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Communication

Phytochemical and Antioxidant Characterization of Extracts from Unexplored Medicinal Plants *Salix schwerinii* and *Salix kochiana*

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Abstract: For a long time, species of the genus *Salix* have been widely utilized and studied as medicinal plants; however, the biological activity and phytochemical composition of *Salix schwerinii* (SS) and *S. kochiana* (SK) have not been studied at all. This study investigated the antioxidant properties of SS and SK extracts and detected phytochemical compounds in the extracts. The results showed that the antioxidant activities (IC_{50}) of SS extract, SK extract, and ascorbic acid (reference) were as follows, respectively: 169.8, 79.8, and 71.2 μg mL^{-1} for ABTS cation radical scavenging and 38.4, 26.2, and 9.3 μg mL^{-1} for DPPH free radical scavenging. The results imply that SK has a high potential as a natural antioxidant. The phytochemical compositions of extracts (mg g^{-1}) were analyzed as follows: SS extracts, 217.7 phenolics (1.54 catechin, 0.86 syringic acid, 0.46 luteolin, and others) and 5.06 salicin; SK extracts, 71.0 phenolics (0.54 catechin, 0.28 myricetin, 0.12 salicylic acid, and others) and 2.11 salicin. Compared to previous studies, the present findings go further to highlight that SS deserves attention as a novel source of salicin. The present study highlights the need for further studies on the aspects of medicinal functions of the extracts, bioprocess design for efficient phytochemical extraction, and applications of bioactive substances.

Keywords: medicinal plant; *Salix schwerinii*; *Salix kochiana*; salicin; catechin; myricetin; luteolin

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1. Introduction

There are 350–520 species belonging to the genus *Salix*, which are known to be medicinal plants [1]. Some species of the *Salix* genus are recognized as valuable bioresources with multiple medicinal functions, particularly for treating rheumatic diseases, ulcers, and parasite skin diseases [2,3]. The beneficial functions of *Salix* spp. are attributed to bioactive compounds such as phenolic glycoside (e.g., salicin), flavonoids (e.g., luteolin, kaempferol, and epicatechin gallate), and other phenolic compounds (e.g., catechin, syringic acid, and salicylic acid) [4–6]. In the case of salicin, it is absorbed after ingestion and metabolized into saligenin (in the intestine), and then into salicylic acid (in the liver), finally blocking inflammatory prostaglandins by inhibiting cyclooxygenase 1 and 2 [7,8]. Luteolin, one of the flavones, is widely contained in medicinal plants and has immunopharmacological activities [9]. Biological functions such as antioxidant, anticancer, and antibacterial activities of other flavonoids and phenolic compounds from *Salix* spp. were also introduced in earlier reports [10,11]. In this context, species of the *Salix* genus are valuable plant biomass for pharmacological uses, and phytochemical research on them is required.

There are unexplored *Salix* plants such as *S. schwerinii* (SS) and *S. kochiana* (SK), although these species are traditionally used as medicinal plants, mainly due to their salicin

being recognized as an anti-inflammatory compound, pain reliever, and febrifuge [4,12]. A few researchers have undertaken taxonomic and species divergence studies of SK and SS [13,14], but studies on their phytochemistry are not available. Wang et al. [14] described SK as an important plant with fast growth and luxuriant foliage, receiving attention for use as woven goods, animal feed, etc. Berlin et al. [15] also described the fast growth of SS, and, in addition, SS has resistance to *Melampsora larici-epitea* leaf rust that causes severe loss yields. SK and SS have potential for medicinal uses and other industrial applications. Thus, bioactivity and phytochemistry studies should be conducted on unexplored *Salix* plants including SS and SK.

Herein, we investigate the antioxidant characteristics and phytochemical compositions of SS and SK. For this purpose, methanolic extracts from SS and SK were prepared through ultrasonic-assisted extraction, and antioxidant properties such as total phenol content (TPC) and antioxidant activity were evaluated. After that, the major known phytochemical compounds of *Salix* species were detected and quantified. There are no studies on the phytochemical and antioxidant characterization of the extracts from SS and SK. This study is the first attempt, and it is expected to contribute to the advancement of biochemical and pharmacological knowledge of the traditional medicinal plant *Salix*.

