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ORIGINAL PAPER

Silver Fir (*Abies alba*) Extracts Inhibit Enzymes Involved in Blood Glucose Management and Protect against Oxidative Stress in High Glucose Environment

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Abstract

The diet rich in fruits and vegetables reduces the risk of metabolic syndrome, including diabetes development by various mechanisms of action, mainly due to the presence of polyphenolic compounds. Extracts from different conifer species are known to be a rich source of various polyphenols. In the present study we elucidated the *in vitro* mechanism of anti-diabetic activity of silver fir (*Abies alba*) wood and bark extracts and compared their activity to non-coniferous sweet chestnut wood extract and standardized maritime pine bark extract. Extracts and lignans were tested for their inhibitory activity of enzymes involved in the regulation of blood glucose *in vitro*. The ability of extracts to protect against oxidative stress in high glucose environment was tested on mouse myoblast cell line. Silver fir wood and bark extracts were shown to be effective inhibitors of α-glucosidase, α-amylase and dipeptidyl peptidase 4, three enzymes involved in the regulation of blood glucose levels. Coniferous extracts also showed protection against oxidative stress generated in high glucose environment. Lignans, particularly pinoresinol diglucoside, isolariciresinol and secolariciresinol were shown to be important contributors of antihyperglycemic activity through inhibition of dipeptidyl peptidase 4. This corroborates previously published *in vivo* results on blood glucose level obtained with silver fir wood extract and supports the use of silver fir wood and bark extracts as food supplements or functional foods in borderline diabetes.

Keywords Silver fir · Digestive enzymes · Oxidative stress · Diabetes · Lignans

Abbreviations

DPP4 Dipeptidyl peptidase 4
ROS Reactive oxygen species

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Introduction

Diabetes mellitus is presently the most common chronic disease, recognized as a major health problem associated with increased morbidity, mortality and health care costs. Type 2, or non-insulin dependent diabetes mellitus, represents over 90% of all cases and is increasing globally with an immense rate [1]. The best treatment strategy is to intervene when very early clinical signs, such as impaired glucose tolerance, first manifest [2]. In addition to regular physical activity and weight reducing diets [3], the use of supplementary treatments, including nutraceuticals and functional foods, will be of great value.

It has become evident that postprandial hyperglycemic spikes associated with high incidence and progression of microvascular and macrovascular complications should be the focus of therapeutic approach in type 2 diabetes [4]. One

way to achieve an improved control of the blood glucose level is by retarding the absorption of glucose by acting on enzymes involved in digestion of dietary carbohydrates in gastrointestinal tract such as α-amylase or α-glucosidase [5]. The same effect can also be achieved by inhibition of dipeptidyl peptidase 4 (DPP4), a peptidase that hydrolyses incretin hormones, thereby increases incretins half-life, which finally reduces the blood glucose level [6]. Namely, incretins are involved in secretion of insulin from pancreatic beta cells in the presence of nutrients. Another strategy is to reduce oxidative stress induced by hyperglycemia [7]. In pancreatic beta cells reactive oxygen species (ROS) induce apoptosis and suppress insulin biosynthesis. Pancreatic beta cells are particularly susceptible to the deleterious effects of ROS since their expression of antioxidant enzymes is low [8].

According to many epidemiological and empirical studies, the diet rich in fruits and vegetables can reduce the risk of metabolic syndrome, including diabetes development [9]. The beneficial health effect has been largely attributed to the polyphenols and low molecular weight phenols found in plant-derived foods and spices [10]. The growing evidence from *in vitro* studies as well as from clinical trials suggests that they can potentially be used to control postprandial hyperglycemia and to prevent long term complications of type 2 diabetes mellitus [11].

