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Quantitative Changes of Flavonol Glycosides from Pine Needles by Cultivar, Harvest Season, and Thermal Process

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ABSTRACT: Five flavonol glycosides including quercetin 3-*O*-β-D-glucoside (QG), kaempferol 3-*O*-β-D-glucoside (KG), quercetin 3-*O*-(6″-*O*-acetyl)-β-D-glucoside (QAG), kaempferol 3-*O*-(6″-*O*-acetyl)-β-D-glucoside (KAG), and quercetin 3-*O*-(3″-*O*-*p*-coumaroyl)-β-D-glucoside (QCG) were isolated and purified from red pine (*Pinus densiflora* Sieb. et Zucc.) needles, and identified by nuclear magnetic resonance and mass spectrometer spectral analyses. In addition, the quantification of the five flavonol glycosides in pine needles was performed by high-performance liquid chromatography analysis according to cultivar, growing district, harvest season, and thermal processing. The red pine needles had higher amounts of the five flavonol glycosides than the black pine needles except for QCG. There were no large differences in flavonoid composition and content among pine needles grown in three different areas. Levels of the five flavonol glycosides in red pine needles harvested during Spring ranged from 6.13 to 27.03 mg/100 g dry weight. Levels of two flavonol glycosides, QG and KG, gradually decreased with increasing harvest time, whereas the acylated flavonol glycoside, QCG, a predominant flavonoid in pine needles, increased gradually with increasing harvest time. Two acetyl flavonol glycosides, QAG and KAG, increased steadily through Spring to Autumn, and then decreased gradually by Winter. Meanwhile heat treatments, such as roasting and steaming, increased the five flavonol glycosides during heating for 3 min, but then slowly decreased these when heating for 10 min. Microwave processing increased to some extent the five flavonol glycosides when heating for 3 min, and remained unchanged during the 10 min heating. These results suggest that the pretreated red pine needles with enhanced flavonoid content may be useful as potential sources for nutraceuticals and cosmeceuticals.

Keywords: flavonoids, pine (*Pinus densiflora* Sieb. et Zucc.) needles, seasonal, thermal processing, varietal

INTRODUCTION

Flavonoids are a group of phenolic compounds that have been known as naturally occurring plant pigments in fruits, vegetables, and other plants. They have been reported to possess a variety of biological and pharmacological effects including anticancer, antidiabetic, antihypertensive, anti-inflammatory, antiaging, and antioxidant activities (Liu, 2002; Pandey and Rizvi, 2009). Many epidemiological studies suggested that intake of dietary flavonoids in fruits and vegetables have been linked to reducing the risk of major chronic diseases including cancer and coronary heart disease (Knekt et al., 1996; Hollman and Katan, 1999; Graf et al., 2005). Thus, flavonoids have received much attention as dietary supplements of functional foods for promoting human health.

Pine (*Pinus densiflora* Sieb. et Zucc., Pinaceae) trees have widely been used in oriental medicines as traditional remedies for hypertension, atherosclerosis, stroke, diabetes, cancer, balding, etc. (Im, 1996; Kim et al., 1997). So many species of pine tree are grown over the world. Among them, red pine (*Pinus densiflora*) and black pine (*Pinus thunbergii*) trees are the most common trees in Korea (Song et al., 2016). In particular, the Guemgang pine trees are very famous for peculiar wood characteristics with straight trunks, red color, and thin bark, which are well-known as one of red pine grown in the Taebaeksan range through Mt. Geumgang to Chengsong and Yeongduk of Gyeongbuk province in Korea (Song et al., 2016).

Pine needles have been found to have a lot of biological properties with anti-microbial, anti-mutagenic, anti-carcinogenic, anti-inflammatory, anti-hyperlipidemic, anti-aging, anti-osteoclastic, and antioxidant activities (Choi et al., 1997; Kwak et al., 2006; Hwang et al., 2014; Jung

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et al., 2014; Venkatesan et al., 2017; Kim, 2018; Shim and Ma, 2018). Pine needles contain various kinds of valuable phytochemicals, such as phenolic acids, flavonoids, proanthocyanidins, lignans, phytosterols, resin, and essential oils (Zhang et al., 2011; Karapandzova et al., 2019; Metsämuuronen and Sirén, 2019). Of flavonoids, several flavonol glycosides, acetylated-, and acylated-flavonol glycosides showing antioxidant, antimicrobial, and anti-inflammatory actions have been reported in pine needles (Otsuka et al., 2008; Kwon et al., 2010; Karapandzova et al., 2019). In particular, the acylated flavonol glycosides including kaempferol- and quercetin 3-*O*-(6′-coumaroyl or feruloyl)-rutinosides have been identified as predominant active components in pine needles (Karapandzova et al., 2019; Metsämuuronen and Sirén, 2019). However, few studies are available on isolation and characterization of flavonol glycosides from domestic red pine (*Pinus densiflora*) trees.

Flavonoids are most frequently found in nature as glycosides, and their types and contents vary with varieties, cultivation, harvest, storage, and processing (DuPont et al., 2000; Häkkinen et al., 2000; Hallmann and Rembiałkowska, 2012). Recent studies reported that major flavonoid compositions and concentrations are considerably different from pine tree species, and the tree parts and ages (Karapandzova et al., 2019; Metsämuuronen and Sirén, 2019). In Scots pine (*Pinus sylvestris*), the most abundant flavonoids are flavonol- and dihydroflavonol-type flavonoids, such as kaempferol, quercetin, and taxifolin and their derivatives. In Norway spruce (*Pinus abies*), the main flavonoids are quercetin and myricetin. In addition, two major *Pinus* species in Korea, red pine (*Pinus densiflora*) and black pine (*Pinus thunbergii*), have been reported to have different flavan 3-ols composition and content (Jung et al., 2003). Pine needles contain flavonol glycosides and acylated flavonol glycosides, while pine barks possess dihydroflavonol and flavan 3-ols (Naeem et al., 2010; Maimoona et al., 2011). To date, much research on flavonoid analysis from foreign pine trees have been performed. However, characterization and quantitation of flavonoids in domestic pine trees are still limited.

The objective of this study was to isolate and identify major flavonoids from Geumgang red pine needles, and further determine their content by high-performance liquid chromatography (HPLC) according to cultivars, harvest seasons, and thermal processes.

