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Anti-cancer Effect of *Saxifraga stolonifera* Meerb

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Abstract

Background: *Saxifraga stolonifera* Meerb (SSM) or Yukinoshita in Japanese has been used as a medicinal plant in China and Japan since ancient times. We conducted this research to evaluate the antitumor effects of SSM and explored antitumor factors.

Methods: An extract of SSM was added to cultured cells of various cancers to assess its anti-proliferative effect. Next, a peritoneal dissemination model was created by intraperitoneally injecting gastric cancer cells (NKN45) to each nude mouse. Then, the extract of SSM was orally administered to the mice to evaluate its antineoplastic effect *in vivo*. In addition, in order to identify antitumor phytoconstituents, isolation and fraction of active $CHCl_3$ extract of SSM was carried out by chromatographic methods.

Results: The extract of SSM showed potent antitumor effects in cultured cells of esophageal, gastric, and breast cancers in a concentration and time-dependent manner. The antitumor activity of SSM was seen in the mouse model with the intra-abdominal dissemination of gastric cancer cells. Its antitumor effect was not compromised by heating leaves at 100°C or drying them. The analysis of the extract revealed that the active antitumor phytoconstituent of SSM was an aggregate of polysaccharides.

Conclusions: Our research demonstrated that SSM had a factor contributing to its antitumor effects in gastric cancers. In conclusion, intake of SSM, drinking decoction of dried leaves, is expected to be effective against gastric cancers.

Keywords: *Saxifraga stolonifera* meerb; Yukinoshita; Anti-tumor growth factor

Abbreviations

SSM: *Saxifraga stolonifera* Meerb; cm: Centimeter

Introduction

Cancer has been the leading cause of death in Japan in recent years, affecting 1 out of 2 people [1]. In the treatment of cancer patients, a multidisciplinary approach combining surgical therapy, radiotherapy, and/or pharmacotherapy is used depending on the biological characteristics of cancer and individual pathological conditions. Although these therapies help improve the outcome of patients with cancer, they are often associated with severe adverse reactions or complications. There is an unmet medical need for safer and more effective treatments.

Kampo medicinal therapy or oriental medicine has developed in China, Korea, Japan, and other Asian countries. Some Kampo medicines have been reported to activate the innate immune system to fight against cancer or to have beneficial effects when used in combination with anticancer agents [2-5]. In Asian countries, cancers are treated with various medicinal herbs and the mechanisms of action of these herbs having anticancer effects have been published [6-8]. Recently, studies have also reported that the herbal compound of Curcumin and Ginger [9] Resveratrol [10], and Genistein [11], possess anticancer effects against different types of human tumors. In mountain areas in Japan, *Saxifraga stolonifera* Meerb (SSM), known as Yukinoshita in Japan, has been used since ancient times as a folk remedy for the treatment of swelling due to cancer or inflammation. Yukinoshita is a perennial plant found in almost all parts of Japan. Although Yukinoshita is known to have anti-inflammatory and antimicrobial activities, its antitumor effects are not fully understood. In this research, we evaluated the antitumor activity of SSM, and revealed that decoction of SSM and its active phytoconstituent; polysaccharide had antitumor effects in various types of cancer cells and induced apoptosis in these cells.

Materials and Methods

Cancer cell culture and Quercetin preparation

Breast cancer cells (MCF7, MDA-MB231), esophageal cancer cells (KYSE520, KYSE790), gastric cancer cells (KATOIII, MKN45) were used in the study. MCF7, KYSE520, KYSE790, KATOIII and MKN45 cells were cultured in DMEM medium containing 5% FCS (GIBCO,

Grand Island, NY, USA). MDA-MB-231was initially cultured in L15 medium (DS Pharma Biomedical, Tokyo, Japan) supplemented with 15% of heat-inactivated FCS. The cell culture was maintained in humidified incubators at 37°C in an atmosphere of 5% CO_2 and 95% air. All culture media were supplemented with antibiotics. Quercetin was purchased from WAKO (Osaka, Japan), and dissolved in DMSO (10-2 g/mL) as a stock stored at -20°C.