2. Materials and Methods

2.1. Chemicals

Folin–Ciocalteu phenol reagent (CAS No. 12111-13-6), sodium nitrate ($NaNO_2$) (CAS No. 7632-00-0), gallic acid ($C_6H_2(OH)_3COOH$) (CAS No. 149-91-7), L-ascorbic acid ($C_6H_8O_6$) (CAS No. 50-81-7), sodium carbonate (Na_2CO_3) (CAS No. 497-19-8), 2,2′-azino-bis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS, AzBTS-$(NH_4)_2$) (CAS No. 30931-67-0), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, $C_{18}H_{12}N_5O_6$) (CAS No. 1898-66-4), potassium persulfate ($K_2S_2O_8$) (CAS No. 7727-21-1), luteolin ($C_{15}H_{10}O_6$) (CAS No. 491-70-3), catechin hydrate ($C_{15}H_{14}O_6 \cdot xH_2O$) (CAS No. 225937-10-0), and salicin ($C_{13}H_{18}O_7$) (CAS No. 138-52-3) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Methanol (CH_3OH) (CAS No. 67-56-1), acetonitrile (CH_3CN) (CAS No. 75-05-8), and phosphoric acid (H_3PO_4) (CAS No. 7664-38-2) were acquired from Samchun Chemical (Seoul, Republic of Korea). Kaempferol hydrate ($C_{15}H_{10}O_6 \cdot xH_2O$) (CAS No. 520-18-3) and myricetin ($C_{15}H_{10}O_8$) (CAS No. 529-44-2) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Epicatechin gallate ($C_{22}H_{18}O_{10}$) (CAS No. 1257-08-5) was acquired from Biofron (La Mirada, CA, USA).

2.2. Plant Collection

S. schwerinii E. Wolf. and *S. kochiana* Trautv. were collected in Batsumber district, Tov province, Mongolia, and identified by Dr. Badamtsetseg Bazarragchaa at the National History Museum of Mongolia in June 2014. A voucher specimen (accession numbers KRIB 0054363 and KRIB 0054350) of the retained material is preserved at the herbarium of KRIBB. Figure 1 shows *S. schwerinii* E. Wolf. and *S. kochiana* Trautv., which were used in this study.

2.3. Extraction, Concentration, and Drying

The leaves, shoots, and flowers of SS (79 g) and SK (91 g) were extracted using 1 L of methanol (99.9%) by sonication at 45 °C. The extraction process was repeated using 15 min of sonication and 2 h of resting for 72 h. The methanolic extracts were filtered with non-fluorescence cottons. Then, the filtered extracts were concentrated using a rotary evaporator (N-1000SWD, EYELA, Tokyo, Japan) under reduced pressure at 45 °C. Finally, totals of 5.12 g and 9.28 g of methanolic extracts from SS and SK were obtained via freeze-drying.

Figure 1. Mongolian plants *Salix schwerinii* (**A**) and *S. kochiana* (**B**).

2.4. Measurement of Antioxidant Activity of Freeze-Dried Extracts

The antioxidant activities of each extract from SS and SK were measured using ABTS cation radicals and DPPH free radicals. We followed the reported procedures for determining each radical scavenging activity [16,17]. All experiments were performed in triplicate, and the results are presented as the average IC_{50} values, which are the required concentrations of the extracts to scavenge 50% of the initial radicals. In addition, in all experiments, ascorbic acid was used as a reference antioxidant compound.

2.4.1. ABTS Cation Radical Scavenging Activity

To formulate ABTS cation radicals, ABTS solution (7 mM) and potassium persulfate (2.45 mM) were mixed in a 1:1 volume ratio and then kept at 25 °C for 12 h. To measure the ABTS cation radical scavenging activity of the extracts, 950 µL of the prepared ABTS cation radical solution was added to 50 µL of each extract (control sample: methanol) and then mixed and stored at 25 °C for the radical scavenging reaction. After 30 min, the sample's absorbance at 734 nm was measured (UV spectrophotometer, DU® 730, Beckman Coulter, Brea, CA, USA).

2.4.2. DPPH Free Radical Scavenging Activity

To evaluate the DPPH free radical scavenging activity of the extract, 0.5 mL 0.25 mM DPPH solution was added to 0.5 mL of each extract (control sample: methanol) and then mixed and stored at 25 °C for the radical scavenging reaction. After 30 min, the sample's absorbance at 517 nm was measured.

