OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2018.

Research Article
Characterization and Quantification of Taxifolin Related Flavonoids in *Larix olgensis* Henry Var. koreana Nakai Extract Analysis and its Antioxidant Activity Assay

[1]Shengxue Zhou, [2,3]Ying Shao, [3]Jinghui Fu, [4]Lan Xiang, [2,3]Yinan Zheng and [3]Wei Li

[1]College of Chinese medicine, Jilin Agricultural Science and Technology College, 132101 Jilin, China
[2]Jilin Sino-ROK Academy of Animal Science, 130600 Changchun, China
[3]College of Chinese Medicinal Materials, Jilin Agricultural University, 130118 Changchun, China
[4]School of Pharmaceutical Sciences, Shandong University, 250012 Jinan, China

Abstract

Background and Objective: Taxifolin or dihydroquercetin, is believed to exhibit superior activity and have great use to the food industry. The present study aimed to quantitatively and qualitatively analyze flavonoids in the extract of *Larix olgensis* Henry var. koreana Nakai (which is widely distributed in Northern China) and investigate its antioxidant activity. **Methodology:** Flavonoid identification was performed using high performance liquid chromatography-mass spectrum/mass spectrum (HPLC-MS/MS) and high performance liquid chromatography-ultraviolet (HPLC-UV) analysis, revealing that the above extract primarily contained taxifolin (92.01%) and small amounts of aromadendrin, eriodictyol, quercetin and kaempferol. Statistical analyses were performed using the SPSS 17.0. **Results:** According to the antioxidant assay, the extract showed strong radical scavenging activity against the antioxidant activity were measured using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), being more potent than butylated hydroxytoluene that was used as a positive control. **Conclusion:** Thus, the extract of *Larix olgensis* Henry var. koreana Nakai contained large amounts of flavonoids and exhibited strong antioxidant activity.

Key words: Taxifolin, *Larix olgensis* Henry var. koreana Nakai, characterization, quantification, antioxidant

Received: Accepted: Published:

Citation: Shengxue Zhou, Ying Shao, Jinghui Fu, Lan Xiang, Yinan Zheng and Wei Li, 2018. Characterization and quantification of taxifolin related flavonoids in *Larix olgensis* Henry var. koreana nakai extract analysis and its antioxidant activity assay. Int. J. Pharmacol., CC: CC-CC.

Corresponding Authors: Shengxue Zhou, College of Chinese medicine, Jilin Agricultural Science and Technology College, 132101 Jilin, China
Tel: +86-18641723176
Lan Xiang, School of Pharmaceutical Sciences, Shandong University, 250012 Jinan, China Tel: +86-531-88382028

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Taxifolin or dihydroquercetin, is a light yellow powder soluble in ethanol, acetic acid and boiling water that was first isolated from the leaves of *Chamaecyparis obtusa* Endl. by Fukui, a Japanese scholar[1]. Compared to other antioxidants, taxifolin exhibits superior activity and can remarkably prolong the shelf life of lard, plant oils, powdered milk and candy. Moreover, it is not embryotoxic and does not lead to malformations, hypersusceptibility or mutations[2]. Exploiting taxifolin extracts of natural origin in terms of their potent antioxidant activity and safety is of great use to the food industry. To date, taxifolin has been detected in >50 plants, such as *Rosa davurica*[3], *Opuntia dillenii*[4], milk thistle[5], *Genista corsica*[6], *Ochna beddome*[7], *Polygonum hydropiper*[8], apple[9], *Rhododendron mucronulatum*[10] and larch[11].

Larch has received much attention in recent years as a potential source of taxifolin. This plant, which is the primary deciduous species in the Northeastern and Southwestern forests of China, belongs to the genus *Larix* (Pinaceae). According to natural distribution and artificial cultivation, larch growing in Northern China comprises five species, i.e., *Larix principis-rupprechtii*, *L. olgensis*, *L. kaempferi, L. gmelini* and *L. olgensis* var. koreana[12,13]. Exhibiting physical properties such as rigidness, straight grain and corrosion resistance, the wood of these conifers is widely used in furniture fabrication and building construction[14], with the large quantities of concomitantly generated sawdust being an important taxifolin source.

As previously reported[15], the extract of *L. gmelini* primarily contains taxifolin, together with a small amount of aromadendrin, eriodictyol, quercetin, kaempferol, naringenin and pinocembrin. The majority of these flavonoids exhibit antioxidant and bacteriostatic properties[16-19] and extracts of *L. gmelini* exhibit DPPH radical scavenging activity and exert inhibitory effects on lipid peroxidation[2,20]. Therefore, the taxifolin extract of *L. gmelini* has been used as a natural antioxidant additive in the food industry[2].

To develop new natural resources and avoid the over exploitation of *L. gmelini*, a new taxifolin source, *L. olgensis* var. koreana was determined, which is widely distributed in Northern China and investigated if the corresponding extract can replace that of *L. gmelini*.

Thus, the present study aimed to characterize flavonoids in the extract of *L. olgensis* var. koreana using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and determine the contents of taxifolin and other flavonoids using HPLC-UV analysis. The HPLC-MS/MS can provide characteristic and clear MS fragmentation patterns of analytes and has already been used for the identification and quantitation of flavonoids in many plant derived sources[21]. Additionally, it was determined the antioxidant capacity of the *L. olgensis* var. koreana extract, laying the foundation for its future development and applications.

MATERIALS AND METHODS

Materials and reagents: The wood of *L. olgensis* var. koreana collected in 2017 from Linjiangin the Jilin province of China was authenticated by Professor Yinan Zheng of the Jilin Agricultural University, with a specimen deposited in the laboratory of the same. Samples were freeze, dried and stored in glass containers at -20°C prior to experiments. Sephadex LH-20 was purchased from Beijing Ruida Henghui Science and Technology Development Co., Ltd. Representative standards of taxifolin, aromadendrin, eriodictyol, quercetin and kaempferol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The purity of the taxifolin standard was confirmed to be higher than 95% by HPLC, with purities of other standards similarly confirmed to exceed 98%. HPLC grade methanol was purchased from TEDIA Co. (Ohio, USA). Analytical grade ethanol and formic acid were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China). Polyamide resin was purchased from Cangzhou Baoen Chemical Reagent Co., Ltd. (Cangzhou, China). KBr (SP) was purchased from Shanghai Rongbai Biotechnology Co., Ltd. (Shanghai, China).

Instrumentation and equipment: The following instrumentation was used: Microplate reader (Spectra Max Plus 384, Molecular Biological Instrument Co., Ltd., CA, USA), rotary evaporators (EYELA, Rikakikai Co., Ltd., Japan), electronic balance (BP211D, Sartorius Co., Ltd., Germany), HPLC system (Accela) equipped with an autosampler, vacuum degasser unit and quaternary pump (ThermoAccela, Thermo Fisher Scientific, USA). Additionally, a mass spectrometer (ThermoFinnigan LTQ-Orbitrap XL, ThermoFinnigan, Germany) operating in positive electrospray ionization (ESI) mode was used for MS and MS/MS experiments. The ionization voltage equaled 4.2 kV and the capillary temperature was set to 275°C. Nitrogen was used as a sheath gas (40 U) and auxiliary gas (10 U). A resolving power of 15,000 and 7,500 was used for full and MS2 scans, respectively. A Shimadzu LC-2010 instrument coupled with an SPD-20A UV-detector and an LC solution workstation (Shimadzu Co., Ltd., Japan) was used for the quantitation of flavonoids and IRPrestige-21 (Shimadzu Co., Ltd., Japan) was used for their identification.

