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Studies on rhubarb processed with liquor —The reason why rhubarb is processed with *huangjiu*: the liquor contains a small amount of alcohol—

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Abstract

In China, rhubarb (*da-huang* in Chinese; *daiou* in Japanese) is processed with liquor. There are two liquors that are commonly used for rhubarb preparations, *huangjiu*, which contains a small amount of alcohol and *baijiu*, which contains a large amount of alcohol. Commonly, rhubarb is processed with *huangjiu* rather than *baijiu*. However, there is little evidence concerning why *huangjiu* is used. In this report, we processed liquor-soaked rhubarb using four liquids, distilled water; 16% ethanol; 50% ethanol; and *shaoxingjiu*, a kind of *huangjiu*, and compared the amounts of the following principal compounds in the processed rhubarb: sennoside A, sennoside B, aloe-emodin, rhein, emodin, chrysophanol, physcion, lindleyin, isolindleyin, and total tannin. We found that the changes in the amounts of the principal compound in the processed rhubarb mostly depended on the alcohol concentration of the soaking solution. Sennosides content decreased while anthraquinones content increased in a balanced manner when the rhubarb was processed with 16% ethanol or *shaoxingjiu*. Therefore, rhubarb is processed with liquors that contain a small amount of alcohol as preparing liquor-soaked rhubarb in this manner may decrease its purgative effect and enhance its antibacterial and anti-inflammatory effect.

Key words rhubarb, processing with liquor, alcohol concentration, sennoside, anthraquinone, lindleyin.

Introduction

In China, crude drugs are sometime processed for specific purposes. Rhubarb has been processed by dipping or soaking it in *huangjiu* (Chinese fermented wine). The medicinal effects of rhubarb were reported, e.g., purgative[1,2] due to sennoside, antibacterial[3] and anti-inflammatory[4] due to anthraquinones, anti-inflammatory[5] due to lindleyin, and radical scavenging[6] due to tannin. By processed, the composition of principal compounds in rhubarb may change. Therefore the medicinal effect of processed rhubarb can be different from unprocessed rhubarb.

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We previously studied the change of principal compound contents in rhubarb after liquor-dipping and liquor-soaking.[7] We processed rhubarb using various dipping times and amounts of ethanol and established a standard method for its preparation. Liquor-dipping rhubarb in 16% ethanol for 30 seconds did not affect its sennoside content. After liquor-soaking rhubarb in ethanol for 12 to 24 hours, its sennosides and total tannin contents decreased, and its anthraquinones content increased. Thus liquor-soaking of rhubarb may increase the effect due to anthraquinones.

There are two liquors that are commonly used for rhubarb preparations, *huangjiu*, which contains 15% to 18% alcohol, and *baijiu*, which contains about 50% alcohol. Commonly, rhubarb is processed with *huangjiu* rather than *baijiu*. However, there is little evidence

concerning why *huangjiu* is used. And it is not clear that the medicinal effect of liquor-soaked rhubarb processed with *huangjiu* is same to the rhubarb processed with ethanol. In this report, we processed liquor-soaked rhubarb using four liquids, distilled water; 16% ethanol; 50% ethanol; and *shaoxingjiu*, which is a kind of *huangjiu*, and compared the contents of the following principal compounds in rhubarb: sennoside A, sennoside B, aloe-emodin, rhein, emodin, chrysophanol, physcion, lindleyin, isolindleyin, and total tannin. We also compared the amounts of these compounds eluted into the four liquids used in the soaking process. Thus we elucidated the difference of composition of principal compounds in liquor-soaked rhubarb processed with ethanol containing several amount of alcohol and the effect of *shaoxingjiu* on those principal compounds content.

Materials and Methods

Crude drug material: Rhubarb was purchased from Tochimoto Tenkaido Co., Ltd. (Lot 007008001, 2008).

Liquor materials: These are summarized in Table 1. *Shaoxingjiu* is one of the *huangjiu*, used for process in China, which made in shaoxing.

Reagents: Sennoside A, sennoside B, and polyamide C-200 were purchased from Wako Pure Chemical Industries, Ltd.; aloe-emodin, rhein, emodin, and chrysophanol were from Funakoshi, Ltd.; physcion and gallic acid were from Nakalai Tesque, Ltd.; Folin-Ciocalteu's reagent was from Merck, Ltd.; and lindleyin and isolindleyin were provided by Tsumura Co., Ltd. All chemicals were of an analytical grade, and the chromatographic solvents were of an HPLC grade.

