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IDENTIFICATION, IN VITRO AND IN VIVO ANTIOXIDANT ACTIVITY, AND GASTROINTESTINAL STABILITY OF LIGNANS FROM SILVER FIR (*ABIES ALBA*) WOOD EXTRACT

Eva Tavčar Benković,[1] Dušan Žigon,[2] Vladimir Mihailović,[3] Tanja Petelinc,[4] Polona Jamnik,[4] and Samo Kreft[1]

[1]Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia
[2]Jožef Stefan Institute, Ljubljana, Slovenia
[3]Department of Chemistry, Faculty of Science, University of Kragujevac, Kragujevac, Serbia
[4]Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Lignans are generally known for their beneficial impact on human health. In this study, we found that the water extract of silver fir (*Abies alba*) wood contains lignans, which constitute approximately 10% of the extract and include isolariciresinol, hydroxymatairesinol, secoisolariciresinol, lariciresinol, pinoresinol, and matairesinol. The antioxidant activity of the extract was measured by several in vitro assays and an assay in a eukaryotic cell model (yeast). The extract had greater in vitro antioxidative activity than ascorbic acid, resveratrol, or butylated hydroxytoluene (BHT) and similar antioxidative activity to epigallocatechin gallate. The intracellular antioxidant effect in the yeast gave indirect evidence that the components of the silver fir wood extract (SFWE) effectively pass through eukaryotic cell membranes and have higher in vivo antioxidative activity than ascorbic acid, resveratrol, tocopheryl succinate, or BHT and similar to epigallocatechin gallate. The in vitro gastrointestinal digestion of the lignans in the extract is not significant; therefore, it is reasonable to expect antioxidative effects from orally applied SFWE.

KEYWORDS. Silver fir wood, *Abies alba*, lignans, antioxidant

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

Isolariciresinol (PubChem CID: 160521), hydroxymatairesinol (PubChem CID: 10948757), secoisolariciresinol (PubChem CID: 65373), lariciresinol (PubChem CID: 332427), pinoresinol (PubChem CID: 73399), matairesinol (PubChem CID: 119205), ascorbic acid (PubChem CID: 54670067), butylated hydroxytoluene (PubChem CID: 31404), resveratrol (PubChem CID: 91745415), epigallocatechin gallate (PubChem CID: 65064).

INTRODUCTION

Conifer bark and wood have become popular sources of polyphenols with known biological, pharmacological, and clinical activities that contribute to human health, such as anti-inflammatory activity, cardiovascular disease prevention, cancer prevention, antidiabetic, and antimicrobial activities.[1–6] One of the oldest and most researched of these extracts is a maritime pine extract, Pycnogenol. An extract similar to Pycnogenol is an extract of the silver fir (*Abies alba*) trunk,

Address correspondence to Eva Tavčar Benković, Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia. E-mail: eva.tavcar@ffa.uni-lj.si
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which exhibited strong antioxidant properties in vitro and in isolated primary mononuclear cells from peripheral human blood. This extract also prevented guinea pig's atherogenesis by preventing the formation of plaques and ameliorating vascular relaxation[7] and protected the heart from the damage caused by ischemia and reperfusion.[8] It was found to contain at least 13 polyphenols. Both Pycnogenol and silver fir trunk extract were obtained using an organic solvent.[7,9] However, the solubility in intestinal fluids is different than that in organic solvents.[10] The bioactivity of polyphenols has been a popular topic over the past few years. Research studies have shown highly variable results because of the high number of tested compounds with different solubilities and the variety of different assays used, including bioavailability assays.[11] Water extracts can also exhibit significant biological activity, but research focusing on water extracts is often neglected.

In this research, we focused on the water-extractable fraction of silver fir wood (SFWE), which was recently shown to lower postprandial glycemic response.[12] The extract, studied in this study, represents a water-soluble fraction of the branches and is not an organic fraction of the silver fir trunk; so, we expected that it contains different types of polyphenols.[7–9]

One important constituent of the water-extractable fractions of some foods is lignans. Lignans are not only present in everyday foods, such as flaxseed, sesame seed, and asparagus,[13] but are also an important fraction of wood extractives.[14] Lignans appear to have activities that are important for human health. Lignans have a two-sided interaction with microbiota: they influence gut microbiota, which constitute the basis of their further metabolism, resulting in other activities, such as antidiabetic and anticancer activities and the reduction of cardiovascular disease risk.[15–18] Furthermore, lignans are precursors of the important bioactive compounds, enterodiol and enterolactone.[19–23]

Here, we identified the compounds of the silver fir wood extract (SFWE), measured in vitro and in vivo antioxidant activities, and studied the gastrointestinal (GI) digestion. We also tested the in vivo bioavailability of the SFWE using yeast cells. We discussed our findings in accordance with its recently discovered antidiabetic action.