Extracts from the bark and the wood of different conifer species are known to be a rich source of various polyphenols, and accordingly possess interesting biological, pharmacological and clinical activities. The most studied among them is Pycnogenol®, the standardized French maritime pine bark extract, widely available in dietary supplements, cosmetics and health products. Its antidiabetic activity has been attributed to the inhibition of α-amylase and α-glucosidase [12] and was confirmed in diabetic patients [13]. We have focused our attention to extracts of conifer species, silver fir (*Abies alba*), indigenous in mountainous regions of Europe. Some aspects of their antioxidative and vasoprotective activities have already been evaluated [14, 15]. In this research, we elucidated the *in vitro* mechanism of anti-diabetic activity of silver fir (*Abies alba*) wood (Belinal®) and bark extracts and compared their activity to non-coniferous sweet chestnut wood extract Farmatan® and maritime pine bark extract Pycnogenol®. We evaluated the ability of the extracts to inhibit enzymes involved in the management of the blood glucose level and ability to protect against oxidative stress generated in high glucose environment. Lignans are known to be an important fraction of wood extractives, including the aqueous extract of silver fir wood [16], thus the inhibitory activities of nine selected lignans identified in Belinal® [17] were further characterized for their inhibitory activity against the three enzymes.

Materials and Methods

Materials

Silver fir bark extract was extracted by a two-step extraction as described previously [14]. Silver fir wood extract, Belinal® was obtained (Alpe Pharma, Ljubljana, Slovenia) by water extraction followed by spray drying [18]. Farmatan®, an aqueous extract of sweet chestnut (*Castanea sativa*) wood, was obtained from Tanin Sevnica (Sevnica, Slovenia). Pycnogenol® was purchased from Biolandes (Le Sen, France). Lignans were purchased from the following suppliers: pinoresinol, lariciresinol, secoisolariciresinol diglucoside and nortrachelogenin (PhytoLab, Vestenbergsgreuth, Germany); isolariciresinol, hydroxymatairesinol, secoisolariciresinol, pinoresinol diglucoside and matairesinol (Sigma-Aldrich, St. Louis, MO, USA). The dried extract powders were dissolved in 25% (v/v) dimethyl sulfoxide in water. Solutions of lignans in concentration 1 mg/mL were prepared in 50% (v/v) methanol in water.

Enzyme Inhibition Tests

Inhibitory activities on α-glucosidase (EC 3.2.1.20; Sigma-Aldrich, MO, USA), α-amylase (EC 3.2.1.1, type IV, Sigma–Aldrich, MO, USA) and dipeptidyl peptidase 4 (EC 3.4.14.5, Abnova, Taipei City, Taiwan) were performed in triplicates as previously described [19]. The data points are expressed as mean ± standard deviation. The IC_{50} value was determined from the dose–response curve.

Cell Culture

C2C12 (ATCC® CRL-1772™) mouse myoblast cell line was cultured in DMEM low glucose (with stable glutamine and sodium pyruvate) medium (Biosera, Boussens, France) supplemented with 10% fetal bovine serum (HyClone, GE Healthcare, Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Biosera, Boussens, France). The cells were cultivated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were passaged twice a week.

Cytotoxicity Test

The extracts dissolved in serum free DMEM Low Glucose medium were added to adherent C2C12 cells in 96-well plates in serum free medium. Final concentrations of 20, 10 and 5 μg/mL of each extract were used. The cells were incubated at 37 °C with 5% CO_2 for 24 h. After the incubation, the cytotoxicity was determined by the Cell Proliferation Reagent WST-1 kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. FLUOstar Omega microplate reader (BMG, Ortenberg, Germany) was employed to measure absorbance.