MTT assay

The antitumor effect was quantitatively analyzed using MTT assay. Cancer cells were plated on 96-well plates at a density of 1×104 per 100 µL. The cells were added with SSM extracts at a concentration of 0.2, 0.66, 2, 6.6, or 20 µg/mL, and incubated at 37°C for 1 to 72 hours. After incubation, 10 µL of Solution I from the MTT cell proliferation assay Kit (Roche Diagnostics, Tokyo, Japan) was added to each well. After incubation at 37°C for 4 hours, visualization solution (100 µL) was added to each well, and the plate was incubated at 37°C overnight. The absorbance at 450 nm was measured in each well using a multi-function plate reader (Filter Max F5; Molecular Devices, Wokingham-Berkshire, UK).

Morphological observation and detection of apoptosis

The antitumor effect of SSM on gastric cancer cells was assessed by observation of individual cell morphology by polarization microscopy. Additionally, apoptosis inducing ability of SSM was analyzed with TUNEL reaction (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics, Tokyo, Japan). Briefly, pelleted cells (2×106) were suspended with 100 µL of Fixation solution (60 min, 25°C). After wash with PBS, cells were re-suspended with 100 µL of Permeabilisation solution for 2 min on ice. Induction of apoptosis was analyzed by flow cytometry (BD AccuriTM C6, BD Biosciences, USA).

Flow cytometry analysis

Trypsinized cells (1×106/mL) were washed twice in ice-cold Hanks and fixed in 75% ice-cold ethanol for at least 1 h. After fixation, cells were washed twice with PBS, then stained in 100 µg/mL PI (Sigma), 10 µg/mL RNase (Sigma) in PBS, and incubated for 30 min at 37°C. Fluorescence emitted from the propidium–DNA complex was quantitated after excitation of the fluorescent dye by flow cytometry.

Animal experiments

Female nude mice at 4 weeks old were purchased from SLC Inc. (Hamamatsu, Japan) and used for the study. 2×106 of MKN45 cells in 1ml of PBS were injected into the peritoneal cavity of 10 mice to establish a model of gastric cancer peritoneal disseminated metastasis, as reported previously [12]. The model mice were divided into two groups of five animals, i.e., SSM and control groups, and orally administered SSM or PBS for 4 weeks. Starting on the following day, SSM or PBS was administered to each mouse by intragastric insertion with an outer sheath of 21 G polycarbonate tube from the mouth. The mice in the SSM group were administered SSM extract (0.002 g in 0.2 mL) once every 24 hours. The mice in the control group were administered PBS (0.2 mL). Each group consisted of five animals, which were kept in individual cages. Mice were weighed once weekly to evaluate weight variation.

Plant material and decoction

The fresh leaves of *S. stolonifera* (8 kg) were collected from Mt. Ooiwa, Toyama, Japan in December 2014, and most of them were dried in an oven (40°C) to give dried leaves (1 kg). Some fresh leaves were crushed and pressed to obtain a decoction.

Preparation of water and $CHCL_3$-soluble portions of SSM

For the preparation of the water and $CHCl_3$-soluble portions, fresh leaves of *S. stolonifera* (910 g) were washed with water to remove the impurities and freeze dried in N_2. The dried leaves were sonicated with 3 L of 70% EtOH for 90 min in three times and filtered. The filtrates were dried under reduced pressure to yield 70% EtOH extract (32 g). 500 mL of $CHCl_3$ was added to the 70% extract and was further sonicated for 3 times in order to separate the $CHCl_3$-soluble and water soluble portions. Each solvent was corrected and evaporated to obtain water-soluble (24 g, 2.63% yield) and $CHCl_3$-soluble (2.7 g, 0.29% yield) portions.

Preparation of large scale $CHCl_3$ extract of SSM and its fractions

The dried leaves of SSM (1 kg) were extracted with $CHCl_3$ under sonication (8 L, 1.5 h, ×3) at 30°C, and the solvent was evaporated under reduced pressure to yield a $CHCl_3$ extract (35 g). The $CHCl_3$ extract (35 g) was chromatographed on silica gel with EtOAc-n-hexane, and EtOAc-MeOH solvent systems to give eight fractions (Fr. 1-8). The antitumor assay was carried out for each fraction and potent Fr. 7 [EtOAc-MeOH (60:40) eluate, 4.08 g] was then rechromatographed on silica gel with EtOAc-$CHCl_3$ and EtOAc-MeOH solvent systems to give eight fractions (Fr. 7-1-7-8). The active fraction against the cancer cells, Fr. 7-7 [EtOAc-CHCl3 (50:50) eluate, 1.85 g] was further subjected to a Cosmosil 75C18-OPN column chromatography with MeOH-H_2O (9:1, 2.7 L) and MeOH (500 mL), to afford 32 subfractions, S-1 to S-32 (100 ml each). Jelly-like substances (60.7 mg) were obtained from S-23 after drying that under reduced pressure.

