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Antibacterial and antifungal activity of phytosterols and methyl dehydroabietate of Norway spruce bark extracts

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ABSTRACT

The current study focuses on the analysis of *in vitro* biological activity of extract from bark of Norway spruce *(Picea Abies)*, which can find potential application in food and cosmetic industry and pharmacology. Milled bark was subjected to Soxhlet extraction and supercritical fluid extraction to obtain two ethanol extracts. These extracts were further used to obtain their pre-extracts to *n*-hexane. It was investigated whether beta-sitosterol exhibits bacteriostatic activity necessary to observe antimicrobial and antifungal activity of methyl dehydroabiatate. This synergic effect and bacteriostatic activity of beta-sitosterol have not been previously reported. The greatest inhibition zone of *n*-hexane pre-extracts was confirmed in bacterium *Pseudomonas aeruginosa* (0,9 – 1,5 cm) and yeast *Alternaria alternata* (0,7 – 1,6 cm). It is novel, the antioxidant, antimicrobial and antifungal activity of spruce bark extracts assessed in terms of food and cosmetic fortification.

1. Introduction

Bark of Norway spruce contains a valuable compounds which concentration is much higher than in wood (Jablonsky et al., 2015; Yang and Jaakkola, 2011). From the spruce bark, a wide range of substances could be obtained. These compounds can be used in various industries such as food, medicine, cosmetics and also in the synthesis of antioxidants BHA (butylhydroxyanisole), BHT (butylhydroxytoluene) and TBHQ (tert-butylhydroquinone) (Jerez et al., 2007).

Nevertheless, tree bark is nowadays considered only as a residual waste biomass and is utilized mainly as a source of a heat, energy and biogas. The wood industry of the European Union produces 6–8 million tonnes of this biomass each year (Ferreira et al., 2015). Besides combustion, this biomass source can also be upgraded in a more advantageous manner by the extraction, or isolation of biologically active substances.

Substances found in spruce bark have significant biological and chemical properties that are effective not only against pests, wood-borne organisms and pathogens (Li et al., 2008; Pan and Lundgren, 1995), but also show positive effects on the human organism (Co et al., 2012; Jablonsky et al., 2015; Kinouchi et al., 2000; Wani et al., 2016). The results of several authors (Ház et al., 2013; Jablonsky et al., 2015; Kreps et al., 2016) confirm that abietic acid and its derivatives, terpenes and fatty acids are mostly represented in the extracts of Norway spruce bark. Resin acids such as abietic, dehydroabietic, methyl ester of dehydroabietic, neoabietic, pimaric, isopimaric, levopimaric, sandrakopimaric, and palustric acids have strong antimicrobial activity against a broad spectrum of gram-positive and gram-negative bacteria, including microorganisms. These microorganisms are resistant to certain types of antibiotics (methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enteroccocus*).

Antifungal activity of the naturally occurring dermatophytes (agents of fungal infections) was also reported (Jokinen and Sipponen, 2016; Rautio et al., 2012; Sipponen and Laitinen, 2011). The antimicrobial mechanism of resin acids is not yet fully understood. Jokinen and Sipponen (2016), found out that these compounds affect the branching

Abbreviations: CCM, Czech Collection of Microoganisms, CZ (Czech Republic); NCTC, National Collection of Type Cultures, UK (United Kingdom); OPT, Collection of Department of Food Technology, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava; BHA, butylhydroxyanisole; BHT, butylhydroxytoluene; TBHQ, tert-butylhydroquinone; TEAC, trolox equivalent antioxidant capacity; DPPH, 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl; TLC, thin layer chromatography; IZ, inhibitory zone; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, international standard for measuring the antioxidant activity of the samples; Rf, retardation factor; GC–MS, gas chromatography–mass spectrometry

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of fatty acids in the cell wall of bacteria and reduce membrane proton gradient, which results in the destruction of the bacterial cell wall and cell agglomerations (Jokinen and Sipponen, 2016).

The antimicrobial activity of these compounds found in bark is also likely to contribute to terpenes, which are the most widespread group of substances in wood and are part of essential oils and resins (Yang and Jaakkola, 2011). It was reported that the synthesis of terpenes (C_{10} - C_{20}) contributed increasingly to the protection of Norway spruce against fungal pathogens and bark beetle (Hammerbacher et al., 2013; Schmidt et al., 2010). The emergence of resistance of pathogenic and potentially pathogenic bacteria as well as the increased incidence of adverse effects of many antibiotics on the human organism led many experts to search for and investigate new antimicrobials of plant origin (Sampedro and Valdivia, 2014).

The bark of spruce trees provides other important biologically active substances. Some authors (Co et al., 2012; Jyske et al., 2014; Kähkönen et al., 1999; Li et al., 2008), propose spruce bark as a potential source of bioactive substances and antioxidants. This conclusion is based on high concentrations of interesting polyphenol compounds such as stilbenes (mainly glycosides and their aglycone), ligands, flavonoids and tannins. Stilbenes serve as a chemical defence of spruce against herbivores and pathogens (Li et al., 2008). They are concentrated mainly in the bark, which contains 5–10 % of stilbenes on dry weight (Co et al., 2012; Mannila and Talvitie, 1992). The major stilbenes of Norway spruce bark responsible for the antileukemic activity, tested in the L1210 leukemia system of the mouse were isorhapontinsol and piceatinol (Mannila and Talvitie, 1992).

