論　文（Original article）

Chemical characterization and efficient extraction of condensed tannin from the bark of juvenile *Salix* species

Rei MAKINO[1)*] and Koh HASHIDA[1)]

Abstract

Short-rotation harvesting of *Salix* species has been studied for potential biomass resources because of their great bud flush. The chemical characteristics and extractability of bark extractives, especially condensed tannin, from 1-2- and 3-5-year-old *Salix pet-susu* and *Salix sachalinensis* trees were investigated. Among the results, the yields of 70% acetone aqueous extractives from the bark of *S. pet-susu* and *S. sachalinensis* were 29.0% and 25.8% (based on oven dried bark), respectively; and the tannin polymer contents of the extractives of *S. pet-susu* and *S. sachalinensis* were 43.4% and 39.4% (based on 70% acetone aqueous extractives), respectively. The chemical characteristics of the tannin polymers were determined by ^{13}C-NMR, Py-GC/MS, and GPC analyses, and the tannins of *S. pet-susu* and *S. sachalinensis* consisted mainly of procyanidin and prodelphinidin. The approximate degree of polymerization was calculated as 6 (*S. pet-susu*) and 5 (*S. sachalinensis*). From the results of total extractives with water extraction at various temperatures, and with organic solvent extraction, the highest yields of total extractives and total polyphenols were obtained with 100 °C water extraction. On the other hand, those of total flavanols were observed with 70% acetone extraction. In order to consider an alternative treatment for debarking and grinding treatment, crush treatment was conducted, and the results indicated that such treatment raised the yields of total extractives, total polyphenols, and total flavanols to those from powder form. Additionally, it is evident that a 2-hour extraction time was the most effective to obtain condensed tannin from the crushed sample of *S. sachalinensis* and a 1-hour extraction time was adequate in the case of *S. pet-susu*. Based on the results, we conclude that short-rotation harvested *Salix* trees are potentially valuable natural resources for useful chemicals, especially condensed tannin.

Key words: bark, condensed tannin, juvenile *Salix* species, crush pretreatment

1. Introduction

Salices are fast growing tree species, with advantages for biomass production because of their great bud flush. Recently, short-rotation harvesting of *Salix* species has been investigated for potential biomass resources, such as fuel wood materials and raw materials for pulp and bioethanol production (Willebrand et al. 1993, Kopp et al. 2001, Sassner et al. 2008). In Japan, short-rotation plantation has been carried out using *Salix pet-susu* and *Salix sachalinensis* in Hokkaido and the harvesting is performed in 3 years cycle (Utsugi et al. 2015). Since *Salix pet-susu* and *Salix sachalinensis* are widely distributed from upstream to downstream along the river in Hokkaido, the short-rotation plantation is possible to be carried out in various site locations. For efficiency concerning cultivation, these tree species are harvested in less than 5 years, and the standard felling age in Hokkaido is politically set to 5 years (Hokkaido government 2017). Therefore, it is important to make clear the characteristics of juvenile *Salix* species for usage of these species in Hokkaido (Orihashi et al. 2014).

The bark of the *Salix* species is well known to contain a variety of useful compounds, including salicin, salicylates, polyphenols, and cinnamic acid derivatives (Pearl and Darling 1970, Kammerer et al. 2005, Forster et al. 2010). Some *Salix* species such as *Salix rorida* contain rich amounts of condensed tannin in the bark (Ohara and Yanagi 1995). Condensed tannin has various useful properties, such as antioxidant activity (Rice-Evans et al. 1996, Yokozawa et al. 1998, Kahkonen et al. 1999), antiherbivore activity (Ayres et al. 1997), antifeedant activity (Ohmura et al. 2000) and antimicrobial activity (Scalbert 1991). Little study has been done on bark extractives from juvenile trees, including short-rotation *Salix* species. Therefore, it is important to characterize bark extractives from juvenile trees of the *Salix* species, with an eye to promoting the utilization of extractives with high added value. In this study, the amounts and chemical characteristics of bark extractives (in particular, condensed tannin) of juvenile *Salix* species, and the extractability of condensed tannins under various conditions, were investigated.