Method of preparation: Sixteen % and 50% ethanols were used as substitutes for *huangjiu* and *baijiu*, respectively.

Ten grams of rhubarb chips were dipped in 40 mL of 1 of 4 kinds of liquid, distilled water, 16% ethanol, 50% ethanol, and three brands of *shaoxingjiu* (liquors 1-3), and were stirred to ensure thorough saturation with the liquid. These liquids are referred to as soaking solutions (SS). Each preparation was then left for 24 hours. Then, the soaked rhubarb chips were removed from the SS, and the excess fluid was absorbed with paper. The chips were dried overnight in an oven set to conditions similar to those of sun-drying (40℃, 4000 lux). In this paper, processed rhubarb is referred to as liquor-soaked rhubarb (LSR). Rhubarb dried overnight in the oven was used as a control. Then, we analyzed the amounts of the principal compounds in the LSR and SS.

HPLC method:

(1) Preparation of LSR sample solution

Each powdered LSR sample (200 mg) was extracted with 50 mL of 50% methanol under ultrasonication for 20 minutes. After centrifugation of the samples at 3000 rpm for 10 minutes, the supernatants were filtered through a 0.45 μm membrane filter constructed of cellulose. The resultant solutions were injected into the HPLC system. The residue remaining after the extraction contained 0.007% of lindleyin, 0.010% of isolindleyin, 0.036% of sennoside A, 0.015% of sennoside B, 0.016% of aloe-emodin, 0.041% of rhein, 0.017% of emodin, 0.026% of chrysophanol, and 0.011% of physcion.

(2) Preparation of SS sample solution

For sennoside and anthraquinone: Each SS was diluted with 50% methanol, and the supernatants were filtered through a 0.45 μm membrane filter constructed of

Table 1 Kind of *shaoxingjiu*

	Brand name	Alcohol concentration (%)	Distribution source
Liquor 1	Zhongguo huadiao laojiu Tapai Shaoxingjiu	16	Zhejiang sheng liangyoushipin jinchukou gufen, Co., Zhejiang, China
Liquor 2	Hongyou Shaoxing Huadiaojiu	16	Zhejiang shaoxing shi donghu jiuye, Co., Zhejiang, China
Liquor 3	Lao shanghai Laojiu	15-16	Yamaya, Co., Miyagi, Japan

cellulose. The resultant solutions were injected into the HPLC system.

For lindleyin and isolindleyin: To remove interfering substances, a solid phase-HPLC method was performed, which was based on the methods described in JP15[8] and those of Yamasaki[9] and Seto *et al.*[10] Using this method, interfering substances were removed, and 92.66 ±1.51% of lindleyin and 99.44±3.07% of isolindleyin was eluted (n=4).

First, 2 g of polyamide C-200 were packed into the packing reservoir of a polyamide column. Each SS was diluted with distilled water, and a 5 mL sample was subjected to the polyamide column. Then, the column was eluted with 5 mL of methanol/0.05% sodium hydrogen carbonate (7:13) and 5 mL of methanol/0.25% acetic acid (1:1). Then, 10 mL of 80% methanol were added to the column, and the eluates were filtered through a 0.45 μm membrane filter constructed of cellulose. These solutions were injected into the HPLC system.

(3) HPLC conditions

Previous studies used HPLC to simultaneously determine the amounts of the principal compounds in rhubarb.[11-13] However, in simultaneous analysis of sennoside A and sennoside B, the separation of the sennoside B peak from neighboring peaks is often difficult.[13] The same is true for lindleyin and isolindleyin.[11,12] Therefore, we developed a reproducible method to analyze these compounds under isocratic conditions, which separated these peaks completely (Rs>1.5). The recoveries achieved by standard addition were in the range of 88.45-102.64% (Table 2).

System 1: The apparatus comprised an L-2400 detector, an L-2130 pump (Hitachi, Tokyo, Japan), a CTO-6A column oven, a C-R6A chromatograph (Shimadzu, Kyoto, Japan), and a Wakopak Handy-ODS column (250 mm×4.6 mm, Wako, Osaka, Japan).

System 2: The apparatus comprised an L-2400 detector, an L-2130 pump, a D-2500 chromatograph, an L-2200 auto sampler (Hitachi, Tokyo, Japan), a CTO-6A column oven (Shimadzu, Kyoto, Japan), and an ODS-120T column (250 mm×4.6 mm, Tosoh, Tokyo, Japan).