Oxidative Stress Protection Test

The amount of 1.8×10^4 C2C12 cells *per* well were seeded into a black 96-well microtitre plate and incubated (37 °C, 5% CO_2) overnight (16 h). Next, the extracts were added in the final concentrations of 10 and 5 μg/mL (these concentrations had no cytotoxic effect on C2C12 cells). After 1-h incubation, 10 μL of glucose (Sigma-Aldrich, Steinheim, Germany) in PBS (Biosera, Boussens, France) was added to a final concentration of 55 mM in samples tested for oxidative stress protection in high-glucose environment. To samples tested for oxidative stress protection in normal-glucose environment 10 μL of PBS was added. Cells were incubated for 4 h (37 °C, 5% CO_2). Next, $DCFH_2$-DA (Sigma-Aldrich, Steinheim, Germany) from stock solution in dimethyl sulfoxide (5 mg/mL) was introduced into the cell medium and added to wells to reach final concentration of 5 μg/mL. The final concentration of dimethyl sulfoxide reached the level of 0.1% (*v*/v) in each well. After 30 min, the determination of ROS production was determined by measuring the intracellular fluorescence of the dichlorofluorescein product on FLUOstar Omega Microplate Reader (BMG Labtech) using λ(ex./em.) = 485/520 nm. The ROS level in low-glucose untreated cells was set up as 100%. The ratio of ROS production was calculated according to low-glucose control wells that did not contain extracts. Differences between groups were analyzed by ANOVA. All differences were considered statistically significant with $p < 0.05$. The results are presented as mean ± SD.

Results and Discussion

We determined the inhibitory activities of the silver fir (*Abies alba*) wood and bark extracts upon the three enzymes involved in the regulation of blood glucose levels and compared them to non-coniferous sweet chestnut wood extract Farmatan® and maritime pine bark extract Pycnogenol® (Fig. 1). Pycnogenol® with proven antidiabetic clinical efficacy [13] and confirmed inhibitory activities upon α-glucosidase and α-amylase [12, 20] was used as reference extract. In this study we additionally revealed its inhibitory effect upon DPP4. After per oral delivery of the extracts, high concentrations would be available in the gut to provide inhibition of the enzymes, therefore high concentrations of extracts (mg/mL) were also used in *in vitro* tests. Both silver fir extracts exhibited great *in vitro* inhibitory potential upon all three enzymes. α-glucosidase was best inhibited by wood extract, followed by bark extract and Pycnogenol®, the least active was Farmatan®. DPP4 was best inhibited by bark extract and the effect of wood extract was comparable to the effect of Pycnogenol®. Again Farmatan® was least effective. The difference among the two extracts was most evident on α-amylase, where bark extract of silver fir and Pycnogenol® were most effective followed by silver fir wood extract. Farmatan® was effective only at very high concentrations.

The lignan content of foods is generally low and usually does not exceed 2 mg/100 g [21]. Since total lignans from the water extract of silver fir wood represent 10% of the extract [17], inhibitory activities upon mentioned enzymes were examined for 9 lignans (lariciresinol, isolariciresinol, matairesinol, nortrachelogenin, pinoresinol, pinoresinol diglucoside, secolariciresinol, secoisolariciresinol diglucoside and hydroxymatairesinol) identified in the water extract of silver fir wood [17]. Table 1 shows the percent of inhibition of each enzyme. While α-glucosidase and α-amylase were only slightly inhibited by a limited number of tested lignans in 1 mg/mL concentration, all of them inhibited DPP4. The best inhibition of DPP4 was achieved by pinoresinol diglucoside and isolariciresinol, followed by secolariciresinol. Pinoresinol inhibited all three enzymes, although the inhibition was not very strong.

In a recent clinical trial, effects of sweet chestnut wood extract (Farmatan®) and silver fir wood extract (Belinal®) on blood glucose level were compared [22]. When taken concomitantly with a consumption of a standard meal, the glycemic index of a meal was significantly lowered by silver fir wood extract, whereas no significant difference was observed with sweet chestnut wood extract. On the contrary, in our study the sweet chestnut wood extract also inhibited α-glucosidase and DPP4, although the inhibition was slightly less potent than with coniferous extracts. The inhibition of α-amylase, however was achieved only at very high concentrations of sweet chestnut wood extract, which could be attributed to the non-specific inhibition or denaturing of the enzyme, commonly anticipated from plant extracts in high enough concentrations [21]. Different bioavailability of polyphenols in the extracts may play additional role in *in vivo* antidiabetic activity [23], particularly in targeting DPP4, which is not a readily accessible target in the gut compared to the other two enzymes.