MATERIALS AND METHODS

SFWE (Belinal) was obtained from Abies Labs d.o.o., Ljubljana, Slovenia. It is a dried aqueous extract from the silver fir (*A. alba*) wood from branches.

All chemicals and reagents used for analyses of total phenolic compounds and antioxidant assays were purchased from Sigma-Aldrich (Deisenhofen, Germany). Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, and bile extract porcine were also purchased from Sigma-Aldrich (Deisenhofen, Germany). Reference standard compounds that were used to confirm identity and for the quantification were purchased from the following suppliers: pinoresinol, lariciresinol, secoisolariciresinol diglucoside, and nortrachelogenin (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany); isolariciresinol, hydroxymatairesinol, secoisolariciresinol, pinoresinol, and matairesinol (Sigma-Aldrich Co. Bratislava, Slovakia). The solvents used for The high-performance liquid chromatography (HPLC) analysis were of HPLC-grade purity: water and acetonitrile (JT Baker; Deventer, Netherlands), and trifluoroacetic acid (Roth, Karlsruhe, Germany). All spectrophotometric measurements were performed on a UV/Vis spectrophotometer Nanocolor (Macherey-Nagel GmbH & Co. KG, Germany).

Identification and Quantification of the SFWE Compounds

For identification via reference standards and quantification of the compounds, the HPLC system (Shimadzu Prominence) was used. It consisted of a system controller (CBM-20A), a column oven (CPO-20AC), and a

solvent delivery pump with a degasser (DGU-20A5) connected to a refrigerated autosampler (SIL-20AC) with a photodiode array detector (SPD-M20A) that monitored the wavelengths 190–800 nm. The responses of the detectors were recorded using LabSolution software version 5.71. The chromatography was performed at 40°C and a flow rate of 2 mL min^{-1} using a Phenomenex Kinetex® C18 column (10 cm × 4.6 mm I.D., 2.7-μm particle size). The following gradient method using water (solvent A) and acetonitrile (solvent B), both containing 0.1% trifluoroacetic acid, was utilized: 0–1 min 5% B, 1–10 min 5–30% B, and 10–15 min 100% B.

For mass spectrometric (MS) analyses, the chromatographic separation was performed on a Waters Acquity ultraperformance liquid chromatograph® (Waters Corp., Milford, MA, USA) with conditions identical to that used for the quantitative HPLC analysis, except that the formic acid was used instead of trifluoroacetic acid in the mobile phase. The flow rate was 0.5 mL min^{-1} and the column temperature was maintained at 40°C. The LC system was interfaced with a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-ToF Premier, Waters, Milford, MA, USA). The compounds were analyzed under negative (ESI(−)) ion conditions. The capillary voltage was set at 3.0 kV, while the sampling cone voltage was 20 V. The source and desolvation temperatures were 120 and 200°C, respectively. The nitrogen desolvation gas flow rate was 500 L h^{-1}. The acquisition range was between *m/z* 50 and 1000 with argon serving as the collision gas at a pressure of 4.5×10^{-3} mbar in the T-wave collision cell. The MS/MS experiments were performed using collision energy of 30 eV to generate the product ion spectra that provided the best structural information. The data were collected in the centroid mode, with a scan accumulation time of 0.2 s and an interscan delay of 0.025 s. The data station utilized the MassLynx v4.1 operating software. Accurate mass measurements were obtained with an electrospray dual sprayer using the reference compound leucine enkephalin ($[M + H]^+ = 556.2271$) at a high mass resolution of 10,000.