The injection volume of the samples was 10 μL, and the column temperature was 40℃ under all conditions.

(1) Sennoside A and sennoside B

The mobile phase used was CH_3CN-0.05M sodium phosphate (pH2.8) (18:82 v/v) at a flow rate of 0.7 mL/min, and the detection wavelength was set at 380 nm using System 1.

(2) Anthraquinones (aloe-emodin, rhein, emodin, chrysophanol, and physcion)

The mobile phase used was CH_3CN-CH_3OH-0.05M H_3PO_4 (50:18:32 v/v) at a flow rate of 0.8 mL/min, and the detection wavelength was set at 290 nm using System 2.

(3) Lindleyin and isolindleyin

The mobile phase used was CH_3CN-CH_3OH-0.05M H_3PO_4 (10:9:81 v/v) at a flow rate of 0.8 mL/min, and the detection wavelength was set at 280 nm using System 1.

Determination of total tannin amount by the Folin-Ciocalteu method[14]: Each powdered LSR sample (100

Table 2 Recovery data obtained after standard addition

Compound	Concentration (μg/mL)	Added (μg/mL)	Obtained (μg/mL)	Recovery (%)
Lindleyin	8.92	2.80	11.26	96.10
		5.60	14.90	102.64
		11.20	20.42	101.49
Isolindleyin	9.17	3.80	12.60	97.15
		7.60	16.71	99.68
		15.20	24.34	99.89
Sennoside A	15.76	2.20	17.97	100.05
		4.40	19.78	98.10
		8.80	24.73	100.67
Sennoside B	5.06	1.80	6.78	98.73
		3.60	8.42	97.22
		7.20	11.83	96.44
Aloe-emodin	1.98	1.00	2.85	95.71
		2.00	3.83	96.16
		4.00	5.78	96.60
Rhein	7.36	2.00	9.18	98.06
		4.00	11.12	97.89
		8.00	14.91	97.07
Emodin	2.27	1.00	3.04	92.93
		2.00	4.06	95.16
		4.00	6.09	97.16
Chrysophanol	3.71	1.10	4.38	91.20
		2.20	5.54	93.81
		4.40	7.87	97.10
Physcion	1.39	0.44	1.62	88.45
		0.88	2.08	91.49
		1.76	3.06	97.06

mg) was extracted with 10 mL of 80% methanol under ultrasonication for 10 min, followed by centrifugation at 3000 rpm for 10 min.[15] And each LSR solution was diluted with 80% methanol.

Each SS was also diluted with 80% methanol.

The reaction mixture was composed of 0.5 mL of sample solution and 0.5 mL of Folin-Ciocalteu's reagent diluted twofold with distilled water, and the mixture was left undisturbed for 3 minutes. Then, 2.5 mL of 0.4 M sodium carbonate were added, and the mixture was left undisturbed for 2 hours. Absorbance was measured at 765 nm. The total phenolic amount was defined as the gallic acid equivalent of the sample solution.

Statistical analysis: The significance of differences was determined by the *t*-test. All data are expressed as mean ± S.D.

Results

We described the sum of sennoside A and sennoside B, 5 kind of anthraquinone (aloe-emodin, rhein, emodin, chrysophanol, and physcion), and lindleyin and isolindleyin as sennosides, anthraquinones, lindleyins, respectively.

Changes in the amounts of the principal compounds in the LSR and SS (distilled water, 16% ethanol, 50% ethanol, and liquor 2):

(1) Sennosides (sennoside A and sennoside B) content (Fig. 1)

Sennosides content decreased in the LSR, showing significant differences in not all samples, while that in the SS significantly increased as the alcohol concentration rose.

