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

QING CHU1,2, KAZUE SATOH3, TAISEI KANAMOTO4, SHIGEMI TERAKUBO4, HIDEKI NAKASHIMA4, QINTAO WANG1 and HIROSHI SAKAGAMI2

1Department of Periodontology and Oral Medicine, School of Stomatology, The Fourth Military Medical University, 145 West Changle Road, Xi'an, P.R. of China
2Divisions of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama;
3Department of Anatomy, School of Medicine, Showa University, Tokyo;
4Department of Microbiology, St. Marianna University School of Medicine, Kanagawa, Japan

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 anthracyle antitumor antibiotic. DB, but not CO and AS, showed weak anti-HIV activity. These data demonstrate several unique antitumor properties of DB.

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, nocardixins, 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 (DETPAC), 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 3000 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 obtaining informed consent from the patients. Since these normal cells were cultured in DMEM (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 (DETPAC), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Dojin, Kumamoto, Japan).

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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, H2O 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 FeSO4 (containing 0.2 mM DETAPAC) 50 μl, 0.1 M phosphate buffer (pH 7.4) 50 μl, 92 mM DMPO 20 μl, sample (in H2O) 50 μl, 1 mM H2O2, 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 CC50>500, >461, >500 μg/ml) and human cancer cell lines (OSCC lines, HSC-2, HSC-3, HSC-4, Ca9-22 and NA: mean CC50>481, >469, >464 μg/ml, respectively; human glioblastoma T98G: mean CC50>500 μg/ml), making it difficult to accurately determine the tumor-specificity index (TS>1.03<, >1.06<, >1.1<, respectively) (Table I). It should, however, be...
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).
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* polysaccharide 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 HSC-2 and oral normal cells (HGF, HPC, HPLF) (Table IV).
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.

<table>
<thead>
<tr>
<th></th>
<th>DMPO-OOH radical intensity (% of control)</th>
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<tbody>
<tr>
<td></td>
<td>50 μg/ml</td>
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<tr>
<td>DB</td>
<td>42.1±3.9</td>
</tr>
<tr>
<td>CO</td>
<td>38.4±2.6</td>
</tr>
<tr>
<td>10 μM vitamin C</td>
<td>95.9±4.5</td>
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</table>

Each value represents mean ±S.D. from triplicate assays. aExpected value from the mean of the DMPO-OOH intensity of DB-treated and that of VC-treated cells. bExpected 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.

<table>
<thead>
<tr>
<th>Herbal extract</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (μg/ml)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (μg/ml)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB</td>
<td>263</td>
<td>57</td>
<td>5</td>
</tr>
<tr>
<td>AS</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;1</td>
</tr>
<tr>
<td>CO</td>
<td>378</td>
<td>&gt;500</td>
<td>&lt;1</td>
</tr>
<tr>
<td>DS (μg/ml)</td>
<td>195</td>
<td>0.59</td>
<td>329</td>
</tr>
<tr>
<td>CRDS (μg/ml)</td>
<td>880</td>
<td>0.17</td>
<td>5111</td>
</tr>
<tr>
<td>AZT (μM)</td>
<td>237</td>
<td>0.014</td>
<td>17109</td>
</tr>
<tr>
<td>ddC (μM)</td>
<td>3301</td>
<td>3.5</td>
<td>934</td>
</tr>
</tbody>
</table>

SI: Selectivity index; DS: dextran sulfate; CRDS: curdlan sulfate; AZT: azidothymidine; ddC: dideoxycytidine.
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 adenyl cyclase activities, the increase of C-myc gene expression, cell cycle arrest at the S/G₂-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 PGE₂ production by activated macrophages). This finding needs to be further investigated to elucidate the active principles for these activities.
Acknowledgements

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References


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.