Spectrophotometric Determination of Phenolic Compound Content

The total phenolics' content in SFWE was assayed using the Folin–Ciocalteu reagent.[24] The results of total phenolics' content in the extract were expressed in milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g extract). The procedure for estimating the total content of phenolic (hydroxycinnamic) acids was adopted from the Polish Pharmacopoeia (2005). First, 5 mL of water was added to 1 mL of extract (1 mg/mL). HCl (1 mL, 0.1 M), Arnow reagent (1 mL, 10% w/v of sodium molybdate and 10% w/v sodium nitrite), and NaOH (1 mL, 1 M) were added to the mixture, and the volume was adjusted to 10 mL. The absorbance at 490 nm was read immediately. The results were expressed as caffeic acid equivalents (mg CAE/g extract).

The total flavonoid content was determined using the aluminum trichloride method[25] and expressed as rutin equivalents (mg RUE/g extract).

The spectrophotometric pH differential method was used to determine the monomeric and total (monomeric plus polymerized) anthocyanin contents in the extract.[26]

In Vitro Antioxidative Activity

The activities of SFWE and four reference substances—resveratrol, tocopheryl succinate, butylated hydroxytoluene (BHT), and epigallocatechin gallate (EGCG)—were compared.

The DPPH radical scavenging activity was assayed according to the method of Takao et al.[27] with the slight modifications of Kumarasamy et al.[28] Initially, 1 mL of a methanol solution of DPPH (80 μg/mL) was mixed with the same volume of the examined extract or the reference antioxidant dissolved in methanol. The reaction mixture was left in the dark at room temperature for 30 min. Then, the absorbance of the mixture was measured at 517 nm. The ability to scavenge the DPPH radical was calculated by the following equation: % inhibition $= [(A_C - A_S)/A_C] \times 100$, where A_C is the absorbance of the control solution and

A_S is the absorbance of the extract in the presence the DPPH solution. The concentration of the extract that is able to achieve 50% scavenging (IC_{50}) was calculated from the sigmoidal dose–response curve using OriginPro8 software in which the scavenging percentage was plotted against the extract concentration (μg/mL).

For the $ABTS^+$ radical scavenging assay, the method of Re et al.[29] was adopted with slight modifications. Briefly, a solution of the ABTS radical cation ($ABTS^+$) was prepared 16 h prior to the experiment by reacting 7-mM ABTS and 2.45-mM potassium persulfate in the dark. The resulting solution of $ABTS^+$ was diluted with 80% ethanol to an absorbance of 0.700 ± 0.005 at 734 nm. A range of extract concentrations (0.1 mL) was mixed with the $ABTS^+$ solution (0.9 mL). The mixture was incubated at 30°C for 30 min before its absorbance at 734 nm was measured. The $ABTS^+$ radical scavenging activity was calculated as the percentage of inhibition: [(AC – AS)/AC] × 100.

To determine the reducing power of the extract, the method based on reduction of Fe_{3+} in ferricyanide complex to Fe_{2+} by presence of antioxidants in the sample was employed.[30] In this method, an increase in absorbance at 700 nm indicates a greater reducing activity of samples.

The nitric oxide radical scavenging activities were assayed using the Griess reaction according to method described by Green et al.[31]

The extracts' ability to inhibit non-site-specific hydroxyl radical-mediated peroxidation was observed according to method described by Hinneburg et al.[32] The superoxide radical scavenging activity (PMS–NADH System) was also measured. Superoxide anions were generated using the PMS/NADH system and subsequently reduced nitroblue tetrazolium, yielding a chromogenic product that was measured at 560 nm. A test solution (1 mL) in 0.1-M phosphate buffer (pH 7.4), 625 µl of a 468-µM NADH solution, 625 µl of a 150-µM NBT solution, and 625 µl of a 60-µM PMS solution were incubated at room temperature for 5 min. The absorbance of the solution was measured at 560 nm.