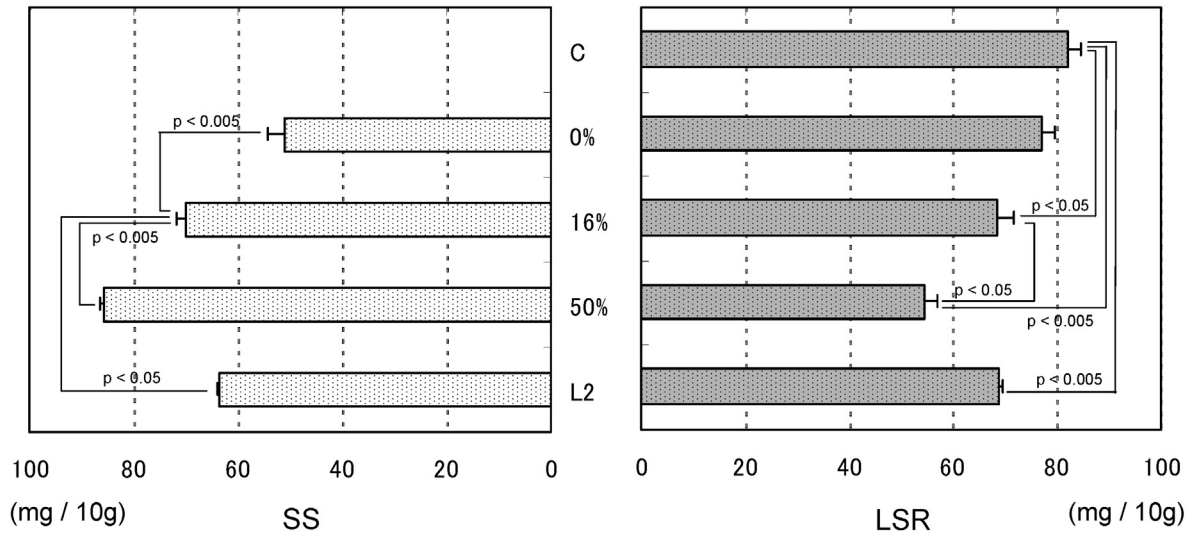
The sennosides content in the LSR processed with liquor 2 was similar to that of the LSR processed with 16% ethanol. However, the amounts of sennosides in the SS obtained using liquor 2 were significantly lowered than those in the SS produced using 16% ethanol.

(2) Anthraquinones (aloe-emodin, rhein, emodin, chrysophanol, and physcion) content (Fig. 2)

Anthraquinones content in the LSR increased, showing significant differences in not all samples, while that in the SS significantly decreased as the alcohol concentration was reduced. When using 50% ethanol, the anthraquinones content in the LSR did not increase. The anthraquinones contents of the LSR and SS obtained using liquor 2 were intermediate of those obtained using 16% ethanol and 50% ethanol.

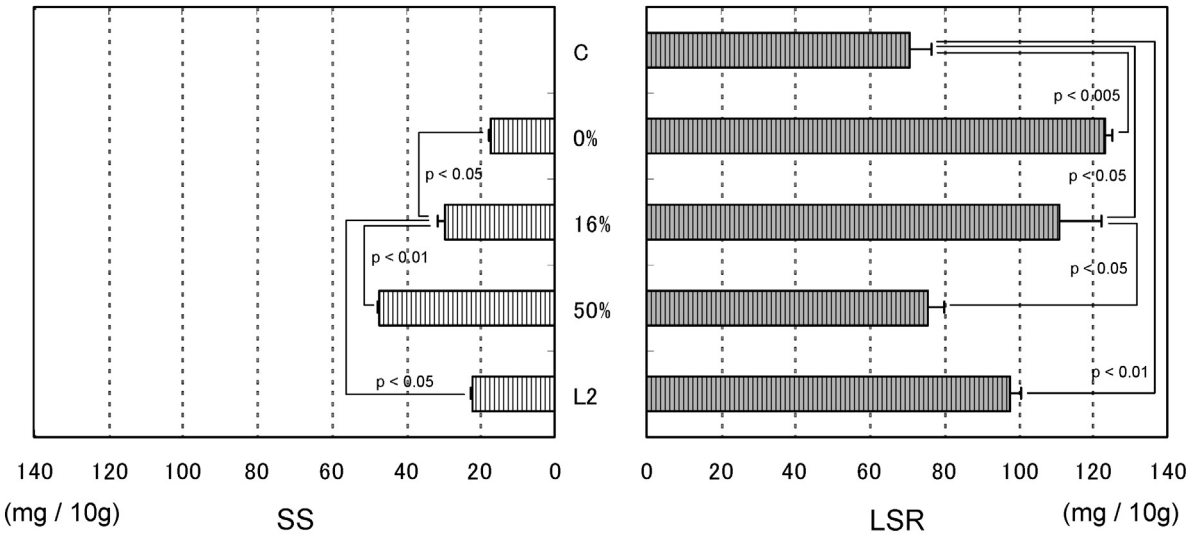
(3) Lindleyins (lindleyin and isolindleyin) content (Fig. 3)