Extraction of total flavonoids: The lyophilized wood of *L. olgensis* var. koreana (200 g) was crushed into a powder and refluxed for 2 h in 80% methanol (2000 mL) 2 times. The extract was filtered and the filtrate was concentrated under reduced pressure using a rotary evaporator. For HPLC analysis, the dried extract was dissolved in methanol and filtered through a 0.45 μm membrane.

Isolation and purification of the taxifolin extract: The concentrated extract was loaded on a Sephadex LH-20 column for isolation[22]. About 0.5 g sample was dissolved in 0.5 mL of ethanol, filtered through a 0.22 μm microfiltration membrane and eluted with ethanol at a rate of 9 drops min^{-1}. The eluate was collected and concentrated for further processing.

The concentrated extract was loaded on polyamide resin for purification[23,24] and was eluted with water followed by 50% ethanol. The eluates, which contained taxifolin according to the results of thin layer chromatography analysis, were combined, concentrated, redissolved in water at 95°C and filtered. The filtrate was crystallized in a refrigerator at 4°C and subjected to multiple recrystallization. The obtained crystals were dried to afford 2.3 g of the taxifolin extract (92.07% taxifolin by HPLC).

Identification of flavonoids in the taxifolin extract: The above taxifolin extract was characterized by HPLC-MS/MS. A Phenomenex Lunar (4.6×150 mm, 5 μm) chromatographic column was chosen for LC separation. The mobile phase consisted of 0.1% (A) aqueous formic acid (B) methanol with the following gradient used: 0-2 min 35% B, 2-12 min linear increase from 35-75% B, 12-17 min linear increase from 75-95% B, 17-23 min 95% B, 23-25 min linear decrease from 95-35% B and 25-30 min 35% B. The following parameters were used: Flow rate = 0.35 mL min^{-1}, temperature = 30°C, detection wavelength = 290 nm, detection time = 30 min, injection volume = 10 μL.

For infrared (IR) spectroscopy characterization, samples were mixed with KBr at a mass ratio of 1:100, ground to uniformity in an agate mortar and pressed into tablets. Spectra were recorded at a resolution of 4 cm^{-1} between 4000 and 400 cm^{-1} with 64 scans per spectrum.

Taxifolin: Compound 1 showed molecular ion peaks at m/z 305.0673 $[M+H]^+$, 322.0939 $[M+NH_3+H]^+$ and 327.0493 $[M+Na]^+$, with MS/MS fragments observed at m/z 287.0545 $[M+H-H_2O]^+$, 259.0545 $[M+H-H_2O-CO]^+$, 195.0284 $[M+H-C_6H_6O_2]^+$ and 153.0178 $[M+H-C_6H_6O_2-C_2H_2O]^+$ allowing this compound to be identified as taxifolin ($C_{15}H_{13}O_7$, calcd. m/z 305.0661 $[M+H]^+$, $C_{15}H_{16}NO_7$, calcd. m/z 322.0927 $[M+NH_3+H]^+$; $C_{15}H_{12}NaO_7$, calcd. m/z 327.0481 $[M+Na]^+$). The following IR (KBr) peaks were observed for compound 1 (cm^{-1}): 3428, 2953, 2833, 1636, 1610, 1510, 1473, 1415, 970, 775. ^{1}H NMR (DMSO-d_6), δ: 11.89 (s, 1 H, OH-5), 10.84 (s, 1H, OH-7), 9.04 (s, 1H, OH-4), 8.99 (s, 1H, OH-3), 6.72 (d, 2H, H-5 , 6), 5.90 (d, 1H, J = 2 Hz, H-8), 5.85 (d, 1 H, J = 2 Hz, H-6), 5.75 (d, 1 H, J = 11 Hz, OH-3), 4.96 (d, 1H, J = 11 Hz, H-2), 4.48 (dd, 1H, J =11, 6.0 Hz, H-3). ^{13}C NMR (DMSO), δ: 197.68 (C4), 16.94 (C7), 163.30 (C5), 162.53 (C9), 145.75 (C3′), 144.92 (C4′), 128.02 (C1′), 119.36 (C6′), 115.33 (C5′), 115.09 (C2′), 100.40 (C10), 95.99 (C6), 95.00 (C8), 83.02 (C2), 71.54 (C3). All of these data matched those reported in literature[25], confirming the identity of compound 1 as taxifolin.

Aromadendrin: Compound 2 showed molecular ion peaks at m/z 289.0696 $[M+H]^+$ and 311.0514 $[M+Na]^+$, with aromadendrin or eriodictyol ($C_{15}H_{13}O_6$, calcd. m/z 289.0707 $[M+H]^+$, $C_{15}H_{12}NaO_6$, calcd. m/z 311.0526 $[M+Na]^+$) proposed as possible structures. Additional MS/MS analysis showed fragmentation peaks at m/z 271.0597 $[M+H-H_2O]^+$, 243.0646 $[M+H-H_2O-CO]^+$ and characteristic fragments at m/z 195.0284 $[M+H-C_6H_6O]^+$ and 153.0178 $[M+H-C_6H_6O-C_2H_2O]^+$. ^{1}H NMR (DMSO-d_6), δ: 11.89 (1H, s, 5-OH), 10.80 (1H, br s, 7-OH), 9.52 (1H, s, 4'-OH), 7.29 (2H, d, J = 8.5 Hz, H-2', 6'), 6.81(2H, d, J = 8.5 Hz, H-3', 5'), 5.77 (1H, d, J = 2.1 Hz, H-8), 5.85 (1H, d, J = 2.1 Hz, H-6), 5.73 (1H, d, J = 6.2 Hz, 3-OH), 5.02 (1H, d, J = 11.4 Hz, H-2), 4.57 (1H, dd, J = 11.4, 6.2 Hz, H-3). ^{13}C NMR (DMSO), δ: 197.40 (4-C), 167.0 (7-C), 164.1 (5-C), 163.3 (8a-C), 158.0 (4′-C), 129.4 (2′,6′-C), 128.2 (1′-C), 115.0 (3′,5′-C), 100.6 (4a-C), 96.2 (6-C), 95.2 (8-C), 83.5 (2-C), 72.2 (3-C). Based on the above data, compound 2 was identified as aromadendrin.

