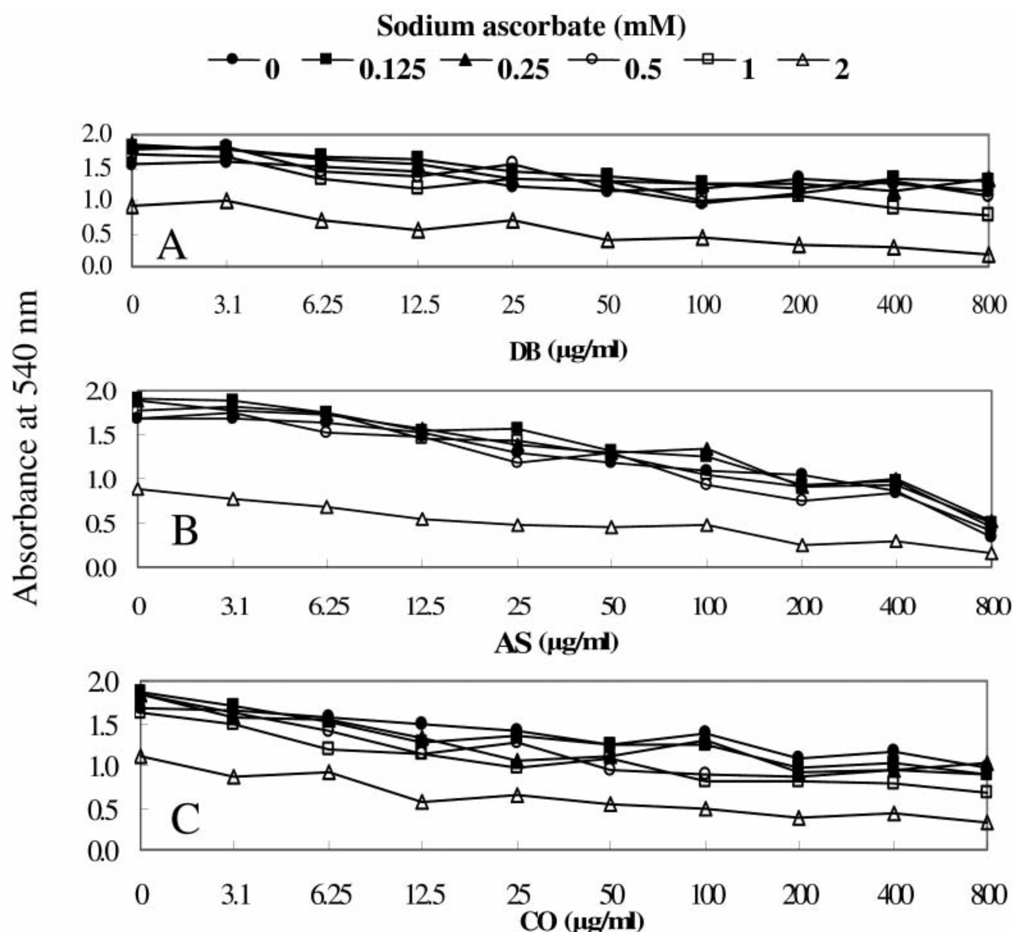

Figure 2. *Combination effect of three herbal extracts (DB, AS and CO) and sodium ascorbate. Near confluent HSC-2 cells were incubated for 48 hours without (control) or with the indicated concentrations of DB, AS or CO, in combination with sodium ascorbate (VC), and the viable cell number was then determined by MTT method. Each value represents the mean from triplicate assays.*

(Figure 6A). DB and CO synergistically enhanced the superoxide anion-scavenging activity of vitamin C (Table III).

All three extracts scavenged OH radical (generated by Fenton reaction) to comparable extents (IC_{50}=1,200, 1,500 and 1,200 µg/ml, respectively) (Figure 6B).

Anti-HIV activity. DB (SI=5), but not CO (SI<1) or AS (SI<1), slightly reduced the cytopathic effect of HIV infection, although its anti-HIV activity was much less than that of dextran sulfate (SI=329), curdlan sulfate (SI=5,111), AZT (SI=17,109) and ddC (SI=934) (Table IV).