Lindleyins content in the LSR was significantly decreased compared with that in the control. These amounts were approximately equal in all cases. Lindleyins content in the SS significantly increased as the alcohol concentration rose. The lindleyins contents of the LSR and SS obtained using liquor 2 were similar



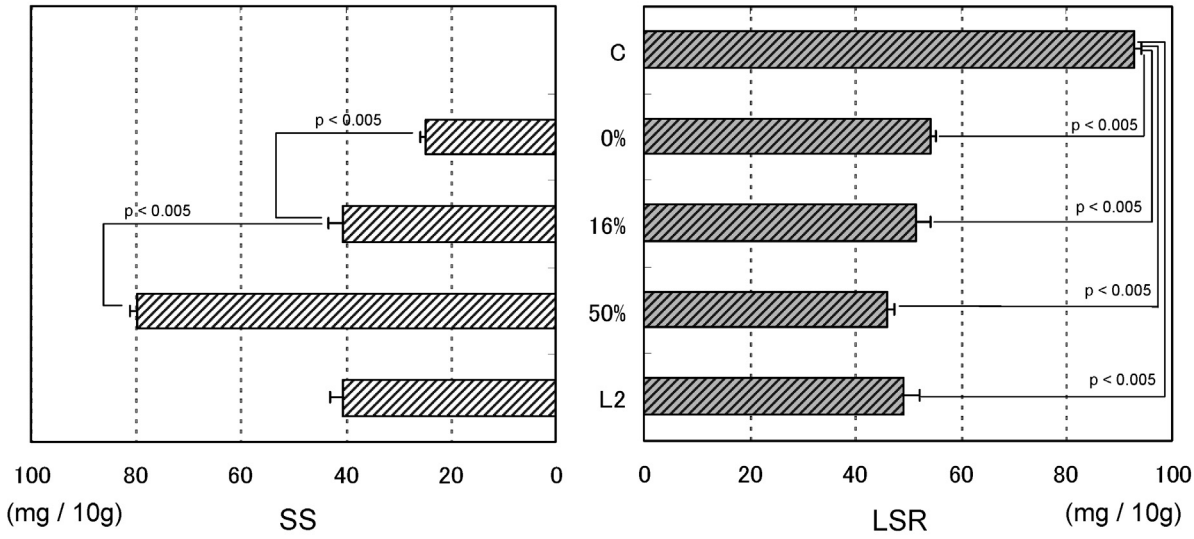
C: control, 0%: distilled water, 16%: 16% ethanol, 50%: 50% ethanol, L2: liquor 2

Fig. 1 Sennoside A and sennoside B content in the LSR and SS (n = 3)



C: control, 0%: distilled water, 16%: 16% ethanol, 50%: 50% ethanol, L2: liquor 2

Fig. 2 Anthraquinones content in the LSR and SS (n = 3)



C: control, 0%: distilled water, 16%: 16% ethanol, 50%: 50% ethanol, L2: liquor 2

Fig. 3 Lindleyin and isolindleyin content in the LSR and SS (n = 3)

to those achieved using 16% ethanol.

(4) Total tannin content (Fig. 4)

Total tannin content in the LSR had a tendency to decrease. The decrease was significantly greater for 50% ethanol than for 16% ethanol. The tannin content in the SS significantly increased as the alcohol concentration rose.

The eluted amount of principal components in SS using liquor 1 or liquor 3 was similar to that using liquor 2 (Data not shown).

Difference in principal compound amounts in the LSR processed with 3 brands of *shaoxingjiu* (Liquors 1-3) (Fig. 5): We determined whether rhubarb processed with ethanol differs from that processed with *shaoxingjiu*. The sennosides content was approximately equal in all conditions except for that of the LSR processed with liquor 1, which was slightly and significantly reduced. The same pattern was observed for tannin content. The anthraquinones content of the LSR processed with *shaoxingjiu* demonstrated a tendency to be lower

