



Research note

In vitro cytotoxic activity of ginseng leaf/stem extracts obtained by subcritical water extraction



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ABSTRACT

Ginseng leaf/stem extract produced by subcritical water extraction at high temperature (190°C) possess higher cytotoxic activity against human cancer cell lines than ethanol extract. Subcritical water extraction can be a great candidate for extraction of functional substance from ginseng leaves/stems.

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Ginseng (*Panax ginseng* Mayer) is a valuable agricultural product used in many traditional medicinal therapies [1]. Although ginseng root sells at a high price, growing ginseng in a forest environment is not a highly lucrative business strategy, because it requires a minimum growth of 5–8 years prior to harvesting [2].

Unlike ginseng root, it is possible to harvest ginseng leaves annually. Furthermore, ginseng leaves and stems were found to be rich in polysaccharides, phenolics, flavonoids, and ginsenosides [3,4]. Previous studies evaluated the bioactivity of extracts of ginseng leaves and stems; however, these extracts were prepared by traditional methods and using organic solvents such as methanol, n-hexane, chloroform, ethyl acetate, and n-butanol [5]. Also, these techniques involve a long extraction period and produce low yields.

Subcritical water (SW) extraction has been utilized extensively in various areas of green engineering and material cycling [6,7]. Under subcritical conditions, the dielectric constant of water can be altered [8]. This study aims to compare the antitumor efficacy of ginseng leaf and stem extracts, prepared by both traditional and SW extraction procedures, in human cancer cell lines.

Ginseng leaves and stems were extracted by ethanol, hot water, and SW extraction. For ethanol extraction, ginseng leaves and stems (20 g) were mixed with 200 mL of 70% (v/v) ethanol and heated for 3 hours at 60°C in a water bath. For hot water extraction, the sample (20 g) was dissolved in 200 mL distilled water and heated for 3 hours at 80°C in a water bath. After extraction, the slurry was filtered through filter paper (Whatman No. 2, GE Healthcare UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, UK), and the solid residue was extracted twice more under identical conditions. The solvent was evaporated using a rotary evaporator (N-1000V; Eyela, Tokyo, Japan). After the evaporation was completed, the extract was transferred to a freeze-drying tube and lyophilized. The dried sample was then weighed and stored at –20°C prior to analysis [9].

SW extraction was performed using an SW extraction system (DIONEX ASE 100; Dionex Corporation, Sunnyvale, CA, USA). The extraction cell (34 mL) was filled with a mixture of ginseng powder and diatomaceous earth in the ratio of 1:3, and placed vertically in the extraction apparatus. And then distilled water flows in a Milli-Q

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Table 1
Mobile phase gradient condition of high performance liquid chromatography analysis for flavonoid content in ginseng leaf and stem extracts

Time (min)	% mobile phase A ¹⁾	% mobile phase B ²⁾
0	90	10
10	80	20
20	80	20
25	70	30
35	67	33
40	60	40
60	40	60

¹⁾ 0.1% formic acid (v/v), pH 2.7

²⁾ 100% acetonitrile

system (Millipore, Bedford, MA, USA) into the apparatus. Working temperature and static time were set at 110°C, 165°C, and 190°C for 15 minutes. During extraction, the pressure was maintained at less than 500 psi. After extraction, fresh water was pumped through the entire pathway, including the cell, for washing. The SW extracts were freeze-dried and stored at –20°C until used.

Human cancer cell lines were purchased from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). The AGS (human stomach adenocarcinoma), HT-29 (human colorectal adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cell lines were maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL). SK-MES-1 (human lung carcinoma) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. HeLa (human cervical adenocarcinoma) cells were grown in Minimum Essential Medium containing 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. All cell lines were cultured in a 37°C, 5% CO₂ incubator. For experimentation, adherent cells in the logarithmic growth phase were harvested using 0.25% Trypsin (Life Technologies, Inc., Carlsbad, CA, USA). Cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA). For cytotoxicity testing, cells were present in new dishes and grown to 80% confluence prior to treatment.

Cytotoxicity was determined by quantifying the relative cell number. In this study, cytotoxic activity was assessed by a modified MTT assay, as described previously [10]. Briefly, 100 µL aliquots of the cell suspension were transferred to 96-well microplates (1.5 × 10⁴ cells/well) and incubated for 24 hours. Ginseng extracts were dissolved in phosphate-buffered saline, and the final concentrations were adjusted to 0.25 mg/well, 0.5 mg/well, 1 mg/well, and 2.5 mg/well. Cells were incubated with the extracts for 44 hours at 37°C. At the end of the incubation, MTT (2.5 mg/mL in phosphate-buffered saline) was added, and the plate was incubated for an additional 4 hours. The supernatant

was then aspirated, and 100 µL of dimethyl sulfoxide was added to the wells, to dissolve the colored formazan crystals produced from the reaction of cells with MTT. Optical density values were then measured using a microplate reader at 570 nm. All conditions were performed in triplicate and the dose causing 50% cell death (IC₅₀) was calculated.