Discussion

The antitumor activity of DB, AS, CO has been reported. *A. sinensis* polyasccharide inhibited the growth of transplanted solid tumor of mice *in vivo* (25). The total polysaccharide prepared from *A. sinensis* (Oliv.) Diels (Chinese Danggui) possessed antitumor effects on experimental tumor models *in vivo* and inhibitory effects on invasion and metastasis of hepatocellular carcinoma cells *in vitro* (11). An extract of *A. sinensis* inhibited the metastasis of B16-BL6 metastatic mouse melanoma cells, possibly through the inhibition of cell adherence to the extracellular matrix (ECM) and the reduction of cell migration (26). *C. officinalis* killed ascites tumor cells *in vitro* (27), suggesting it as a potential candidate chemopreventive agent against hepatocellular carcinoma through antioxidant and antineoplastic effects (12).

The present study demonstrated that three herbal extracts (DB, AS, CO) showed very weak cytotoxicity against the human OSCC cell lines and human glioblastoma cell line (T98G), as compared with human oral normal cells (HGF, HPC, HPLF), suggesting that their antitumor mechanism may not be mediated by their cytotoxic action. We found a very low level of hormetic effects of these extracts on both

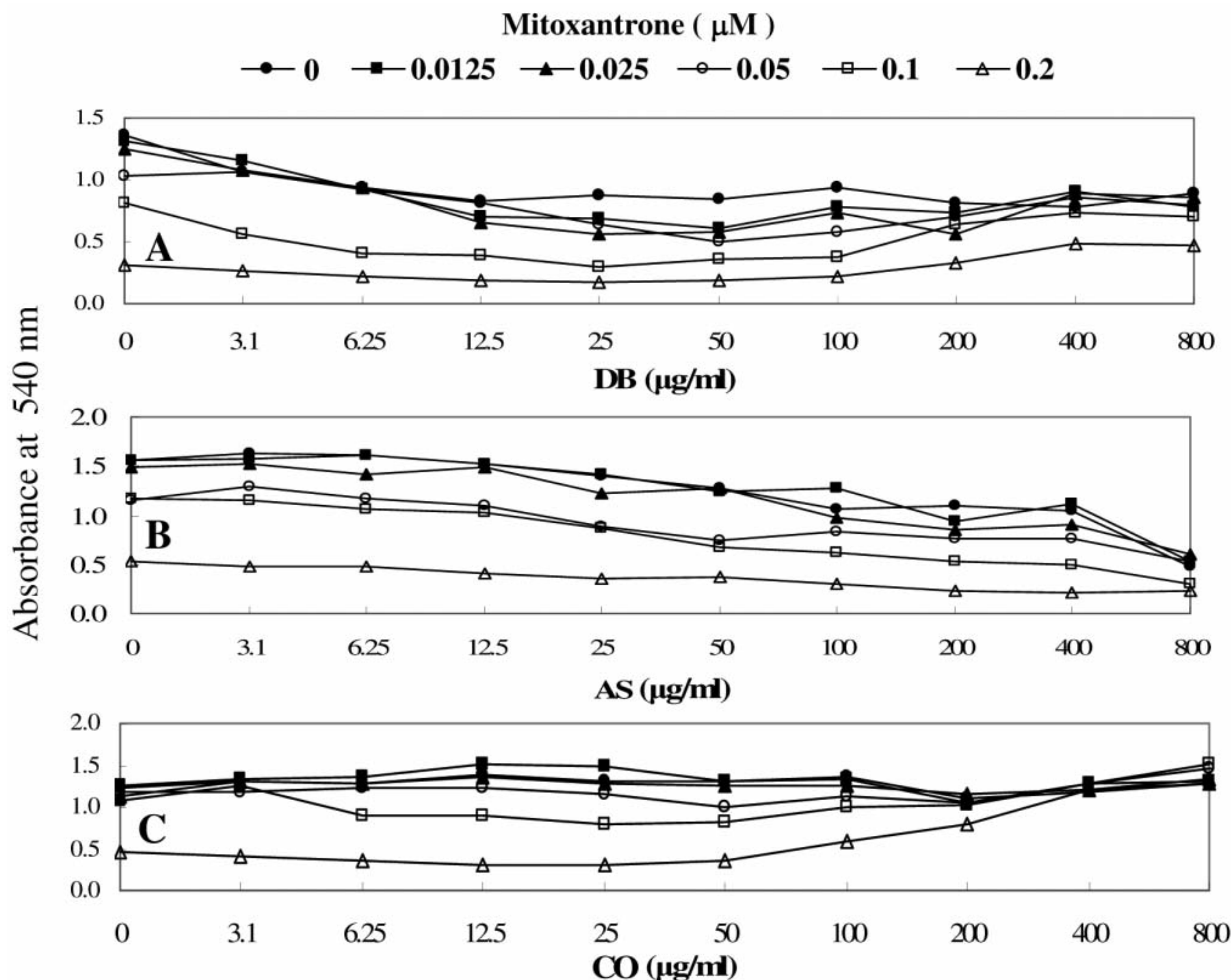

Figure 3. *Combination effect of three herbal extracts (DB, AS and CO) and mitoxantrone. Near confluent HSC-2 cells were incubated for 48 hours without (control) or with the indicated concentrations of DB, AS or CO, in combination with mitoxantrone (MITO), and the viable cell number was determined by MTT method. Each value represents the mean from triplicate assays.*