Eriodictyol: Compound 3 showed the same molecular weight (m/z 289.0696 $[M+H]^+$) as compound 2. However, it showed MS/MS signals at m/z 271.0596 $[M+H-H_2O]^+$, 163.0385 $[M+H-C_6H_6O_3]^+$ and characteristic fragments at m/z 179.0335 $[M+H-C_6H_6O_2]^+$ and 153.0178 $[M+H-C_6H_6O_2-C_2H_2]^+$. IR (KBr), cm^{-1}: 3396 (OH), 1637 (C = O), 1604, 1259, 1083, 823. ^{1}H NMR (DMSO-d_6), δ: 12.14 (1H, s, 5-OH), 7.37-7.45 (2H, m, H-2 , 6), 7.01 (1H, d, J = 8 Hz, H-5), 6.83 (1H, d, J = 2 Hz, H-8), 5.93 (1H, d, J = 2 Hz, H-6), 5.35 (1H, dd, H-2), 3.12 (1H, t, H-3α), 2.71 (1H, dd, H-3β). ^{13}C NMR (DMSO), δ: 43.4 (C-3), 79.8 (C-2), 95.8 (C-8), 96.6 (C-6), 102.9 (C-10), 114.5 (C-2), 115.8 (C-5), 119.0 (C-6), 131.2

(C-1), 145.9 (C-3), 146.3 (C-4), 164.2 (C-9), 164.8 (C-5), 167.5 (C-7), 197.1 (C-4)[26]. Based on the above data, compound 3 was identified as eriodictyol.

Quercetin: Compound 4 showed molecular ion peaks at m/z 303.0489 $[M+H]^+$ ($C_{15}H_{11}O_7$, calcd. m/z 303.0505 $[M+H]^+$) and 325.0336 $[M+Na]^+$ ($C_{15}H_{10}NaO_7$, calcd. m/z 325.0324 $[M+Na]^+$). IR (KBr), cm^{-1}: 1663.10 (C=O stretch), 1610.89(C=C stretch), 3408.74(C-O-H stretch) and 1382.03 (C-C stretch, OH in-plane bend). ^1H NMR (DMSO-d_6), δ: 12.48 (1H, s, 5-OH), 10.76 (1H, br s, 7-OH), 9.58 (1H, s, 3′-OH), 9.35 (1H, s, 4′-OH), 9.29 (1H, s, 3′-OH), 7.66 (1H, d, J = 2.1 Hz, H-2'), 7.53 (1H, dd, J = 8.5, 2.1 Hz, H-6'), 6.88 (1H, d, J = 8.5 Hz, H-5'), 6.39 (1H, d, J = 2.0 Hz, H-8), 6.17 (1H, d, J = 2.0 Hz, H-6). Comparison with a known standard allowed compound 4 to be identified as quercetin.

Kaempferol: Compound 5 showed molecular ion peaks at m/z 287.0566 $[M+H]^+$ and 304.2499 $[M+NH_3+H]^+$. IR (KBr), cm^{-1}: 3411.19 (OH), 2815.59 (CH), 1659.15 (C=O), 1616.45 (C=C), 1571.12 (C=C), 1380.99 (δ-OH), 1225.48, 1176.26, 1088.98, 976.674, 884.56, 797.234, 703.226, 566.847, 501.824. ^1H NMR (DMSO-d_6), d: 12.49 (1H, s, 5-OH), 8.03 (2H, d, J = 8.9 Hz, H-2', 6'), 6.92 (2H, d, J = 8.9 Hz, H-3', 5'), 6.41 (1H, d, J = 1.9 Hz, H-8), 6.17 (1H, d, J = 1.9 Hz, H-6). Comparison with a known standard allowed compound 5 to be identified as kaempferol ($C_{15}H_{11}O_6$, calcd. m/z 287.0550 $[M+H]^+$)[27].

Quantitation of flavonoids in the taxifolin extract

Preparation of sample and standard solutions: A taxifolin extract sample (10 mg) and individual standard samples were precisely weighed and dissolved in 10 mL of methanol-water (4:6) to obtain sample and standard solutions of 1 mg mL^{-1}.

Quantitation of taxifolin, aromadendrin and eriodictyol: Flavonoids present in the taxifolin extract were quantified using HPLC as an external standard method. Since the high intensity of the taxifolin peak affected the detection and quantitation of minor peaks, this compound was partially removed by preparative HPLC before quantitation of other compounds. The removed taxifolin was accounted for when calculating the mixture composition. A COSMOSIL 5C18-MS-(4.6×150 mm, 5 µm) column was used for separation, with other HPLC conditions being identical to those described in section 2.5. The described method enabled the separation of taxifolin, aromadendrin and eriodictyol, however, quercetin and kaempferol could not be separated. Therefore, quantitation of these two compounds was performed under different conditions.

Quantitation of quercetin and kaempferol: The above mentioned COSMOSIL 5C18-MS-(4.6×150 mm, 5 µm) column was used in this experiment and elution was performed using a mixture of 0.1% aqueous formic acid and methanol (63:37). The following parameters were used: Flow rate = 1 mL min^{-1}, column temperature = 25°C, detection wavelength = 360 nm, detection time = 20 min, injection volume = 20 µL.

The contents of the above five flavonoids were calculated as follows.

$$w(\%) = \frac{Co \times Ss \times Vs}{So \times M1} \quad (1)$$

Where:

C_0 = Concentration of the standard
S_s = Peak area of a given flavonoid in the test sample
V_s = Volume of the test sample
S_0 = Peak area of the standard and
M_1 = Total mass of the taxifolin extract

Methodology evaluation: Linearity, recovery, precision, repeatability and stability were evaluated to ensure the validity of this newly developed HPLC-ELSD method.The linear relationship between concentration and peak area was determined using 0.05, 0.1, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1} standard solutions. Precision was determined by measuring intraday variabilities of all standard solutions by performing consecutive injections 6 times/day. Repeatability was determined by flavonoid quantification in five identical extract samples and relative standard deviation (RSD) calculation. Flavonoid stability was evaluated by injecting the same sample solution at time points of 0, 1, 4, 8, 10 and 12 h and calculating chromatographic peak areas and RSDs. Recoveries were determined by adding standard solutions of low, medium and high concentrations (0.8, 1.0 and 1.2 µg mL^{-1}) to the taxifolin extract with known contents of the above five analytes, followed by extraction. The resulting solutions were analyzed and measurements for each concentration were performed in triplicate. The recoveries were calculated as:

$$\text{Recovery (\%)} = \frac{\text{Total amount detected-Original amount}}{\text{Spiked amount}} \times 100$$

Antioxidant activity measurement

Sample solution preparation: The taxifolin extract and butylated hydroxytoluene (BHT, positive control) were diluted to concentrations of 0.001, 0.005, 0.01, 0.05, 0.1 and 0.2 mg mL^{-1} with 70% alcohol.

