Spruce Bark as a Source of Antioxidant Active Substances

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The antioxidant potential of extracts from spruce bark was studied after Soxhlet extraction with ethanol and n-hexane. Ethanol spruce bark extracts were pre-extracted with a mixture of ethanol and n-hexane in a ratio of 1:5. Residues of the extracts and pre-extracts were added to lard (200 mg/kg) to examine its influence on oxidation stability of lard. The composition of the bark extractives was analyzed by GC/MS. The highest antioxidant activity was observed in the original ethanol extracts (15.0 mmol/mg), which had greater antioxidant activity than alpha-tocopherol (13.9 mmol/mg). The n-hexane extract from the spruce bark had 70% less antioxidant activity than the ethanol extract. The high antioxidant activity of the ethanol extract was due to the presence of resin acids (35%) and stilbenes (12%). These antioxidant-active substances increased the oxidation stability of the lard by 5 h, while the n-hexane extract increased the oxidation stability by only 0.5 h. The spruce bark was found to be an alternative feedstock of compounds with potential for use in foodstuffs as antioxidant.

Keywords: Spruce bark; DPPH; Trolox; Antioxidant activity; Induction period; Protection factor

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INTRODUCTION

The bark of spruce is a renewable source of biologically active compounds that can serve as an alternative to fossil-based raw materials (Sládková *et al.* 2016; Kreps *et al.* 2017). Norway spruce (*Picea abies*) contains potentially bioactive compounds such as fatty acids, terpenes, monoterpenes, sesquiterpene, diterpenes, waxes, sterols, saccharides, and phenolic compounds including stilbenes, tannins, and flavonoids, which display health-promoting properties including anticancer activity (Li *et al.* 2008; Tanase *et al.* 2013; Kreps *et al.* 2017). According to Li *et al.* (2008) more than 60 antioxidant phenolic compounds were isolated from spruce bark. Compounds such as piceid, astringin, quercetin, abietic acid (resin acid), piceid, and resveratrol aglycone have been identified. Resveratrol is an antioxidant also found in red wine and is currently being used extensively on account of its positive effects on human health and the recognition that resveratrol affects the prolongation of cell life even in mammals. Bioactive polyphenols of spruce bark could become potential active ingredients for cosmetics, functional foods, or pharmaceuticals (Co *et al.* 2012; Francezon *et al.* 2017). Various studies have indicated that the polyphenolic compounds can be mainly found in fruits,

vegetables, coffee, tea, and red wine (Miranda *et al*. 2016). Some studies showed that the forestry residues have an important number of polyphenolic compounds (Burčová *et al.* 2018; Ház *et al*. 2018; Coșarcă *et al.* 2019). For example, Coșarcă *et al*. (2019) have shown that these polyphenols can also be extracted from bark and could be utilized as food antioxidants.

More than 60 phenolic substances with antioxidant effects have been isolated from spruce bark (Li *et al.* 2008). Among these compounds, peptide, astringin, quercerin, abietic acid, and piceid resveratrol aglycon were identified. Stilbenes (resveratrol, astringin, and pterostilbene) that are isolated from spruce bark are potent anti-inflammatory, anti-tumor, antioxidant, antiaging, and chemoprotective agents (Mannila and Talvitie 1992; Jyske *et al.* 2014). This work focused on the determination of the antioxidant activity of extractives from spruce bark. Extracts and pre-extracts were added to lard to examine their protection factor, which was expressed as the resistance of the lard with added antioxidants to accelerated oxidation at 110 °C. This work is devoted to prolonged oxidative stability of lard, which is usually stabilized in the food industry by adding synthetic antioxidants such as BHT. The aim of this work was to replace synthetic antioxidants with antioxidants derived from natural renewable sources such as spruce bark.

EXPERIMENTAL

Materials

Spruce bark characterization

Spruce (*Picea abies* (L.) H. Karst) bark was obtained from Bioenergo Ltd. (Ružomberok, Slovakia). The bark was air-dried, ground, and separated into particle sizes that ranged from 1 mm to 1.5 mm.

Lard characterization

Lard was obtained from JAV-AKC Ltd. (Vlčany, Slovakia). The lard was made without synthetic antioxidants. The peroxide value of lard was 2.0 meqO/kg, and the content of free fatty acids was 0.68 % w/w.