Table III. *Synergistic superoxide scavenging activity of herbal extracts and vitamin C.*

	DMPO-OOH radical intensity (% of control)	
	50 μg/ml	25 μg/ml + 5 μM vitamin C
DB	42.1±3.9	53.0±5.7<69.0 [(42.1+95.9)/2][a]
CO	38.4±2.6	43.7±4.0<67.2 [(38.4+95.9)/2][b]
10 μM vitamin C	95.9±4.5	

Each value represents mean ±S.D. from triplicate assays. [a]Expected value from the mean of the DMPO-OOH intensity of DB-treated and that of VC-treated cells. [b]Expected value from the mean of the DMPO-OOH intensity of CO-treated and that of VC-treated cells.

Table IV. *Anti-HIV activity of herbal extracts.*

Herbal extract	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
DB	263	57	5
AS	>500	>500	><1
CO	378	>500	<1
DS (μg/ml)	195	0.59	329
CRDS (μg/ml)	880	0.17	5111
AZT (μM)	237	0.014	17109
ddC (μM)	3301	3.5	934

SI: Selectivity index; DS: dextran sulfate; CRDS: curdlan sulfate; AZT: azidothymidine; ddC: dideoxycytidine.

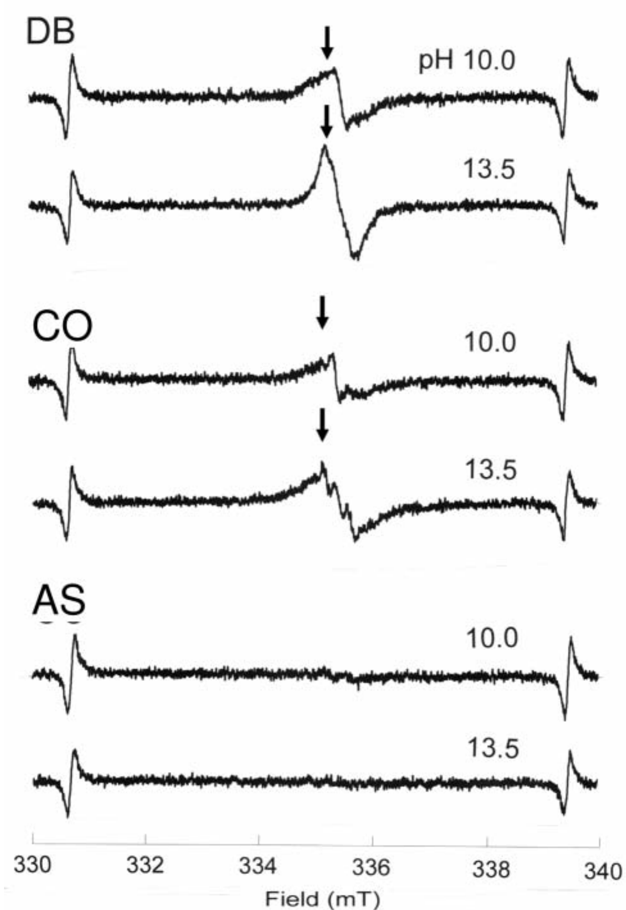

Figure 4. *Radical production by herbal extracts. Samples (20 mg/ml) were mixed with an equal volume of 0.2 M buffer solution at the indicated pH, and the radical intensity was then measured by ESR. DB and CO produced a broad radical peak (indicated by arrow) under alkaline conditions, and its radical intensity increased with increasing pH, whereas AS did not produce any detectable radical intensity.*