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2. Materials and methods

2.1 Plant materials

Bark samples were collected from *Salix pet-susu* and *Salix sachalinensis* grown in Hokkaido. Branches of these *Salix* trees were separated into 1-2-year-old and 3-5-year-old groups. The bark samples were air-dried, ground in a cutting mill, and passed through a 2 mm mesh.

2.2 Extraction and purification of condensed tannin polymers

Condensed tannin polymers were prepared according to Ohara et al. (1994). Milled samples (10 g) were extracted four times with 200 ml of 70% acetone aqueous solution at room temperature for 6-12 hours, and the extractives were recovered by centrifugation. Then, acetone was removed on a rotary evaporator, and the solutions were freeze-dried to produce the corresponding 70% acetone extractives.

The portion of 70% acetone extractives was dissolved in water and extracted successively with *n*-hexane and ethyl acetate (EtOAc). The EtOAc-soluble fractions were dried on a rotary evaporator to yield EtOAc extractives. The water-soluble portion was freeze-dried, then dissolved in 50% methanol aqueous solution, and applied to a Sephadex LH20 column. The column was eluted with 50% methanol aqueous solution until the eluate was colorless, and then eluted with 50% acetone aqueous solution. The 50% acetone eluate was evaporated and freeze-dried to obtain tannin polymers (Fig. 1).

2.3 Characterization of condensed tannin polymers

^{13}C-NMR spectra ^{13}C-NMR spectra were recorded on an ALPHA-500 spectrometer (JEOL, Japan) using acetone-d_6-D_2O (9/1, v/v) as a solvent.

Pyrolysis-GC/MS analysis (Py-GC/MS) Py-GC/MS analysis was performed with a Curie-point pyrolyzer (JHP-3, Japan Analytical Industry, Japan). Py temperature: 500ºC; time: 4 s. The pyrolyzer was interfaced (interface temperature 270ºC) with a GC/MS system consisting of a Shimadzu GC-17A and QP5000 (Shimadzu, Japan) operating under the conditions as reported by Ohara et al. (2003). The molar ratios of pyrogallol type B-ring to catechol type B-ring was calculated according to the following equation:

Pyrogallol/catechol=[pyrogallol yield (mol)+5-methypyrogallol yield (mol)]/[catechol yield (mol)+4-methylcatechol yield (mol)]

Gel permeation chromatography (GPC) analysis Condensed tannin polymers were acetylated with pyridine-acetic anhydride (1/1, v/v) at room temperature. The average molecular weights of the acetyl derivatives were measured by GPC analysis. GPC analysis was performed with a LC-VP HPLC system (Shimadzu, Japan). Analysis conditions: column, Shodex KF-802, KF-803, KF804 column (i.d. 8 mm x 300 mm, Showa denko, Japan); column temperature, 40 °C; mobile phase, THF; flow rate, 1 ml min^{-1}; detection, UV absorbance at 280 nm; molecular weight standards, polystyrenes.

2.4 Water extraction

Milled samples (1 g) were extracted with 200 ml of water at 25, 50, 80, and 100 °C, in a reflux condenser for three hours. The extractives were then recovered by filtration through a 1G3 glass filter. The total amount of extractives was determined by weighing the freeze-dried filtrate. The filtrate was used as the test solution for quantitative analyses of total polyphenols and total flavanols.

2.5 Organic solvent extraction

Milled samples (1 g) were extracted with 200 ml of 70% acetone or 50% ethanol aqueous solution at room temperature for three hours. The extractives were then recovered using the same procedure as in water extraction, and the filtrate was used for quantitative analyses.