The inhibitory activity toward lipid peroxidation was determined using the ferric thiocyanate method in linoleic acid–water emulsion system, as described by Hsu et al.[33]

In Vivo Antioxidative Activity

Measurement of Level of Reactive Oxygen Species (ROS) Within Yeast Cells. The yeast *Saccharomyces cerevisiae* ZIM 2155 was obtained from the Culture Collection of Industrial Microorganisms (ZIM) of the Biotechnical Faculty of the University of Ljubljana in Ljubljana, Slovenia. The yeast cells were cultivated in yeast extract (10 g L^{-1}; Biolife), peptone (20 g L^{-1}; Biolife), and glucose (20 g L^{-1}; Merck) (YEPD) medium at 28°C and 220 rpm until achieving the stationary phase. The cells were then centrifuged for 3 min at 4000 *g*, washed once with phosphate-buffered saline (PBS) (Merck), and resuspended in PBS at a concentration of 1×10^8 cells mL^{-1}. The cells were further incubated at 28°C and 220 rpm for 96 h.[32] The yeast cells (*S. cerevisiae)* were exposed to SFWE, the reference standards (ascorbic acid, resveratrol, and EGCG) were dissolved in water, and BHT and tocopheryl succinate were dissolved in ethanol. The concentrations of SFWE and each standard in the cell suspension were 0.05 and 0.1 g/L After a 2-h incubation at 28°C and 220 rpm, samples were taken and their intracellular oxidation levels were measured.

Intracellular oxidation was measured using 2′,7′-dichlorofluorescein (H_2DCF), which reacts with oxidants, thus revealing the presence of ROS. This compound was administered to the cells as 2′7 ′-dichlorofluorescein diacetate (H_2DCFDA), which easily penetrates the plasma membrane and is hydrolyzed inside the cells by non-specific esterases. The nonfluorescent H_2DCF can then be oxidized by the oxidants present in the cell to the fluorescent compound 2′,7′-dichlorofluorescein (DCF), which can be measured.[34]

The cells from the 2-mL incubations were sedimented by centrifugation (14,000 *g* for 5 min) and washed thrice with 50-mM potassium phosphate buffer (pH 7.8). The cell

pellets were resuspended in nine volumes of 50-mM potassium phosphate buffer (to make a 10%, v/v solution) and incubated at 28°C for 5 min. The ROS-sensing dye H_2DCFDA (Sigma) was added from a 1-mM stock solution in 96% ethanol (Merck) to a final concentration of 10 μM. After a 20-min incubation at 28°C and 220 rpm, the fluorescence of the yeast cell suspension was measured using the kinetic mode of a Safire II microplate reader (Tecan). The excitation and emission wavelengths of DCF are 488 and 520 nm, respectively. All tests were conducted in triplicate. The results are presented as an average of the relative reductions of intracellular oxidant level ±SD with respect to the control (untreated culture).

In Vitro GI Digestion Assay

The assay was performed according to the procedure described in the literature.[35] To simulate the digestion process, SFWE was dissolved in simulated salivary fluid (SSF) at a concentration of 2 mg/mL. The studies are performed in two consecutive steps including incubation for 2 h with simulated gastric and small intestinal digestive fluids, respectively. Changes in chemical composition and antioxidant activity of SFWE were determined in samples after 1, 2, 3 and 4 h of start of digestion process.

RESULTS AND DISCUSSION

The compounds contained in the SFWE were identified and quantified via their MS fragmentation patterns, high-resolution mass measurements, ultraviolet (UV) spectra, retention time, and comparison with reference standard compounds (Table 1; Figure 1). Six lignans were identified, representing the six highest

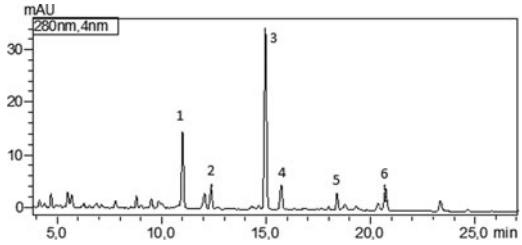

FIGURE 1. Chromatogram of SFWE with identified lignans.

peaks on the HPLC chromatogram. Among them, only two lignans were previously found in the silver fir bark extract (secoisolariciresinol and laricinresinol).[9] The following three reference standards were also purchased and compared, but corresponding substances were not found in the SFWE: secoisolariciresinol diglucoside, nortrachelogenin, and matairesinol.

