Antioxidant Activity of Pine Bark Constituents

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A modified *in vitro* lipid peroxidation inhibition assay was used to guide the fractionation and the isolation of antioxidative principles of Finnish pine bark extract. This approach yielded 3,4-dihydroxybenzoic acid (protocatechuic acid) and taxifolin-3-O-β-glucopyranoside as major antioxidative compounds from the plant material. The structural elucidation of these compounds was undertaken with the help of HPLC-DAD and HPLC-ESI-MS analyses. Their IC_{50} values, in comparison to trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), were: trolox (1.78 ± 0.56 μM) < protocatechuic acid (5.77 ± 1.63 μM) < taxifolin-3-O-β-glucopyranoside (16.30 ± 1.98 μM). The method for the determination of antioxidant activity proved reproducible and quick for routine analyses with 96 well plates.

Key words: Pinus sylvestris L., Protocatechuic Acid, Taxifolin-3-O-β-glucopyranoside

Introduction

The bark of Scots pine (*Pinus sylvestris* L.) is composed of a highly complex array of tissues and rich in polyphenolic compounds (Peltonen, 1981). Though French maritime (*Pinus maritima*) bark has been investigated for its procyanidins that shows strong antioxidant properties (Wood *et al.*, 2002; Liu *et al.*, 1998) and widely used as a remedy for various degenerative diseases, no such data are available in scientific literature about *P. sylvestris* bark. This encouraged us to study the extract for its antioxidative phenolics by a modified *in vitro* lipid peroxidation inhibition assay.

We developed a method for the determination of the antioxidant activity of pine bark phenolics by modifying the one previously used (Saleem *et al.*, 2001) keeping in view the fact that there is an ever increasing demand of rapid and reliable methods for screening large number of plant extracts and their antioxidative constituents. The method for the determination of antioxidant activity proved suitable to guide the isolation of antioxidant principles from the crude plant extract of complex chemical nature.

Experimental

Plant material

Freshly prepared pine bark extract was kindly provided by Ravintorengas Oy. The material was transported to the Department of Chemistry, University of Turku for further studies.

Materials

Na_2CO_3, and EDTA were purchased from Fluka BioChemica, (Buchs, Switzerland). Butylated hydroxytoluene (BHT), trolox, α-tocopherol and t-butyl hydroperoxide (t-BuOOH) (70% aqueous solution); were from Sigma Chemical Co. (St. Louis, MO, USA) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was from Bio-Orbit Ltd. (Turku, Finland). All chemicals for extraction and HPLC analysis were of HPLC grade except acetone. The 96 well microtiter plates were from Cliniplate, Lab-Systems OY, (Helsinki, Finland).

Sample preparation and column chromatography

The 500 ml of extract was vacuum filtered through Buechner funnel containing a filter paper (110 mm) (Scheicher & Schuell, Dassel, Germany) into a 1500 ml Erlenmeyer filtering flask, and concentrated with a rotary evaporator at 35 °C to 30 ml. The insoluble portion was separated by centrifugation and the soluble part was introduced into a Sephadex LH-20 column (40 × 2.5 cm I. D., 5–11 μm), Pharmacia (Uppsala, Sweden). The extract was fractionated into 10 fractions (F1–F10) which were evaporated to dryness and re-dissolved in de-ionized water at 40 mg/ml.

Isolation of protocatechuic acid and taxifolin-3-O-β-glucopyranoside

The sub-fractions 4.1–4.10 were obtained from fraction F4 (methanol 50%) and 4.9 (methanol 48%) was re-dissolved in water and subjected to LiChroprep RP-18 column (44 × 3.7 cm I. D., 40–63 μm) with 0–30% acetonitrile gradient in HCOOH (1% aqueous). The five sub-fractions 2–12% with acetonitrile gradient and three with 12–15% acetonitrile gradient were pooled on the basis of their similar chromatographic profiles and evaporated to dryness to yield 16.0 mg protocatechuic acid and 13.3 mg taxifolin-3-O-β-glucopyranoside.

HPLC-DAD analysis

The extract was filtered through Millex-HV_{13} filter, 0.45 μm (Millipore, Bedford, MA) before injecting into the HPLC column. Separations were performed on Spherisorb 5 ODS-2 column (250 × 4.6 mm I. D., 5 μm, Phase Separations Ltd., UK). HPLC system consisted of Pump L-7100 connected with Diode Array Detector L-7455 and a programmable autosampler L-7250, (Hitachi Ltd., Tokyo, Japan). Two solvents were used for elution: (A), acetonitrile; (B), 5% formic acid. The elution profile was 0–5 min., 100% B (isocratic); 5–60 min., 0–30% A in B (linear gradient). The flow rate was maintained at 1.0 ml min^{-1} with column back pressure of 70–136 bar. The wavelength of UV detector was fixed at 280 nm with automatic acquisition in UV-VIS detection range of the apex.