2.6 Total polyphenols and flavanols in extractives

Total polyphenols and flavanols in the extractives were determined according to the Folin-Ciocalteu method (Julkunen

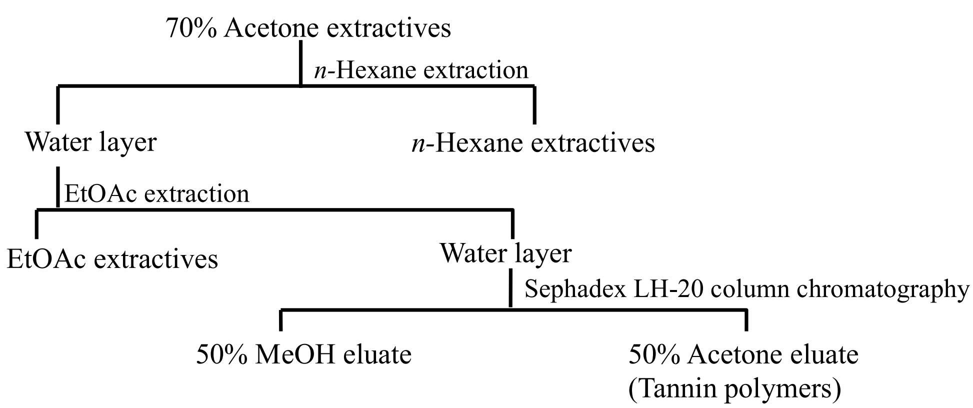


Fig. 1. Purification of tannin polymers

Fig. 2. Untreated, crushed, and ground branch samples of 1-2-year-old *Salix* species

1985) and the vanillin-HCl method (Broadhurst and Jones 1978), respectively. The calibration curve was determined using catechin as the standard sample.The experiment was carried out in duplicate.

2.7 Crush treatment of branch as a pre-treatment for extraction

The branch samples with bark of 1-2-year-old *Salix* species were cut into a 3-cm length chip and then beaten with a hammer, or ground in a Wiley mill to pass through a 2 mm pass (Fig. 2). Three grams of the sample were added to 200 ml of water. Then 100 °C water extraction was performed for three hours.

3. Results and discussion

3.1 Chemical characteristics of condensed tannin in bark of *Salix pet-susu* and *Salix sachalinensis*

As is seen in Table 1, the yields of 70% acetone aqueous extractives from the bark of *S. pet-susu* and *S. sachalinensis* were 29.0% and 25.8%, respectively. The yields of 70% acetone aqueous extractives from benzene extracted barks of four Japanese *Salix* species varied between 27.2% and 34.9% (Ohara and Yanagi 1995). In the present study, the 70% acetone aqueous extractives from juvenile trees of the *Salix* species showed a similar range to reported amounts. The tannin polymer contents of these extractives from *S. pet-susu* and *S. sachalinensis* were 43.4% and 39.4%, respectively. The yields of EtOAc extractives, which contain mainly low-molecular weight polyphenols, from *S. pet-susu* and *S. sachalinensis* were 15.0% and 16.0%, respectively. The two studied *Salix* species contained higher amounts of tannin polymers than the amounts of low-molecular weight polyphenols. From the results of 50% methanol eluates, *S. pet-susu* and *S. sachalinensis* can be assumed to contain high amounts of sugar and glycosides as well as the amounts of condensed tannin.

The chemical characteristics of the tannin polymers were determined by ^{13}C-NMR, Py-GC/MS, and GPC analyses, and these results are listed in Table 2. Through comparison of the chemical shifts in the ^{13}C-NMR spectrum with corresponding data in the literature (Foo 1984, Ohara et al. 1994, Ohara and Yanagi 1995), the tannins of *S. pet-susu* and *S. sachalinensis* consisted mainly of procyanidin and prodelphinidin (Fig. 3). The molar ratios of pyrogallol type B-ring to catechol type B-ring of *S. pet-susu* and *S. sachalinensis* calculated by Py-GC/

Table 1. Yields of 70% acetone extractives from the bark of 3-5-year-old *Salix* species and fractionation of the extractives

