

## Preparation of Clinically Useful Sennoside-reduced Rhubarb

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**Objective:** The aim of this study was to develop a method of removing sennoside to reduce the cathartic effect of rhubarb while conserving its other pharmacological activities.

**Methods:** Rhubarb powder was steam autoclaved at 121°C and 0.14 MPa for 20, 60, or 120 minutes, and HPLC analysis was conducted to determine levels of rhubarb components. Mice were fed non-autoclaved or 20-minute-autoclaved rhubarb extracts. Feces were collected and weighed over a 24-hour period. India ink was orally administered to determine the distance of fecal migration through the intestinal tract.

**Results:** Autoclaving 20, 60, and 120 minutes decreased sennoside A and B to trace levels but only autoclaving 20 minutes conserved most of the (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate contents (i.e., 69%, 90%, 88%, respectively). Therefore only rhubarb autoclaved for 20 minutes was used in subsequent experiments. Fecal output (in g) in mice treated with water (control), autoclaved rhubarb, and non-autoclaved rhubarb was  $2.78 \pm 0.07$ ,  $3.30 \pm 0.13$  ( $p = 0.348$ ), and  $3.81 \pm 0.07$  ( $p = 0.005$ ). India ink migration was far less in mice treated with autoclaved rhubarb vs non-autoclaved rhubarb.

**Conclusion:** Steam autoclaving the rhubarb for 20 minutes reduces sennoside levels and its cathartic activity while conserving its other pharmacological activities.

**Key words:** Rhei Rhizoma, rhubarb, sennoside, cathartic effects, autoclave

### INTRODUCTION

Sennoside is a major component of rhubarb, is classified as an irritant purgative, and is utilized widely as a purgative not only in Kampo medicine but also in western medicine. In recent years, rhubarb has been shown to have pharmacological activities other than cathartic activity. However, it is often not possible to take advantage of these other effects because the cathartic component of rhubarb (sennoside) is so overwhelming. Therefore, the aim of this study is to find an efficient method of selective sennoside inactivation to permit clinical application of rhubarb's other active components.

### MATERIALS AND METHODS

#### Reagents and materials

The reagents and materials included lyophilized rhubarb (produced in Qinghai, China) from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan); sennoside A from Nacalai Tesque, Inc. (Kyoto, Japan); sennoside B from YONEYAMA YAKUHIN KOGYO CO., LTD. (Osaka, Japan); emodin from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); rhein from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA); (+)-catechin, (-)-epicatechin gallate, and (-)-epicatechin from AppliChem GmbH (Darmstadt, Germany).