MATERIALS AND METHODS

Materials and reagents

Geumgang pine (*Pinus densiflora* Sieb. At Zucc., red pine) from the lower branches of a single 15～18 year old tree was harvested in the mountains at an elevation of 580 m, Uljin, Gyeongbuk, Korea. The successive harvestings occurred in May (spring), August (summer), October (autumn), and January (winter) in 2020. Haesong pine (*Pinus thunbergii* Parlatore, black pine) was also harvested in the seashore of Sokcho, Uljin, and Yeongduk, Korea. Two pine trees were identified by President in Southern Regional Office of Forest Service, National Institute Forest Science (Uljin, Korea), and a voucher specimen was deposited at the Department of Food Science and Nutrition, Catholic University. The needles were separated from the branches and instantly freeze-dried. Nuclear magnetic resonance (NMR) solvents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All solvents for HPLC analysis were of Merck HPLC grade (Merck, Kenil worth, NJ, USA). All other reagents used in this study were of analytical grade.

Thermal processing

Fresh pine needles were steamed, roasted, and microwaved according to the method described previously (Lee and Choi, 2012). Freshly harvested pine needles were steamed in a domestic stainless steel steamer (Kitchenart Co., Inc., Incheon, Korea) for 3, 5, and 10 min, and cooled. Fresh pine needles were roasted in an electric roaster (Dongkwang Oil Machine Co., Seoul, Korea) with constant stirring at 180±20°C for 3, 5, and 10 min. Finally, fresh pine needles were placed in a rotating glass container (dimensions 290 mm i.d.) of a domestic microwave oven (Samsung RE-C200T, Samsung Electronics, Suwon, Korea) and heated for 3, 5, and 7 min. Three heat pretreated pine needles were dried overnight in a drying oven (JISICO J-300M, JISICO BLDG, Seoul, Korea) at 45±5°C and milled to 20 mesh by a mechanical coffee mixer. All samples (10 g) in each treatment were extracted with 80% aqueous (aq.) ethanol (EtOH, 100 mL) under an ultrasonic cleaner (Power Sonic 420, Hwashin Tech Co., Ltd., Gwangju, Korea), and HPLC analysis was performed to quantify the flavonoids.

Isolation and identification of flavonoids

Dried pine needles (5 kg) harvested in the middle of May were extracted twice with 80% aq. EtOH (100 L) at 40°C under an ultrasonic cleaner (Power Sonic HT-300 L, Hwashin Tech Co., Ltd., Gwangju, Korea) overnight. The filtrate was concentrated and yielded the dark residue (683.60 g). The residue was suspended in distilled water and then filtered to remove a sticky resin in the lower layer. The solubilized extract was successively partitioned with petroleum ether (PE), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH), and evaporated to obtain the fractions: PE (232.40 g), EtOAc (84.04 g), and *n*-BuOH (151.47 g). The EtOAc fraction was put onto a silica gel (70～230 mesh, 2.5 kg, Merck, Damstadt, Germany) column (9×60 cm), and gradiently eluted with chloroform

($CHCl_3$)-methanol (MeOH)-H_2O (10:1, 7:1, 5:1, 3:1, and 1:0.5:0.1, v/v, each volume 1.5 L) to obtain 7 fractions; fraction (Fr.) 1 (0.12 g), Fr. 2 (1.34 g), Fr. 3 (3.97 g), Fr. 4 (4.03 g), Fr. 5 (9.94 g), Fr. 6 (12.73 g), and Fr. 7 (17.48 g). All fractions were monitored by ultraviolet (UV)-visible (Vis) spectrophotometer and HPLC was performed to ascertain flavonoids. The Fr. 5 was chromatographed on silica gel (70~230 mesh) column (4×40 cm) gradiently eluted with $CHCl_3$-MeOH (7:1, 5:1, and 3:1 v/v, each volume 1 L) to yield six fractions; Fr. 5-A (0.75 g), Fr. 5-B (2.20 g), Fr. 5-C (1.75 g), Fr. 5-D (0.76 g), Fr. 5-E (0.05 g), and Fr. 5-F (0.05 g). The Fr. 5-B and Fr. 5-D were successively chromatographed on a Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column (2×80 cm) with 90% aq. MeOH and a octadecyl-silica (ODS)-A (YMC America, Inc., Devens, MA, USA) column (3×30 cm) with 40% aq. MeOH, respectively; this yielded component (Comp.) 1 [retention time (Rt)=31.6 min, 98.28 mg] from Fr. 5-B and Comp. 2 (Rt=39.6 min, 57.80 mg) from Fr. 5-D. The Fr. 6 and Fr. 7 were also chromatographed using Sephadex LH-20 (90% aq. MeOH; Sigma Chemical Co.) and ODS-A (40% aq. MeOH; YMC America, Inc.) columns, respectively to yield Comp. 3 (Rt=25.8 min, 100.35 mg) and Comp. 4 (Rt=26.8 min, 395.46 mg) from Fr. 6, and Comp. 5 (Rt=21.5 min, 122.85 mg) from Fr. 7. A schematic procedure for isolation and purification of five flavonoids from red pine needles is presented in Fig. 1.

Identification of flavonoids

UV absorption spectra of isolated five flavonoids (in MeOH) were obtained with a photodiode array UV-Vis spectrophotometer (S-1100, Scinco, Seoul, Korea). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of flavonoids were measured in CD_3OD on a spectrometer (Jeol 500, JEOL Ltd., Tokyo, Japan), and chemical shifts are given as δ value with TMS as an internal standard. Fast-atom bombardment mass spectrometry (FAB-MS)

