Original Research Article

Establishment of optimal conditions to extract bioactive substances from Gamazumi using supercritical carbon dioxide

ABSTRACT

The fruits of Gamazumi (*Viburnum dilatatum* THUNB) have been previously reported to suppress the adverse effects of oxidative stress in rats. To reduce time and cost, we attempted to establish optimal conditions for extracting bioactive components from Gamazumi fruits using supercritical carbon dioxide. To verify whether the conventional hexane extraction may be replaced by the supercritical carbon dioxide extraction, component analysis results and antigenotoxic potential in mice were used to compare bioactivity of the supercritical carbon dioxide and hexane extracts.

At the same extraction pressure, the extraction rate was maximized when supercritical carbon dioxide with a low temperature was used, and extraction efficiency was improved. GC/MS analysis revealed that vitamins E and stigmasterols were included in supercritical carbon dioxide and hexane extracts, and that no qualitative differences between supercritical carbon dioxide and hexane extracts were observed. The antigenotoxic potential of Gamazumi extracts was studied in mice exposed to cigarette smoke inhalation. Mice received single or 5 consecutive oral administrations of Gamazumi extracts at 0, 3, and 6 h prior to smoke inhalation. Although single administration decreased nuclei tail length in the stomach when both administration and the intervals of smoke inhalation were short, five consecutive administrations decreased tail length in the lung and stomach regardless of the interval. At short intervals, inhaled cigarette smoke and orally administered extracts may be simultaneously present in the gastric cavity, and direct reaction between [1]cigarette smoke and extract is possible. The antioxidant activity of Gamazumi extracts may result in antigenotoxic potential. There were no differences in component analysis and antigenotoxic potential between supercritical carbon dioxide and hexane extracts of Gamazumi; thus, it is possible to replace the conventional hexane extraction with the supercritical carbon dioxide extraction.

*Keywords: [*Gamazumi*, supercritical carbon dioxide extract, hexane extract, antogenotoxic potential, smoking, comet assay, antioxidant potential]*

1. INTRODUCTION

The supercritical fluid extraction method is an energy-saving process with many advantageous features [1-6]. The extraction of long-chain unsaturated fatty acids with bioactive effects, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), from natural products (Alaska pollack milt, mackerel, and squid) using supercritical carbon dioxide ($scCO_2$) has been well studied [7-9]. The common solvent extraction method, i.e., Soxhlet extraction using hexane, leads to the denaturation of unsaturated fatty acids, which are heat-sensitive compounds, during solvent removal

through heating. In contrast, during the $scCO_2$ extraction, gaseous CO_2 is easily removed from the extracts by decreasing the pressure without heating, thereby protecting the compounds from denaturation. Natural products contain many bioactive compounds, but their physical properties are similar to each other, and it is difficult to separate specific bioactive compounds and directly refine them from the natural products. Therefore, it is important to develop an easy method to extract and separate bioactive compounds using the characteristics of $scCO_2$. The power of the supercritical fluid extraction method changes markedly with extraction condition. Therefore, it is necessary to develop optimal extract conditions to reduce time and cost.

Gamazumi (*Viburnum dilatatum* THUNB) is a deciduous small tree of the *honeysuckle* family and has a wide habitat ranging from the flatlands to the high mountains in Japan. Gamazumi fruits (Fig. 1) have a red color and are sometimes used for fruit liquor and pickles. In Nambu Town, San-nohe District, Aomori Prefecture (Japan), attempts are being made to develop Gamazumi as a regional specialty. Regarding the bioactivity of Gamazumi, it has been reported that Gamazumi suppresses the adverse effects of oxidative stress in rats [10]. It has been suggested that administration of Gamazumi to rats suppresses the consumption of antioxidant enzymes and that the antioxidant components in Gamazumi directly suppress the production of oxidative damage. Generally, in the analysis of pharmacological components of a plant extracts such as juice, fat-soluble and water-soluble components are separated by extraction using an organic solvent such as hexane. However, here, $scCO_2$ having a function equivalent to that of hexane was used as an extractant. Extraction of fat-soluble components using ScCO2 is currently attracting attention as a clean and efficient method. To verify whether the conventional hexane extraction may be replaced by the $scCO_2$ extraction, component analysis results and antigenotoxic potential in mice (i.e. bioactivity) were compared between the two extraction methods.