Flavonoid was extracted following a previous method, with some modifications [11]. The ginseng extracts (100 mg) were weighed and dissolved in 50 mL of 50% aqueous methanol containing 80 mg of ascorbic acid as an antioxidant. The mixture was sonicated for 5 minutes, and 2M HCl (10 mL) was slowly added to it within 5 minutes. This mixture was incubated at 90°C for 2 hours. After cooling, the mixture was filtered through a 0.45 µm syringe filter (25 mm i.d., GD/X 25 nylon syringe filter; Whatman Inc., Piscataway, NY, USA), and the filtrate was evaporated at 50°C using a rotary evaporator to remove the solvent, freeze-dried, and stored at –20°C until use.

Concentrations of flavonoids in the ginseng extracts were determined by high performance liquid chromatography (HPLC) [12]. The ginseng extract was dissolved in 50% dimethyl sulfoxide in methanol (5 mg/mL). After filtering through a 0.45 µm syringe filter, 20 µL was injected into an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The separation was carried out on a DuPont Zorbax ODS C₁₈ column (150 × 4.6 mm², i.d. 5 µm). Table 1 shows the mobile phase condition of HPLC analysis. The peaks were identified using UV absorbance at 360 nm. Calibration curves were obtained by plotting peak area versus concentration of quercetin and kaempferol. The linear regression of calibration curve was more than 0.99.

The concentration of ginseng leaf and stem extracts affecting normal cell (Raw 264.7) viability was evaluated using an MTT assay. The extracts, at all concentrations, were found to have no cytotoxic activity on Raw 264.7 cells (<20%, data not shown). The IC₅₀ value and cytotoxic activity of ginseng leaf and stem extracts were presented in Table 2 and Fig. 1., respectively. The extract prepared by the SW extraction method at 190°C showed the highest cytotoxicity. As shown in Table 2, the IC₅₀ value of the SW 190°C extract was the lowest among all preparations. Importantly, the cytotoxic effect of this extract was the most pronounced in the AGS and MCF-7 cell lines, with the IC₅₀ values for these cell lines being lower than 0.25 mg/mL.

First, the cytotoxic activity of the SW 190°C extract on AGS was high (>80%) at concentrations ranging between 0.25 mg/mL and 2.5 mg/mL (Fig. 1A). The extracts prepared by ethanol, hot water, or SW extraction at 110°C, 165°C, and 190°C, when added at a concentration of 2.5 mg/mL, inhibited the growth of HT-29 cells by 95.20%, 96.78%, 65.67%, 91.49%, and 85.51%, respectively (Fig. 1B). Although the SW 190°C extract exhibited slightly lower activity than ethanol, hot water, and SW 165°C extracts at a concentration of 2.5 mg/mL, it showed the highest activity at 0.5 mg/mL (80.53%), while interestingly, the other extracts, when used at this concentration, lost their cytotoxic activity (≤20%).

Table 2
Concentration of the ginseng leaves and stems extracts that inhibited 50% of the cell growth (IC₅₀) of different cancer cell lines

Extract condition	IC ₅₀ value (mg/mL)				
	AGS	HT-29	HeLa	MCF-7	SK-MES-1
Ethanol	0.718 ± 0.04	0.979 ± 0.11	1.004 ± 0.14	1.034 ± 0.23	1.461 ± 0.05
Hot water	0.952 ± 0.09	0.990 ± 0.08	1.578 ± 0.29	1.094 ± 0.22	2.239 ± 0.28
SW 110°C	1.115 ± 0.03	1.914 ± 0.02	1.784 ± 0.23	2.707 ± 0.17	2.840 ± 0.19
SW 165°C	0.602 ± 0.08	1.062 ± 0.01	1.169 ± 0.20	1.563 ± 0.19	2.065 ± 0.17
SW 190°C	<0.25	0.327 ± 0.08	0.715 ± 0.05	<0.25	0.211 ± 0.11

Data are presented as mean ± standard deviation

AGS, human stomach adenocarcinoma; HeLa, human cervical adenocarcinoma; HT-29, human colorectal adenocarcinoma; IC₅₀, dose causing 50% cell death; MCF-7 human breast adenocarcinoma; SK-MES-1, human lung carcinoma; SW, subcritical water