HPLC-ESI-MS

HPLC-ESI-MS analysis was performed with Perkin Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). The instrument was equipped with pneumatically assisted ion spray interface and Macintosh data system. HPLC system consisted of Perkin-Elmer Series 200 with a UV/VIS-detector. Samples were introduced into the system by a Perkin-Elmer Series 200 Autosampler (Perkin-Elmer, Norwalk, CT, USA). The separation of individual compounds was achieved on Spherisorb 5 ODS-2 column (250 × 4.6 mm I. D., 5 μm, Phase Separations Ltd., UK). The HPLC solvent system consisted of acetonitrile and formic acid-water (0.4:99.6, v/v). The elution profile was same as used in HPLC-DAD analysis. The injection volume was 20 μl. The flow rate was 1.0 ml min^{-1}. Before the ESI, main part of the flow was split and only minor part was introduced into the ion source. The mass spectrometer was operated in negative ion mode and the data were acquired by scanning between *m/z* 100 and 1100 using a 0.30-u step size. The ion spray voltage was – 4000 V while the orifice plate voltage was – 35 V. Ring voltage was – 220 V and the nebulizer gas was set at 8 and curtain gas at 10. The nitrogen gas temperature was 300° C.

Fluoroscan ascent FL 374

The Fluoroscan *Ascent* FL II (version 1.3., 1998) manufactured by Labsystems (Helsinki, Finland) is a microplate fluorometer equipped with both fluorometric and luminometric detection technologies. It has luminometric sensitivity of 5 femtomol with white 96-well plates. The instrument is equipped with on-board incubator for the temperature control and a built-in orbital shaker with adjustable speed and diameter to speed up the reaction times by effective shaking. The microplates are moved into and out of the instrument with a robotic arm that allows fast and accurate sample loading with a facility to use 1–96 and 384 wells. In the kinetic measurement type, 50 measurements were taken from each measurement point using a defined interval time of 1 sec. The results were calculated by processing the data in the following forms: the average rate (reaction slope calculated from raw data and the time stamps for each well with least square method), maximum rate (the highest reaction rate calculated for each well), time to maximum rate (highest reaction rate calculated as the time taken from the first reading to the mid point of the sliding window) and time to change (the time required to reach a defined change in the signal). The kinetic rate was measured in sec.

Lipid peroxidation assay

Rat liver microsomes were prepared as mentioned in Saleem *et al.* (2001). Samples were simultaneously analyzed in 96 well microtiter plates by automatic dispension of t-BuOOH through the dispensor tip at the lower-dispension position. A 100 μl of buffer (50 mM Na_2CO_3 + 0.1 mM EDTA,

pH = 10.2), 20 µl of 0.5 mg/ml luminol (5 mg luminol + 0.5 ml 0.1 M NaOH + 9.5 ml 0.1 M H_3BO_3), 20 µl of rat liver microsomes in 0.15 M KCl (final concentration, 1.5 µg protein/ml), and 20 µl of the samples were pipetted into reaction cuvettes. The reaction was initiated by 200 µl (0.9 mM) t-BuOOH at 33 °C. A 20 µl of buffer was used as blank. The chemiluminescence was measured in relative luminescence units (RLU) in kinetic mode. Each well was measured 50 times with an integration time of 1.0 sec. The integral luminescence was calculated by adding RLU values of 50 successive measurements during 41.6 min runs. The percent inhibition of luminescence was calculated according to the following formula:

$$\text{Inhibition } \% = \frac{L_b - L_s}{L_b} \times 100\% = (1 - \frac{L_s}{L_b}) \times 100\%$$

where L_s = integral luminescence of sample, L_b = integral luminescence of blank sample.

The IC_{50} (concentration that inhibits lipid peroxidation by 50%) was calculated on the basis of integral (area under the kinetic reaction curve). The starting concentration for further dilutions was always maintained at 40 mg/ml. For selecting a reference antioxidant, the IC_{50}s were determined for vitamin-E, trolox (a water-soluble analogue of vitamin-E) and BHT. The vitamin-E did not show dose dependence at six data points and BTH was a weaker antioxidant than the pine bark extract. However, trolox was an active antioxidant in our test system, and was chosen for further work.

Statistical analyses

The IC_{50} values were calculated by plotting inhibition% vs concentration into $y = y_o + A\ (1 - e^{-x/B})$ for trolox and protocatechuic acid using Origin 6.1 (OriginLab Corporation, MA, USA), while for taxifolin-3-O-β-glucopyranoside linear fit ($y = A + Bx$) was used, where A and B are fitting the parameters. y = inhibition% and x = concentration of the inhibitor.