Methods

Sample preparation

Soxhlet extraction was used to isolate active biological substances in the spruce bark (*Picea abies* (L.) H. Karst). Ethanol and n-hexan, separately, were used as extraction solvents. Milled bark (50 g) was extracted with 500 mL of extraction solvents for an extraction time of 8 h. The solvent was evaporated, and 5 g of residues were pre-extracted with 60 mL of ethanol and n-hexane mixture in a ratio 1:5. The separated phases were divided into a separating funnel.

Qualitative analysis of extracts using GS-MS

The analysis of the extractives was carried out using a gas chromatograph with a detector MS (MS 5975C, Agilent, Santa Clara, CA, USA) and column HP-5MS (30 m x 0.32×10^{-3} m; film thickness of 0.25×10^{-6} m). The injection volume of the sample was 1

x 10^{-6} L with a concentration of 5 mg/mL of n-hexane. The carrier gas was helium (316 mL min^{-1}), and the feed temperature was 250 °C. The split was set to 25:1. The column temperature was 80 °C for 2 min; then, it was raised by 15 °C min^{-1} to 320 °C for 50 min. The oven outlet was set to 300 °C, the MS detector source at 250 °C, and the MS quadrupole at 150 °C. The electron energy was 70 eV *m/z* and ranged from 30 to 780.

Antioxidant capacity of extracts and isolated fractions

The antioxidant activities of the spruce bark extract and its pre-extracts were determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical. The analysis was carried out on a microplate reader Epoch 2 (City, Country) at 517 nm. The concentration of the sample in ethanol for UV was 0.1 mg/mL. DPPH (500 µM) in the amount of 75 µL was added to 200 µL of the sample (dissolved in ethanol for UV). Ethanol for UV (75 µL) was used as the control. The calibration curve was prepared by diluting the trolox solution and was in the range of 80 µM to 1 µM.

Protective factor in lipid oxidation

Antioxidant activity was measured by the protection factor (PF), as calculated by Eq. 1,

$$PF = IP_A/IP_0 \quad (1)$$

where IP_A is the induction period of lard with the addition of antioxidant and IP_0 is the induction period of lard without the addition of antioxidant.

Statistical analysis

All analysis were performed three times. Mean value of experimental data was determined with confidence interval (95%), calculated with single-variable analysis of data.

RESULTS AND DISCUSSION

This study presented biologically active compounds extracted from the bark of Norway spruce from a new alternative feedstock with an antioxidant potential. The yields of ethanol and n-hexane with Soxhlet extraction were 6.9% and 5.4%, respectively. This result correlated with previous literature (Jablonský *et al.* 2017; Kreps *et al.* 2017). The antioxidant activity (Table 1) and stabilization of the oxidation of the lipids (Fig. 1) by adding bark extracts were investigated. The highest antioxidant activity was observed in the original ethanol extracts (15.0 mmol/mg). The experimental results showed that separation of the non-polar substances from the original ethanol extract by pre-extraction with n-hexane and ethanol had no significant effect in terms of antioxidant activity. This finding was advantageous in terms of economic, time, and technological demands. The separation of non-polar substances from the original ethanol extract reduced the antioxidant activity of the extract by approximately 5%. Nonpolar substances that were obtained by n-hexane extraction achieved 70% lower antioxidant activity in comparison to the ethanol extract. The results correlated with the findings of Co *et al.* (2012), which stated that, in terms of the recovery of extracts and the antioxidant activity of extracts, it

is better to use polar solvents, *e.g.* ethanol or water. The extraction of a particular group of substances, *e.g.* the antioxidants, is determined by the extraction conditions (time, temperature, pressure, extraction technique, solvent), which must be chosen optimally according to the nature of the substances extracted. According to Co *et al.* (2012), Moraes *et al.* (2013), and Xu *et al.* (2017), the most suitable method of extracting antioxidants is Soxhlet extraction using accelerated organic solvent extraction (ASE) with ethanol or water. According to the results of antioxidant analysis of spruce extracts in Talmaciu *et al.* (2016), supercritical extraction with CO_2 and ethanol as a co-solvent is more effective than supercritical extraction with CO_2 without ethanol. Based on these various results and claims, it is clear that extraction techniques and the solvent used are the key factors affecting the amount and variability of spruce isolated substances.