Statistical Methods

All data were expressed as mean ± S.D. Comparisons between multiple groups were made by the student's t-test. P values less than 0.05 were assumed to indicate significance. All analyses were done with JMP11.0 software.

Results

Inhibition of cancer cell growth by SSM

20 µg/mL of the SSM decoction was individually incubated with esophageal cancer (KYSE550 and KYSE 790), gastric cancer (KATO III and MKN45) or breast cancer cell lines (MCF 7 and MDA-MB231). After 60 hours, the variation in cell growth capacity was analyzed by MTT assay. The results demonstrated that the treatment with the decoction inhibited the growth of all tested cancer cell lines, as compared with the control treatment. In particular, KYSE550, KATOIII and MKN45 cells were inhibited their growth significantly (Figure 1).
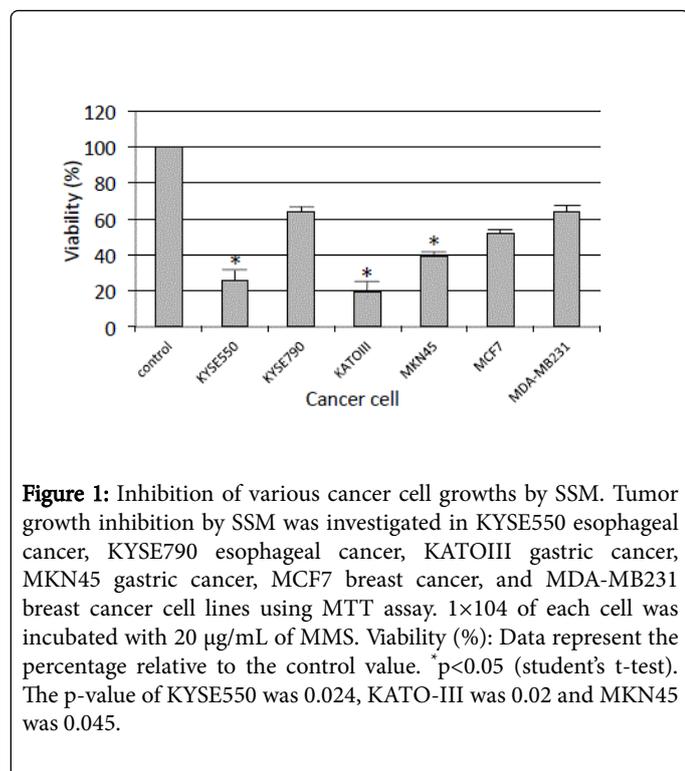
Figure 1: Inhibition of various cancer cell growths by SSM. Tumor growth inhibition by SSM was investigated in KYSE550 esophageal cancer, KYSE790 esophageal cancer, KATOIII gastric cancer, MKN45 gastric cancer, MCF7 breast cancer, and MDA-MB231 breast cancer cell lines using MTT assay. 1×104 of each cell was incubated with 20 µg/mL of MMS. Viability (%): Data represent the percentage relative to the control value. *p<0.05 (student's t-test). The p-value of KYSE550 was 0.024, KATO-III was 0.02 and MKN45 was 0.045.

Induction of apoptotic change into MKN45 gastric cancer cell by SSM

MKN45 gastric cancer cells were treated with the decoction of SSM and tumor growth inhibition was assessed by MTT assay. The results indicated that the growth of gastric cancer cells was inhibited by the incubation with SSM significantly in a time-and concentration-dependent manner (Figures 2a and 2b).