Spectrophotometric Determination of Phenolic Compound Content

The total phenolic content (TPC) was 249.6 mg GAE/g dry extract. The flavonoid content was 4.1 mg RUE/g dry extract. The total phenolic acid content was 10.6 mg CAE/g dry extract, and the total anthocyanin content was 0.015 mg CGE/g. The TPC is expressed in gallic acid equivalents and does not necessarily reflect the actual amount of phenolic compounds in the extract. A phenolic content of 25% would mean that 75% of the extract should consist of non-phenolic components. Carbohydrates are a known non-phenolic component of conifer wood extract.[12] The contents of flavonoids (4.1 mg/g) and anthocyanins (0.015 mg/g) in this SFWE were very low, unlike in the maritime pine bark extract which contains 65–75% procyanidins, so antioxidative activity, discussed further, should be a consequence of other compounds.[36]

TABLE 1. Identification and quantification of the compounds found in SFWE

	Compound	Elemental composition	*m/z* (ESI(−))	% (g compound/100 g SFWE)
1	Isolariciresinol	$C_{20}H_{24}O_6$	359	1.78
2	Hydroxymatairesinol	$C_{20}H_{22}O_7$	373, 355, 327	0.89
3	Secoisolariciresinol	$C_{20}H_{26}O_6$	361, 346, 315	5.51
4	Lariciresinol	$C_{20}H_{24}O_6$	359	0.79
5	Pinoresinol	$C_{20}H_{22}O_6$	357	0.24
6	Matairesinol	$C_{20}H_{22}O_6$	357	0.50

TABLE 2. Antioxidant activities of SFWE and the reference compounds expressed as IC_{50}s. Lower numbers correspond to higher activities

	IC_{50} (μg/mL)						
Samples	$ABTS^{\bullet+}$ scavenging activity	DPPH scavenging activity	NO radical scavenging activity	OH radical scavenging activity	Superoxide radical scavenging activity	Inhibitory activity toward lipid peroxidation	Reducing power[a] (absorbance et 700 nm)
SFWE	32.10	35.46	73.52	4.66	163.96	240.42	1.76
Ascorbic acid	24.67	7.62	14.19	—[b]	—	—	2.01
BHT	24.76	11.58	—	9.24	—	17.00	2.21
Resveratrol extract	11.29	16.62	36.64	5.84	>500	73.35	1.92
EGCG	12.24	7.06	12.37	18.29	12.28	—	2.48

[a]Absorbance at concentration of 125 μg/mL, higher absorbance indicates a higher reducing activity.
[b]The test was not performed (e.g., because of insufficient solubility in the test medium or interaction with the test components).

In Vitro Antioxidant Activity

Antioxidant activity has to be tested through numerous test models, as the mechanism of antioxidant action is highly dependent on the features of the individual molecule, its structure, and its environment. Most tests used to measure a substance's antioxidant activity also measure the substance's ability to scavenge free radicals. DPPH and ABTS tests are the most common antioxidant activity assays. ABTS assay is applicable for both hydrophilic and lipophilic antioxidants.[37]

Table 2 summarizes the antioxidant activities of SFWE and standard antioxidants. IC_{50} is defined as the concentration (in μg/mL) required to achieve 50% inhibition.

In the DPPH and ABTS tests, the antioxidant activity of SFWE was lower than that of BHT, resveratrol, EGCG, and vitamin C but IC_{50} value of SFWE was only 23% lower than IC_{50} values of vitamin C and BHT. However, DPPH and ABTS are artificial free radicals that are not found in the human body.

Therefore, we also used more relevant tests and probed the extract's ability to scavenge biologically relevant radicals: nitric oxide, hydroxyl radical, and superoxide, as well as the lipid peroxidation test. Nitric oxide is an important cellular signaling molecule involved in many physiological and pathological processes; for example, it is a powerful vasodilator. Low levels of nitric oxide production are important in protecting organs from ischemic damage; so, strong nitric oxide scavenging activity of the exogenous antioxidant is generally not desired. Superoxide is biologically used by the immune system to kill invading microorganisms, but the oxidative damage it may cause contributes to the pathogenesis of many diseases and aging. Lipid peroxidation is the oxidative degradation of lipids, the main builders of the cell membranes. End products of lipid peroxidation may be mutagenic and carcinogenic.

In the NO radical assay, the superoxide radical scavenging activity assay, the inhibitory activity toward lipid peroxidation assay, and the reducing power tests, the antioxidant activity of SFWE was lower than those of the tested standards. Testing the inhibition of lipid peroxidation showed that SFWE had higher IC_{50} values compared to other applied methods, suggesting that this extract possesses a lower antioxidant potential in the lipid system and contains more antioxidants which dissolve better in water.