Enzyme inhibition was tested with two silver fir extracts. Whereas bark extract was obtained by extraction with organic solvent [14], wood extract is an aqueous extract [18]. Lignans are important constituent of the water-extractable fractions, although in smaller amount they were also present in organic solvent bark extract [14]. The antidiabetic activity of total lignans has been recognized in the literature before [24]. There is also intriguing but not yet compelling evidence from epidemiological studies that lignans decrease coronary heart disease and cardiovascular disease mortality. Interventional studies using higher doses of lignans have found positive associations with some cardiovascular risk factors [20]. In this study, lignans were shown to assist in the antidiabetic activity of the silver fir wood extract *in vitro*. However, the lignans are

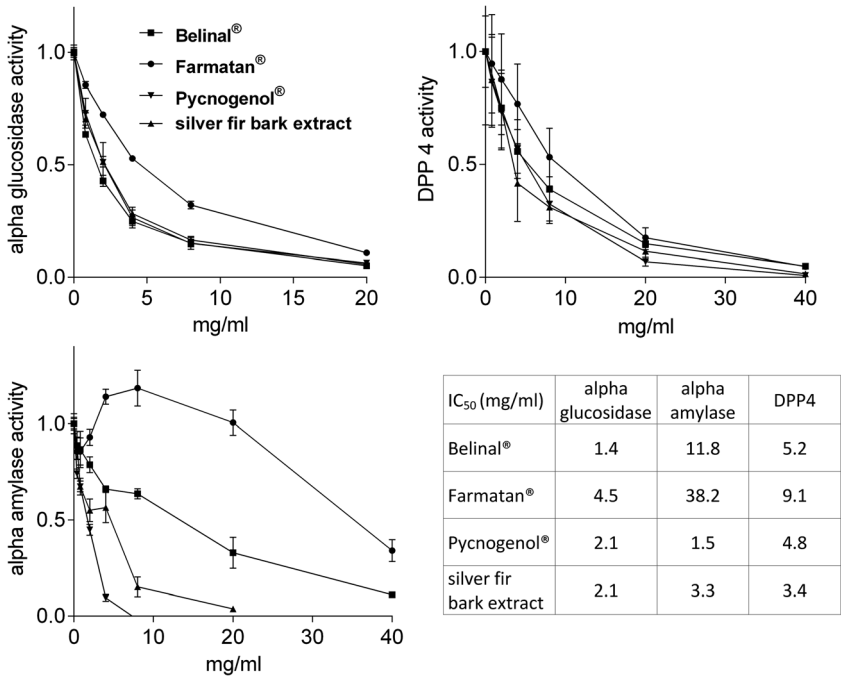

Fig. 1 Residual enzyme activity plotted against inhibitor concentrations for four tested extracts. IC_{50} values are given in the table

IC_{50} (mg/ml)	alpha glucosidase	alpha amylase	DPP4
Belinal®	1.4	11.8	5.2
Farmatan®	4.5	38.2	9.1
Pycnogenol®	2.1	1.5	4.8
silver fir bark extract	2.1	3.3	3.4

not solely responsible for the inhibitory effect of the extracts. Silver fir extracts were shown to contain other components with important role in anti-diabetic activity [14]. As shown in our previous work, where some components of silver fir extracts were individually tested, gallic acid, protocatehuic acid and *p*-hydroxybenzoic acid showed moderate inhibition of α-amylase while gallic acid and *p*-coumaric acid inhibited α-glucosidase [19].