The morphological changes of MKN45 gastric cancer cells were analyzed by polarization microscopy and compared to control cells. The living cell was obviously decreased in the SSM treated MKN45 cell culture, and it was contained some cells exhibiting of apoptotic characteristics whose nuclei were more shrank than control culture (Figure 2c). Furthermore, MKN45 cell culture was analyzed with TUNEL reaction to investigate apoptotic cells. After 24 hours treatment, it was indicated that 10.3% of apoptotic cell was exhibited in the SSM -treated culture, and it was clearly more than control culture (0.1 %; Figure 2d). The cell cycle analysis was performed by flow cytometry by using PI staining (Figure 2e). The amount of G1 cell that was 89.4% in control cell was obviously decreased to 39.8% in SSM-treated cell. On the other hand, 6.2% of G2/M phase cells in control were increased to 14.1% after the SSM-treatment. These data showed that the treatment with SSM induced G2 arrest of cell cycle and apoptotic change into the MKN45 gastric cancer cell.

Inhibition of peritoneal dissemination metastasis by SSM

A model of gastric cancer peritoneal disseminated metastasis was created by injecting MKN45 cells at an amount of 2×106 per animal into the peritoneal cavity of 4-week-old mice. The model mice were divided into SSM and control groups, and orally administered SSM or PBS for 4 weeks. Mice in the SSM group had feces with similar appearance and consistency as that in the control group, and the SSM-treated mice did not exhibit diarrhea, reduced body movement, or decreased appetite. After 4 weeks, the mice were sacrificed and subjected to laparotomy and counting of peritoneal disseminations. The results demonstrated a significant reduction in the number of peritoneal disseminations in the SSM treated group compared with the control group (p=0.025) (Figures 3a and 3b).
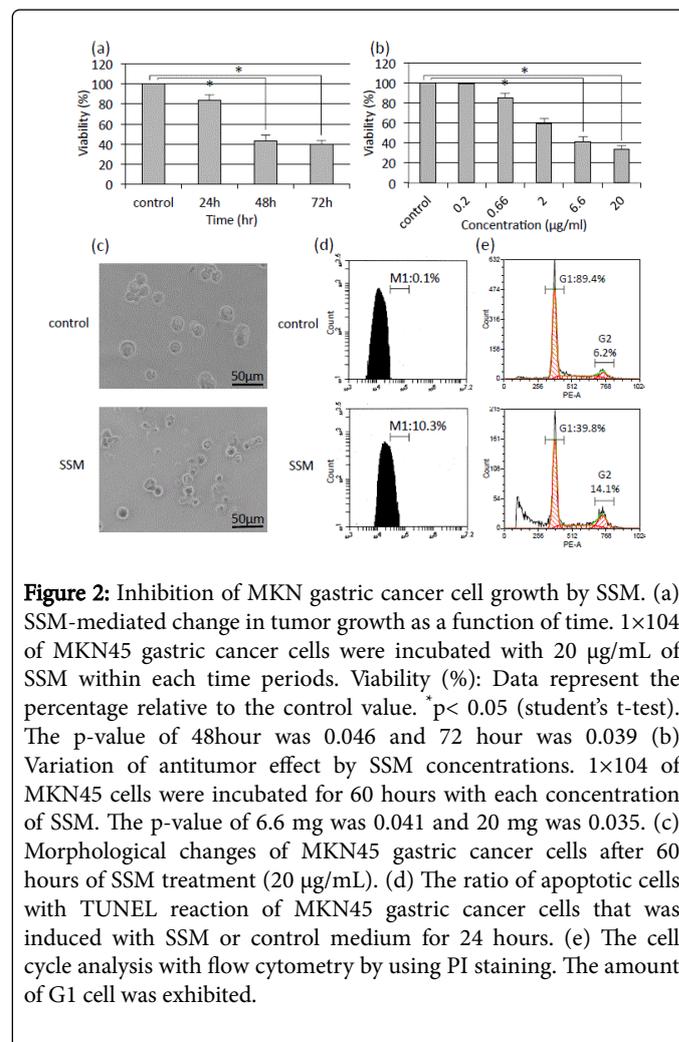