Scavenging capacity against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•): A modified methodology of Luo *et al.*[28]. was used, employing a 96 well plate for rapidly determining absorbance values. DPPH (128 mg) was dissolved in anhydrous ethanol in a 50 mL volumetric flask. A 10 mL aliquot of the above solution was removed and diluted to 50 mL to yield a final concentration of 1.3×10^{-4} M. Sample (80 µL) or BHT solutions were mixed with the DPPH• solution (80 µL) and kept for 30 min in the dark at 37°C. Sample absorbance (A_{sample}) was determined at 517 nm using anhydrous ethanol as a blank. The absorbance (A_1) of sample or BHT solutions (80 µL) mixed with 70% ethanol (80 µL) was used as background absorbance. The absorbance of a mixture of DPPH• solution (80 µL) with 70% ethanol (A_0) was recorded as the total DPPH• absorbance. All measurements were performed in triplicate. The scavenging capacity (SC) against DPPH• was determined as follows.

$$SC\ (\%) = (1-\frac{A_{sample}-A_1}{A_0})\times100 \quad (2)$$

BHT was used as a reference standard antioxidant. The effective concentration required to achieve 50% scavenging was recorded as the EC_{50} value, which was determined by regression analysis of the dose-response curve plotted as inhibition percentage vs. sample concentration.

Scavenging capacity against 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{+\cdot}$): This experiment was conducted using a slight modification of a previously reported by method of Re *et al.*[29]. A stock solution of $ABTS^{+\cdot}$ was prepared by mixing 86 µL of potassium persulfate solution (2.45 mM) with 5 mL of $ABTS^{+\cdot}$ solution (7 mM) and allowing the mixture to stand in the dark for 12-16 h at ambient temperature. The $ABTS^{+\cdot}$ working solution was obtained by diluting the stock solution with 70% ethanol to a final absorbance of 0.7±0.02 at 734 nm. About 50 µL aliquot of sample or BHT solutions was mixed with 200 µL of the $ABTS^{+\cdot}$ working solution and the absorbance of the mixture at 734 nm was recorded as A_{sample}. The $ABTS^{+\cdot}$ working solution (200 µL) was mixed with 70% ethanol (50 µL) and the absorbance of the mixture was recorded as A_0. Both sample and $ABTS^{+\cdot}$ working solutions were freshly prepared. All measurements were performed in triplicate. The SC against $ABTS^{+\cdot}$ was determined according to the following equation, with EC_{50} calculated as previously described:

$$SC\ (\%) = (1-\frac{A_{sample}}{A_0})\times100 \quad (3)$$

Statistical analysis: All data were presented as Mean±SD. Statistical analyses were performed using the SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical package and significance was verified using one-way ANOVA followed by the Student's t-test. $p<0.01$ was taken as a criterion of statistical significance.

RESULTS AND DISCUSSION

Characterization of flavonoids by HPLC-MS/MS: The HPLC-MS/MS spectrum of the *L. olgensis* var. koreana extract is shown in Fig. 1, with 2 major (1, 2) and three minor peaks (3-5) identified as flavonoids based on extracted Ion chromatograms-MS/MS analysis and comparison with known standards (Fig. 2).

Quantitation of flavonoids by HPLC-UV analysis: As shown in Fig. 3, taxifolin (1), aromadendrin (2) and eriodictyol (3) could be fully separated using the COSMOSIL 5C18-MS-II (4.6×150 mm, 5 µm) column and the first set of HPLC conditions, whereas quercetin and kaempferol could not be separated under these conditions. Therefore, two compounds quercetin (4) and kaempferol (5) were separated by employing another set of HPLC conditions with 0.1% aqueous formic acid and methanol (63:37) (Fig. 4).

According to our calculations, the extract of *L. olgensis* var. koreana contained 92.01% taxifolin, 2.36% aromadendrin, 0.19% eriodictyol, 0.053% quercetin and 0.045% kaempferol.

Methodology evaluation

Linearity and range: The obtained calibration curves exhibited good linearity for concentrations between 0.05 and 1.00 mg mL^{-1} for each analyte. A detailed description of the obtained results is presented in Table 1.

Recovery, precision and repeatability: The accuracy of the developed method was confirmed by a recovery experiment, wherein five different concentrations of the five analytes were evaluated in triplicate, with recoveries (%) and RSDs shown in Table 1. The mean recoveries ranged from 98.9-102.90%, with RSDs being less than 2.11%, indicating that the established method had acceptable precision and accuracy.

The data in Table 1 demonstrated that the developed method was sufficiently accurate for detecting the above mentioned analytes. Moreover, the precision of this method