#### Instrumentation and operating conditions

The HPLC system consisted of a HITACHI L-7100

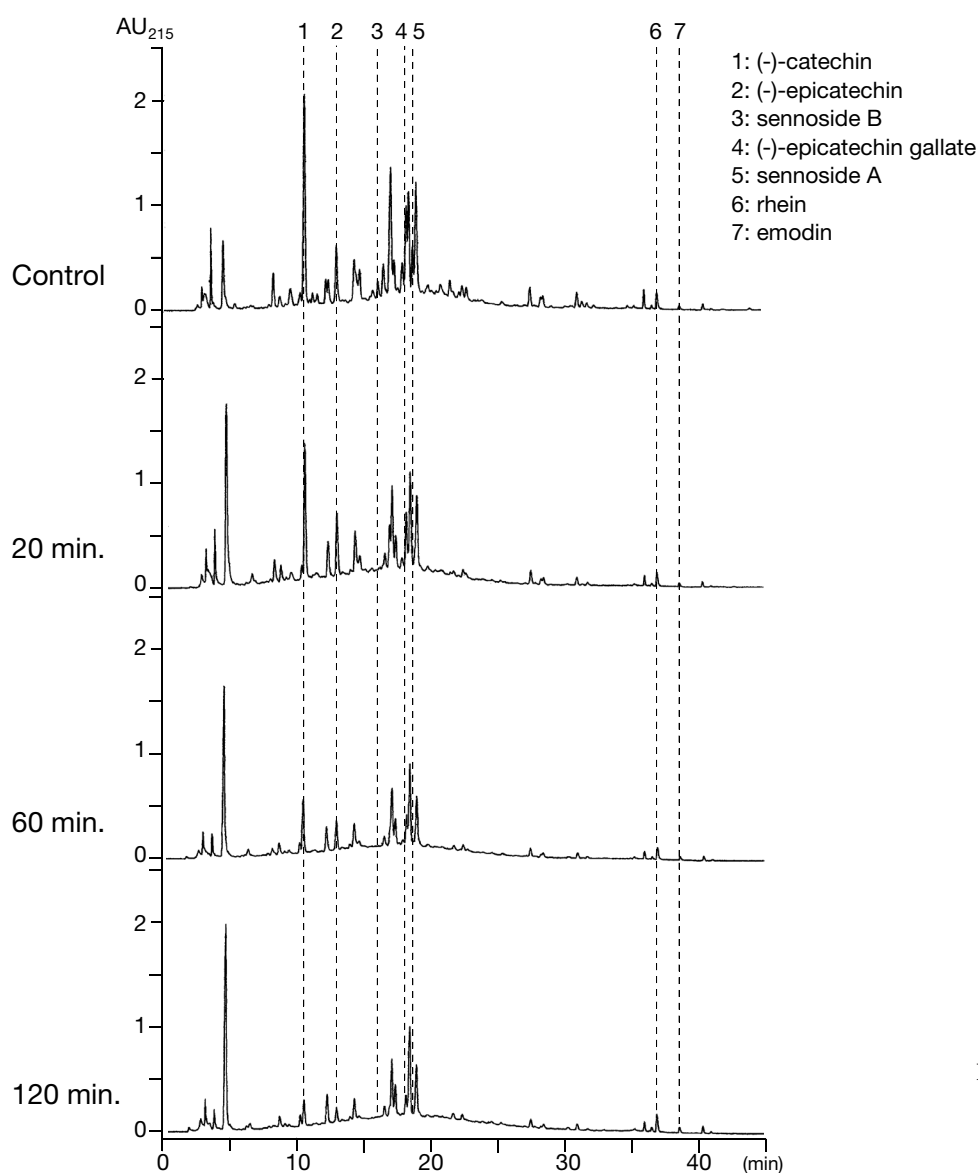
liquid chromatograph. HPLC separation was carried out on a Mightysil RP-18 GP column (250 mm × 4.6 mm, KANTO CHEMICAL CO., INC., Tokyo, Japan). The elution was performed using a mobile phase gradient of H<sub>2</sub>O/CH<sub>3</sub>CN (90:1-0:100) at a flow rate of 1.0 ml/min. Rhubarb components in the column effluent were quantified by ultraviolet detection at a wavelength of 215 nm.

#### Sample preparation for HPLC analysis

The rhubarb was wrapped in aluminum foil, steam autoclaved at 121°C and 0.14 MPa for 20 min, 60 min, or 120 min, macerated in deionized water, soaked in boiling water for 40 min, and centrifuged. The supernatants contained sennoside-reduced rhubarb components. The components of non-autoclaved rhubarb (control) were extracted in the same way.

#### Animals

C57BL/6 mice were housed under a 12-h light/dark cycle in a controlled environment (22-24°C, 55% relative humidity) with free access to food and water. All procedures were carried out in accordance with Guidelines for the Care and Use of Animals for Scientific Purposes at Tokai University and were approved by the Institutional Review Board of Tokai University School of Medicine. (Permit Number: 151030).



**Fig. 1** HPLC charts showing the components of autoclaved-rhubarb extracts.

#### Measurement of fecal output in mice

Sennoside is a major component of rhubarb, is metabolized to rheinanthrone by some bifidobacterium strains, and is an activator of intestinal tract peristalsis. To determine the effects of autoclaving on rhubarb components, the mice were first treated orally with 0.1 ml of *Bifidobacterium LKM 512* solution (0.1 g/ml in 0.9% NaCl) (to metabolize sennoside to rheinanthrone in the intestines) and then with either non-autoclaved rhubarb (200 mg/kg,  $n = 4$ ), 20 minute-autoclaved rhubarb (200 mg/kg,  $n = 4$ ), or water as a control ( $n = 4$ ). These treatments were repeated one more time after a 24-hour interval. The feces of each mouse were collected for 24 hours after the final treatment, dried in a desiccator, and then weighed.

#### Measurement of intestinal peristaltic movement in mice

After oral administration of *LKM 512*, mice were treated orally for 4 hours with either rhubarb extract ( $n = 2$ ) or autoclaved-rhubarb extract ( $n = 2$ ) prepared as described above, for 1.5 hours with 0.4 ml of 5% India ink suspended in 10% gum arabic solution, sacrificed with isoflurane (2.5%), and dissected to remove

their gastrointestinal tracts. Intestinal peristaltic activity was measured by ruler as the distance from the India ink to the terminus of the ileum (i.e., the base of the caecum or start of the colon).