Indeed, the extract showed good antioxidant activity in hydroxyl radical assay. Hydroxyl radical is highly reactive and can damage all types of biologically relevant molecules, such as carbohydrates, nucleic acids, lipids, and amino acids. It cannot be eliminated by an enzymatic reaction; so, endogenous and exogenous antioxidants become of high importance. The hydroxyl radical scavenging activity of SFWE was higher than those of any other tested standards: slightly higher than that of

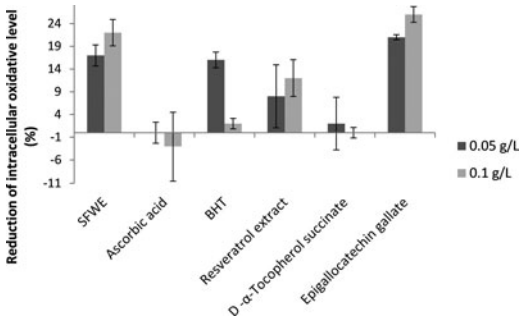

FIGURE 2. Intracellular antioxidant activitity of tested compounds in *Saccharomyces cerevisiae*.

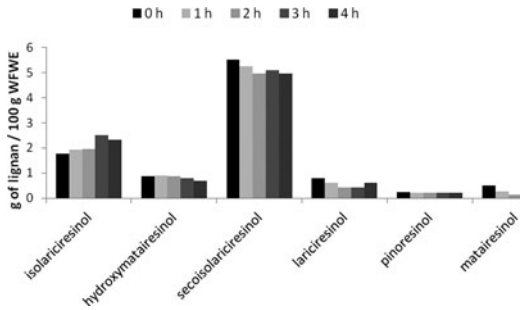

FIGURE 3. Content of identified lignans in SFWE during in vitro digestion.

resveratrol, twice as high as that of tocopheryl succinate, and almost fourfold higher than that of EGCG.[38]

In Vivo Antioxidative Activity

SFWE had a greater antioxidant activity in the intracellular antioxidative assay in *S. cerevisiae* than the other tested standard antioxidants (ascorbic acid, resveratrol, BHT, and tocopheryl succinate), except EGCG, which had a comparable activity (Figure 2). The antioxidant activity of SFWE (non-significantly, $p = 0.09$) increased at higher concentration, whereas the antioxidant activity of some other substances decreased at higher concentration (for BHT, $p = 0.02$) or even a pro-oxidative activity appeared. These results also indirectly indicate that the components of SFWE successfully pass through eukaryotic cell membranes.

In Vitro GI Digestion

Digestion could effect the chemical composition and antioxidant activities of polyphenols due to body temperature, pH value, and digestive enzymes.[35] Therefore, in vitro simulated-digestion studies are additional indicators of the biological significance of examined extracts. The stability of the individual compounds present in the extract under digestive conditions without gut microbiota was assessed using a simulated gastric and small intestinal model. Variations in the concentrations of the main extract constituents were determined by HPLC every hour during the 4-h digestion (Figure 3). All the examined

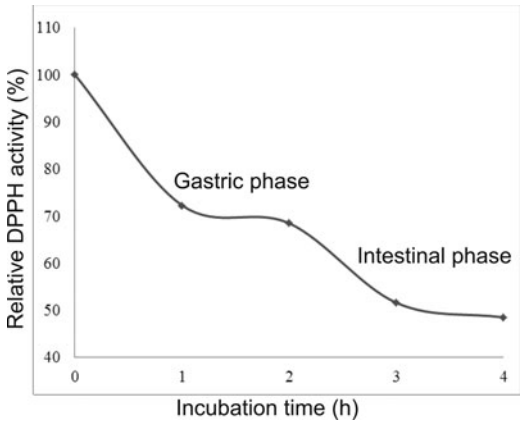

FIGURE 4. Changes in the antioxidant activity of SFWE during in vitro digestion measured by a DPPH assay (% inhibition of DPPH radicals relative to % inhibition on the beginning of digestion).

compounds showed high stability under gastric conditions during the first 2 h of digestion and during the second 2 h of intestinal digestion. During the digestion, the variations in the antioxidant activities of SFWE were monitored using a DPPH method (Figure 4). The results showed that the antioxidant activity of SFWE decreased by only approximately 30% during gastric digestion and by an additional 20% during intestinal digestion. The lignan content did not decrease proportionally, indicating that lignans are not the only substances responsible for the observed antioxidant activity of SFWE.