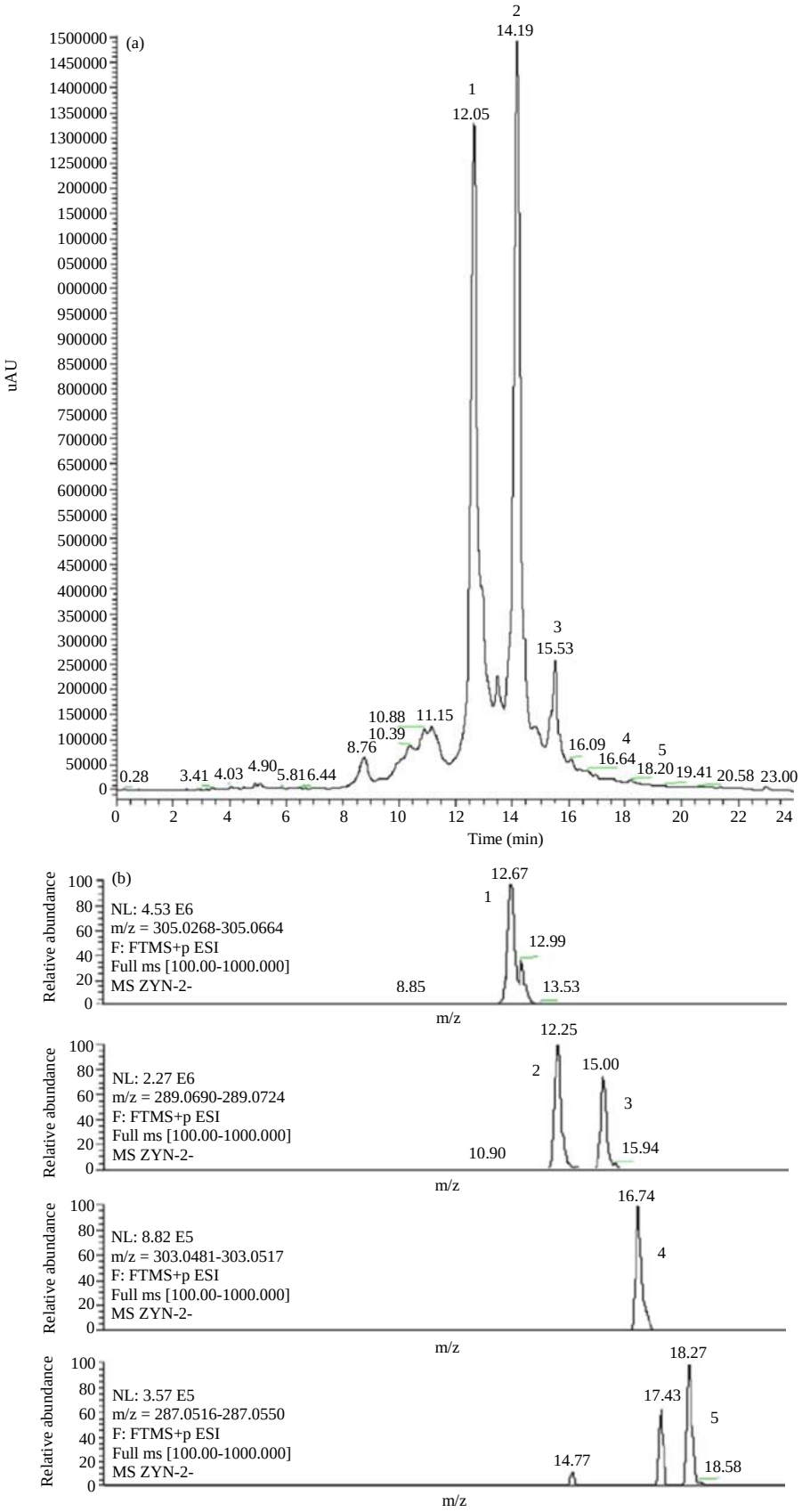

Fig. 1(a-c): Continue

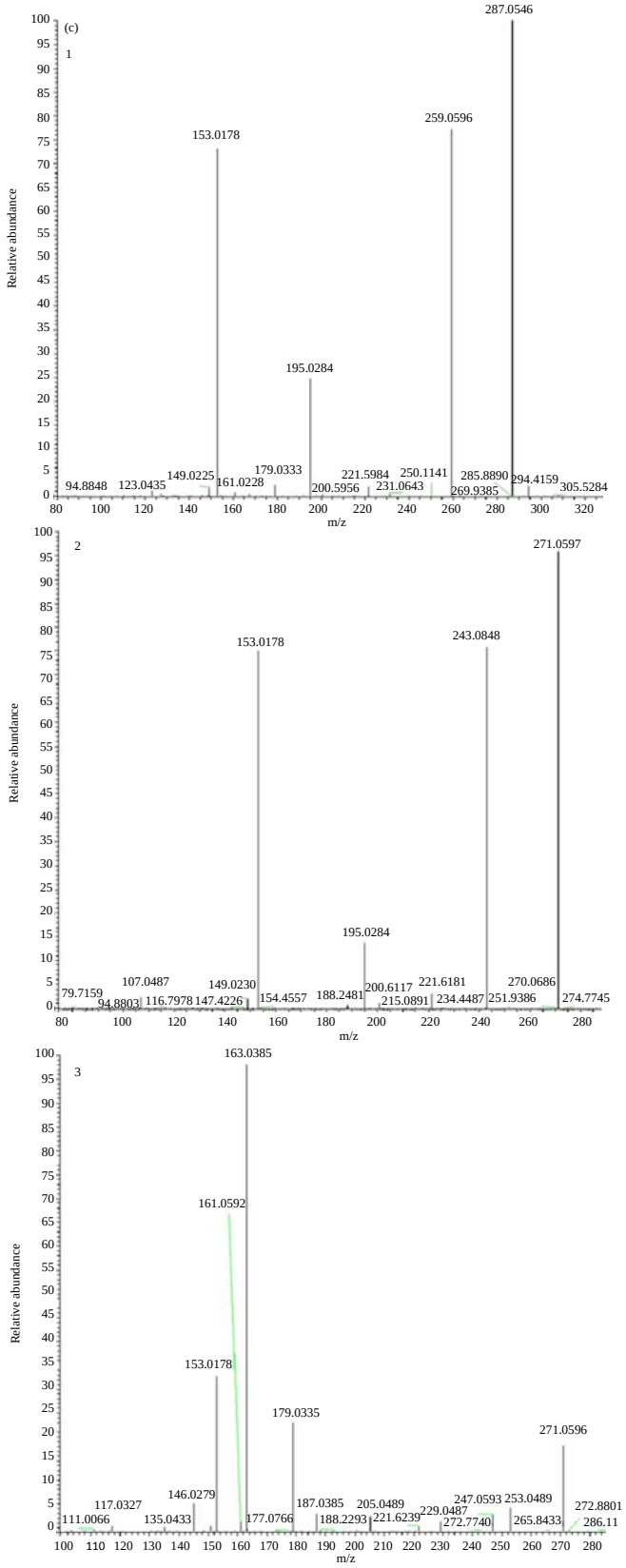

Fig. 1(a-c): HPLC-MS/MS spectra of the *L. olgensis* var. koreana extract, (a) HPLC-UV spectrum, (b) Extracted ion chromatograms of peaks 1-5 and (c) MS/MS spectra of peaks 1-3

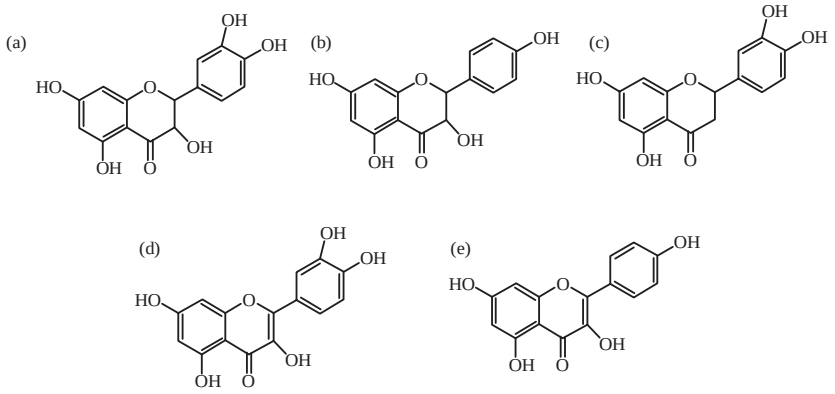

Fig. 2(a-e): Structures of flavonoids 1-5 identified in the extract of *L. olgensis* var. koreana, (a) Taxifolin, (b) Aromadendrin, (c) Eriodictyol, (d) Quercetin and (e) Kaempferol