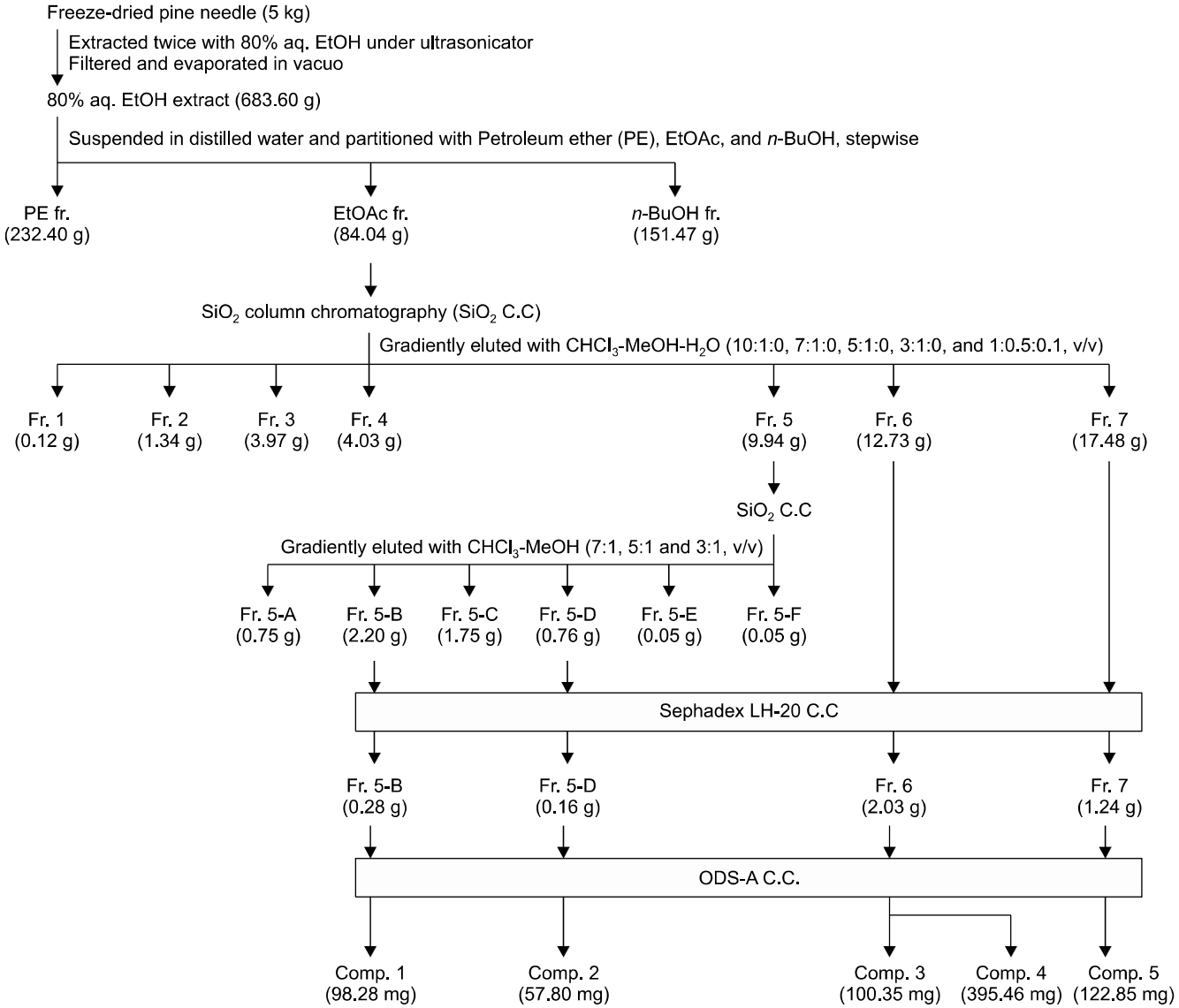


Fig. 1. Schematic procedure for isolation and purification of the five flavonol glycosides from red pine needles.

was recorded on a JMS-700 mass spectrometer (ion source, Xe atom beam; accelerating voltage, 10 kV; JEOL Ltd.), using nitrobenzyl alcohol as a mounting matrix.

Quantification of flavonoids by HPLC

Dried pine needle powder (10 g) was extracted twice with 100 mL of 80% aq. EtOH by an ultrasonic cleaner for 1 h, filtered and evaporated under reduced pressure. The EtOH extract was further redissolved in 100 mL of 80% aq. EtOH and left to stand overnight at room temperature. The upper layer was taken and brought to 100 mL with the same solvent. The aliquot was passed through 0.45 μm membrane filter (polyvinylidene fluoride syringe filter, Finetech Research and Innovation Corp., Taichung, Taiwan) and finally injected into an analytical HPLC. HPLC was performed on a Waters e2690/5 HPLC system equipped with 2998 photodiode array detector (Waters Corporation, Milford, MA, USA) at 350 nm and autosampler. HPLC analysis was carried out using a YMC-Pack Pro C_{18} column (46 mm i.d.×250 mm, YMC America, Inc.) with a Guard-Pak C18 precolumn (Waters Corporation) insert. The separation was conducted using a linear gradient (0～10 min: 10～20% B, 10～15 min: 20～20% B, 15～30 min: 20～30% B, 30～35 min: 30～40% B, 35～40 min: 40～50% B, 40～50 min: 50～60% B, 50～55 min: 60～90% B, and 55～60 min: 90～100% B) of two solvent systems: solvent A, 0.05% H_3PO_4 in H_2O; solvent B, CH_3CN at a flow rate of 0.8 mL/min. Individual flavonoids were identified by a comparison of their Rt. with those of the five standard flavonoids isolated previously. Linear correlation coefficients were superior to 0.995 for each flavonoid. Levels of flavonoids were determined by calibration curves of five standard flavonoids [3-*O*-β-D-glucoside (QG): y=1.5173x+1.0044, kaempferol 3-*O*-β-D-glucoside (KG): y=1.3803x+4.3434, quercetin 3-*O*-(6″-*O*-acetyl)-β-D-glucoside (QAG): y=1.1311x +0.1397, kaempferol 3-*O*-(6″-*O*-acetyl)-β-D-glucoside (KAG): y=1.1177x+0.2087, and quercetin 3-*O*-(3″-*O*-*p*-coumaroyl)-β-D-glucoside (QCG): y=1.2033x+0.4858] and expressed as mg per 100 g of dried weight of pine needles. Recovery rates of five flavonoids were above 95%. The typical HPLC profiles of the five standard flavonoids and the 80% aq. EtOH extract of pine needles are shown in Fig. 2.

Statistical analysis

All data were expressed as the mean±standard deviation (SD) of three determinations and their statistical analyses were performed using IBM SPSS Statistics 19.0 software (IBM Corp., Armonk, NY, USA). The significant difference ($P<0.05$) between the means was identified by using one-way ANOVA followed by Duncan's multi-range test.