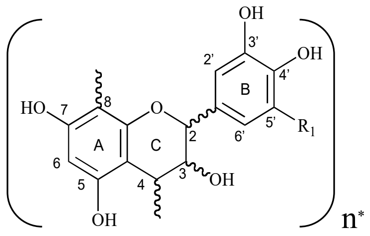
Species	70% Acetone aqueous extractives[a]	*n*-Hexane extractives[b]	EtOAc extractives[b]	50% MeOH eluates[b]	Tannin polymers[b]
			(%)		
S. pet-susu	29.0	1.7	15.0	42.2	43.4
S. sachalinensis	25.8	1.5	16.0	43.6	39.4

[a] % based on the oven-dried bark
[b] % based on the 70% acetone aqueous extractives

Table 2. Abundant structures of condensed tannin polymers, ratio of pyrogallol type B-ring to catechol type B-ring, and average molecular weight of their acetates

Species	Abundant proanthocyanidins[a]	P/C[b]	Mn[c]	Mw[d]	Mw/Mn
S. pet-susu	Procyanidin and prodelphinidin	1.1	3200	8000	2.5
S. sachalinensis	Procyanidin and prodelphinidin	0.9	2600	7900	3.0

[a] Determined by ^{13}C-NMR spectroscopy
[b] [pyrogallol yield (mol)+ 5-methylpyrogallol yield (mol)]/[catechol yield (mol)+ 4-methylcatechol yield (mol)] determined by Py-GC/MS
[c] Number-average molecular weight of acetylated tannin polymers
[d] Weight-average molecular weight of acetylated tannin polymers



Procyanidin :R_1=H
Prodelphinidin :R_1=OH

*n: the number of repeating unit

Fig. 3. Chemical structure of condensed tannin from bark of *Salix* species

MS were 1.1 and 0.9, respectively, indicating that the relative abundance of pyrogallol type B-ring and catechol-type B-ring is almost the same. The characteristics of the tannin polymers obtained by Py-GC/MS were consistent with those obtained by ^{13}C-NMR spectroscopy. From the results of GPC analysis of the acetylated tannins, the approximate degree of polymerization can be calculated as 6 (*S. pet-susu*) and 5 (*S. sachalinensis*). The dispersivity (Mw/Mn) of *S. pet-susu* was close to that of *S. sachalinensis*, and their Mw/Mn values show a larger molecular weight distribution than that of *Salix rorida* (Liu et al 2008).

3.2 Water extraction at various temperatures

In the present extraction conditions (Fig. 4), the yields of total extractives of *S. pet-susu* and *S. sachalinensis* were similar, at 22.1-33.5%. The yields of total extractives with water extraction depended on the extraction temperature. The highest yields of total extractives were obtained with 100 °C water extraction: 33.5% and 30.8% for *S. pet-susu* and *S. sachalinensis*, respectively. In the case of organic solvent extraction, the yields of total extractives with 70% acetone were slightly higher than those with 50% ethanol, for both *Salix* species. Extraction with 70% acetone was effective for obtaining tannin (Ohara et al. 1994), while ethanol is considered preferable for food utilization due to its safety for humans. The yields of organic solvent extractives were similar or less than those of 80 °C water extraction. Therefore, 100 °C water extraction was found to be most effective for the juvenile trees of the *Salix* species.

Like the yields of total extractives, the yields of polyphenols depended on the extraction temperature (Fig. 5). The highest yields of total polyphenols were obtained with 100 °C water extraction: 12.4% and 10.7% for *S. pet-susu* and *S. sachalinensis*, respectively. In contrast to the results for total extractives and total polyphenols, the highest yields of total flavanols were obtained with 70% acetone extraction: 14.1% and 12.1% for *S. pet-susu* and *S. sachalinensis*, respectively (Fig. 6). However, the overall extraction results for total flavanols and total polyphenols were similar to each other. Therefore, it is suggested that the polyphenols consist mainly of flavanol units such as condensed tannin. Next, 100°C water extractives for the two tree-age ranges were examined.