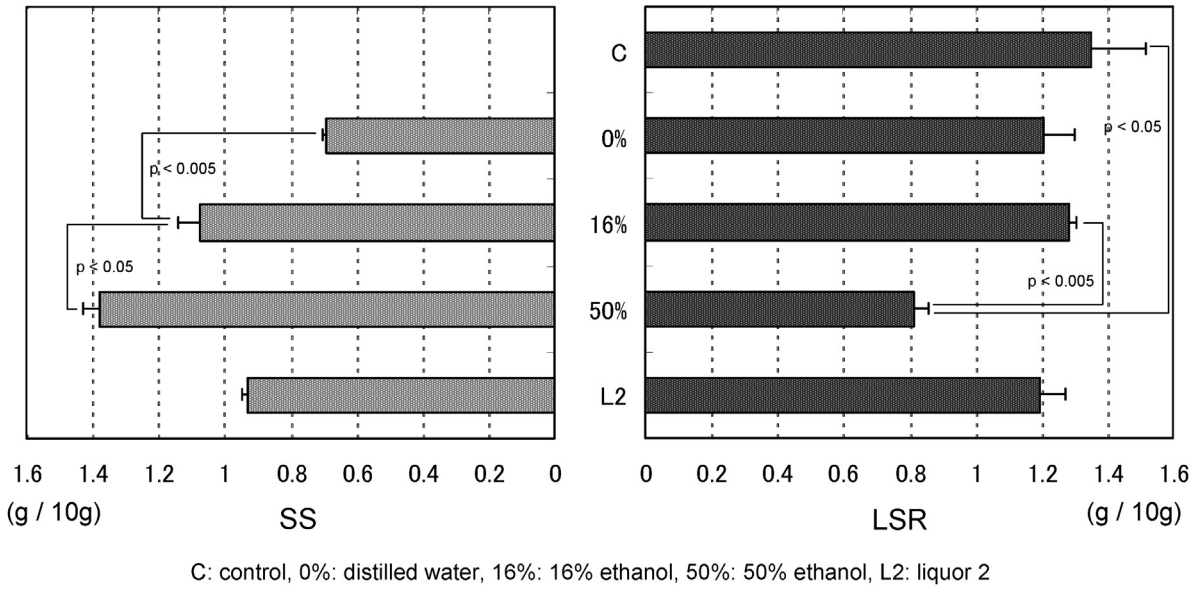


Fig. 4 Total tannin content in the LSR and SS (n = 3)