Coniferous extracts (Belinal® and Pycnogenol®) were able to reduce ROS production, typical for high glucose environment [25]. On the other hand, no such effect was observed with Farmatan®. Polyphenols which are considered the most effective constituents to ameliorate metabolic syndrome are absorbed very poorly and their bioavailability is low, therefore, much lower doses compared to enzyme inhibition test were used. In cytotoxicity test on C2C12 mouse myoblast cell line concentration 5 and 10 μg/mL of Belinal®, Farmatan® and Pycnogenol® had no significant effect on cell viability (Online resource 1), therefore these two concentrations were used in oxidative stress protection tests. High glucose level is able to increase the production of ROS in different cell types *in vitro* [26, 27]. The C2C12 cells in the presence of the two extracts from coniferous species (Belinal® and Pycnogenol®) showed significantly less intracellular ROS production in high glucose environment compared to non-coniferous

Table 1 The inhibition of α-glucosidase, α-amylase and dipeptidyl peptidase 4 (DPP4) by 9 lignans (1 mg/mL) present in silver fir wood extract (Belinal®)

	Inhibition (%)		
Lignans (1 mg/ml)	α-glucosidase	α-amylase	DPP4
Lariciresinol	NI	NI	7.9 ± 7.2
Matairesinol	NI	NI	7.0 ± 14.1
Nortrachelogenin	16.6 ± 6.2	NI	18.9 ± 11.9
Pinoresinol	13.8 ± 5.4	9.2 ± 3.4	7.0 ± 7.0
Secoisolariciresinol diglucoside	NI	NI	4.3 ± 7.6
Hydroxymatairesinol	1.8 ± 6.6	NI	28.8 ± 17.0
Isolariciresinol	NI	NI	49.2 ± 7.0
Pinoresinol diglucoside	15.8 ± 14.1	NI	49.6 ± 10.2
Secolariciresinol	NI	17.2 ± 7.6	42.9 ± 20.4
Belinal® (1 mg/ml)	39.5 ± 2.2	15.1 ± 5.9	32.0 ± 9.5