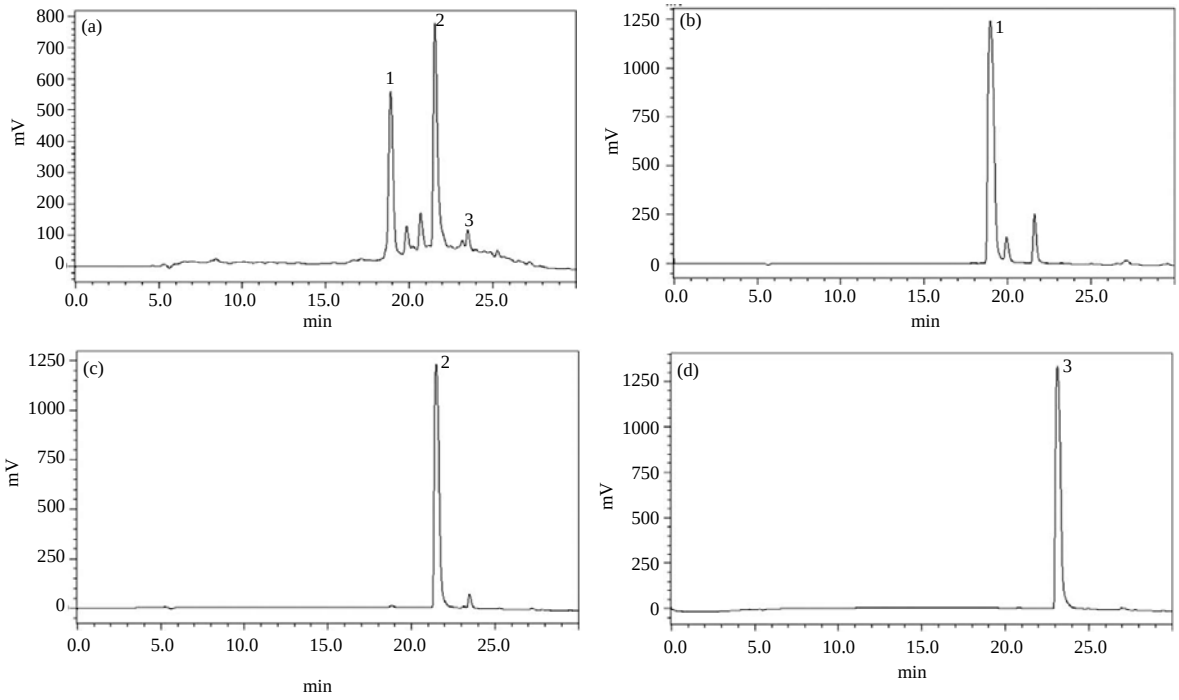

Fig. 3(a-d): HPLC chromatograms of the (a) *L. olgensis* var. koreana extract, (b) Taxifolin, (c) Aromadendrin and (d) Eriodictyol standards, obtained using the first set of conditions (1: Taxifolin, 2: Aromadendrin, 3: Eriodictyol)

Table 1: Evaluation of HPLC-UV methodology for flavonoid quantitation in the *L. olgensis* var. *koreana* extract

Flavonoids	Linear range (mg mL^{-1})	Linear equation x (mg mL^{-1}) y (mg mL^{-1})	r	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)	Recovery	
							Mean (%)	RSD (%)
Taxifolin	0.05-1.00	y = 255178095.9739x-3462608.3596	0.9998	1.31	2.90	1.60	99.5	1.37
Aromadendrin	0.05-1.00	y = 245392781.3721x-3619329.5171	0.9986	1.29	2.11	1.73	100.9	2.11
Eriodictyol	0.05-1.00	y = 271836955.2573x-3939326.5719	0.9991	1.51	1.73	1.93	99.4	1.56
Quercetin	0.05-1.00	y = 329938357.9991x-4756891.7328	0.9993	2.13	1.99	1.57	102.9	1.97
Kaempferol	0.05-1.00	y = 357892681.3357x-3178343.9791	0.9997	1.77	2.51	1.35	98.9	1.99

RSD: Relative standard deviation

was evaluated by performing intra-day tests for 5 different concentrations of five analytes. Intra-day tests were conducted on the mixed standard solution 5 times a day for three consecutive days (1, 3, 5 days) and a day. Thus determined intra-day precision was expressed as RSDs, which equaled 1.29-2.13% (Table 1).

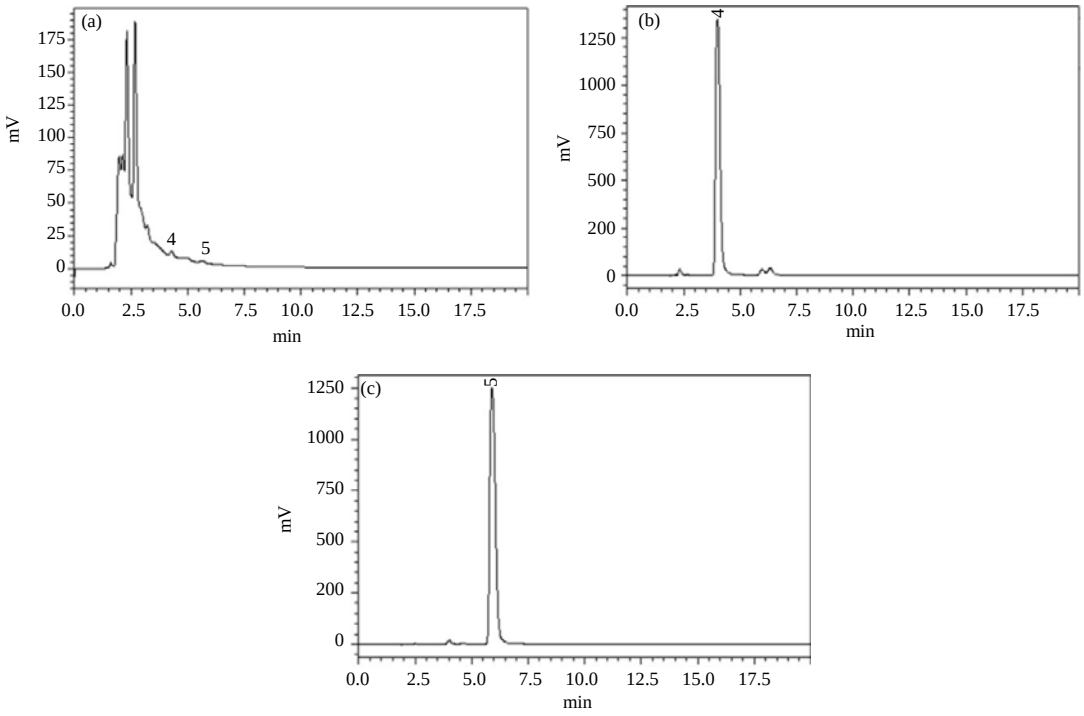

Fig. 4(a-c): HPLC chromatograms of the (a) *L. olgensis* var. koreana extract (b) Quercetin and (c) Kaempferol standards using the second set of conditions (4: Quercetin, 5: Kaempferol)

Repeatability was determined for each analyte, with RSD values shown in Table 1. As injections were performed on three different days, the repeatability assay featured higher RSD values than other data. For analyte concentrations of 10-100 mg L^{-1}, RSDs of 2.9-1.73 are acceptable. Thus, RSD values determined for repeatability indicate an acceptable precision of the developed flavonoid quantification method.

Stability: In the 24 h stability test, the RSD of the relative retention time (RRT), defined as the ratio of the retention time of the individual peak to that of the reference peak was less than 1.93%.

Antioxidant assay: The $DPPH^{\cdot}$ scavenging assay is the most frequently used antioxidant screening method, since this radical directly and rapidly reacts with antioxidants in a simple manner[30]. The scavenging capacities of taxifolin and BHT (positive control) against $DPPH^{\cdot}$ are shown in Fig. 5a, revealing that taxifolin was significantly more potent than BHT ($p<0.01$), with the respective EC_{50} values equaling 1.50 ± 0.37 and 5.05 ± 0.51 μg mL^{-1}, respectively. Thus, the hydrogen donating ability of taxifolin exceeded that of BHT.