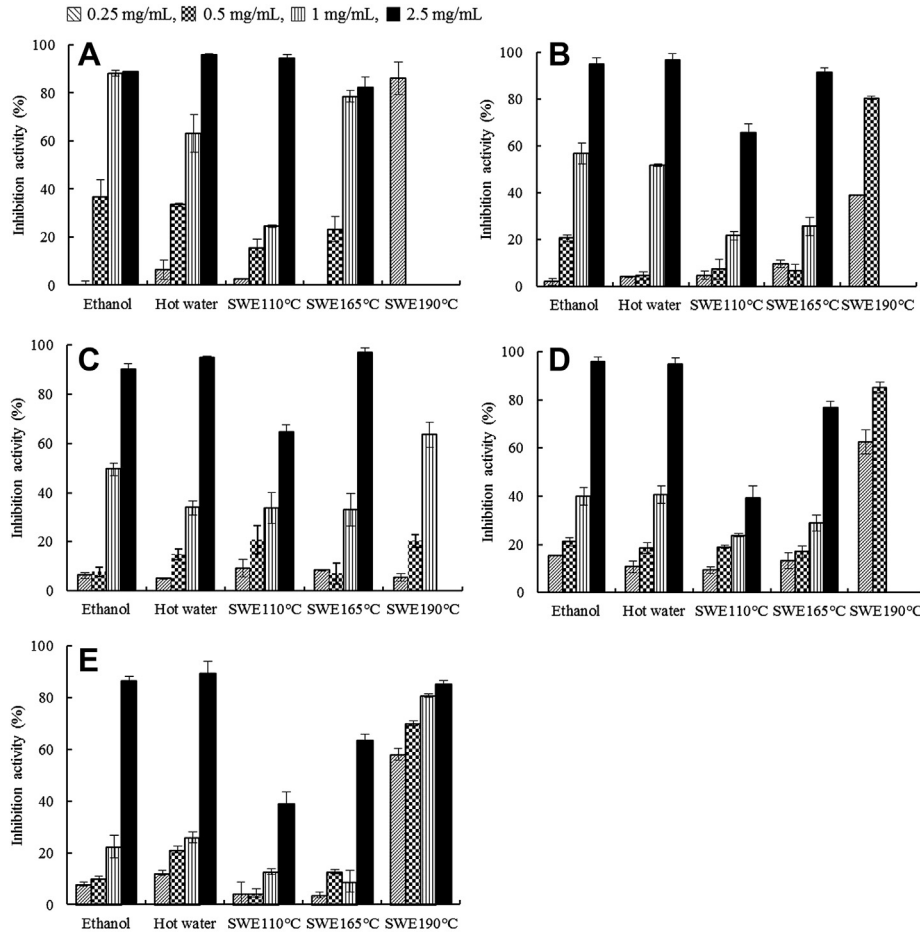


Fig. 1. Cytotoxic activities of ginseng leaf and stem extracts against five different cancer cell lines in an MTT assay: (A) AGS, (B) HT-29, (C) HeLa, (D) MCF-7, and (E) SK-MES-1. Concentration of extracts was adjusted at 0.25 mg/mL (▨), 0.5 mg/mL (▩), 1 mg/mL (▧), and 2.5 mg/mL (■). Data are presented as mean ± standard deviation. AGS, human stomach adenocarcinoma; HeLa, human cervical adenocarcinoma; HT-29, human colorectal adenocarcinoma; MCF-7 human breast adenocarcinoma; SK-MES-1, human lung carcinoma.

Among the cell lines, HeLa cells were resistant to the cytotoxic effect of the SW 190°C extract (Fig. 1C). The inhibitory activity of the ethanol, hot water, SW 110°C, SW 165°C, and SW 190°C ginseng leaf/stem extracts at 1 mg/mL were 49.47%, 33.82%, 33.64%, 33.00%, and 63.62%, respectively. The inhibitory activity of the SW 190°C extract in the MCF-7 (Fig. 1D) cell line was greater than 60% at 0.25 mg/mL, whereas at the same concentration, the extracts produced by ethanol, hot water, SW extracts at 110°C and 165°C inhibited the cell viability by less than 20% (Fig. 1D). SK-MES-1 cancer cells showed the maximal cell death (>80%) when treated with ethanol, hot water, and SW 190°C extracts at 2.5 mg/mL, whereas the viability of this cell line was minimally affected by the SW 110°C extract (Fig. 1E). Upon increasing the concentration from

Table 3
Correlation coefficients (*r*) between total flavonoid content and cytotoxic activities on human cancer cell lines.