Fig. 1. Image of Gamazumi fruits

2. MATERIAL AND METHODS

2.1 Supercritical carbon dioxide extraction

The $scCO_2$ extraction was conducted in accordance with a previous study [5]. The fruit of Gamazumi (kindly provided by Gamazuni Ruby Institute, Nambu, Aomori) was dried well to inhibit the adverse effect of water against the extraction, and then crushed to powder using a mixer. Powdered sample (25 g) was placed into an extraction chamber and the air in the chamber was replaced with CO_2. Next, CO_2 was sent into the extraction chamber using a high pressure pump, and the chamber was then equilibrated at the target temperature and pressure. After the temperature and pressure reached equilibrium, CO_2 was sent into the extraction chamber continuously to extract components from the sample. The extraction temperature and pressure were set at 35 – 45 °C and 12.5 – 20.0 MPa, respectively, and changes in the extraction rate due to the differences in extraction conditions were investigated. Soxhlet extraction using hexane was also conducted for comparison with the $scCO_2$ extraction.

2.2 Antigenotoxicity analysis of Gamazumi extract

Male ddY mice were obtained from SLC Japan Co. (Shizuoka, Japan) at 7 weeks of age, and were used after 1 week of acclimatization. Mice were fed basal diet (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* throughout the

acclimatization and experiment period. Mice were housed 3 per cage in an air-conditioned animal room. The animal room was maintained at 20 - 24°C and 50 - 70% humidity with a 12-h light-dark cycle.

Three mice were randomly assigned to each treatment group. Gamazumi extracts were suspended in 0.5% carboxymethyl cellulose (CMC) solution. Gamazumi extract was orally administered to each mouse once or 5 times with an interval of 24 h at 1000 or 2000 mg/kg.

Mice were exposed to cigarette smoke inhalation at 0, 3, and 6 h after the administration of single or 5 consecutive administrations of Gamazumi extract. For exposure to cigarette smoke, three mice (combined body weight, ca. 120 g) were put into an 1800 mL polypropylene whole-body inhalation chamber containing 4 inlet pores at the top and an exhaust pore at the side. Smoke was generated by one 2-s, 35-mL puff per cigarette through a 50-mL glass syringe equipped with a cigarette holder from an unfiltered commercial cigarette (Piece, Japan Tobacco, Tokyo; containing 15 mg nicotine and 1.3 mg tar, according to the manufacturer).

Smoke from eight cigarettes was introduced to the chamber from the inlet pores and the pores were closed immediately. After 1 min, the mice were removed from the chamber and returned to the original cage. The fold dilution of the smoke by the air was calculated using the equation: fold dilution = (1800–120)/smoke volume. The smoke concentration (6.4-fold dilution) was a little higher than that used for a previous long-term inhalation study (8-fold dilution) [11]. From shortly after treatment until just before euthanasia, the mice were carefully observed for pharmacotoxic signs.

DNA damage induced by smoke inhalation in the lung and stomach was investigated using the comet assay as described by Sasaki et al. [12]. Mice were sacrificed 15 min after smoke inhalation, and glandular stomach and lung were removed. Lung tissues were minced, suspended in 4 mL chilled homogenizing solution (pH 7.5) containing 0.075 M NaCl and 0.024 M Na_2EDTA, and then homogenized gently using a Potter-Elvehjem type homogenizer at 500 - 800 rpm, on ice. Glandular stomach tissues were opened and rinsed with physiological saline; mucosa was then scraped into 4 mL chilled homogenizing buffer and homogenized gently using a Potter-Elvehjem type homogenizer at 500 - 800 rpm, on ice. To obtain nuclei, the homogenates were centrifuged at 700 g for 10 min at 0°C, and the precipitates were re-suspended in chilled homogenizing buffer at 1 g organ weight per mL.