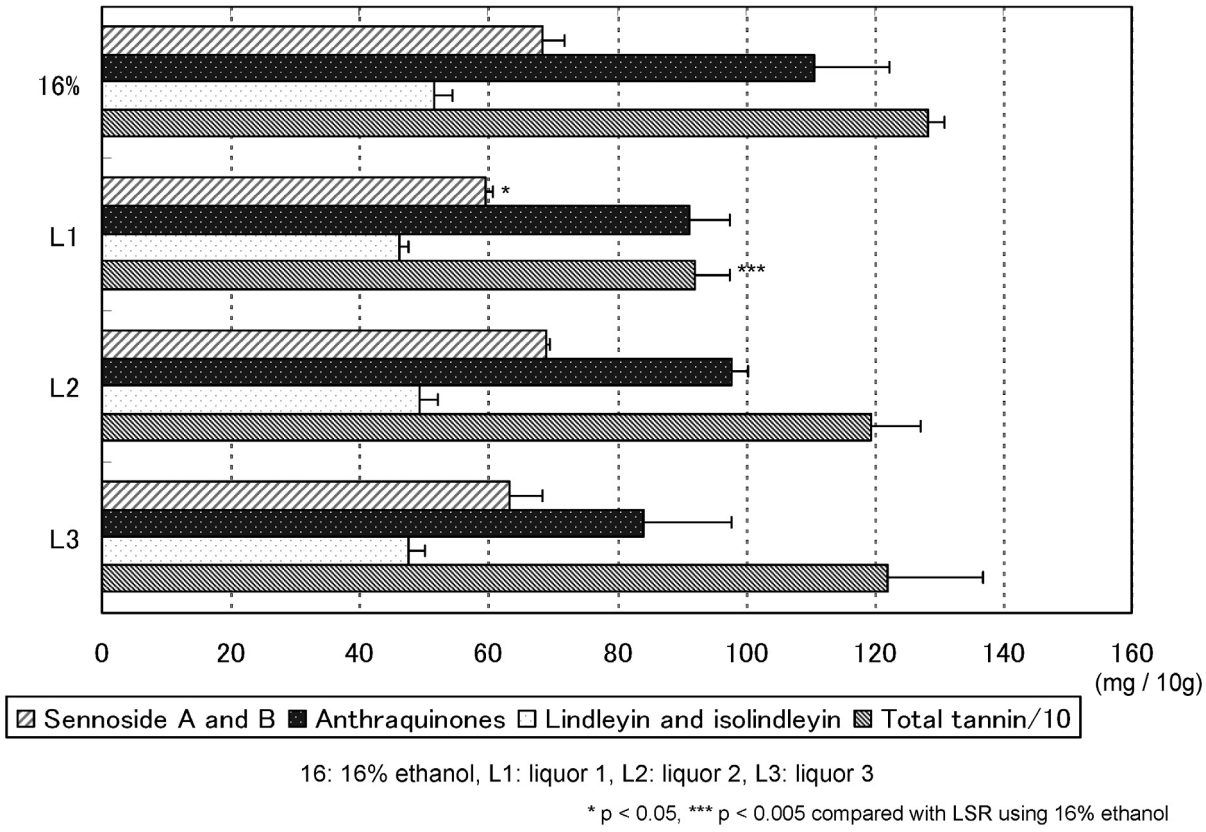


Fig. 5 Comparison of the principal compound amounts in the LSR processed using several liquors containing the same amount of alcohol (n = 3)

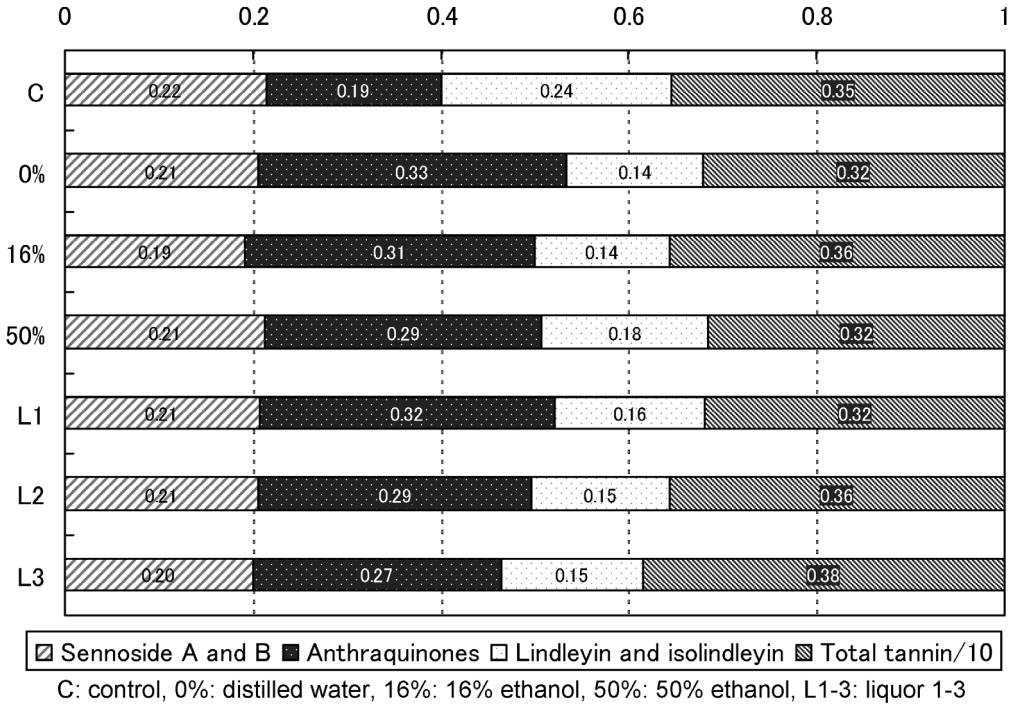


Fig. 6 Relative amounts of the principal compounds in the LSR processed with several liquids

than that of the LSR produced with 16% ethanol. Lindleyins content was approximately equal in all cases.

Difference in the relative amounts of the principal compounds in LSR processed with several liquors (Fig. 6): After liquor soaking, the anthraquinones content increased greatly, while the sennosides content decreased slightly in all cases. Lindleyins content decreased; however, the content in the LSR processed with 50% ethanol was higher than that in the LSR made with 16% ethanol. The anthraquinones contents of the LSR processed with liquor 2 and liquor 3 were lower than that of the LSR made with 16% ethanol. The composition of the LSR processed with liquor 1 was similar to that produced with 50% ethanol.

Discussion

1. Liquor-soaked rhubarb (LSR) refers to rhubarb soaked in alcohol for 24 hours. Under this process, the main principal compounds are eluted into the soaking solution (SS), while a proportion of these compounds remain in the LSR. However, the sum of the compounds in the LSR and SS is larger than the amount of the compounds in the control. It may be possible to increase the polarity and extract efficiency of this process, which needs to be explored.