RESULTS AND DISCUSSION

Isolation and identification of five flavonol glycosides from red pine needles

Five flavonol glycosides were isolated and purified from

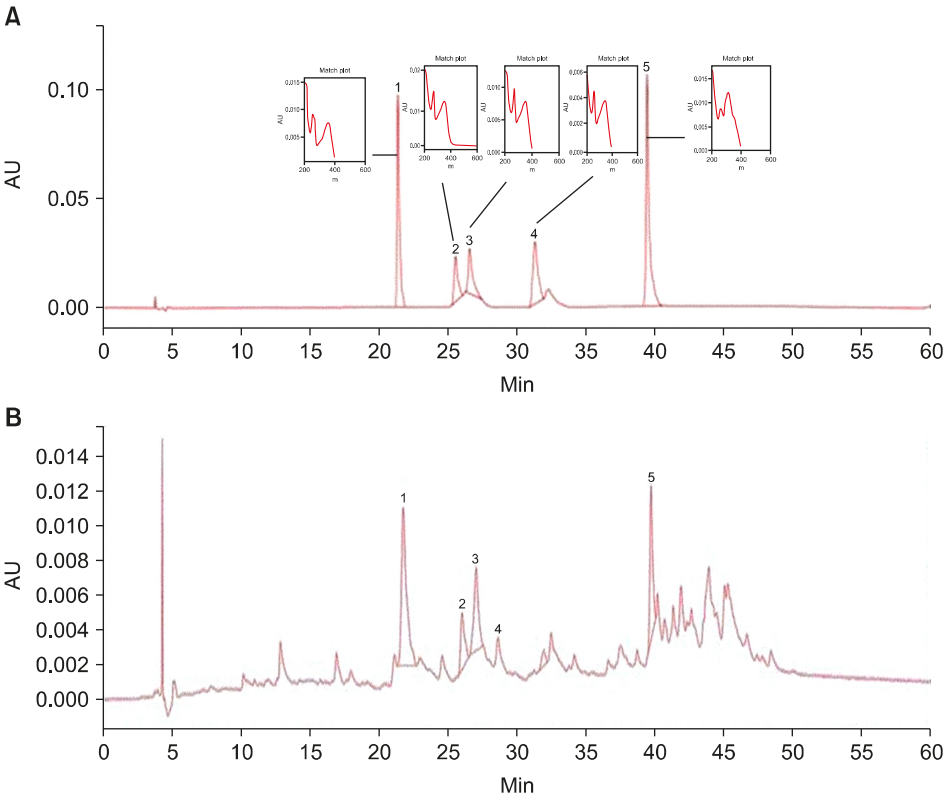


Fig. 2. HPLC chromatograms of the five standard flavonol glycosides (A) and the ethanolic extract (B) of Geumgang red pine needles.

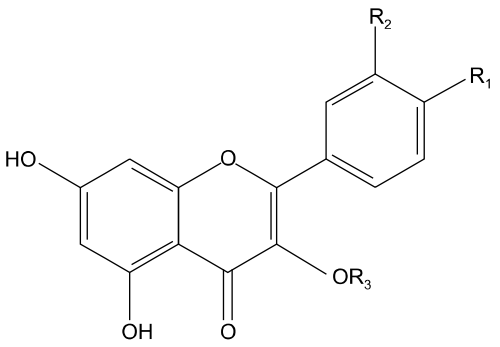
red pine needles by a series of isolation procedures including silica gel, ODS-A, and Sephadex LH-20 column chromatograhy. The structures of the five flavonoids were identified by MS and NMR spectrometry, and by comparison of published spectral data. Comp. 5 and Comp. 3 were easily characterized as QG (isoquercitrin) and KG (astragalin), respectively, which have already been found in pine needles (Karapandzova et al., 2019; Metsämuuronen and Sirén, 2019) and mulberry leaves (Lee and Choi, 2012). Comp. 4 showed a protonated molecule

Table 1. ^{1}H- and ^{13}C-NMR spectral data of three flavonol glycosides isolated from Geumgang red pine needles

Position	QAG	KAG	QCG
^{1}H-NMR			
H-6	6.21 (H, d, J=2.0 Hz)	6,21 (H, d, J=2.0 Hz)	6.21 (H, d, J=2.0 Hz)
H-8	6.40 (H, d, J=2.0 Hz)	6.44 (H, d, J=2.0 Hz)	6.40 (H, d, J=2.0 Hz)
H-2′	7.62 (H, d, J=2.0 Hz)	8.00 (H, d, J=8.9 Hz)	7.71 (H, d, J=2.0 Hz)
H-3′		6,87 (H, d, J=8.9 Hz)	
H-5′	6.84 (H, d, J=2.0 Hz)	6.87 (H, d, J=8.9 Hz)	6.89 (H, d, J=2.0 Hz)
H-6′	7.60 (H, dd, J=2.0, 8.6 Hz)	8.00 (H, d, J=8.9 Hz)	7.59 (H, dd, J=2.0, 8.3 Hz)
H-1″	5.11 (H, d, J=7.7 Hz)	5.35 (H, d, J=7.5 Hz)	5.42 (H, d, J=8.0 Hz)
H-3″			5.12 (H, t, J=9.3 Hz)
H-2″~H-5″	3.30~3.48	3.19~3.60	3.35~3.72
H-6″a	4.06 (H, m)	3.96 (H, m)	3.73 (H, m)
H-6″b	4.17 (H, m)	4.09 (H, m)	3.61 (H, m)
H-2‴			6.43 (H, d, J=16.0 Hz)
H-3‴			7.69 (H, d, J=16.0 Hz)
H-5‴ and H-9‴			7.48 (H, d, J=8.3 Hz)
H-6‴ and H-8‴			6.81 (H, d, J=8.3 Hz)
Acetyl H	1.84	1.74	
^{13}C-NMR			
C-2	158.54	156.35	158.61
C-3	135.57	133.30	135.66
C-4	179.50	177.40	179.56
C-5	163.09	160.92	163.22
C-6	100.09	98.67	100.05
C-7	166.26	164.31	166.17
C-8	94.93	93.72	94.86
C-9	159.48	156.50	159.06
C-10	104.59	103.75	105.85
C-1′	123.18	120.80	123.34
C-2′	117.54	130.90	116.90
C-3′	149.92	114.98	150.04
C-4′	146.01	159.90	146.08
C-5′	115.94	114.98	116.98
C-6′	123.51	130.90	123.16
C-1″	104.31	101.11	104.07
C-2″	75.76	72.70	74.27
C-3″	78.04	76.97	79.11
C-4″	71.46	69.66	69.65
C-5″	75.87	73.90	78.45
C-6″	64.41	63.12	62.38
C-1‴			169.18
C-2‴			116.21
C-3‴			146.82
C-4‴			127.46
C-5‴ and C-9‴			11.31
C-6‴ and C-8‴			116.97
C-7‴			161.40
Acetyl (C=O)	172.85	169.84	168.74
Acetyl (CH_3)	20.62	20.17	25.47
Carboxyl (COOH)			169.07

Chemical shift in δ ppm, coupling constant (*J*) expressed in Hz in parenthesis and measured in the solvent CD_3OD-d_6, TMS used as an internal standard.