NI, No inhibition

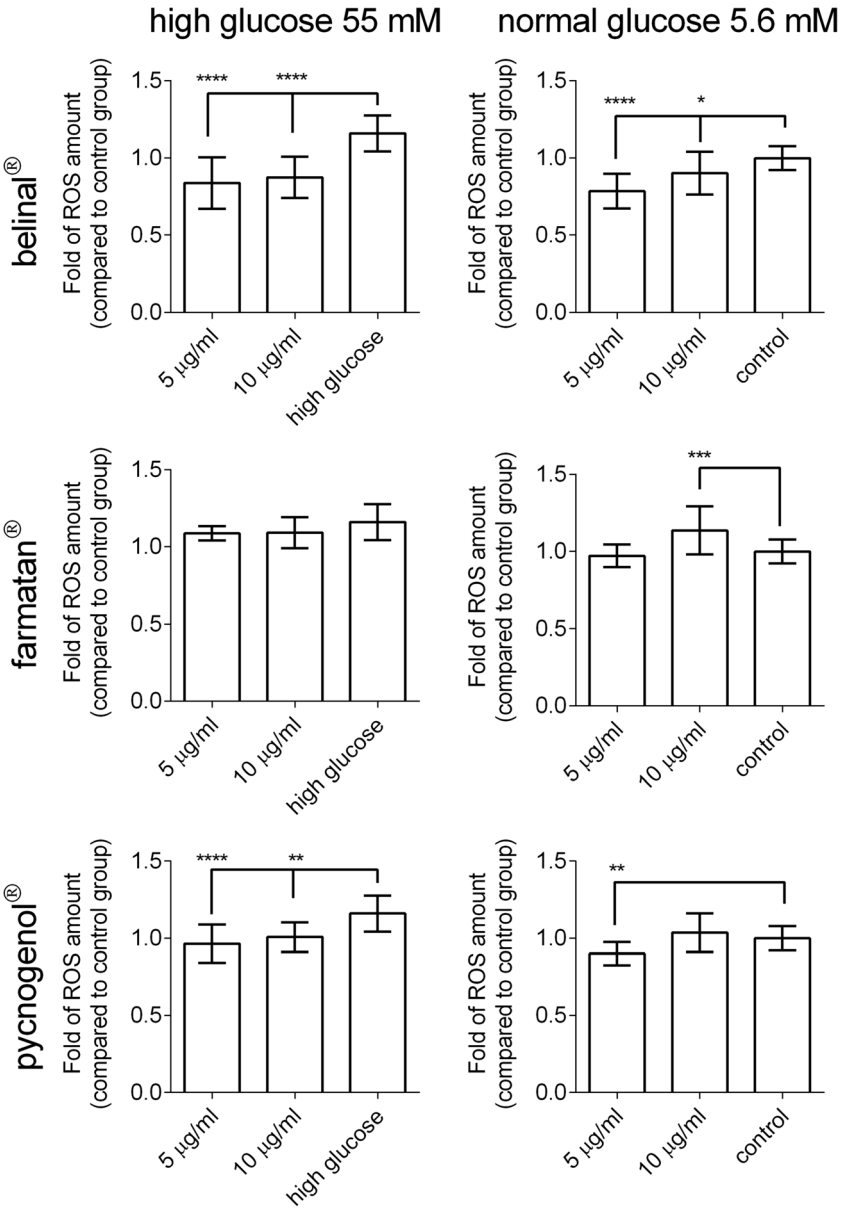

Fig. 2 Oxidative stress protection tests. The cells in the presence of the two extracts from coniferous species (Belinal® and Pycnogenol®) showed significantly less intracellular ROS production in high glucose (55 mM) environment compared to non-coniferous sweet chestnut wood extract Farmatan® after 4 h incubation. The results are presented as mean ± SD of the ratio to untreated low-glucose (*control*) cell group. * Indicates a significant difference in comparison with the high glucose/control group $p < 0.05$, ** indicates a significant difference in comparison with the high glucose/control group $p < 0.01$, *** indicates a significant difference in comparison with high glucose/control group cells $p < 0.001$, and **** indicates a significant difference in comparison with high glucose/control group cells $p < 0.0001$

sweet chestnut wood extract Farmatan® (Fig. 2). In normal glucose environment (5.6 mM) Pycnogenol® and Belinal® also reduced intracellular ROS production; however, the effect was more pronounced at lower concentration of both extracts. Interestingly, higher concentration of Farmatan® in normal glucose environment slightly (but significantly) increased ROS production. Previous studies showed that pine bark extract Pycnogenol® is able to attenuate hyperglycemia-induced oxidative stress on different models [28]. In our study the silver fir wood extract Belinal® was able to attenuate ROS generation more effectively than Pycnogenol®. The lower efficiency of higher concentration of both coniferous extract could be explained by stronger prooxidant effect, as was described for a standardized lignan composition from *Cedrus deodara* [29] or for flavonoids [30] present in these extracts. Possible precipitation of lipophilic compounds from the solution could also be the reason for lower effect of more concentrated samples.

Conclusions

In summary, this study elucidates the *in vivo* results on blood glucose level obtained with silver fir wood extract [22] and supports the use of silver fir extracts as food supplements and functional foods in borderline diabetes. Clean industrial scale production of efficient silver fir extracts, safe for human consumption is feasible by excluding organic solvents, as in the case of wood extract

[18]. Silver fir wood and bark extracts efficiently inhibited enzymes involved in the regulation of blood glucose levels. Lignans, particularly pinoresinol diglucoside, isolariciresinol and secolariciresinol were shown to be important contributors of antihyperglycemic activity through inhibition of DPP4. Antidiabetic activity is also reinforced by protection against oxidative stress generated in high glucose environment that was shown for both coniferous extracts (Belinal® and Pycnogenol®).

Compliance with Ethical Standards

Conflict of Interest M. Lunder, I. Roškar and B. Štrukelj are co-authors of Patent SI 24984 A.

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