The scavenging capacities of taxifolin and BHT against ABTS are shown in Fig. 5b, revealing that the effects of taxifolin and BHT were similar at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1 and 0.2 mg mL^{-1}, however, at concentrations of 0.01 and 0.05 mg mL^{-1}, taxifolin was superior to BHT ($p< 0.01$). The EC_{50} values of the extract and BHT equaled 7.49 ± 1.11 and 12.38 ± 2.63 μg mL^{-1}, respectively, indicating that the former had a higher ABTS radical scavenging ability than the latter.

The antioxidant capacity of a given compound depends on its structure[31], the number and location of phenolic hydroxyls are important factors determining the antioxidant activity of flavonoids[32,33]. For example, the antioxidant capacity of flavonoids increases with the increasing number of phenolic hydroxyl groups. Moreover, flavonoids with phenolic hydroxyls ortho to each other are more potent than those with meta-hydroxyls, since the former structural motif allows the formation of semiquinone-type free radicals.

Thus, taxifolin is the primary flavonoid in the extract of *L. olgensis* var. koreana, with the presence of five phenolic hydroxyl groups in its structure being responsible for the potent antioxidant effects of this extract.

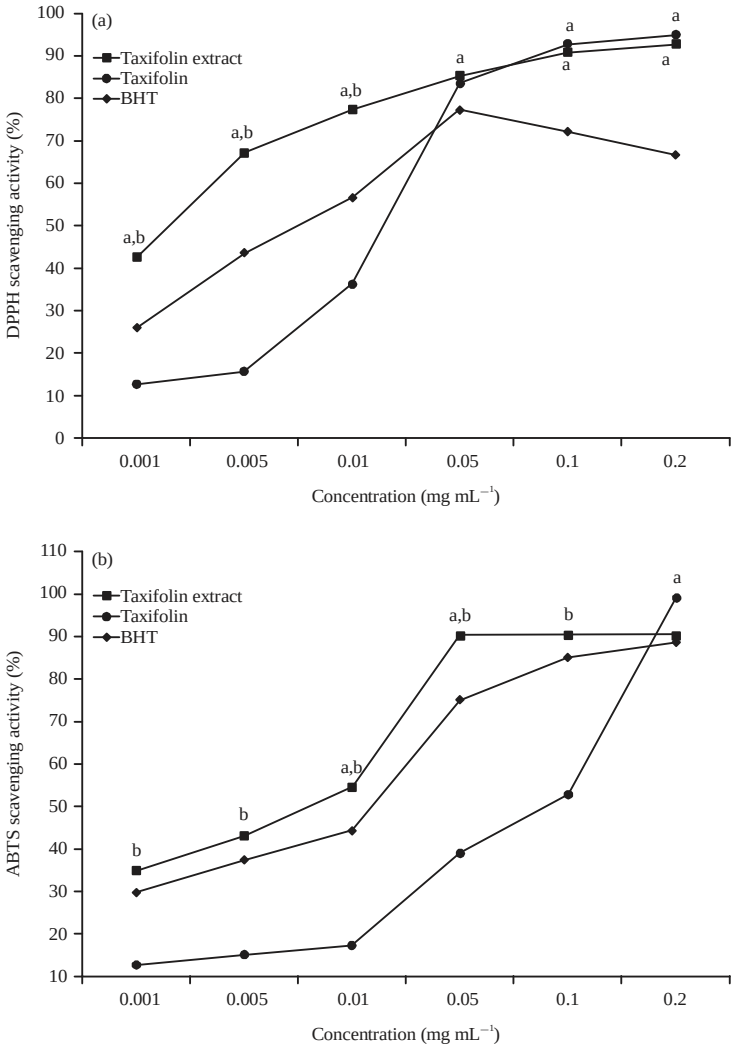

Fig. 5(a-b): Scavenging activities of taxifolin extracted from *L. olgensis* var. koreana, taxifolin standard and BHT (positive control) against (a) DPPH and (b) ABTS radicals

a: $p<0.05$, DPPH and ABTS scavenging activities of extracted taxifolin and the taxifolin standard vs. those of BHT, b: $p<0.05$, DPPH and ABTS scavenging activities of extracted taxifolin vs. those of the taxifolin standard

CONCLUSION

Flavonoids in the extract of *L. olgensis* var. koreana were characterized using HPLC-MS/MS, with quantitation performed using HPLC-UV. The primary ingredient of the extract was identified as taxifolin (92.07%), with aromadendrin (2.39%), eriodictyol (0.19%), quercetin (0.053%) and kaempferol (0.045%) also detected. The above extract exhibited strong antioxidant activity, surpassing that of BHT, a well known synthetic antioxidant, which was primarily attributed to its high flavonoid content. The extract of *Larix olgensis* var. koreana provides a new resource for taxifolin antioxidant in the food industry.

SIGNIFICANCE STATEMENT

This study discovers flavonoids taxifolin contained in *Larix olgensis* Henry var. koreana Nakai that can be beneficial for strong radical-scavenging activity against DPPH• and ABTS•+. This study help the researchers to uncover the critical areas of new natural resources of taxifolin antioxidant activity that many researchers were not able to explore. Thus a new theory on new resource for taxifolin antioxidant in the food industry may be arrived at.

ACKNOWLEDGMENTS

Authors would like to give thanks for the staff of Chinese Medicine College of Jilin Agricultural University. Also thanks

to Chinese Medicine key advantages of the provincial subjects (Screening and chemical modification of Chinese medicine target active ingredient against cerebral ischemic stroke disease.) for supporting this project (JNYHZ 2015-X035).