2.5. Measurement of Total Phenol Content (TPC) in Freeze-Dried Extracts

TPC values of the extracts were measured by following the reported protocols [18,19]. First, 10 µL of extract, 790 µL of deionized water, and 50 µL Folin–Ciocalteu phenol reagent were mixed, and then stored at 30 °C. After 8 min, 150 µL of sodium carbonate (20%, w v^{-1}) was additionally added and then further stored at 25 °C. After 1 h, the absorbance at 765 nm was measured. In the procedure for TPC measurement, gallic acid with different concentrations was used as a standard phenolic compound for calculating TPC in the extracts. All experiments were performed in triplicate, and the results were represented as the average mg gallic acid equivalent (GAE) per g of extract.

2.6. HPLC Analysis for Quantification of Phytochemical Compounds

The contents of selected known phytochemicals (salicin, catechin, syringic acid, salicylic acid, luteolin, kaempferol, epicatechin gallate, and myricetin) in the extracts from SS and SK were quantified using a high-performance liquid chromatography (HPLC) system. The detector of the HPLC system was a diode array detector (G7117C, Agilent, Santa Clara, CA, USA), and the column used was an INNO column C18 (5 µm, 4.6 mm × 250 mm). The operation conditions were [20] solvents, phosphoric acid in deionized water (0.03 vol%, solvent A) and acetonitrile (solvent B); flow rate of solvents, 0.8 mL min^{-1}; column temperature, 25 °C; injection, 5 µL sample; and monitoring wavelengths, 280, 300, and 360 nm. The gradient elution method was adopted (min, % of solvent A:B): 0 min, 90:10%; 15 min, 80:20%; 28 min, 60:40%; 36 min, 25:75%; 38 min, 90:10%; and 50 min, 90:10%. For the quantification of phytochemicals, calibration curves were produced by analyzing standard compounds (HPLC grade) with different concentrations.

3. Results

3.1. Antioxidant Characteristics of Extracts from Salix schwerinii and S. kochiana

The antioxidant characteristics (such as antioxidant activities and total phenol content) of the extracts from SS and SK were analyzed, and the results are shown in Figure 2A. In the ABTS assay, SS and SK extracts showed IC_{50} values of 169.8 ± 3.3 and 79.8 ± 5.4 µg mL^{-1}, respectively, and SK's value was similar to that of the reference antioxidant ascorbic acid (71.2 ± 1.1 µg mL^{-1}). In the results of the DPPH assay, the extracts from SS and SK showed IC_{50} values of 38.4 ± 0.9 and 26.2 ± 5.6 µg mL^{-1}, respectively, which means that 312.9% and 181.7% more were required than ascorbic acid (9.3 ± 1.3 µg mL^{-1}) for scavenging 50% of the radicals. Further, the TPC values in the extracts from SS and SK were measured because phenolics are significant contributors to scavenge radicals. TPC values of SS and SK extracts were 217.7 ± 4.5 and 71.0 ± 0.6 mg GAE g^{-1} extract, respectively, implying that 21.8% and 7.1% of each extract were phenolic compounds (Figure 2B).