Regular agarose GP-42 (Nacalai Tesque, Kyoto, Japan) diluted to 1% in physiological saline (total 75 µL) was quickly layered on a slide (Matsunami Glass Ind., Ltd., Osaka, Japan) coated with agarose GP-42 and covered with another slide. The slide sandwiches were placed horizontally to allow the agarose to solidify. The nucleus suspension was mixed 1:1 (v/v) with 2%, 45°C, low melting point agarose (Nacalai Tesque), and 75 µL of the nucleus mixture was quickly layered in the same manner after removal of the covering slide. Finally, another 75 µL of agarose GP-42 was quickly layered on again. Slides prepared from nuclei isolated by homogenization were placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept at 0 °C in the dark overnight, and then in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 10 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 15 min at 25 V (0.96 V/cm) and approximately 250 mA. The slides were neutralized and then stained with 50 µL of 20 µg/mL ethidium bromide (Wako Pure Chemical Industries, Ltd.). We examined and photographed 50 nuclei per slide at 200 x magnification with the aid of a fluorescence microscope. The length of comet tail was measured for 50 nuclei per organ per animal. Mean migration of 50 nuclei from each organ was calculated for each individual animal. The differences between the averages of Gamazumi extract treated-mice and control mice (mice exposed to cigarette smoke without treatment with Gamazumi extracts) were compared using the Dunnett test after one-way ANOVA. A p-value less than 0.05 was considered significant.

2.3 Antioxidant activity assay (DPPH assay):

Gamazumi extract aliquots (0.1 mL) were mixed with 8.9 mL of DPPH reaction solution (0.1 M acetate buffer, 0.016% 1,1-diphenyl-2-picrylhydrazyl in ethanol, 84% xylene) and absorbance at 510 nm was measured. The inhibition ratio was calculated using the following formula: Inhibition (z)=100×(1-AS/AC). AS is the absorbance of the sample and AC is the absorbance of the control, in which there are no antioxidant reagents.

3. RESULTS

The extraction rate is shown as a function of the integrated flow rate of CO_2 (Fig. 2). The integrated flow rate of CO_2 is the volume of CO_2 obtained at 25 °C and 0.1 MPa after extraction. At 45 °C, the extraction rate was maximum (approximately 7%) when the extraction pressure was 20.0 MPa. The extraction rate was higher at a higher extraction pressure (20 MPa) than at a lower extraction pressure (12.5 MPa). At the higher extraction pressure, the solubility of the components in scCO2 increased because the density of $scCO_2$ is high under high pressure conditions [3]. At 15.0 MPa, the extraction rate was maximum (approximately 8.5%) when the extraction temperature was 35 °C. The extraction rate was higher at a lower extraction temperature (35 °C) than at a higher extraction temperature (45 °C) because the density of $scCO_2$ is high under low temperature conditions [3]. Upon increasing the pressure under a constant temperature and decreasing the temperature under a constant pressure, the slopes of the graphs increased, indicating that the efficiency of the extraction was improved. The extraction efficiency was thus improved under the conditions of high pressure and low temperature.

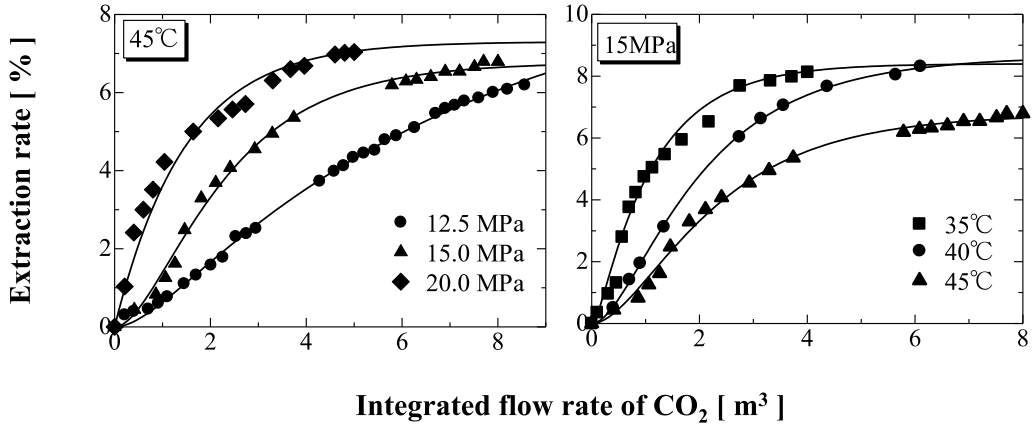

Fig. 2. Extraction rate of the $scCO_2$ method under various conditions

The components in the extracts were analyzed using GC/MS (Table 1). Vitamin E and stigmasterols were detected in both the $scCO_2$ and Soxhlet extracts. When the $scCO_2$ extraction was performed at a high pressure, flavonoids were detected at 20 MPa. However, flavonoids were not detected during the Soxhlet extraction. The other components were either denatured or not analyzed. During the Soxhlet extraction, the components were denatured due to heating, and the amounts of the other components were increased compared with the corresponding components during the $scCO_2$ extraction.