REFERENCES

1. Fukui, Y., K. Nakadome and H. Ariyoshi, 1966. Studies on the monomer flavonoides of the plants of Coniferae. II. Isolation of a new taxifolin glucoside from the leaves of *Chamaecyparis obtusa* Endlicher. Yakugaku Zasshi: J. Pharmaceut. Soc. Jpn., 86: 184-187.
2. Wang, Y., Y. Zu, J. Long, Y. Fu and S. Li *et al*., 2011. Enzymatic water extraction of taxifolin from wood sawdust of *Larix gmelini* (*Rupr.*) Rupr. and evaluation of its antioxidant activity. Food Chem., 126: 1178-1185.
3. Yoshida, T., X.J. Zhe and T. Okuda, 1989. Taxifolin apioside and davuriciin M_1, a hydrolysable tannin from *Rosa davurica*. Phytochemistry, 28: 2177-2181.
4. Dok-Go, H., K.H. Lee, H.J. Kim, E.H. Lee and J. Lee *et al*., 2003. Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from *Opuntia ficus-indica* var. *saboten*. Brain Res., 965: 130-136.
5. Halim, A.F., S.M. Khafagi and A.A. Gohar, 1982. Flavonoids from fruits of *Silybum marianum* var. *albiflora*. Planta Med., 45: 163-163.
6. Pistelli, L., I. Giachi, D. Potenza and I. Morelli, 2000. A new isoflavone from *Genista corsica*. J. Nat. Prod., 63: 504-506.
7. Jayaprakasam, B., A.G. Damu, K.V. Rao, D. Gunasekar, A. Blond and B. Bodo, 2000. 7-*O*-methyltetrahydroochnaflavone, a new biflavanone from *Ochna beddomei*. J. Nat. Prod., 63: 507-508.
8. Miyazawa, M. and N. Tamura, 2007. Inhibitory compound of tyrosinase activity from the sprout of *Polygonum hydropiper* L. (Benitade). Biol. Pharm. Bull., 30: 595-597.
9. Vega-Villa, K.R., C.M. Remsberg, Y. Ohgami, J.A. Yanez, J.K. Takemoto, P.K. Andrews and N.M. Davies, 2009. Stereospecific high-performance liquid chromatography of taxifolin, applications in pharmacokinetics and determination in tu fu ling (*Rhizoma smilacis glabrae*) and apple (*Malus*× *domestica*). Biomed. Chromatogr., 23: 638-646.
10. Ahn, J.Y., S.E. Choi, M.S. Jeong, K.H. Park and N.J. Moon *et al*., 2010. Effect of taxifolin glycoside on atopic dermatitis like skin lesions in NC/Nga mice. Phytother. Res., 24: 1071-1077.
11. Diwakar, G., J. Rana and J.D. Scholten, 2012. Inhibition of melanin production by a combination of Siberian larch and pomegranate fruit extracts. Fitoterapia, 83: 989-995.
12. Wang, H., W. Liu, W. Wang and Y. Zu, 2013. Influence of long-term thinning on the biomass carbon and soil respiration in a larch (*Larix gmelinii*) forest in Northeastern China. Scient. World J. 10.1155/2013/865645.
13. Zhou, Y.F., H.S. He, R.C. Bu, L.R. Jin and X.Z. Li, 2008. [Modeling of forest landscape change in Xiaoxinganling mountains under different planting proportions of coniferous and broadleaved species]. J. Applied Ecol., 19: 1775-1781, (In Chinese).
14. Yang, L., X. Sun, F. Yang, C. Zhao, L. Zhang and Y. Zu, 2012. Application of ionic liquids in the microwave-assisted extraction of proanthocyanidins from *Larix gmelini* bark. Int. J. Mol. Sci., 13: 5163-5178.
15. Tiukavkina, N.A., I.A. Rulenko and I.A. Kolesnik, 1997. Taxifolin from dahurian larch-application for the approval as novel food. Voprosy Pitaniia, 6: 12-15.
16. Haraguchi, H., Y. Mochida, S. Sakai, H. Masuda and Y. Tamura *et al*., 1996. Protection against oxidative damage by dihydroflavonols in *Engelhardtia chrysolepis*. Biosci. Biotechnol. Biochem., 60: 945-948.
17. Nakayama, T., M. Yamaden, T. Osawa and S. Kawakishi, 1993. Suppression of active oxygen-induced cytotoricity by flavonoids. Biochem. Pharmacol., 45: 265-267.
18. Sugihara, N., T. Arakawa, M. Ohnishi and K. Furunko, 1999. Anti-and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with α-linolenic acid. Free Rad. Biol. Med., 27: 1313-1323.
19. Yun, B.S., I.K. Lee, J.P. Kim, S.H. Chung, G.S. Shim and I.D. Yoo, 2000. Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of *Eucalyptus globulus*. Arch. Pharmacal Res., 23: 147-150.
20. Luo, S., X. Zhang, X. Zhang and L. Zhang, 2014. Extraction, identification and antioxidant activity of proanthocyanidins from *Larix gmelinii* Bark. Nat. Prod. Res., 28: 1116-1120.
21. Choi, J.Y., S.J. Lee, S.J. Lee, S. Park and J.H. Lee *et al*., 2010. Analysis and tentative structure elucidation of new anthocyanins in fruit peel of *Vitis coignetiae* pulliat (meoru) using lc-ms/ms: contribution to the overall antioxidant activity. J. Sep. Sci., 33: 1192-1197.
22. Rayyan, S., T. Fossen, H.S. Nateland and O.M. Andersen, 2005. Isolation and identification of flavonoids, including flavone rotamers, from the herbal drug 'crataegi folium cum flore' (hawthorn). Phytochem. Anal., 16: 334-341.
23. Wybranowski, T., B. Ziomkowska and S. Kruszewski, 2013. Antioxidant properties of flavonoids and honeys studied by optical spectroscopy methods. Med. Biol. Sci., 27: 53-58.
24. Tyukavkina, N.A., K.I. Lapteva, V.A. Belyaeva and V.A. Kulichkova, 1968. A study of sorption processes on a polyamide sorbent. I. The sorption of quercetin and dihydroquercetin. Chem. Nat. Compd., 4: 294-296.
25. Zhang, W.P., W. Liu, J.H. Fu, J. Chai, W.C. Liu and Z.Y. Nan, 2013. Structural identification and quantitative analysis of taxifolin in *Larix olgensis* Henry var. koreana Nakia. Food Sci., 34: 293-296.

26. Wagner, H., V.M. Chari and J. Sonnenbichler, 1976. ^{13}C-NMR-spektren naturlich vorkommender flavonoide. Tetrahedron Lett., 17: 1799-1802.
27. Xu, M.Y., Y.L. Han, Y.Z. Dong and L.J. Zhang, 2007. [Separation, purification and spectrum analysis of total flavonoids from *Cercis chinensis*]. J. Chin. Med. Mater., 30: 1252-1255, (In Chinese).
28. Luo, W., M. Zhao, B. Yang, G. Shen and G. Rao, 2009. Identification of bioactive compounds in *Phyllenthus emblica* L. fruit and their free radical scavenging activities. Food Chem., 114: 499-504.
29. Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol. Med., 26: 1231-1237.
30. Bozin, B., N. Mimica-Dukic, I. Samojlik, A. Goran and R. Igic, 2008. Phenolics as antioxidants in garlic (*Allium sativum* L., Alliaceae). Food Chem., 111: 925-929.
31. Das, S., I. Mitra, S. Batuta, M.N. Alam, K. Roy and N.A. Begum, 2014. Design, synthesis and exploring the quantitative structure-activity relationship of some antioxidant flavonoid analogues. Bioorg. Med. Chem. Lett., 24: 5050-5054.
32. Tsuji, P.A., K.K. Stephenson, K.L. Wade, H. Liu and J.W. Fahey, 2013. Structure-activity analysis of flavonoids: Direct and indirect antioxidant and antiinflammatory potencies and toxicities. Nutr. Cancer, 65: 1014-1025.
33. Tumer, T.B., P. Rojas-Silva, A. Poulev, I. Raskin and C. Waterman, 2015. Direct and indirect antioxidant activity of polyphenol- and isothiocyanate-enriched fractions from *Moringa oleifera*. J. Agric. Food Chem., 63: 1505-1513.