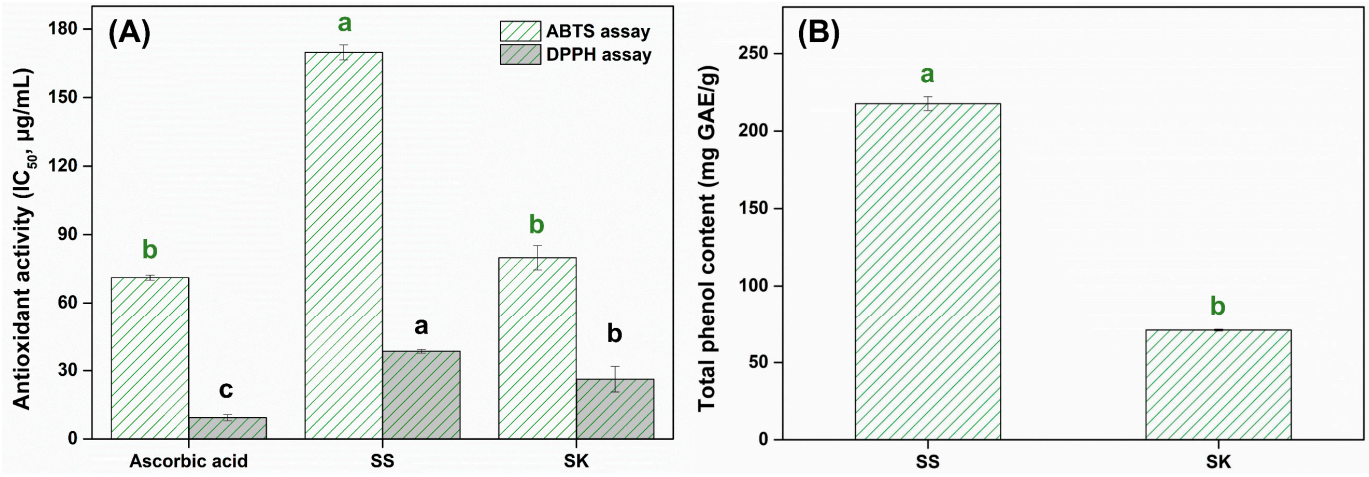


Figure 2. Antioxidant activities (**A**) and total phenol content (**B**) of the extracts (dry weight basis) from *Salix schwerinii* (SS) and *S. kochiana* (SK). Ascorbic acid was used as a reference antioxidant in antioxidant activity assays. Data with different letters are significantly different ($p < 0.05$).

3.2. Detection of Phytochemical Compounds in Extracts from Salix schwerinii and S. kochiana

Several major phytochemicals of *Salix* species, such as salicin, catechin, syringic acid, salicylic acid, luteolin, kaempferol, epicatechin gallate, and myricetin, were detected in SS or SK extracts by HPLC-DAD analysis (Figures 3 and A1). Table 1 lists the content of known phytochemicals in a 1 g extract from SS or SK. Vanillic acid, rutin, and quercetin were not detected in either extract. High amounts of salicin were identified in the extracts from SS (5.06 mg g^{-1} extract) and SK (2.11 mg g^{-1} extract), and considerable amounts

of catechin were detected in both extracts (1.54 mg g^{-1} SS extract and 0.54 mg g^{-1} SK extract). In the case of other phytochemicals, the SS extract contained 0.86 mg syringic acid, 0.46 mg luteolin, 0.28 mg kaempferol, 0.14 mg epicatechin gallate, and 0.08 mg myricetin per g of extract. The extracts from SK contained relatively low amounts of syringic acid (0.02 mg g^{-1} extract) and epicatechin gallate (0.10 mg g^{-1} extract) and no luteolin or kaempferol, whereas salicylic acid was detected only in the SK extract, and the SK extract contained relatively high amounts of myricetin (0.28 mg g^{-1} extract).

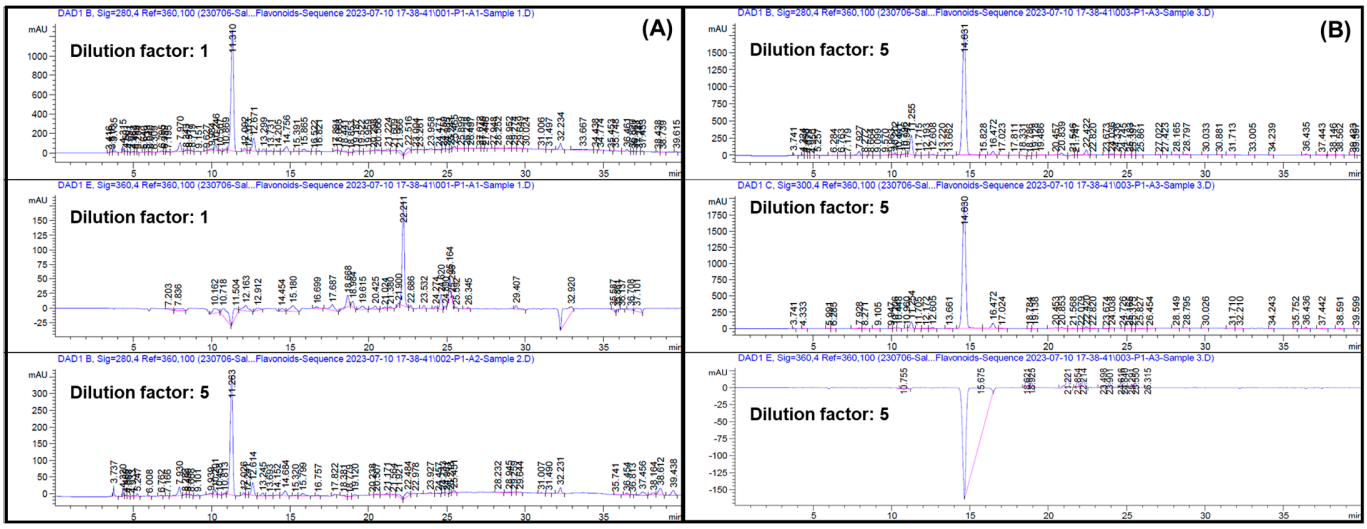
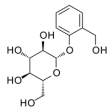
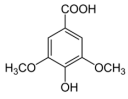
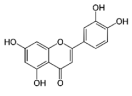
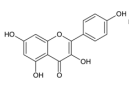
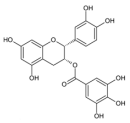
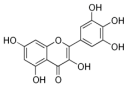