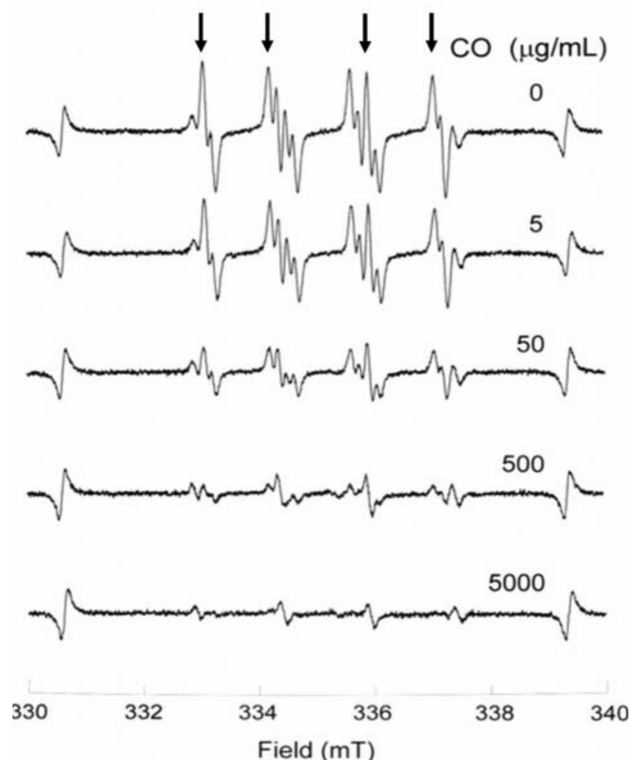

Figure 5. *ESR spectra of DMPO-OOH adduct produced in the HX-XOD reaction mixture with the indicated concentration of CO. When the superoxide anion was reacted with DMPO, four radical peaks (arrows) of the spin adduct (DMPO-OOH) were generated. The height of the superoxide anion peak was diminished in the presence of increasing concentrations of CO.*

normal and tumor cells, in contrast to previous reports (15, 16). Establishment of optimal treatment times may be necessary to achieve the hormetic effect.

The present study demonstrates that DB and CO, produced broad radical peak under alkaline condition, and enhanced both the cytotoxicity and superoxide anion scavenging activity of vitamin C. We found also that only DB, but not AS nor CO, showed anti-HIV activity (Table IV). Since all these three properties of DB are similar to those of lignin (17), DB may contain some lignin-like components.

Previous studies have shown that sodium ascorbate has antineoplastic effects, such as the inhibition of proteinase K and adenylyl cyclase activities, the increase of *C-myc* gene expression, cell cycle arrest at the S/G_2-phase, and induction of apoptosis of tumor cells (28). Higher concentrations of vitamin C can induce apoptotic cell death in various tumor cell lines including OSCC and salivary gland tumor cell lines, possibly *via* its prooxidant action (24). The previous studies suggested that effective components from *Angelica* protected the cells from hypoxic injury, mainly through the alleviation of free radical-mediated injury, and the alterations of gene expression, caspase activation and NO concentration (29). An extract of *C. officinalis* has been reported to reduce the NO concentration and the activity of iNOS (30). The radical-scavenging activity of herbal extracts demonstrated here are consistent with the previous reports.

Considering the lower cytotoxicity and synergism with vitamin C, herbal extracts (DB, CO) may be applicable as adjuvant medicines combined with some anticancer drugs. We have recently found that DB showed the potent anti-inflammatory activity (measured by the inhibition of NO and PGE_2 production by activated macrophages). This finding needs to be further investigated to elucidate the active principles for these activities.