Figure 2: Inhibition of MKN gastric cancer cell growth by SSM. (a) SSM-mediated change in tumor growth as a function of time. 1×104 of MKN45 gastric cancer cells were incubated with 20 µg/mL of SSM within each time periods. Viability (%): Data represent the percentage relative to the control value. *p< 0.05 (student's t-test). The p-value of 48hour was 0.046 and 72 hour was 0.039 (b) Variation of antitumor effect by SSM concentrations. 1×104 of MKN45 cells were incubated for 60 hours with each concentration of SSM. The p-value of 6.6 mg was 0.041 and 20 mg was 0.035. (c) Morphological changes of MKN45 gastric cancer cells after 60 hours of SSM treatment (20 µg/mL). (d) The ratio of apoptotic cells with TUNEL reaction of MKN45 gastric cancer cells that was induced with SSM or control medium for 24 hours. (e) The cell cycle analysis with flow cytometry by using PI staining. The amount of G1 cell was exhibited.

Tumor growth inhibitory phytoconstituents of SSM

In order to elucidate tumor growth inhibitory phytoconstituents of SSM, decoction prepared from boiled leaves were incubated with MKN45 gastric cancer cells, and the tumor growth inhibitory effect was compared with that of non-boiled one using MTT assay. After boiling, the red color of the backside of the leaf was changed to green (Figure 4a).

The antitumor effect of the boiled SSM decoction was same as control in dose-dependent manner (Figure 4b). Furthermore, anti-tumor activity of quercetin for the MKN45 gastric cancer cell was estimated. Quercetin was one of the important ingredients of SSM, because of the anti-cancer effect that was reported before [13,14]. We examined the tumor growth inhibition effect of liquid component of Quercetin by MTT assay. The results showed that the quercetin had the weaker tumor growth inhibition activity than the whole of SSM preparation (Figures 2a and 4c). Then, a comparison of the antitumor

activity of the decoction and the 70% extract of SSM demonstrated that the 70% extract shows significant potent activity at the level comparable to that of the decoction. However, no water-soluble portion of the 70% extract showed any potent activity, whereas the CHCl3-soluble portion exhibited significant antitumor activity against the MKN45 gastric cancer cells. These observations encouraged us to investigate the fractions of CHCl$_3$-soluble portion from SSM. The 32 fractions were thus prepared from the CHCl$_3$ extracts by using the silica gel column chromatography and the tumor growth inhibited activities of each fraction were analyzed by MTT assay (Figure 4d). Among them, fraction S-23, which was obtained from rechromatographic separation of Fr. 7-7, had the strongest activity, and the other several fractions show moderate activities. Interestingly, the substances of fraction 23 showed jelly-like physical property.

Figure 3: Inhibition of peritoneal dissemination metastasis by SSM. (a) Inhibition of peritoneal dissemination metastasis by SSM treatment. Sacrificed nude mice were subjected to laparotomy for comparison of the number of disseminations in the mesenterium. (b) Number of peritoneal disseminations in the SSM and control groups. *p<0.05 relative to control (student's t-test). The p-value was 0.025.

From the above findings, oral administration of SSM had a tendency to suppress the metastasis of gastric cancer without apparent adverse reactions in mice.

Discussion

SSM is an evergreen perennial native to areas such as moist, semi-shaded rocky places in Japan, South Korea and China. It is an edible wild plant known as Yukinoshita in Japan. As a folk remedy handed down from a time when medicine and drugs were not well developed, SSM is said to relieve cuts, burns, frostbite, insect bites, tumors,