Both Co *et al.* (2012) and Talmaciu *et al.* (2016) studied the effects of extraction conditions on the recovery of antioxidant-active substances from spruce bark. The antioxidant activity of the extracts was not significantly influenced by the extraction time, but temperature and pressure were the dominant factors. In the temperature range of 160 to 180 ° C the highest antioxidant activity of ethanol extracts was detected. This finding was consistent with those of other authors, who report that the yield of antioxidant active ingredients as well as the antioxidant activity of the extracts increases with increasing extraction temperature. With a higher temperature, the solubility of the polyphenols in the solvent increases, and thus the diffusion of the extracted substances increases.

Table 1 shows that the antioxidant activity of ethanol extract was similar to the antioxidant activity of synaptic acid and caffeic acid, which are known as antioxidant-active substances. The original ethanol extract showed about 63% and 7% higher antioxidant activity than β-carotene and α-tocopherol, respectively. These are commonly found antioxidants in foods. Paulova *et al.* (2004) and Moraes *et al.* (2013) found similar results. From the analysis of the antioxidant activity of spruce extracts, spruce bark can be judged to be a prospective source of natural antioxidants that can be applied in various sectors, for example in the food or pharmaceutical industries.

Table 1. Antioxidant Activity of Spruce Bark Extracts and Selected Antioxidants

Antioxidant capacity of bark extractives TEAC[a] mmol/mg[b]			
ethanol extract			**n-hexane extract**
original extract	*ethanol pre-extract*	*n-hexane pre-extract*	*original extract*
15.0 ± 0.4	14.3 ± 0.3	8.3 ± 0.3	4.5 ± 0.2
Antioxidant capacity of selected antioxidants TEAC[a] mmol/mg[b]			
synaptic acid			14.6 ± 0.4
α-tocopherol			13.9 ± 0.5
caffeic acid			14.9 ± 0.3
β-carotene			5.5 ± 0.3

[a] TEAC- Trolox equivalent antioxidant capacity; [b] Dry matter

Natural antioxidants isolated from spruce bark exhibit a variety of biological effects, including anti-inflammatory, anti-cancer, or further beneficial effects in skin aging or prevention of atherosclerosis (Xu et al. 2017).

The different antioxidant activities of the extracts were dependent on the individual substances present in the extracts. GC-MS analysis was performed to identify

the compounds that contributed to the antioxidant activity of spruce extract (Table 2). The most abundant compound (31.7 rel.%) identified in ethanol spruce extract was dehydroabietic acid methyl ester. Dehydroabietic acid belongs to the diterpenes, and together with abietic acid it forms the primary part of resin acids. These acids are particularly important due to their antimicrobial properties (Burčová *et al.* 2018). The antioxidant activity of these substances has not been further analyzed in the available literature. In view of the majority of the methyl esters of acids (dehydroabietic, abietic, 7-oxodehydroabietic) and the relatively low level of stilbenes and sterols identified in the extract with highest antioxidant activity, there may be some synergism between substances. Methyl esters of abietic acids could act in the presence of antioxidants as synergists for their antioxidant effect. However, this assumption should be further examined and analyzed.

The second dominant group of substances in the ethanol were stilbenes, which have several positive biological effects on human health. They have strong anti-inflammatory, anti-cancer, antioxidant and chemoprotective effects. According to several authors, stilbene glucosides (piceid, astringin, isorhapontin), present in spruce, have an important function of chemical protection against pathogens (Jyske *et al.* 2014)

According to Kreps *et al.* (2017) and Burčová *et al.* (2018), the antioxidant capacity of the plant extracts was affected mainly by the composition and the dominant group of substances in these extracts.

Table 2. GC/MS Analysis of Compounds in Spruce Bark Extracts

		Original ethanol extract	Original n-hexane extract
Class	**Compound**	**Yield of compounds [%]**	**Yield of compounds [%]**
Terpene	β-sitosterol	5.5 ± 0.2	34.1 ± 0.5
	γ-sitosterol	1.2 ± 0.3	18.0 ± 0.5
	Thunbergol	6.8 ± 0.3	-
	Kauren-18-ol, acetate, (4.beta.)	2.0 ± 0.5	-
	Longifolene	-	6.0 ± 0.4
	13-Epimanool	-	15.4 ± 0.5
Stilbene	Piceid	6.2 ± 0.5	1.0 ± 0.3
	Astrignin	5.8 ± 0.3	0.8 ± 0.3
Resin acids	Methyl dehydroabietate	31.7 ± 0.5	4.3 ± 0.5
	7-Oxodehydroabietic acid, methyl ester	1.2 ± 0.5	2.2 ± 0.5
	Dehydroabietic acid	1.8 ± 0.4	-