Figure 3. HPLC chromatograms for the methanolic extracts from *Salix schwerinii* (**A**) and *S. kochiana* (**B**). Monitored wavelengths were 280, 300, or 360 nm.

Table 1. Phytocompounds detected in the methanolic extracts from *Salix schwerinii* (SS) and *S. kochiana* (SK).

Phytocompound		Salicin	Catechin	Syringic acid	Salicylic acid	Luteolin	Kaempferol	Epicatechin gallate	Myricetin
Structure									
Content (mg g^{-1} Extract, DW)	**SSE**	5.06	1.54	0.86	ND	0.46	0.28	0.14	0.08
	SKE	2.11	0.54	0.02	0.12	ND	ND	0.10	0.28

DW, dry weight; SSE, SS extract; SKE, SK extract; ND, not detected.

4. Discussion

As findings in Figure 2 illustrate, SS extracts contain more phenolic compounds than SK extracts, but the antioxidant activities of SK extracts were higher than those of SS extracts (Figure 2A). This could be due to the different radical scavenging activities of each single compound in all phenolics of the extract. In fact, a previous study reported that the antioxidant activities were high in the order of myricetin, quercetin, and catechin [21]. Regarding Table 1 (lists of phytochemical compositions), the higher radical scavenging activity of SK extracts can be attributed to having 3.5 times more myricetin. On the other hand, it has been reported that salicin (the most abundant compound in SS extracts) does not show radical scavenging activity, and thus it is presumed that it did not contribute to the antioxidant activity of the SS extracts [22]. In addition to these reasons, unknown compounds present in the crude extract from SK are probably contributing to the robust antioxidant activity of the extracts. Research to identify unknown compounds of SS and SK

should be addressed in a follow-up study, and the present study highlights the potential of SK extracts to be comparable to ascorbic acid as a natural antioxidant.

Table 2 summarizes the antioxidant characteristics of extracts from various *Salix* species, such as *S. mucronata*, *S. alba*, *S. amplexicaulis*, *S. babylonica*, *S. fragilis*, *S. purpurea*, and *S. triandra* [23,24]. The TPC values ranged from 5.6 to 131.4 mg g^{-1} of *Salix* extract. The TPC values of SS and SK extracts showed relatively high levels compared to the previous studies, and in particular, SS's value was the highest. The extracts from *S. mucronata* show relatively high IC_{50} (i.e., relatively low antioxidant activity), but the extracts from other species show relatively low IC_{50}. The remarkable antioxidant activity of the other species can be attributed to either (i) inherent properties or (ii) differences in extraction methods. To study this point, microwave-assisted extraction (MAE) will be applied to SS and SK extraction processes for efficiently recovering phytochemicals in a short time and then compared to the present process based on the ultrasonic-assisted system.

Table 2. Summary of the antioxidant characteristics of extracts from the species of the genus *Salix*.