hemorrhoids, otitis media, otitis externa, tonsillitis and pharyngitis by reducing inflammation and draining pus when fresh leaves or juice from them is applied to the affected site.
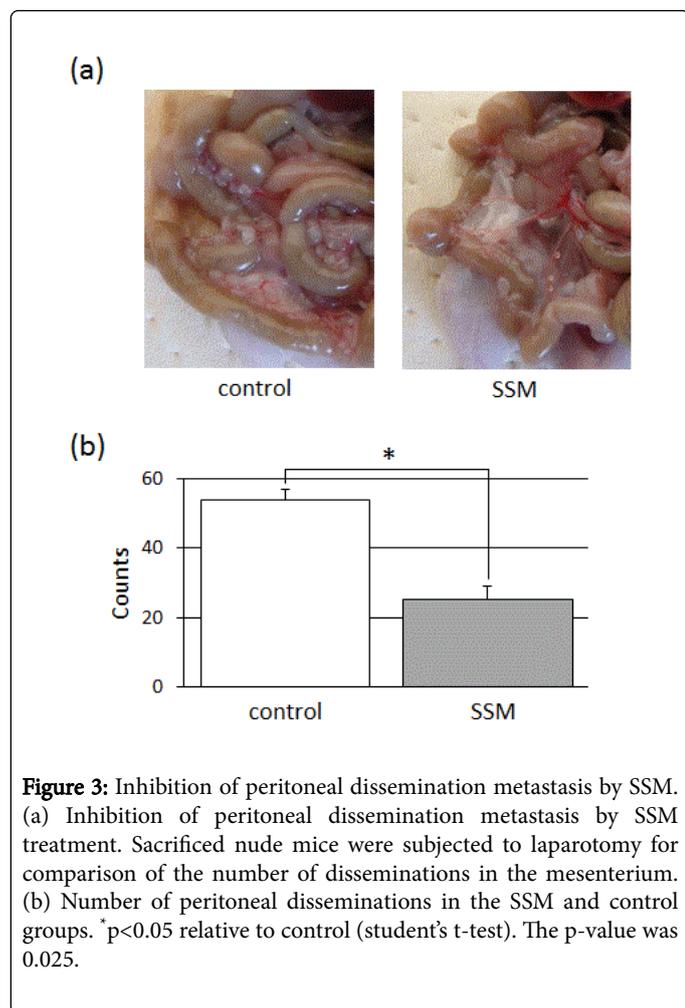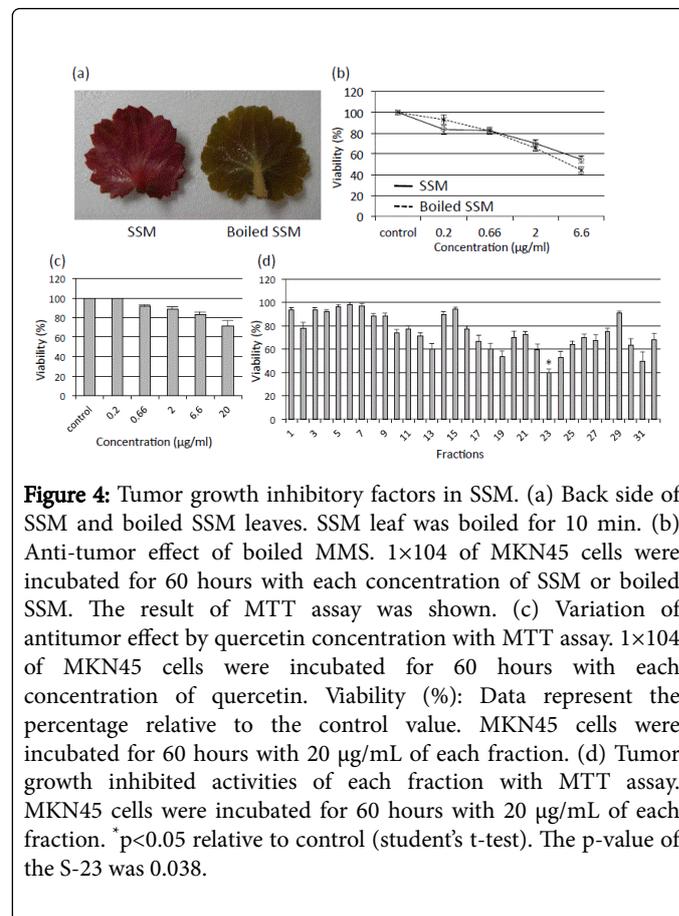

Figure 4: Tumor growth inhibitory factors in SSM. (a) Back side of SSM and boiled SSM leaves. SSM leaf was boiled for 10 min. (b) Anti-tumor effect of boiled MMS. 1×104 of MKN45 cells were incubated for 60 hours with each concentration of SSM or boiled SSM. The result of MTT assay was shown. (c) Variation of antitumor effect by quercetin concentration with MTT assay. 1×104 of MKN45 cells were incubated for 60 hours with each concentration of quercetin. Viability (%): Data represent the percentage relative to the control value. MKN45 cells were incubated for 60 hours with 20 μg/mL of each fraction. (d) Tumor growth inhibited activities of each fraction with MTT assay. MKN45 cells were incubated for 60 hours with 20 μg/mL of each fraction. *p<0.05 relative to control (student's t-test). The p-value of the S-23 was 0.038.