#### Statistical analysis

Values are expressed as the mean  $\pm$  SEM. All statistical analyses were done by using GraphPad Prism6 (GraphPad Software Inc., San Diego, CA). First, Bartlett's test was used to determine whether the data in each analysis had equal variance. However, because our data were non-normally distributed, non-parametric tests were used. The Kruskal-Wallis test was used to determine whether our samples originate from the same distribution and Dunn's test was used to further analyze the specific sample pairs for stochastic dominance.

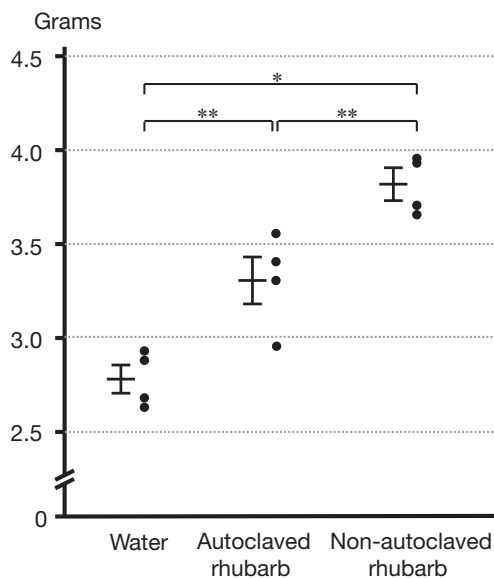
## RESULTS

#### Preparation of autoclaved rhubarb

Fig. 1 shows the HPLC charts of the autoclaved (20, 60 or 120 minutes) and non-autoclaved rhubarb (control) extracts. Table 1 shows the amount of each major component in 5 grams of crude rhubarb.

**Table 1** HPLC analysis of the content of each major component of rhubarb before and after autoclaving.

	Control	20 min.	60 min.	120 min.
(+)-catechin	55.0 mg	38.0 mg (69%)	17.0 mg (31%)	9.0 mg (16%)
(-)-epicatechin	19.3 mg	17.4 mg (90%)	10.0 mg (52%)	8.1 mg (42%)
Sennoside B	1.4 mg	trace	trace	trace
(-)-epicatechin gallate	15.9 mg	14.0 mg (88%)	7.6 mg (48%)	5.2 mg (33%)
Sennoside A	2.1 mg	trace	trace	trace
Rhein	1.4 mg	1.4 mg (100%)	1.4 mg (100%)	1.4 mg (100%)
Emodin	trace	trace	trace	trace

**Fig. 2** The total fecal output in 24 hours of mice treated with autoclaved and non-autoclaved rhubarb. Dunn's test,  $n = 4$  (each group), \* $p = 0.005$ , \*\*n.s. ( $p = 0.348$ )

Only trace amounts of sennoside A and B remained after samples were autoclaved for 20–120 minutes. In contrast, (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate were at 69%, 90% and 88% of their levels before autoclaving, respectively, after 20 minutes of autoclaving; 31%, 52%, and 48% after 60 minutes of autoclaving; 16%, 42%, and 33% after 120 minutes of autoclaving, and significantly higher than the corresponding percentages of sennoside A and B. The contents of rhein and emodin were not significantly changed by autoclaving. Therefore, we concluded 20 minutes of autoclaving was adequate for preparing rhubarb components largely depleted of sennoside.

#### The feces output of mice treated with autoclaved-rhubarb

We investigated the effect of autoclaved rhubarb on fecal output. The dry weight of feces excreted during the 24-hour period of treatment with water (the no-rhubarb control), 20-minute-autoclaved rhubarb, and non-autoclaved rhubarb was  $2.78 \pm 0.07$  g,  $3.30 \pm 0.13$  g, and  $3.81 \pm 0.07$  g, respectively. Food and water intakes were similar among these three groups. Differences among these three groups were statistically significant by the Kruskal-Wallis test ( $p = 0.0002$ ). Notably, the total feces output by mice treated with non-autoclaved rhubarb was significantly greater than that excreted by mice treated with water ( $p = 0.005$ ).