CONCLUSIONS

The compounds identified in this article, lignans, have proven antioxidant as well as antidiabetic actions.[39,40] We showed that

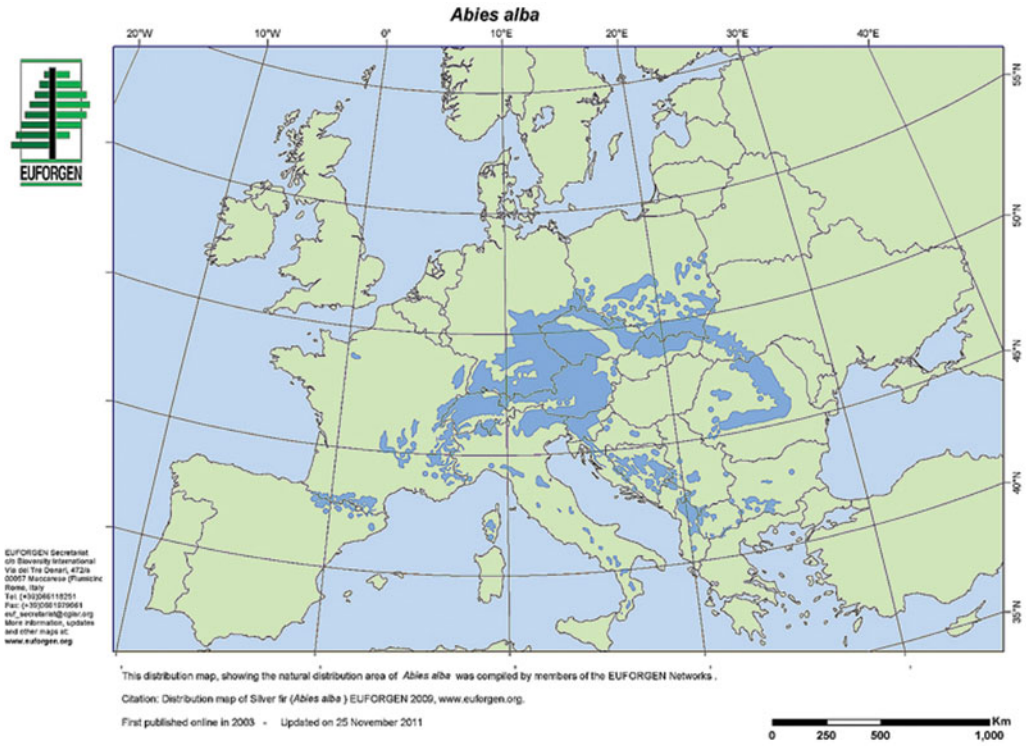

FIGURE 5. The importance of Silver fir in Central Europe is shown on natural distribution map42.

SFWE exhibits in vitro and in vivo antioxidant activities due to lignans and also some other, unidentified compounds. This is in accordance with the findings of our another study, published recently, which showed that SFWE lowers post-prandial glycemic response. The antioxidant activity is one of the known mechanisms that contribute to controlling blood glucose levels through the diminution of inflammation.[12] Finally, the amount of lignans available for further metabolism by gut microbiota was sufficient to support the high bioactive potential of SFWE.

The silver fir has an important economic significance in Central Europe with 11% in the annual felling of timber (Figure 5).[41] As the raw material for the extract (wood from branches) represents a leftover material at wood processing, the production of such an extract holds an economic as well as ecological entity. Such an extract can be used as an organic source of antioxidants and as an antidiabetic agent in a variety of products including pharmaceuticals, food supplements, and cosmetics.

ABBREVIATIONS

SFWE	silver fir wood extract
EGCG	epigalocatechin gallate
DPPH	1,1-diphenyl 2-picryl hidrazin
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
TPC	total phenolic content
CAE	caffeic acid equivalents
RUE	rutin equivalents
BHT	butylated hydroxytoluene
SSF	simulated salivary fluid

ORCID

Eva Tavčar Benković http://orcid.org/0000-0003-0983-5525

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