Salix Species (Part)	Extraction Process					Extract Type	Antioxidant Characteristics		Ref.
	Method	Solvent	Temp.	Time	Biomass Loading		TPC (mg GAE g^{-1} Extract)	IC_{50} (μg mL^{-1})	
S. mucronata (leaf)	SLE	MeOH (pure)	-	-	-	dried	128.1	131.6 [a]	[23]
S. mucronata (leaf)	SLE	MeOH (85%)	-	-	-	dried	131.4	98.8 [a]	
S. mucronata (leaf)	SLE	MeOH (70%)	-	-	-	dried	130.0	102.5 [a]	
S. mucronata (leaf)	SLE	Water	-	-	-	dried	89.5	209.8 [a]	
S. alba (leaf)	MAE (850 W)	Water	-	5 min	-	dried	7.6	6.7	[24]
S. amplexicaulis (leaf)	MAE (850 W)	Water	-	5 min	-	dried	21.5	13.0	
S. babylonica (leaf)	MAE (850 W)	Water	-	5 min	-	dried	5.6	24.9	
S. fragilis (leaf)	MAE (850 W)	Water	-	5 min	-	dried	8.4	8.8	
S. purpurea (leaf)	MAE (850 W)	Water	-	5 min	-	dried	38.1	12.8	
S. triandra (leaf)	MAE (850 W)	Water	-	5 min	-	dried	47.5	3.1	
S. schwerinii (leaf, shoot, and flower)	USLE	MeOH (pure)	45 °C	8 h	79 g L^{-1}	freeze-dried	217.7	38.4 [b]	This study
S. kochiana (leaf, shoot, and flower)	USLE	MeOH (pure)	45 °C	8 h	91 g L^{-1}	freeze-dried	71.0	26.2 [b]	

[a] DPPH IC_{50} of ascorbic acid was 13.6 μg mL^{-1} [23]. [b] DPPH IC_{50} of ascorbic acid was 9.3 μg mL^{-1} in this study. SLE, solid–liquid extraction; MAE, microwave-assisted extraction; USLE, ultrasonic-assisted SLE; TPC, total phenol content.

The present study reported the phytochemical compositions of the extracts from SS and SK. Quantitatively, SS extracts contained 217.7 mg of phenolic compounds per g of extract, of which 1.54, 0.86, 0.46, 0.28, 0.14, and 0.08 mg were catechin, syringic acid, luteolin, kaempferol, epicatechin gallate, and myricetin, respectively (Figure 2B and Table 1). A measure of 1 g of SK extracts contained 71.0 mg of phenolic compounds, of which 0.54, 0.02, 0.12, 0.10, and 0.28 mg were catechin, syringic acid, salicylic acid, epicatechin gallate, and myricetin, respectively. The earlier report of El-Wakil et al. [25] observed myricetin, rutin, luteolin, kaempferol, and quercetin, or their derivates, in the methanolic extracts from *Salix tetrasperma*. Gaafar et al. [26] also investigated phytochemical compositions of *Salix* species (*S. mucronata*) and reported that the methanolic extracts contained about

0.17 mg catechin, 0.01 mg myricetin, 0.05 mg luteolin, and 0.04 mg kaempferol per g of extract. The present study showed relatively high contents of phytochemical compounds, and it can be attributed to the use of efficient extraction methods (i.e., ultrasonic-assisted extraction system) or to the nature of SS and SK. On the other hand, salicin (one of the phenolic glycosides) was present at 5.06 mg and 2.11 mg in 1 g of SS and SK extracts, respectively. According to Gligorić et al. [24], leaf extracts from *Salix* species contained 2.0–20.1 mg salicin g^{-1} extract; the studied species were (mg salicin g^{-1} extract) *S. alba* (3.2), *S. amplexicaulis* (14.9), *S. babylonica* (2.0), *S. fragilis* (2.1), *S. purpurea* (20.1), and *S. triandra* (2.4). In addition, Kenstavičienė et al. [27] investigated salicin contents of various *Salix* species: *Salix purpurea* cl. 04132 (10.05%), *Salix purpurea* "Lutea" cl. 9731 (6.53%), *Salix caspica* (3.87%), *Salix mollissima* (0.26%), etc. The present study highlights the comparable potential of SS as a novel source of salicin (5.06 mg g^{-1} SS extract). In order to improve the utilization feasibility of SS, a study using statistical methods to determine how best to efficiently recover salicin from SS in an industrial setting could be conducted [28,29], and we are planning that.