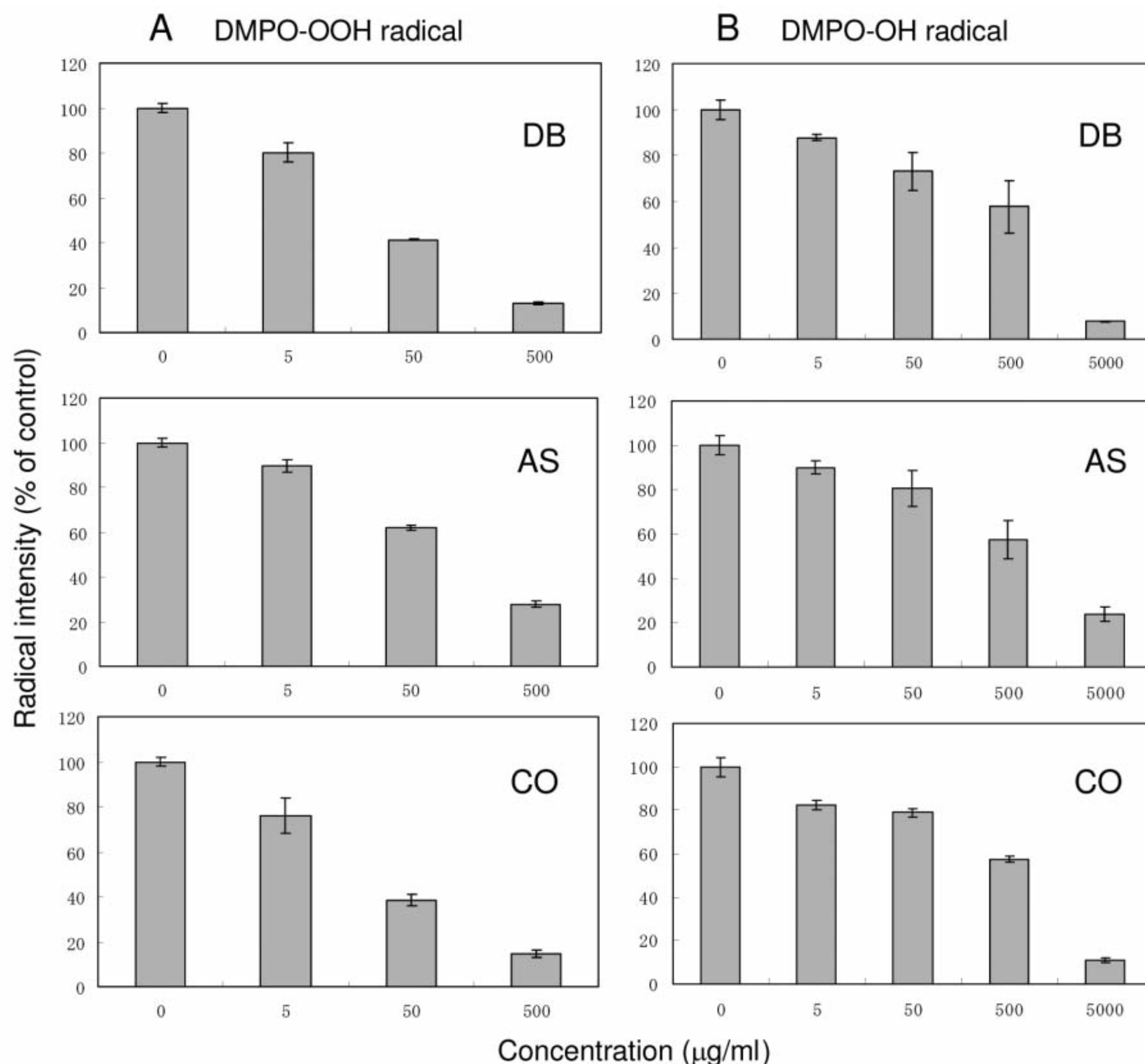

Figure 6. *Superoxide anion and hydroxyl radical scavenging activity of three herbal extracts (DB, AS and CO). Radical intensity of the first peak of DMPO-OOH (produced from HX-XOD reaction) (A) and DMPO-OH radical (produced by Fenton reaction) (B) in the presence of increasing concentrations of each extract was shown. Each value represents the mean±S.D. from triplicate assays.*

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