Table 1 The ratio of components in the extracts

	$scCO_2$ extracts at 45 °C			Soxhlet
	12.5 MPa	15.0 MPa	20.0 MPa	Hexane extracts
Vitamin E (%)	89.2	71	71.8	52.2
Stigmasterols (%)	8.2	18.1	18.5	24.1
Flavonoids (%)	-	0.4	1.4	-
Others (%)	1.4	10.5	8.3	23.7

Five consecutive administrations of the $scCO_2$ and hexane extracts at 0, 3, and 6 h prior to smoke inhalation significantly decreased nuclei tail length in the lung and stomach. Single administration of both extracts, however, tail length decreased significantly when mice received the extracts at 0 and 3 h but not at 6 h before smoke inhalation in the stomach; there were no significant decreases in the lung (Figs. 3 and 4). These results show that the effect on the induction of comet tails of the $scCO_2$ extract is almost the same as that of the hexane extract.

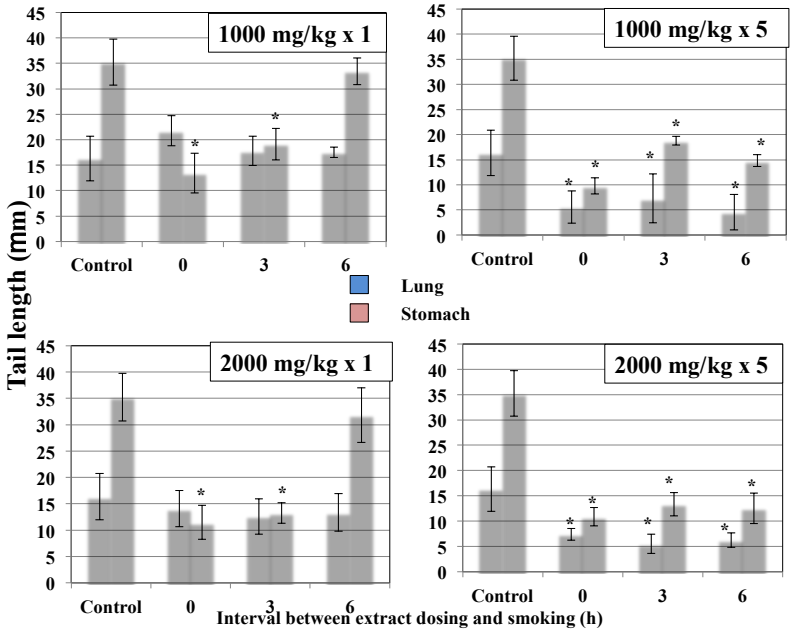

Fig. 3. Effects of feeding of Gamazumi $scCO_2$ extract on the induction of comet tail in mice exposed to cigarette smoke.

*Mice in the control group were exposed to cigarette smoke without receiving Gamazumi extract. Significantly lower than the Gamazumi-untreated control, * P < 0.05; Mean ± S.D = Mean values ± standard diviation of three mice.*