Sterols (34.1 %) were dominant in n-hexane extracts, and stilbenes (< 2 %) or resin acid (6.5 %) were less represented. A similar composition of spruce bark extract (Soxhlet extraction, ethanol), was identified in the study performed by Jablonsky *et al.* (2015). The results showed that, in particular, stilbenes and resin acid were responsible for the antioxidant activity of the ethanol extract. The results of GC-MS analysis, antioxidant activity, and percentages of stilbenes and resin acids of the ethanol extract from spruce bark indicated the potential for the application of extracts in a wide range of industries such as the foodstuff, cosmetics, and pharmaceutical industries. One possible application is the stabilization of fat oxidation in the food industry.

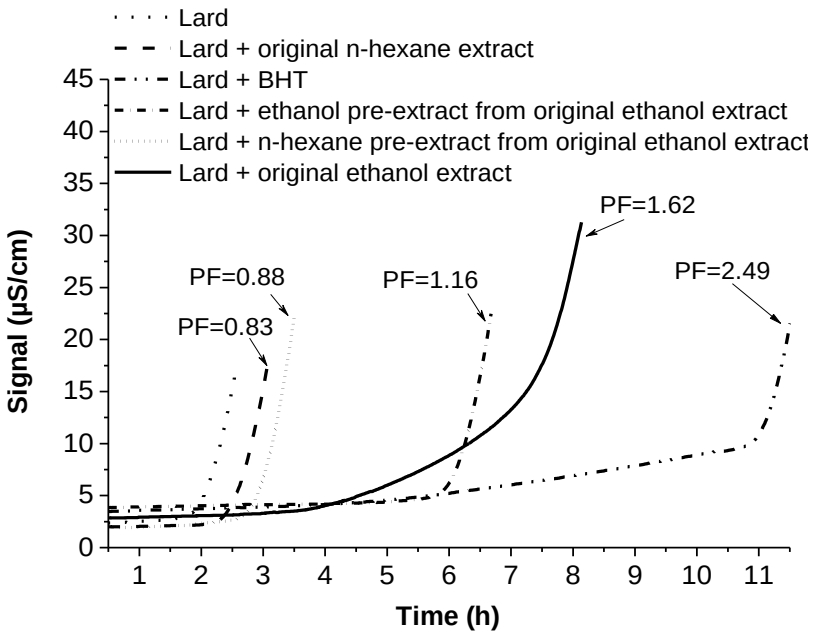

Fig. 1. Protective factor (PF) of lard without addition of antioxidant and with addition of extracts from bark of Norway spruce and synthetic BHT antioxidant (butylated hydroxytoluene)

All of the determined extracts were added to lard (200 mg/kg) to examine their protection factor (PF). The protection factor was stated as the resistance of lard with antioxidant to accelerated oxidation at 110 °C. Butylated hydroxytoluene- BHT (synthetic antioxidant; used to stabilize lard oxidation; PF = 2.49) was used to compare its antioxidant effect with extractive compounds from the bark of Norway spruce. The ethanol extract (antioxidant potential of 15.0 mmol/mg) had the best protective factor. This was only 35% lower than the BHT synthetic antioxidant. The ethanol pre-extract from the original ethanol extract had a good protective factor compared to the original ethanol extract. This also supported the results of antioxidant activity (Table 1). However, its factor was about 50% lower compared to BHT. Nonpolar extracts obtained by extraction with n-hexane had a low protection factor. This was similar to the n-hexane pre-extract from the ethanol extract. These protection factors were lower than the protection factor of BHT by 65%.

CONCLUSIONS

1. The highest antioxidant activity was observed in the original ethanol extracts (15.0 mmol/mg), which had greater antioxidant activity than alpha-tocopherol 13.9 mmol/mg. The ethanol extracts also increased the oxidation stability of the lard by 5 h (PF = 1.62).
2. The antioxidant activity of the ethanol extract was influenced by the presence of resin acids (34.7%) and stilbenes (12%). These substances were present only in a small amount in the n-hexane extract. The n-hexane extract had low antioxidant

activity and achieved about 70% lower antioxidant activity compared to the ethanol extract.

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