**SUPPLEMENTARY MATERIAL**

**Extraction, identification and antioxidant activity of proanthocyanidins from *Larix gmelinii* Bark**

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This study was intended to extract and identify the proanthocyanidins from Larix gmelinii bark. Different extraction methods and degreasing methods were investigated. The content of proanthocyanidins, antioxidant activities and FT-IR analysis were used to evaluate and identify these extracts. The ultrasonic extracts showed a higher content of proanthocyanidins and antioxidant activity than supercritical carbon dioxide extracts. The defatted extracts showed a higher content of proanthocyanidins and antioxidant activit~~i~~y than un-defatted extracts. DPPH radical scavenging capacity of extracts (29.88 μg mL-1) was higher than VC (36.04 μg mL-1), and the inhibition effect of lipid peroxidationof extracts(15%) was higher than Vc(13%) and VE(11%). The FT-IR analysis revealed that the main phenolic compounds were almost the same as proanthocyanidins standards.

**Keywords:** Proanthocyanidins; Larix gmelinii bark; antioxidant activity; FT-IR

**Experimental**

***Material***

Larix gmelinii bark material used in the study was kindly given by neimenggu and was authenticated by Dr. Zhao Guang-jie, professor of wood science (College of Materials Science and Technology, Beijing Forestry University, Beijing, China). The voucher specimen was deposited in Beijing Forestry University Herbarium numbered as LX-1960-001.The Larix gmelinii bark was dried at room temperature. The standards used for FT-IR analysis were Proanthocyanidins (Purity: ≥98%, UV) (purchased from Beijing H&Q Chemical Institute and Beijing Aoke biological technology Co.,LTD), Methanol (HPLC Solvent) was purchased from J.T. Barker (USA). Ethanol (absolute ethanol, AR) which was used as a co-solvent, hydrochloric acid (AR), glacial acetic acid (AR), trichloromethane (AR), petroleum ether (AR) and n-butanol (AR) were obtained from Beijing Chemical Works. 2, 2-Diphenyl-1-picrylhydrazyl (free radical, 95%), Vitamin C (≥98%) was obtained from Alfa Aesar (USA).

***Methods***

*Sample preparations*

Larix gmelinii bark, which was kept at room temperature, then was ground into small pieces by crushing machine. The bark was crushed for 1min, after every 30 s. Crushed bark was then sieved and the particles of the size between 380 and 250 um were selected. 10 g of the sieved bark was weighed and was Soxhlet extracted with 100ml petroleum ether for 6 hours, and then the solvent was evaporated; sample 1was prepared. 10 g of the sieved bark was weighed and was bathed in 100ml petroleum ether for 2days, and then the cake was obtained from vacuum filtering the solution through a filter, sample 2 was prepared. 10 g of the sieved bark (sample 3) was weighed and was put into the extraction vessel, and then the extract was defatted with 100ml petroleum ether by separatory funnel. 10 g of the sieved bark (sample 4) was weighed and was put into the extraction vessel, un-defatted. All the samples were divided equally into two parts for supercritical carbon dioxide extraction and ultrasonic extraction.

*Supercritical carbon dioxide extraction of* *Larix gmelinii bark*

The samples were extracted by Supercritical carbon dioxide extractor (SFE-2, Applied Separations Inc., USA). The instrument was equipped with automated back pressure regulator, 50 mL extraction vessel, 50 mL collection vessel, temperature controller, high pressure P-50 series pump, cooling systems filled with glycol, and a series III pump for co-solvent. The co-solvent pump was purged before each extraction in order to ensure that co-solvent entered the system.

The carbon dioxide flow rate was fixed to 5 g/min,the ethanol concentration was 80% (vol %), the material-solution ratio was 1∶3.78, the pressure was 300 bar and the temperature was 35 °C. First was0.5h static extraction,then dynamic extraction was also used for 0.5h. After extraction the collection vessel was washed with 20 mL of ethanol to minimize the losses. The solution was dried in a vacuum oven at 35 °C. All samples were analyzed in triplicate.

*Ultrasonic extraction of Larix gmelinii bark*

The samples were extracted by ultrasonic cleaning equipment (KQ 5200DB, Kunshan Ultrasonic Instrument Co.,Ltd , China). 80% (vol %) ethanol was selected as solvent. Ratio of the material-solutionwas 1∶15, temperature was 35 °C and extraction for 1h. After the extraction the bark extracts solution was filtered by a buchner funnel. The filtrate was dried in a vacuum oven at 35 °C. All samples were analyzed in triplicate.

*Determination of proanthocyanidins*

Proanthocyanidins were determined by a butanol-HCl assay (Hagerman et al., 2000). Briefly, 10 mg of the proanthocyanidins extract was weighed and was dissolved with methanol at the concentration of 0.2mg/mL. 1.0 ml of the proanthocyanidins methanol solution was mixed with 6.0 mL of butanol-HCl (95:5 v/v) and 0.2 mL ferric reagent (2% ferric ammonium sulfate in 2.0 M HCl). After 40 min at 95°C in a water-bath, the absorbance was measured at 546 nm against a blank containing methanol.

Method: To adopt the ultraviolet spectrophotometry, the absorbability was used as the Y-axis and the concentration as the X-axis by drawing standard curve for determination of the content of proanthocyanidins.