2. When rhubarb was processed with ethanol, the sennosides and tannin contents in the LSR tended to decrease as the alcohol concentration rose, while anthraquinones content increased as the alcohol concentration reduced. However, the lindleyins content in the LSR was unchanged under all conditions. The amounts of these compounds in the SS increased as the alcohol concentration rose.

The lindleyins content in the LSR processed with distilled water was significantly smaller than control, lindleyins were soluble in SS. It can be connect with water-soluble character of lindleyin. However we did not resolve the reason why the lindleyins content in LSR hardly changed.

The anthraquinones content in LSR processed with distilled water or 16% ethanol significantly increased than control. We think the soaking process increased the content of hydrophobic substances, e.g., anthraquinone.

The total tannin content in the LSR processed with 16% ethanol was larger than that processed with

distilled water. However it was error range, therefore tannin content in these two rhubarbs were nearly-unchanged, and that content in LSR processed with 50% ethanol decreased significantly.

3. When rhubarb was processed with *shaoxingjiu* (a kind of *huangjiu*), the changes in the amounts of the principal compound in the LSR mostly similar to that of the LSR produced using 16% ethanol. However, there was a slightly different. Only using liquor 1, the sennoside and tannin contents in the LSR were lower than those in the LSR processed with 16% ethanol. The anthraquinone content in the LSR processed with 3 kind of liquor had a tendency to be lower than that in the LSR produced with 16% ethanol. Otherwise, the amounts of sennosides, anthraquinones, and tannins in the SS produced with liquor 2 were lower than those of the SS produced with 16% ethanol.

Huangjiu is brewed from grains that contain many components, including sugars and amino acids.[16] These components may reduce the amounts of the principal compounds eluted into the liquor when rhubarb is soaked with liquor, and this phenomenon may affect extract efficiency.

4. Processing with 16% ethanol or *shaoxingjiu* was expected to increase anthraquinones content and decrease the effect of sennosides. Both sennosides and anthraquinones have a purgative effect.[2,17] Oshio *et al.*[2] reported ED_{50} (mg/kg) values indicating purgative effects of sennoside A, sennoside B, aloe-emodin, rhein, emodin, chrysophanol, and physcion (13.5, 13.9, 59.6, 97.5, >500, >500, and >500, respectively). The purgative effects of sennosides are much stronger than those of anthraquinones. Therefore, we think that processing with *huangjiu* may decrease the purgative effect due to sennosides, may produce a rhubarb extract with mainly antibacterial[3] and anti-inflammatory effects[4] due to anthraquinones.

We previously studied descriptions of the medicinal effects of LSR in medicinal literatures published since the Jin and Yuan Dynasties.[18] We found that it was improved blood stasis. In this experiment, LSR processing with 16% ethanol or *shaoxingjiu* increased anthraquinones content. Inflammation of the blood vessels is one of the causes of blood stasis.[19,20] Sometimes it has a bacterial cause.[21] The LSR is possible to improve blood stasis by removing the inflammation, as description of medicinal literatures. This explains the use of *huangjiu* as it contains a small amount of alcohol.

5. The lindleyin content in the LSR processed with 50% ethanol was similar to that observed in the LSR produced with 16% ethanol, and the anthraquinone content was similar to that of the control. In such preparations, we can expect an anti-inflammatory effect due to lindleyin[5] and anthraquinones when a large amount of LSR processed with 50% ethanol is decocted. Therefore, rhubarb processed with *baijiu*, which contains a large amount of alcohol, is expected to have anti-inflammatory effects due to lindleyin and anthraquinones.

6. The tannin content in the LSR demonstrated a tendency to be lower than that of the control. The Folin-Ciocalteu method, which we used, is able to analyze condensed tannin, gallic acid, catechin, (-)-epicatechin 3-*O*-gallate, *etc*. It is reported that tannin and gallic acid are strong radical scavengers,[6] (-)-epicatechin 3-*O*-gallate is an antioxidant of LDL,[22] and catechin is an anticoagulant.[23] Thus, we could not elucidate whether decreasing the total tannin content of LSR affects its medicinal effect.

Conclusion

The changes in the amounts of the principal compound in the LSR mostly depended on the alcohol concentration of the SS. Only LSR processed with 16% ethanol or *shaoxingjiu* decreased sennosides content while increased anthraquinones in a balanced manner. However LSR processed with ethanol slightly differed from that processed with *shaoxingjiu*.

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