$[M+H]^+$ at *m/z* 507 in the positive FAB-MS spectrum, together with a fragment ion peak at *m/z* 303 [M^+-204 (acetylglucose)], indicating the presence of a quercetin skeleton linked to an acetylglucose moiety. The ^{1}H- and ^{13}C-NMR spectra of Comp. 4 showed quercetin acetylglucoside, which the acetyl group was attached to the C-6″ of the glucosyl moiety. This fact was secured by heteronuclear multiple bond correlation (HMBC) cross peak between H-6″ (δ4.06, 4.17) of glucose moiety and the acetyl ketone carbon (δ172.85). Additionally, the HMBC spectrum showed a correlation between the proton at H-1″ (δ5.11) and C-3 (δ135.57), indicating glucose should be attached to C-3 of the quercetin skeleton. From these results, Comp. 4 was identified as quercetin 3-*O*-(6″-*O*-acetyl)-β-D-glucopyranoside. In contrast, Comp. 1 showed a protonated molecule $[M+H]^+$ at *m/z* 491 in the positive FAB-MS spectrum, together with a fragment ion peak at *m/z* 287 [M^+-204 (acetylglucose)], implying that this compound was kaempferol acetylglucoside. ^{1}H- and ^{13}C-NMR spectral data of Comp. 1 were very similar to those of Comp. 4 except for quercetin skeleton of Comp. 5. Thus, Comp. 1 was easily elucidated to be kaempferol 3-*O*-(6″-*O*-acetyl)-β-D-glucopyranoside. Comp. 2 showed a protonated molecule $[M+H]^+$ at *m/z* 611 in the positive FAB-MS spectrum, together with two significant fragment ion peaks at *m/z* 447 [M^+-164 (*p*-coumaric acid)] and 303 [447-144 (hexose)], which was attributed to quercetin coumaroyl glucoside. The ^{1}H- and ^{13}C-NMR spectra of Comp. 2 exhibited quercetin acylated glycoside, which *p*-coumaric acid [H-2‴ (δ6.43), H-3‴ (δ7.69), H-5‴ and H-9‴ (δ7.48), H-6‴ and H-8‴ (δ6.81), C-1‴ (δ169.18), C-2‴ (δ116.21), C-3‴ (δ146.82), C-4‴ (δ127.46), C-5‴ and C-9‴ (δ131.31), C-6‴ and C-8‴ (δ116.97), and C-7‴ (δ161.40)] was attached to the C-3″ of the glucosyl moiety. This fact was supported by HMBC cross peak between H-3″ (δ5.12) of glucose moiety and C-1‴ (δ169.18) of *p*-coumaroyl ketone moiety, and the proton (H-3″, δ5.12) and carbon [(C-2″, δ74.27), (C-4″, δ69.65)] of the glucose moiety. From MS and NMR spectral analysis, Comp. 2 was characterized as QCG for the first time in pine needles, although the three flavonoid (Comp. 1, 2, and 4) derivatives have been previously elucidated in pine needles (Karapandzova et al., 2019; Metsämuuronen and Sirén, 2019). Furthermore, acetylated and acylated flavonol glycosides, such as Comp. 1, 2, and 4, were found to exert strong antioxidant and antibacterial activities (Otsuka et al., 2008; Karapandzova et al., 2019). Additionally, Metsämuuronen and Sirén (2019) isolated and identified acylated flavonol rutinosides, such as quercetin-3-*O*-(6″-*O*-*p*-coumaroyl)-rutinoside (QCR) and kaempferol-3-*O*-(6″-*O*-feruloyl)-rutinoside (KFR), from pine needles, of which QCR was the major abundant flavonol glycoside. Kaffarnik et al. (2005) previously reported that flavonol 3-*O*-glucosides esterified with ferulic or *p*-coumaric acid at positions 3″ and 6″ are the major UV-B screening pigments of the epidermal layer of Scots pine needles. Thus, we first isolated and identified the five flavonol glycosides from Geumgang red pine needles, of which QCG was found to be major flavonoid in red pine needles grown in Korea. The detailed ^{1}H- and ^{13}C-NMR spectral data of the three flavonol glycosides are given in Table 1. The chemical structures of the five flavonol glycosides from red pine needles are presented in Fig. 3.



	R_1	R_2	R_3
Comp. 1	OH	H	6"-Acetyl-glucoside
Comp. 2	OH	OH	3"-*p*-coumaroyl-glucoside
Comp. 3	OH	H	Glucoside
Comp. 4	OH	OH	6"-Acetyl-glucoside
Comp. 5	OH	OH	Glucoside

Fig. 3. Chemical structures of the five flavonol glycosides isolated from red pine needles.