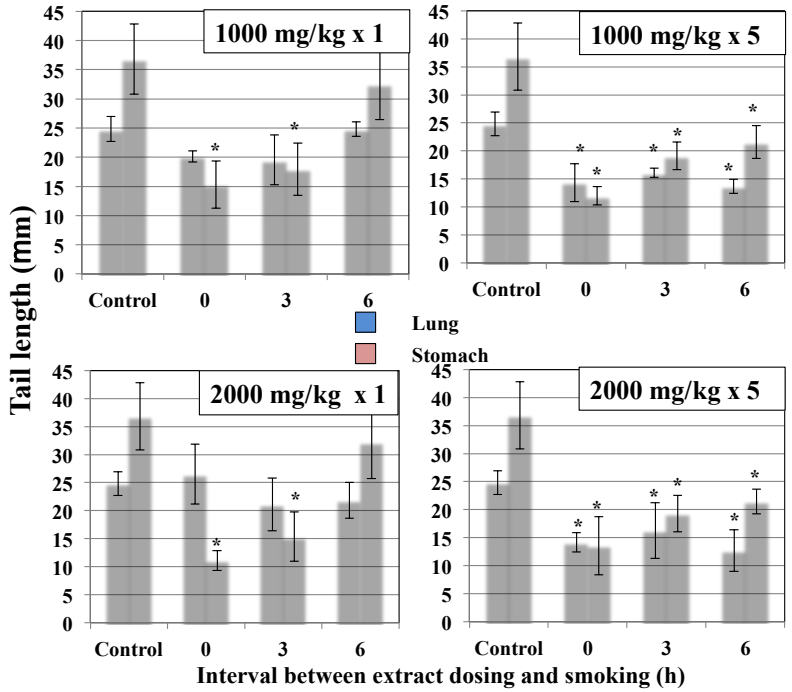

Fig. 4. Effects of feeding of Gamazumi hexane extract on the induction of comet tail in mice exposed to cigarette smoke.

*Mice in the control group were exposed to cigarette smoke without receiving Gamazumi extract. Significantly lower than the Gamazumi-untreated control, * P < 0.05; Mean ± S.D = Mean values ± Standard deviation of three mice.*

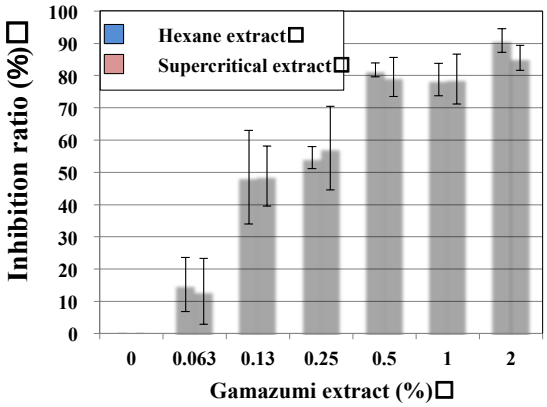

Fig. 5. Antioxidant effects of Gamazumi extracts

The ordinate axis indicates the inhibition ratio of DPPH radicals. Each bar represents mean values ± standard deviation of three experiments

4. DISCUSSION

Although single administration decreased nuclei tail length in the stomach when the administration and intervals of smoke inhalation were short, five consecutive administrations decreased tail length regardless of the interval. On the other hand, in the lung, only five consecutive administrations decreased nuclei tail length. At short intervals, swallowed cigarette smoke and orally administered extracts may be simultaneously present in the gastric cavity, whereby direct reaction between cigarette smoke and extract is possible. DNA damage caused by smoking in gastric mucosal cells is caused by reactive active oxygen species [13]. The antioxidant activity of hexane and $scCO_2$ extracts in Gamazumi that was demonstrated in our study could be attributed to the presence of vitamin E, a well-known antioxidant, in these Gamazumi extracts. Therefore, the observed antigenotoxic potential of Gamazumi extracts in the stomach may be due to inactivation of smoking-induced reactive oxygen species through direct reaction in the gastric cavity. On the other hand, it is unlikely that orally-administered extracts react directly with suctioned cigarette smoke in the trachea. It is possible that five consecutive administrations of extracts elevate blood concentration of antioxidants, which results in an increase in serum antioxidants that exhibit antigenotoxic effects in the lung as well as stomach. In this study, the antigenotoxic potential of the $scCO_2$ extract was shown to be similar to that of the hexane extract.

No large qualitative differences in analyzed components or antigenotoxic potential in mice were observed between the $scCO_2$ and hexane extracts. Carbon dioxide acts as a non-polar solvent, and when it is used as an extractant even in a supercritical state, a fat-soluble substance is extracted. One of the advantages of this extraction method is that carbon dioxide returns to gas after extraction and can be completely removed by returning the setup to normal pressure; thus, no special operation is required to separate it from the extract and no solvent remains. In our Gamazumi fruit extraction experiment, both the $scCO_2$ and commonly used hexane extractions were conducted, and the extraction components of both methods were compared. Since there was no difference between the two extraction methods, it is considered that the conventional hexane extraction method may be replaced with the supercritical carbon dioxide extraction method.

ETHICAL APPROVAL

All procedures were approved by the Animal Experiment Ethics Committee, National Institute of Technology, Hachinohe College, and were conducted under the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but

for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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