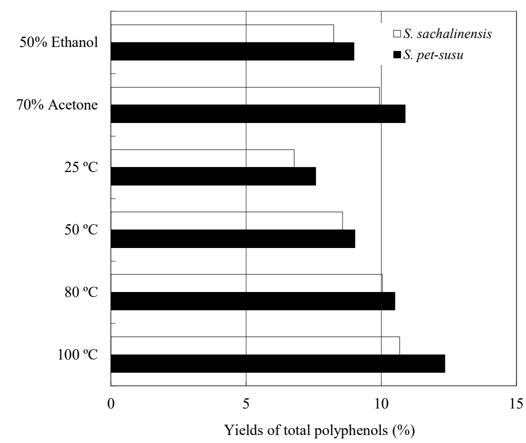


Fig. 5. Yields of total polyphenols with water extraction and organic solvent extraction from bark of 3-5-year-old *Salix* species

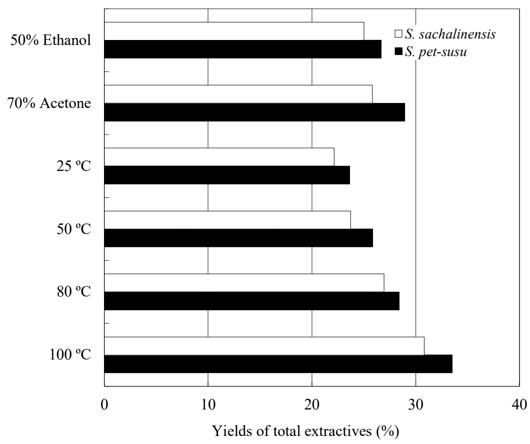


Fig. 4. Yields of total extractives with water extraction and organic solvent extraction from bark of 3-5-year-old *Salix* species

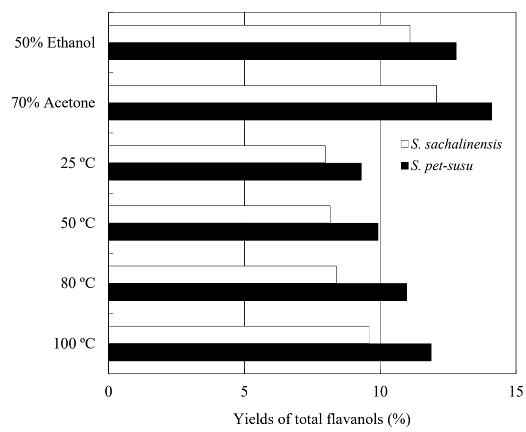


Fig. 6. Yields of total flavanols with water extraction and organic solvent extraction from bark of 3-5-year-old *Salix* species

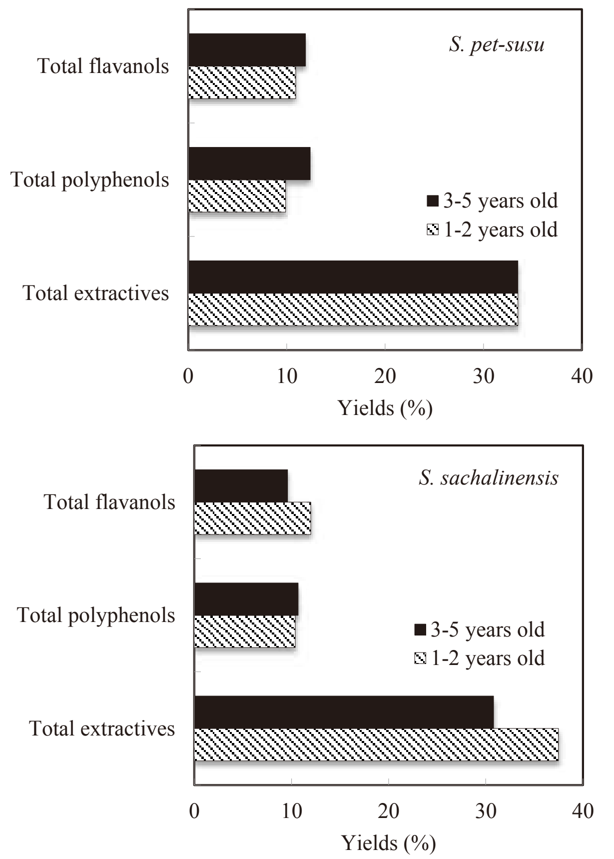


Fig. 7. Yields of total extractives, total polyphenols, and total flavanols obtained from different tree-age samples with 100°C hot-water extraction

The results for both species showed that the yields of total extractives, total polyphenols, and total flavanols were similar for samples with less than six years of growth (Fig. 7).