Quantitative change of five flavonoids according to cultivars, growing district, harvest seasons, and thermal processes

Quantification of the five flavonoids in red pine needles was performed by HPLC analysis, as previously described in the MATERIALS AND METHODS. As shown in Fig. 2, the five flavonol glycosides were clearly detected from the 80% aq. EtOH extract from red pine needles by comparison with retention time of each standard flavonoid isolated previously: QG (Rt: 21.5 min), KG (Rt: 25.8 min), QAG (Rt: 26.8 min), KAG (Rt: 31.6 min), and QCG (Rt: 39.6 min). The HPLC profiles of the Geumgang red pine needles was similar to that of the Haesong black pine needles (data not shown), but levels of flavonoid between the two pine cultivars was considerably different. As shown in Table 2, Geumgang red pine needles possessed five flavonol glycosides, while Haesong black pine needles had very small amounts of four flavonol glycosides except for QCG. In general, black pine needles (25.46～40.28 mg/100 g) have higher amounts of QCG than red pine needles (25.54～27.03 mg/100 g) ($P<0.05$). QCG level of black pine needle was significantly higher in the Sokcho than in the Uljin and Yeongduk ($P<0.05$). In addition in the Uljin location the concentra-

Table 2. Quantitative changes of the five flavonol glycosides from pine needles harvested in May according to cultivars and growing districts (unit: mg/100 g, dry weight)

Cultivar growing	District	Flavonol glycoside				
		QG	KG	QAG	KAG	QCG
Red pine (*Pinus densiflora*)						
	Uljin	18.35±2.10^{a}	6.46±0.50^{a}	16.27±1.42^{b}	6.13±0.42^{a}	27.03±1.22^{b}
	Cheongsong	20.31±0.80^{a}	5.72±0.49^{a}	18.12±1.84^{b}	5.92±0.71^{a}	26.34±1.70^{b}
	Gyeongsan	14.84±0.88^{b}	5.94±0.47^{a}	22.49±1.91^{a}	5.73±0.40^{a}	25.54±1.61^{b}
Black pine (*Pinus thunbergii*)						
	Sokcho	Tr	Tr	Tr	ND	40.28±3.02^{a}
	Uljin	Tr	Tr	Tr	ND	35.86±2.81^{a}
	Yeongduk	Tr	Tr	Tr	ND	25.46±1.59^{b}

QG, quercetin 3-*O*-β-D-glucoside; KG, kaempferol 3-*O*-β-D-glucoside; QAG, quercetin 3-*O*-(6″-*O*-acetyl)-β-D-glucoside; KAG, kaempferol 3-*O*-(6″-*O*-acetyl)-β-D-glucoside; QCG, quercetin 3-*O*-(3″-*O*-*p*-coumaroyl)-β-D-glucoside.
Data are mean±SD of triplicate determinations.
Values with different superscript letters (a,b) within a column indicate significant differences (P<0.05) by Duncan's multiple range test.
ND, not detected; Tr, trace (<0.1 mg/100 g, dry weight).

tions were higher than in Yeongduk (P<0.05). There are no big differences in flavonoid composition and content among red and black pine needles grown in three different areas, respectively.

On the other hand, levels of total flavonoid and five different flavonol glycosides of Geumgang red pine needles were determined by four harvest seasons (Table 3). Total flavonoid concentration was significantly higher (P< 0.05) in Autumn (96.50±1.44 mg/100 g) than in other seasons (Spring: 74.24±1.13 mg, Summer: 84.91±1.18 mg, and December: 58.72±0.81 mg/100 g). Additionally, levels of two flavonol glycosides, QG and KG, decreased gradually as harvest period went from Spring (18.35 and 6.46 mg/100 g dry weight, respectively) to Winter (11.28 and 0.95 mg/100 g, respectively). The acylated flavonol glycoside, QCG, increased steadily from 27.03 to 46.15 mg/100 g through May to October. The contents of two acetyl flavonol glycosides, QAG and KAG, increased gradually from 16.27 and 6.13 to 25.54 and 9.71 mg/100 g by Autumn, respectively, and then decreased slowly for Winter (10.84 and 5.03 mg/100 g, respectively). Thus, pine needles showed different levels and compositions of flavonoids according to cultivars, growing region, and harvest season. These results were consistent with an earlier report that levels of flavonoid in pine needles are affected by variety, harvesting time, and growing district (Routa et al., 2017). It is interesting to note that several acylated flavonol glycosides like QCG were major predominant flavonoids in black pine needles native to eastern seashores of Korea, supporting that the acylated flavonol glycosides, such as quercetin-3-*O*-(6″-*O*-feruloyl)-glucoside, quercetin-3-*O*-(6″-*O*-feruloyl)-rutinoside, kaempferol-3-*O*-(3″,6″-*O*-di-*p*-coumaroyl)-glucoside, kaempferol-3-*O*-(3″-*O*-*p*-coumaroyl, and 6″-*O*-feruloyl)-glucoside, were the most abundant flavonol glycosides in Scots and Macedonia pines (Karapandzova et al., 2019; Metsämuuronen and Sirén, 2019). In addition, the acylated flavonol glycoside derivatives in pine needles were recently found to be anti-methicillin resistant *Staphylococcus aureus* compounds (Rauha et al., 2000; Otsuka et al., 2008). Thus, red and black pine needles from Korea has renewed interest as potential antibiotic ingredients.

Thermal processing in foods is generally used to extend the shelf life and improve food quality, but can cause changes in nutritional value and attenuate bioactive compounds (Lu et al., 2018). Recent studies demonstrated

Table 3. Quantitative changes of the five flavonol glycosides from Uljin Geumgang red pine needles according to harvest seasons (unit: mg/100 g, dry weight)

Harvest season (month)	Flavonol glycoside				
	QG	KG	QAG	KAG	QCG
Spring (May)	18.35±2.10^{a}	6.46±0.50^{a}	16.27±1.42^{c}	6.13±0.42^{c}	27.03±1.22^{d}
Summer (August)	15.79±1.05^{a}	4.26±0.31^{b}	20.38±1.28^{b}	7.49±0.55^{b}	36.99±2.71^{b}
Autumn (October)	13.44±0.84^{b}	1.66±0.16^{c}	25.54±2.49^{a}	9.71±0.70^{a}	46.15±3.02^{a}
December (January)	11.28±0.78^{b}	0.95±0.15^{c}	10.84±1.48^{d}	5.03±0.38^{c}	30.62±1.27^{c}

QG, quercetin 3-*O*-β-D-glucoside; KG, kaempferol 3-*O*-β-D-glucoside; QAG, quercetin 3-*O*-(6″-*O*-acetyl)-β-D-glucoside; KAG, kaempferol 3-*O*-(6″-*O*-acetyl)-β-D-glucoside; QCG, quercetin 3-*O*-(3″-*O*-*p*-coumaroyl)-β-D-glucoside.
Data are mean±SD of triplicate determinations.
Values with different letters (a-d) within a column indicate significant differences (P<0.05) by Duncan's multiple range test.