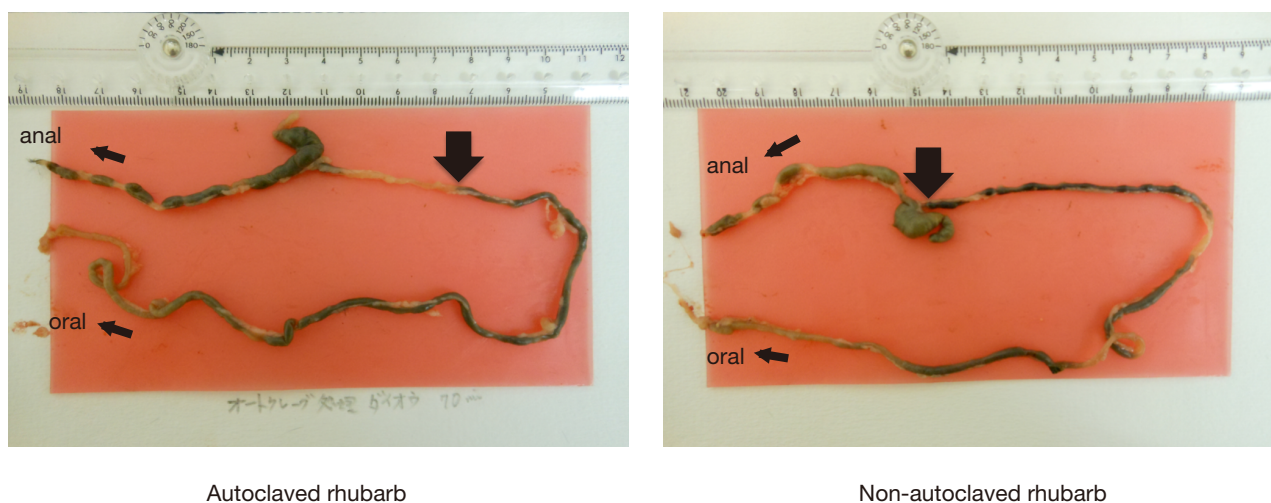
However, fecal output was similar between mice treated with water and mice treated with autoclaved rhubarb ( $p = 0.348$ ), and between mice treated with autoclaved rhubarb and mice treated with non-autoclaved rhubarb ( $p = 0.348$ ) (Fig. 2).

#### Effect of autoclaved-rhubarb on intestinal peristaltic movement

The India ink at 24 hours after the administration was located 42 mm above the lower end of the ileum in mice treated with 20-minute-autoclaved rhubarb and 1 mm above the lower end of the ileum in mice treated with non-autoclaved rhubarb. The movement of India ink was much less in the mice treated with autoclaved-rhubarb (Fig. 3).

#### DISCUSSION

Rhubarb is a crude drug and defined in the Japanese Pharmacopoeia 16th edition published in 2011 as the rhizome of *Rheum palmatum* Linné, *Rheum tanguticum* Maximowicz, *Rheum officinale* Baillon, *Rheum coreanum* Nakai or their interspecific hybrids (*Polygonaceae*). It contains not less than 0.25% of sennosides A ( $C_{42}H_{38}O_{20}$ ; 862.74) calculated on a dried basis [1]. To date, A, B, C, D, E, F, and G are the only identifiable sennosides in rhubarb, and sennoside A and B (its major components) have cathartic activity [2, 3]. The cathartic effect of daiokanzoto, which is composed



**Fig. 3** The distance that India ink moved along the intestinal tract of mice treated with autoclaved and non-autoclaved rhubarb.

of rhubarb (daio in Japanese) and licorice (kanzo), has been demonstrated clinically in a double-blind randomized controlled trial [4]. Therefore, it is common for current Japanese doctors to use rhubarb powder or formulas containing rhubarb as a purgative in not only Kampo treatment but also western medicine treatment.

Rhubarb has other pharmacologic effects (such as diuresis, relief of jaundice, improvement of blood stasis, and anti-inflammation), and its cathartic activity has been regarded as an adverse effect for a long time [5, 6]. In recent years, rhubarb components other than sennoside were shown to have various pharmacological activities. And many reports have associated the pharmacological activities of rhubarb with specific ingredients, for example, the antibacterial activity with rhein and emodin [7, 8], hypoglycemic activity with aconitan [9], anti-inflammatory action with lindleyin [10], nitrogen metabolism improving action with rhatannin [11], renal insufficiency improving action with epicatechin gallate [12], and psychotropic activity with RG tannin [13]. Furthermore, rhubarb components have recently been shown to prolong life in nematodes [14] and reduce cholesterol levels in rats [15].

However, these pharmacological activities of rhubarb cannot be fully realized clinically because of the overwhelming cathartic effect of sennoside. Accordingly, various methods have been devised since ancient times to reduce the cathartic effect of rhubarb, such as dry-heat and soaking in liquor. Several recent studies reported methods of producing sennoside-reduced rhubarb extracts. Liquor-dipping (i.e., dipping rhubarb in 16% ethanol for 30 seconds) had no effect on sennosides content. However, liquor-soaking (i.e., soaking rhubarb in ethanol for 12 to 24 hours) decreased the content of sennosides and tannins and increased the content of anthraquinones [16]. In the hot water treatment of chopped rhubarb, sennoside A levels in the extract peaked at 40 minutes but decreased to 19% of the peak level after 240 minutes, while the content of rhein tended to increase with the extraction time [17]. Dry heating rhubarb at 132°C in an autoclave for 10 minutes reduced the content of sennoside A to