3.3 Efficiency of crush treatment for extraction

The extractability of the extractives with crush treatment was examined. The yields of 100˚C water extractives from untreated, crushed, and ground branch samples are shown in Fig. 8. The yields of total extractives from ground samples of *S. pet-susu* and *S. sachalinensis* were 11.1% and 14.4%, respectively; and the yields from untreated samples of the two species were very low. On the other hand, the yields from crushed samples were 10% and 12.5%, respectively, indicating that crush treatment raised the yields of the extractives to levels near those from powder form. In the same manner, the yields of total polyphenols and total flavanols from crushed samples and ground samples were broadly similar for *S. pet-susu* and *S. sachalinensis*. These results demonstrate that crush treatment has the advantage of obtaining condensed tannin, probably because the crushing helps the solvent penetrate more easily into the bark and wood. In addition, the effect of extraction time was examined for crushed samples (Fig. 9). The yields of total extractives for both *Salix* species showed the yields increased

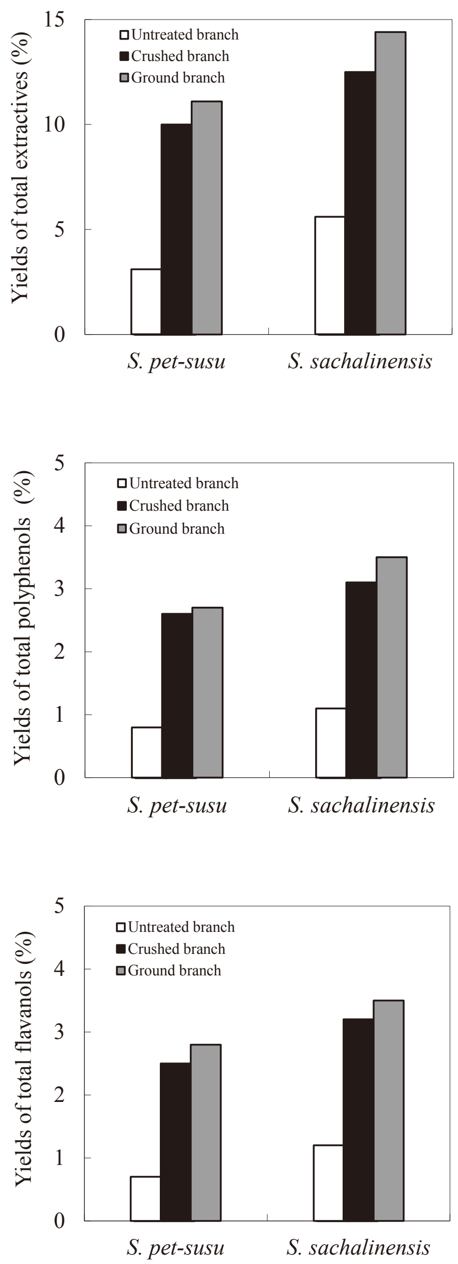


Fig. 8. Yields of total extractives, total polyphenols, and total flavanols obtained from untreated, crushed, and ground branch samples of 1-2-year-old *Salix* species with 100°C hot-water extraction

from 1-hour to 2-hour extraction; however, little difference between 2-hour and 3-hour extraction. Although the yields of total polyphenols and flavanols for *S. sachalinensis* showed a slight increase with 2-hour extraction, those for *S. pet-susu* were roughly the same. Therefore, it is evident that a 2-hour extraction time was the most effective to obtain condensed tannin for *S. sachalinensis* and a 1-hour extraction time was adequate for *S. pet-susu*.