Table 4. Quantitative changes of the five flavonol glycosides from red pine needles by three different thermal processes (unit: mg/100 g, dry weight)

Thermal process	Time (min)	Flavonol glycoside				
		QG	KG	QAG	KAG	QCG
Control		18.16±1.22^{cd}	6.47±0.48^{d}	16.76±1.61^{d}	6.14±0.55^{c}	27.27±1.30^{c}
Roasting						
	3	25.97±1.37^{a}	19.43±1.20^{b}	35.75±2.20^{b}	10.76±1.02^{b}	38.42±2.90^{ab}
	5	18.75±1.02^{cd}	10.78±0.87^{c}	32.67±1.85^{c}	8.90±0.98^{bc}	37.35±2.80^{ab}
	10	16.56±1.11^{d}	6.98±0.68^{d}	30.04±2.02^{c}	7.34±0.67^{c}	34.76±1.89^{b}
Steaming						
	3	24.08±1.47^{ab}	23.98±1.04^{a}	39.57±2.21^{a}	12.56±1.28^{a}	38.40±2.30^{ab}
	5	22.87±1.39^{b}	21.07±1.09^{ab}	37.39±1.78^{ab}	12.67±1.32^{a}	39.86±2.40^{a}
	10	19.53±1.29^{c}	19.75±1.35^{b}	35.92±1.89^{b}	9.50±1.03^{b}	40.49±1.63^{a}
Microwaving						
	3	23.01±1.28^{ab}	17.93±0.93^{b}	32.74±1.81^{c}	9.63±1.42^{b}	38.04±2.98^{ab}
	5	25.90±1.39^{a}	18.32±1.21^{b}	31.96±1.20^{c}	9.57±1.37^{b}	38.39±1.39^{ab}
	7	23.71±1.34^{ab}	17.63±0.86^{b}	32.94±1.89^{c}	9.77±1.40^{b}	37.24±1.64^{ab}

QG, quercetin 3-*O*-β-D-glucoside; KG, kaempferol 3-*O*-β-D-glucoside; QAG, quercetin 3-*O*-(6˝-*O*-acetyl)-β-D-glucoside; KAG, kaempferol 3-*O*-(6˝-*O*-acetyl)-β-D-glucoside; QCG, quercetin 3-*O*-(3˝-*O*-*p*-coumaroyl)-β-D-glucoside.
Data are mean±SD of triplicate determinations.
Values with different letters (a-d) within a column indicate significant differences (P<0.05) by Duncan's multiple range test.

that heat processing enhanced the functional constituents including phenolics and lycopene, and increased antioxidant activity in fruits and vegetables by a variety of physico-chemical changes (Dewanto et al., 2002a; Dewanto et al., 2002b; Turkmen et al., 2005). We examined the effects of thermal processing including roasting, steaming, and microwaving on functionality of pine needles through HPLC analysis. As shown in Table 4, most heat processing methods, except for a microwaving, moderately increased flavonoid levels for the short 3 min heating, but then these levels decreased steadily for the 10 min treatment. In particular, steaming and roasting processes significantly increased KG and KAG contents about 3.0～3.7 and 1.8～2.0 times, respectively, as compared to control (untreated) (P<0.05). Roasting and steaming treatments for a short time facilitate extracting slightly more soluble flavonoid in pine needles by splitting the tissue during heating. However, microwaving moderately increased levels of flavonoids for the short heating time (3 min), and remained at a constant level for 7 min as compared to other heating processes. Particularly, QCG did not show any less degradation at the long thermal treatment in comparison to other flavonoids. The increase in flavonoid content for the microwave-treated pine needles could be due to easier solubilization of flavonoids bound within cell tissues (Ashraf et al., 2012). It is noteworthy that the heat processes are effective for enhancing the levels of phytochemical flavonoids in pine needles. These results support earlier reports that the thermal treatment increased flavonoid content in safflower leaf and mulberry leaf (Lee et al., 2005; Lee and Choi, 2012). Some researchers offered a possible explanation for the heat-induced increase in levels of phenolic compounds in foods, indicating that the thermal treatment releases and degrade bound phenolic compounds in cell walls (Dewanto et al., 2002b; Rakić et al., 2007). However, Ismail et al. (2004) found that thermal treatment decreased the total phenolic content in some vegetables. Thus, the thermal processing increased or decreased antioxidant phenolics depending on the type of plants but not type of cooking. It is necessary to consider an appropriate heating method for improving quality of pine needles before manufacturing of processed products.

In conclusion, we first isolated and identified the five flavonol glycosides from Geumgang red pine needles. Their content and composition were considerably influenced by cultivars, harvest times, and heat processes. Specifically, a suitable heat treatment was found to raise functionality of Geumgang red pine needle through enhancing bioactive flavonoid content. This study provides basic information for establishing standardization and commercialization of Geumgang pine needle extracts. Further study on screening the biological properties of isolated flavonol glycosides from red pine needles is now in progress.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

Ashraf S, Saeed SMG, Sayeed SA, Ali R. Impact of microwave treatment on the functionality of cereals and legumes. Int J Agric Biol. 2012. 14:356-370.

Choi MY, Choi EJ, Lee E, Rhim TJ, Cha BC, Park HJ. Antimicrobial activities of pine needle (*Pinus densiflora Seib et Zucc*.) extract. Kor J Appl Microbiol Biotechnol. 1997. 25:293-297.

Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem. 2002a. 50:3010-3014.

Dewanto V, Wu X, Liu RH. Processed sweet corn has higher antioxidant activity. J Agric Food Chem. 2002b. 50:4959-4964.

DuPont MS, Mondin Z, Williamson G, Price KR. Effect of variety, processing, and storage on the flavonoid glycoside content and composition of lettuce and endive. J Agric Food Chem. 2000. 48:3957-3964.

Graf BA, Milbury PE, Blumberg JB. Flavonols, flavones, flavanones, and human health: epidemiological evidence. J Med Food. 2005. 8:281-290.

Häkkinen SH, Kärenlampi SO, Mykkänen HM, Törrönen AR. Influence of domestic processing and storage on flavonol contents in berries. J Agric Food Chem. 2000. 48:2960-2965.

Hallmann E, Rembiałkowska E. Characterisation of antioxidant compounds in sweet bell pepper (*Capsicum annuum* L.) under organic and conventional growing systems. J Sci Food Agric. 2012. 92:2409-2415.