5. Conclusions

Herein, we investigated the antioxidant properties and phytochemical compositions of SS and SK extracts for the first time. The methanolic extracts from SK showed impressive ABTS cation radical scavenging activity (79.8 μg mL^{-1}), which is similar to that of ascorbic acid (71.2 μg mL^{-1}), implying the high potential of SK extracts as natural antioxidant materials. In the case of the SS extract, it contained a high amount of salicin (5.06 mg g^{-1} extract), confirming its high potential as a novel source of salicin. In addition, the extracts from SS and SK contained several known phytochemicals of *Salix* species, such as catechin, syringic acid, epicatechin gallate, and myricetin. This study provides a preliminary result for further phytochemical and pharmacological studies on SS and SK. In the near future, follow-up studies on in vivo activity evaluation, further characterization of the unknown phytochemicals, and application are required to advance knowledge in the field of traditional medicines.

Author Contributions: Conceptualization, J.L. and H.Y.Y.; methodology, S.K. and K.H.L.; validation, H.Y.Y.; formal analysis, J.L., Y.S. and H.S.; investigation, H.S., Y.S. and C.L.; resources, B.B. and C.L.; writing—original draft preparation, J.L.; writing—review and editing, H.Y.Y.; visualization, J.L.; supervision, H.Y.Y.; project administration, H.Y.Y.; funding acquisition, H.Y.Y. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

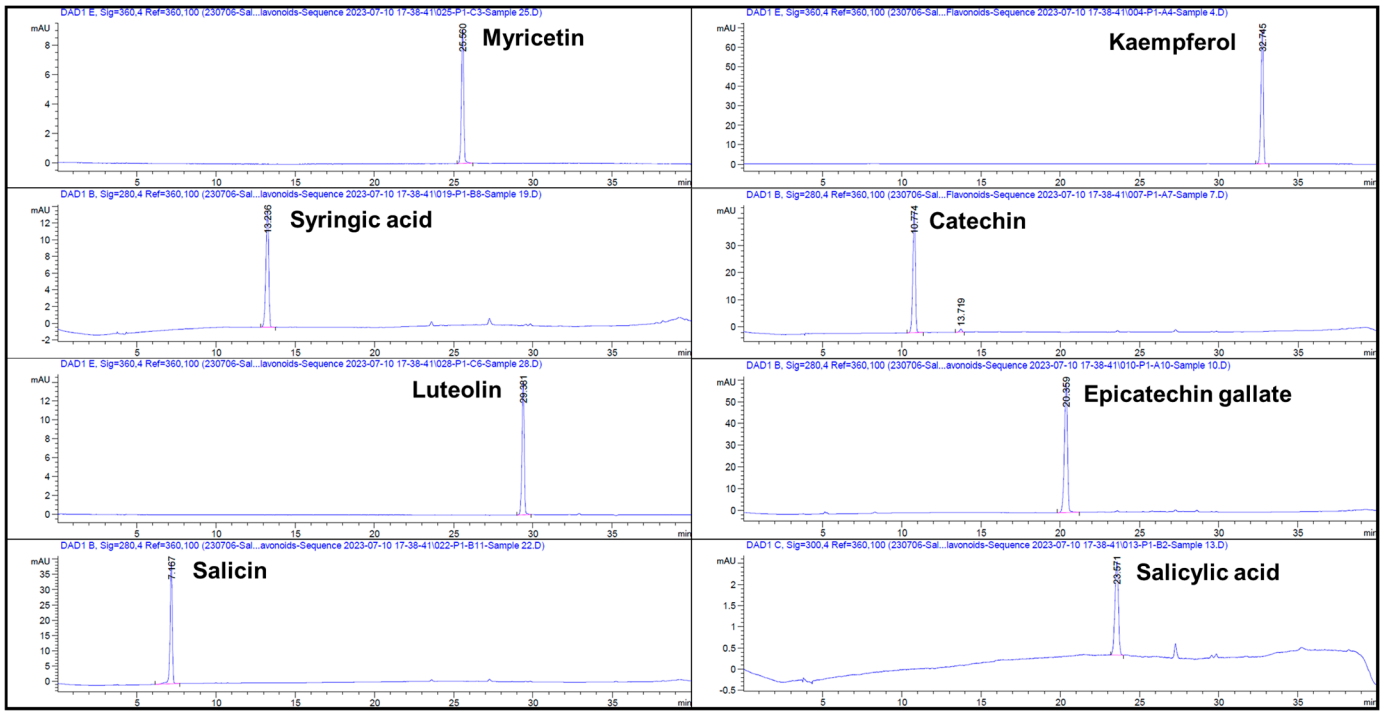

Figure A1. HPLC chromatograms for the eight standard compounds.

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