The standard curve was done with proanthocyanidins standard for calculating the content of proanthocyanidins in the Larix gmelinii bark extract. Determination of proanthocyanidins was calculated as follows:

Y=2.636X-0.0026, R2=0.9992 (1)

Where X is the concentration of the Larix gmelinii bark extract (mg mL-1), Y is the absorbance value of the control.

*Antioxidant activity*

This activity was evaluated by the effect to scavenge DPPH radical. The hydrogen atoms or electrons donation ability of the corresponding extracts were measured from the bleaching of purple coloured methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) suggested by the method of Hanato et al.(1988). One milliliter of various concentrations (1-70 μg mL−1) of the Larix gmelinii bark extract in methanol was added to 3 mL of 0.2 mM DPPH radical solution in methanol. The mixture was shaken vigorously and allowed standing for 30 min in the dark. The absorbance of the resulting solution was measured at 517 nm. Inhibition of DPPH radical was calculated as follows:

DPPH• scavenging effect (%) = [(A0 −A1)/A0] ×100 (2)

where A0 is the absorbance value of the control, A1 is the absorbance values of the 3 mL of 0.2 mM DPPH radical solution in methanol was added to 1 mL methanol at 30 min, respectively. The antiradical activity was expressed as IC50 (μg mL-1), the Larix gmelinii bark extract dose required to cause a 50% decrease of the absorbance at 517 nm. All samples were analyzed in triplicate. A lower IC50 value corresponds to a higher antioxidant activity.

*Inhibition of lipid peroxidation*

20 g of the [edible](app:ds:vegetable" \t ") [oil](app:ds:oil) was weighed and was put into the beaker. 0.006g of the additives (Larix gmelinii bark extract, Vitamin C, Vitamin E and none) were dissolved in a small amount of alcohol, and then added in the weighed [edible](app:ds:vegetable) [oil](app:ds:oil) respectively, fully mix. 20 g of the e[dible](app:ds:vegetable) [oil](app:ds:oil) was weighed and was put into the beaker without any additive. These 4 beakers were putted into the incubator at temperature of 60 °C. Peroxide number (POV) of the [edible](app:ds:vegetable) [oil](app:ds:oil) was determined for every other day. Inhibition of lipid peroxidation was calculated as follows:

Peroxide number (POV) = [(V1-V2) × c × 0.1269]/m × 78.8 ×100 (2)

where POV (meq kg-1) is peroxide number of the sample, V1 (mL) is the volume of the Na2S2O3 standard volumetric solution that the sample consumed, V2 (mL) is the volume of the Na2S2O3 standard volumetric solution that the reagent blank consumed, c (mol L-1) is the concentration of the Na2S2O3 standard volumetric solution, m (g) is the weight of the sample, 0.1269 (g) is the same weight of iodine as 1.00 mL of the Na2S2O3 standard volumetric solution (c=1.000mol L-1), 78.8 is the conversion coefficient.

*FT-IR analysis*

The FT-IR measurements were performed on a Spectrum GX FT-IR spectrometer (Perkin Elmer, USA) equipped with a DTGS detector, in the 400-4000 cm-1 range with a resolution of 4 cm-1. A pre-established program controlled the whole process of increasing temperature, the temperature was raised stepwise. Spectra were calculated from a total of 32 scans. The IR spectra were obtained by IR analysis software.

*Statistical analysis*

All data analysis was performed using excel 2007.



Figure S1. The DPPH free radical scavenging effect of the supercritical carbon dioxide extraction at different concentrations



FigureS2. The DPPH free radical scavenging effect of the ultrasonic extraction at different concentrations



Figure S3. The DPPH free radical scavenging effect of VC at different concentrations



Figure S4.The inhibition of lipid peroxidation effect of Larix gmelinii bark extract compared with that of Vc and VE

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Figure S5.IR spectra of Proanthocyanidins standard

Table S1.The content of proanthocyanidins in Larixgmelinii bark extracts.

|  |  |  |
| --- | --- | --- |
|  | Proanthocyanidins (%) | Yield (%) |
| **Supercritical fluid extraction** |  |  |
| Soxhlet extracted | 68.40 ± 1.1 | 19.40 |
| bathed | 68.47 ± 1.5 | 19.02 |
| extracted | 75.97 ± 2.3 | 18.15 |
| without defatted | 65.06 ± 1.0 | 21.80 |
| **Ultrasonic extraction** |  |  |
| Soxhlet extracted | 73.38 ± 0.6 | 16.51 |
| bathed | 72.18 ± 1.1 | 15.35 |
| extracted | 72.18 ± 2.0 | 14.79 |
| without defatted | 67.66 ± 0.9 | 15.82 |

Table S2. the IC50 of each extract.

|  |  |
| --- | --- |
|  | **IC50 (μg mL-1)** |
| **Supercritical carbon dioxide extraction** |  |
| Soxhlet extracted | 34.39 |
| bathed | 34.45 |
| extracted | 36.95 |
| without defatted | 37.01 |
| **Ultrasonic extraction** |  |
| Soxhlet extracted | 30.88 |
| bathed | 29.88 |
| extracted | 33.18 |
| without defatted | 35.38 |
| **VC** | 36.04 |

**References**

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