Results and Discussion

The pine bark extract (IC_{50} = 129.0 µg/ml) was separated by size exclusion chromatography through a Sephadex LH-20 column and the following fractions were obtained: F1 (water), F2 (water pH = 2), F3–F6 (methanol 30%, 50%, 80% and 100% respectively), F7–F10 (acetone 30%, 50%, 80% and 100% respectively) (Fig. 1). The antioxidant activity was mainly found in F2 (water pH 2) and methanol fractions F3–F6 (92.8–96.7% inhibition) while F1, F9 and F10 were less active (below 56% inhibition). The IC_{50} (µg/ml) were of the following order: pine bark extract = 129.0, F2 = 34.3, F3 = 31.0, F4 = 21.6, F5 = 85.1, and F6 = 83.3. The strongest antioxidative fraction F4 (methanol 50%, IC_{50} = 21.6 µg/ml) was further separated into 10 fractions (4.1–4.10) by a Sephadex LH-20 column and their antioxidant activities were checked. Fraction 4.9 yielded protocatechuic acid and taxifolin-3-O-β-glucopyranoside. The IC_{50} values for these compounds were determined in comparison to trolox and the following order was obtained: trolox 1.78 ± 0.56 µM > protocatechuic acid 5.77 ± 1.63 µM > taxifolin-3-O-β-glucopyranoside 16.30 ± 1.98 µM. The identification of the isolated compounds was undertaken by HPLC-DAD and HPLC-ESI-MS (Table I).

Conclusions

The study presents a systematic approach towards the discovery of antioxidative phenolics of pine bark extract by a modified *in vitro* lipid peroxidation assay. The modified method is relatively faster and accurate for the determination of antioxidant activity tests by chemiluminescence with 96 well microtiter plates and can prove suitable for high throughput screening in pharmaceutical and research laboratories.

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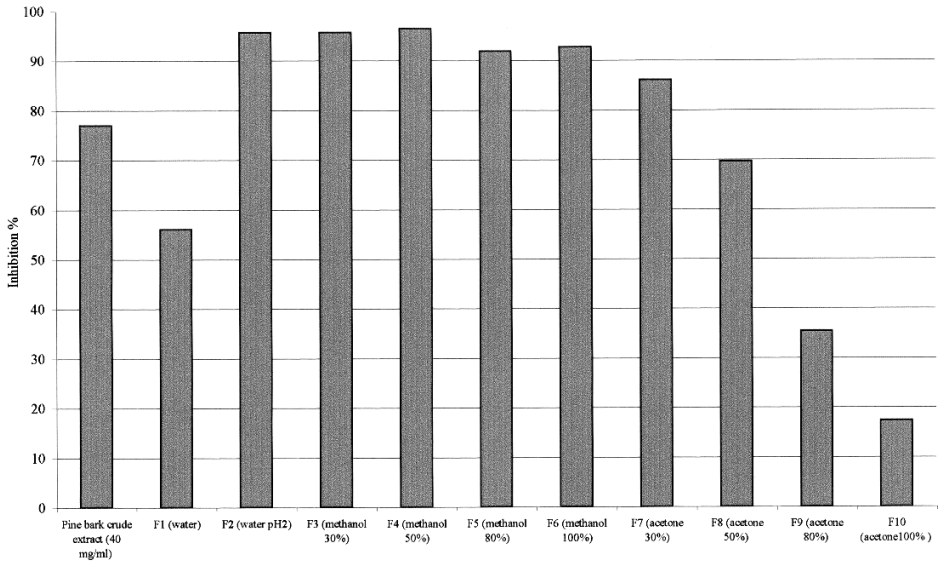
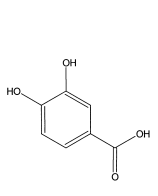


Fig. 1. Antioxidant activities for crude pine bark extract (Havupuu-uutejuoma) and its fractions F1-F10 measured as their inhibition percentage of chemiluminescence from oxidation (compared to blank). Luminescence was measured from a mixture with 100 μl buffer, 20 μl sample (0.40 mg/ml), 20 μl luminol, 20 μl microsomes and 200 μl t-BuOOH (aqueous). Blank luminescence was measured with 20 μl buffer instead of sample.

Table I. HPLC-DAD and HPLC-ESI-MS (*m/z* values in negative ion mode) data of identification of antioxidative compounds of pine bark extract.

Compound	Retention time [min]	UV maxima [nm]	*m/z* values		
Protocatechuic acid (**I**)	16.11	265.300	109.0 [M-COOH]$^-$	152.8 [M-H]	307.3 [2M-H]
Taxifolin-3-O-β-glucopyranoside (**II**)	28.56	295	302.8 [M-glucose]$^-$	465.1 [M-H]	931.3 [2M-H]



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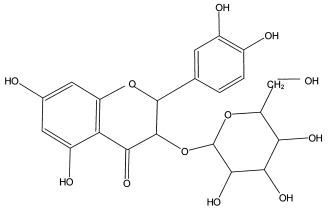

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