Hollman PC, Katan MB. Dietary flavonoids: intake, health effects and bioavailability. Food Chem Toxicol. 1999. 37:937-942.

Hwang YJ, Wi HR, Kim HR, Park KW, Hwang KA. *Pinus densiflora* Sieb. et Zucc. alleviates lipogenesis and oxidative stress during oleic acid-induced steatosis in HepG2 cells. Nutrients. 2014. 6:2956-2972.

Im WK. Plant resource. Seoil Publishers, Seoul, Korea. 1996. p 59-63.

Ismail A, Marjan ZM, Foong CW. Total antioxidant activity and phenolic content in selected vegetables. Food Chem. 2004. 87: 581-586.

Jung HY, Shin JC, Park SM, Kim NR, Kwak W, Choi BH. *Pinus densiflora* extract protects human skin fibroblasts against UVB-induced photoaging by inhibiting the expression of MMPs and increasing type I procollagen expression. Toxicol Rep. 2014. 1:658-666.

Jung MJ, Chung HY, Choi JH, Choi JS. Antioxidant principles from the needles of red pine, *Pinus densiflora*. Phytother Res. 2003. 17:1064-1068.

Kaffarnik F, Heller W, Hertkorn N, Sandermann H Jr. Flavonol 3-*O*-glycoside hydroxycinnamoyltransferases from Scots pine (*Pinus sylvestris* L.). FEBS J. 2005. 272:1415-1424.

Karapandzova M, Stefkov G, Karanfilova IC, Panovska TK, Stanoeva JP, Stefova M, et al. Chemical characterization and antioxidant activity of mountain pine (*Pinus mugo* Turra, Pinaceae) from Republic of Macedonia. Rec Nat Prod. 2019. 13:50-63.

Kim CM, Shin MG, Ahn DG, Lee GS. Encyclopedia of oriental herbal medicine. Jungdam, Seoul, Korea. 1997. p 2488-2489.

Kim JS. Evaluation of *in vitro* antioxidant activity of the water extract obtained from dried pine needle (*Pinus densiflora*). Prev Nutr Food Sci. 2018. 23:134-143.

Knekt P, Jarvinen R, Reunanen A, Maatela J. Flavonoid intake and coronary mortality in Finland: a cohort study. BMJ. 1996. 312: 478-481.

Kwak CS, Moon SC, Lee MS. Antioxidant, antimutagenic, and antitumor effects of pine needles (*Pinus densiflora*). Nutr Cancer. 2006. 56:162-171.

Kwon JH, Kim JH, Choi SE, Park KH, Lee MW. Inhibitory effects of phenolic compounds from needles of *Pinus densiflora* on nitric oxide and PGE2 production. Arch Pharm Res. 2010. 33: 2011-2016.

Lee JY, Park KS, Choi SW. Changes in flavonoid contents of safflower leaf during growth and processing. J Food Sci Nutr. 2005. 10:1-5.

Lee WJ, Choi SW. Quantitative changes of polyphenolic compounds in mulberry (*Morus alba* L.) leaves in relation to varieties, harvest period, and heat processing. Prev Nutr Food Sci. 2012. 17:280-285.

Liu RH. Health benefits of dietary flavonoids: flavonols and flavones. NY Fruit Q. 2002. 10:21-24.

Lu Q, Peng Y, Zhu C, Pan S. Effect of thermal treatment on carotenoids, flavonoids and ascorbic acid in juice of orange cv. Cara Cara. Food Chem. 2018. 265:39-48.

Maimoona A, Naeem I, Saddiqe Z, Ali N, Ahmed G, Shah I. Analysis of total flavonoids and phenolics in different fractions of bark and needle extracts of *Pinus roxburghii* and *Pinus wallichiana*. J Med Plants Res. 2011. 5:2724-2728.

Metsämuuronen S, Sirén H. Bioactive phenolic compounds, metabolism and properties: a review on valuable chemical compounds in Scots pine and Norway spruce. Phytochem Rev. 2019. 18:623-664.

Naeem I, Taskeen A, Mubeen H, Maimoona A. Characterization of flavonols present in barks and needles of *Pinus wallichiana* and *Pinus roxburghii*. Asian J Chem. 2010. 22:41-44.

Otsuka N, Liu MH, Shiota S, Ogawa W, Kuroda T, Hatano T, et al. Anti-methicillin resistant *Staphylococcus aureus* (MRSA) compounds isolated from *Laurus nobilis*. Biol Pharm Bull. 2008. 31:1794-1797.

Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev. 2009. 2:270-278.

Rakić S, Petrović S, Kukić J, Jadranin M, Tešević V, Povrenović D, et al. Influence of thermal treatment on phenolic compounds and antioxidant properties of oak acorns from Serbia. Food Chem. 2007. 104:830-834.

Rauha JP, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, et al. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int J Food Microbiol. 2000. 56:3-12.

Routa J, Brännström H, Anttila P, Mäkinen M, Jänis J, Asikainen A. Wood extractives of Finnish pine, spruce and birch—availability and optimal sources of compounds. Nat Resour Econ Stud. 2017. 73. https://jukuri.luke.fi/handle/10024/540829

Shim KS, Ma JY. Pine needles attenuate receptor activator for nuclear factor-B ligand (RANKL)-induced trabecular bone loss by inhibiting osteoclast differentiation. Integr Med Res. 2018. 7:374-380.

Song WY, Kwon HA, Kim BR, Shin SJ. Hemicelluloses comparison between Korean red pine and Geumgang red pin. J Korea TAPPI. 2016. 48:86-91.

Turkmen N, Sari F, Velioglu YS. The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. Food Chem. 2005. 93:713-718.

Venkatesan T, Choi YW, Lee J, Kim YK. *Pinus densiflora* needle supercritical fluid extract suppresses the expression of pro-inflammatory mediators iNOS, IL-6 and IL-1β, and activation of inflammatory STAT1 and STAT3 signaling proteins in bacterial lipopolysaccharide-challenged murine macrophages. Daru. 2017. 25:18. https://doi.org/10.1186/s40199-017-0184-y

Zhang JM, Shi XF, Ma QH, He FJ, Fan B, Wang DD, et al. Chemical constituents from pine needles of *Cedrus deodara*. Chem Nat Compd. 2011. 47:272-274.