Based on this knowledge, we have focused our research on the antioxidant and antimicrobial potential of extracts of spruce bark, which contains compounds that are not well understood. Their bioactive compounds have a potential in functional foods or in medicine and cosmetics.

2. Material and methods

2.1. Chemicals

The bark of Norway spruce (*Picea Abies*) was obtained as waste produced during industrial debarking of spruce wood in Bioenergo Inc. (Ružomberok, Slovakia). Solvents and reagents used for the extraction and microbial analysis were of analytical grade (90–99 %) and were purchased from Sigma Aldrich (Slovakia) and VWR (Austria). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which was used for determination of trolox equivalent antioxidant capacity (TEAC) was obtained from Sigma Aldrich (Slovakia) with purity 97%. Purity of nitrogen was 99.998%, the spraying agent phosphomolybdic acid hydrate had purity of 99%.

2.2. Sample preparation

The bark was disintegrated by hammer mill and separated bark flour was fractionated by sieves to the particle size in the range of 1 – 1,5 mm. The extraction was carried out by supercritical and Soxhlet extraction to obtain two ethanol extracts from 50 g of milled bark. Supercritical Fluid Extraction SFT 150 (Newark, USA) was used under the following conditions: 7000 psi (48 MPa), 80 °C, CO_2 solvent, 200 ml of ethanol cosolvent, time of 1 h. Soxhlet apparatus was filled with 500 ml of ethanol which extracted compounds from milled bark for 8 h. The solvent was evaporated, and 5 g of residues was pre-extracted with 60 ml of a mixture of ethanol and *n*-hexane in a of ratio 1:5. Extracts, pre-extracts and fractions were subjected to the analysis of antioxidant capacity. Based on the results of antioxidant capacity and GC–MS, only selected extracts, pre-extracts and model samples were subjected to antimicrobial/antifungal analysis.

2.3. Yield of extractives

The yield of extractives (Y, %) was determined after each experiment by drying the samples at 105 °C to a constant weight. The results were expressed on the basis of dry matter before and after extraction as shown in Eq. (1),

$$Y\ (\%) = 100 \times m_{extr} / m_i, \quad (1)$$

where, m_i and m_{extr} are the mass (g) of the samples before and after extraction and drying, respectively.

2.4. Isolation and TLC analysis of compounds in n-hexane pre-extract

Compounds present in the 1.5 g residue of *n*-hexane extract of the bark were separated by flash chromatography (BÜCHI C-601 pump, C-610 controller) with a cartridge (BÜCHI 44815) that contained 80 g of silica gel (40–63 µm). Analytes were eluted by four mobile phases consisting of 300 ml mixture of *n*-hexane and diethyl ether in ratios of 95:5, 90:10, 80:20, 60:40. The last mobile phase was only comprised of 500 ml of methanol. According to the test model, we showed that the average retention of isolated compounds in the column was 15–20 %. Fractions eluted from flash chromatography were qualitatively analyzed by TLC according to literature (Kreps et al., 2015). Fractions with the same Rf factor were collected, solution was evaporated and fractions were stored for further analysis in the freezer at −18 °C.

2.5. Qualitative analysis of extracts using GS–MS

The analysis of extractive substances was done using gas chromatograph with a detector (MS 5975C, Agilent). 7 mg of each sample was dissolved in 1 cm^3 of *n*-hexane. The column HP-5MS was 30 m in length, 0.32 mm in diameter and had a film thickness of 0.25 microns. The GC–MS settings were as follows: carrier gas helium (316 cm^3 min^{-1}), feed temperature of 250 °C, 1 µl injection, split 25:1, column temperature was set at 80 °C for 2 min, then the column was heated at 15 °C min^{-1} to 320 °C for 52 min, oven outlet at 300 °C, MS detector source at 250 °C, MS quadrupole at 150 °C, and the electron energy was 70 eV *am/z* range 30 – 780.

2.6. Antioxidant capacity of extracts and isolated fractions

The antioxidant capacity/scavenging activity of the spruce bark extract and its fractions were determined using DPPH as a free radical. 6 mL of ethanol (UV purity) was added to 1 mg of the sample, from which 3 mL was used as a blank. To the remaining 3 mL solution we have added 300 µL of DPPH (0,5 mM in ethanol) in a second test-tube. The decreased absorbance of the sample with DPPH was compared with the absorption of blank at 517 nm after 5 min., which is the optimal reaction time. The determined absorptions were compared with the absorption of trolox exposed to DPPH assay at different concentrations of trolox. From the calibration curve the trolox equivalent antioxidant capacity (TEAC) was expressed in mmol/L trolox on 1 mg dry weight (d_w) of sample.

2.7. Antimicrobial activity of extracts and isolated fractions

To determine the antimicrobial activity of the spruce bark extract, we used a qualitative method - a diffusion agar assay. For bacterial strains we selected BHI soft agar medium and Sabouroud soft agar for fibrous fungi and yeast. The indicator microorganism (bacteria, fibrous sponge, yeast) was added to the agar medium after sterilization in the autoclave (overpressure 0.1 – 0.15 MPa, temperature 121–128 °C). The inlet concentration of the added microorganisms was 106 KTJ / ml. Inoculated medium was poured into sterile and labelled Petri dishes. Paper disks (Rotilabo - Test Blattchen) with a diameter of 12 mm were