A decoction of SSM is believed to be effective against colds, pertussis, coughing in children and acne through its stomachic, detoxifying, antipyretic and antitussive effects [15]. The temple where we collected SSM, located at the base of the Tateyama mountains, Toyama Prefecture, was built around 1400 years ago, in which a suitable environment for this herb, the raw material of the folk remedy, to grow has been maintained for a long period of time. SSMs in this place are characterized by a vivid deep red color in the back of the leaves. We analyzed the antitumor activity of SSM, which has been used as a folk remedy. Our study has shown that it has the ability to inhibit the tumor-growth in various types of cancer cells, and induce G2 arrest and apoptosis to gastric cancer cells in vitro. It has also been found to inhibit tumor growth in a murine model of peritoneal dissemination of gastric cancer in vivo when administered in drinking the extract.

Pharmacological experiments have indicated that extracts of SSM have a wide range of biological activities, including bacteriostatic [15], antioxidant [16] and antitumor activities. [17]. It has been reported that the main phytochemical constituents of SSM are flavonoids, organic acids and phytosterols such as bergenin, gallic acid, β-sitosterol and quercetin [18]. Bergenin has been known to show the antioxidant activity and to possess ability to trap free radicals [19]. Gallic acid was reported to have an anticancer effect on human lung cancer cell and enhance the anti-tumor activities of cisplatin via the reactive oxygen

species (ROS)-dependent mitochondrial apoptotic pathway [20]. Several reports showed that ß-sitosterol had an apoptosis inducing activity and reduced the growth of cancer cells [21,22]. Quercetin was exhibited the G2 arrest and apoptosis inducing effect on BGC-823 gastric cancer cells [23]. It was also reported that increasing the expression of quercetin was reduced the expression of survivin and activated caspase3 in breast cancer cell line [14]. In the present study, none of the compounds such as quercetin, gallic acid and bergenin had been isolated from the activity-guided active fraction. And the tumor-growth inhibition activity of quercetin seemed to be weaker than whole extract of SSM, suggested that the SSM contained not only quercetin, but also other tumor inhibition factors, working with quercetin for induction of G2 arrest and apoptosis, in partly by reduction of survivin and activated-caspase3 to MKN45 gastric cancer cells.

In this study, we attempted to isolate the antitumor phytoconstituents contained in SSM. The major polyphenolic constituents of SSM are reportedly proanthocyanidins, which are abundant on the back of the leaves, producing the red pigment [23]. However, when the leaves were boiled, the backside color was turned from red to green, but the antitumor activity remained intact (Figures 4a, 4b), suggesting that the red color substance(s) are not antitumor component of SSM against the MKN45 gastric cancer cells, as well as exclusion of proteins. This hypothesis is also supported when 70% ethanol was used for efficiently extracting the red color substances from SSM, and the 70% ethanol extracts were further partitioned with water and $CHCl_3$. Despite the red color substances moved to the 70% extract and water-soluble portion, the anti-tumor activity was not observed in those ones. Hence, the possibility of antitumor effect by the red color substance(s), presumably proanthocyanidins, was completely excluded. In contrast, antitumor activity was obtained from the $CHCl_3$ soluble portion prepared from dried *S. stolonifera*. Furthermore, subfraction S-23, the most active fraction prepared from the $CHCl_3$ extract, was shown to contain jelly-like substances. Interestingly, the substances of fraction 23 were hard to dissolve in most of the solvents. It is thus assumed that the jelly-like substances are an aggregate of polysaccharides, suggesting that the antitumor phytoconstituent of SSM was a mixture of polysaccharides. These findings suggested that the antitumor activity of SSM does not result from a single factor, but from a combination of multiple factors. Further investigation is warranted to elucidate its antitumor mechanism.

Conclusions

In this research, we demonstrated that SSM had a factor contributing to its antitumor effects in many types of cancers. And SSM induced apoptotic change to these cancer cells. In conclusion, intake of SSM, drinking an extract of dried leaves, is expected to be effective against gastric cancers.

Acknowledgements

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