Factor	Total flavonoid content (mg/g extract)	AGS	HT-29	HeLa	MCF-7	SK-MES-1
Total flavonoid content (mg/g extract)	—	0.596	0.842	0.983	0.922	0.902

AGS, Human Stomach Adenocarcinoma; HeLa, Human Cervical Adenocarcinoma; HT-29, Human Colorectal Adenocarcinoma; MCF-7 Human Breast Adenocarcinoma; SK-MES-1, Human Lung Carcinoma

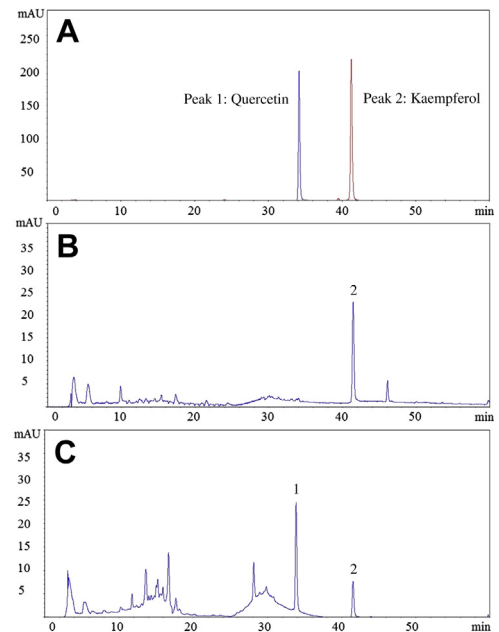


Fig. 2. High performance liquid chromatography chromatograms of ginseng leaves and stems extracts. (A) Standard (quercetin and kaempferol), (B) ethanol extract, and (C) SW extract at 190°C. SW, subcritical water.

Table 4
Concentration of quercetin and kaempferol in ginseng leaf and stem extracts produced by Ethanol and SW at 190°C extraction

Extracts	Quercetin (mg/g extract)	Kaempferol (mg/g extract)
Ethanol extract	—	4.48 ± 0.06
SW extract (190°C, 15 min)	1.98 ± 0.01	4.94 ± 0.05

Data are presented as mean ± standard deviation
SW, subcritical water

1 mg/mL to 2.5 mg/mL, the inhibitory activity of the SW 190°C extract did not increase in all cell lines; no difference was observed in the cytotoxic activity of the SW 190°C extract when used at 1 mg/mL or 2.5 mg/mL. When all the samples tested were pooled to perform correlation analysis, total flavonoid content was found to be significantly correlated with the cytotoxic activity.

Correlation coefficients (r) between the cytotoxic activity and the content of total flavonoid ranged from 0.596 to 0.983 (Table 3). Cytotoxic activity of the extracts of ginseng leaves and stems on MCF-7 and SK-MES-1 were highly correlated with the content of total flavonoid ($r = 0.922$ and $r = 0.902$, respectively). The cytotoxic activity on the HeLa cell line showed the highest correlation with the total flavonoid content of ginseng leaf and stem extracts ($r = 0.983$). These results show that the cytotoxic activities of ginseng leaf and stem extracts are greatly influenced by the flavonoid composition of the sample.

Overall, the extract prepared by the SW extraction method at 190°C demonstrated greater cytotoxic activities than that prepared by ethanol extraction. The high temperature used during the SW extraction process may have increased the cytotoxic potential of the extract. These results correlate with our previous studies, which demonstrated the antioxidant effects of extracts derived from leaves and stems of the ginseng (not published). Previous studies have reported that apoptosis was related to the levels of reactive oxygen species in cells and demonstrated that much higher levels of apoptosis were observed in American ginseng extract-treated cancer cells with low reactive oxygen species levels [13]. Therefore, ginseng leaf and stem extracts with antioxidant activity may be a potential source of natural anticancer agents.

In the present study, extracts of ginseng leaves and stems showed potent anticancer activity in MTT assays. These results demonstrated that SW extraction was a good technique for extracting natural anticancer agents from ginseng leaves and stems. SW extract at 190°C showed the highest cytotoxic activities in human cancer cell lines. For example, SW extraction at 190°C had more than 50% cytotoxic activity on human cancer cell line, except HeLa cells, at a concentration of 0.5 mg/mL; however, other extracts showed lower than 50% activity at the same concentration. The ethanol extract and SW extract at 190°C were analyzed by HPLC to identify the major flavonoid compounds. The HPLC profile revealed that kaempferol was the main flavonoid of ginseng leaf and stem

extracts, and that quercetin was detected only in the SW 190°C extract (Fig. 2). Concentrations of kaempferol were not significantly different between the ethanol and SW 190°C extracts (Table 4). Accordingly, the strong cytotoxic activity of SW extract at 190°C may be due to flavonoid–flavonoid interaction. Our results demonstrate that ginseng leaf and stem extracts produced by SW extraction at a high temperature exhibited anticancer properties *in vitro* and also suggest that the SW extraction technique, as an environment-friendly extraction process, can be employed to utilize ginseng leaves and stems effectively.

Conflict of interest

All contributing authors declare no conflicts of interest.

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