Based on the results of chemical characterization, extractives yields, and crush treatment efficiency, we conclude that short-rotation harvested *Salix* trees are a potentially

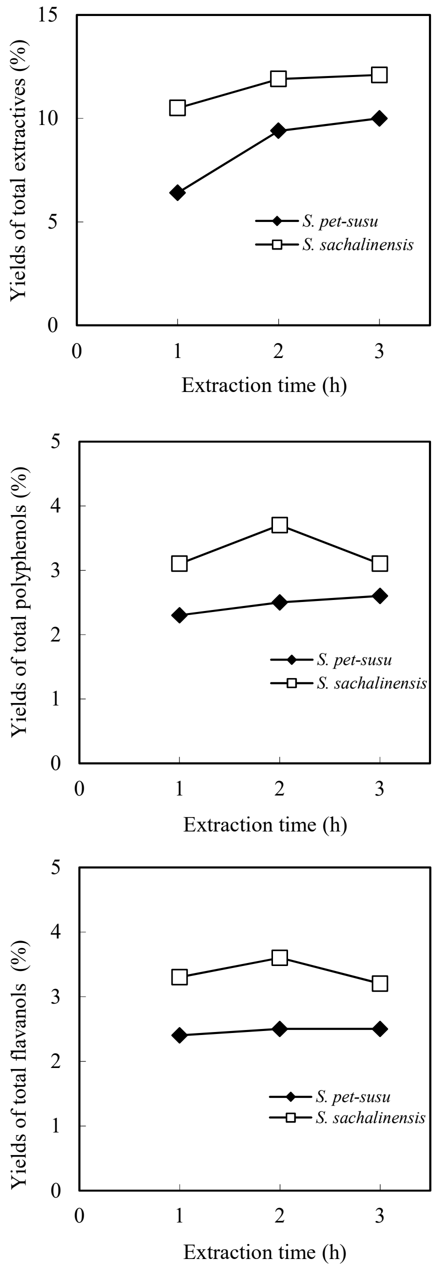


Fig. 9. Yields of total extractives, total polyphenols, and total flavanols obtained from crushed branch samples of 1-2-year-old *Salix* species with 100°C hot water extraction for 1, 2, and 3 hours

valuable natural resource for useful chemicals, especially condensed tannin; and the findings of this study may help to promote the utilization of high added-value extractives.

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低樹齢ヤナギ属樹木の樹皮タンニンの化学特性及び効率的抽出法

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要旨

早生樹であるヤナギは、挿し木栽培が可能であり、萌芽再生能が高いことから、バイオマス資源としての利用が着目されている。ヤナギ樹皮には、ポリフェノール等の有用成分が多く含まれるため、エネルギー利用だけではなく、より高付加価値な利活用の可能性が考えられる。本研究では、低樹齢のエゾノキヌヤナギ及びオノエヤナギについて、樹皮タンニンの化学特性及び効率的抽出法を検討した。樹皮を 70% アセトン水で抽出し、タンニンを精製した後、各種機器分析に供した。エゾノキヌヤナギ、オノエヤナギの樹皮に含まれる 70% アセトン抽出物量は 29.0%、25.8% であり、その中でタンニン量はそれぞれ 43.4%、39.4% に達し、高いタンニン含有量であることが分かった。タンニンの主要構成単位は、procyanidin 及び prodelphinidin であった。有機溶媒抽出と 25-100℃の水抽出の抽出効率を比較した結果、100℃の水抽出で最も高い抽出物量及びポリフェノール量が得られ、水抽出で効率的に抽出物が得られることが分かった。また、抽出工程を簡便にするため、樹皮と木部を分別せず圧潰する処理を前処理として検討した。その結果、圧潰処理では、粉砕処理と同程度の抽出物量が得られた。低樹齢ヤナギ樹木では、圧潰処理を用いることで、樹皮付の試料で効率的に抽出成分を得られることが明らかになった。

キーワード：樹皮、タンニン、低樹齢ヤナギ属樹木、圧潰前処理

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