60% of its level before heating [18]. Decocting rhubarb in hot water for 24 hours decreased the content of sennoside A in the extract to 6.3% of peak levels, but decreased (+)-catechin and (–)-epicatechin gallate levels relatively less (down to 46.1% and 33.0%, respectively, of maximum). Clinically, patients with chronic renal failure could not tolerate this decoction of rhubarb continuously because of diarrhea [19]. Some trials of rhubarb extracts with reduced cathartic activity have been reported as described herein, but unfortunately none have been applied clinically.

Therefore, we examined the effectiveness of another method to reduce the content of sennoside without reducing the contents of other ingredients. We tried to steam autoclave the rhubarb because of a report that the sennoside A level in rhubarb autoclaved under dry heat conditions for 10 minutes was decreased to 60% of its level before autoclaving [18]. In our study, sennoside A and B had almost disappeared from the extract while the contents of other ingredients were relatively well maintained after autoclaving rhubarb for 20 minutes, suggesting that 20 minutes was the most suitable processing time for preparation of clinically useful sennoside-reduced rhubarb. Because of the relatively long time it takes for an autoclave to reach the required temperature and pressure (121°C, 0.14 MPa), no difference was expected between sennoside levels after extraction by autoclaving for 10 minutes and 20 minutes. Therefore, in this study, rhubarb was not autoclaved for 10 minutes as reported in the previous study [18].

Furthermore, our comparison of the dry weight of excreted feces among groups of mice treated with water (control), autoclaved rhubarb, or non-autoclaved rhubarb showed significantly greater 24-h fecal output by mice treated with non-autoclaved rhubarb than by control mice, but similar 24-h fecal output between mice treated with autoclaved rhubarb and mice treated with non-autoclaved rhubarb. These findings suggest that 20 minutes of autoclaving reduces rhubarb's cathartic activity (Fig. 2).

Actually fecal output might be greater in autoclaved-rhubarb-treated group than the water-treated

group even though no significant difference was detected between them. This finding suggests that cathartic activity persists despite the almost complete removal of sennoside A and B by 20 minutes of autoclaving. We propose two reasons for this paradox: 1) the amount of residual sennoside after autoclaving (though undetectable on HPLC) is enough to cause catharsis, 2) the observed cathartic activity is due to rheinoside, another laxative constituent of rhubarb [20].

Then we compared the between-group difference in the distance of India ink migration down the intestinal tract to assess intestinal peristaltic activity. In the intestinal tract, sennoside is metabolized to rheinanthrone (the actual purgative) by several bifidobacteria such as *Bifidobacterium lactis*. Therefore constipation can be improved without reliance on intestinal bacterial flora by using a laxative and probiotic containing bifidobacteria capable of generating rheinanthrone from sennoside in the intestine [21]. Because intestinal floras differ substantially between individual mice, we administered *Bifidobacterium KLM 512* containing *Bifidobacterium lactis* to all mice before the administration of rhubarb. The distance that India ink moved along the intestinal tract was shorter after autoclaved-rhubarb treatment than non-autoclaved-rhubarb treatment, suggesting that autoclaving had reduced the purgative effect.

The present study has some limitations. Rheinosides A, B, C, and D (which have cathartic properties) and other pharmacologically active ingredients such as RG tannin could not be examined because reference standards for them were difficult to obtain. In addition, we did not examine shorter times of autoclaving to determine whether our rhubarb preparation could be optimized further.

To obtain a clinically useful preparation of autoclaved-rhubarb, it is necessary to confirm not only the decrease in its purgative activity, but also the conservation of its other pharmacological activities.

## CONCLUSION

Steam autoclaving of rhubarb for 20 min at 121°C and 0.14 MPa markedly decreased the quantity of sennoside A and B and level of cathartic activity in mice while conserving other ingredients in rhubarb. Therefore the 20-minute-autoclaved rhubarb we prepared in this study is expected to have pharmacological activities other than cathartic, clinically.

## COMPETING INTERESTS

The authors of this study have no competing financial or non-financial interests to declare, however, the Department of Kampo Medicine, Tokai University School of Medicine, did receive a grant from Tsumura, a Japanese manufacturer of Kampo medicine.

## AUTHORS' CONTRIBUTIONS

MA conceived the study and wrote the manuscript. MA and KK participated in the animal experimentation, data collection, data analysis, and interpretation of the data. YN, MK and NI carefully revised the manuscript. All authors read and approved the final draft.

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