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The Influence of Ecological Factors on the Phytochemical Characteristics of *Pinus cembra* L.

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Abstract: *Pinus cembra* L., also known as Swiss pine, is one of the lesser studied coniferous species, despite literature pointing out its great potential to be used for medical purposes due to its high contents in active phytochemicals. The aim of this study is to phytochemically assess rhytidome and periderm extracts obtained from Swiss pine from various locations and altitudes, so as to be able to deduce the best locations for harvesting samples with the highest biological activity. After the plant was analyzed histo-anatomically, hydroalcoholic extracts were obtained using ultrasounds, a rotary evaporator, and dry freezing. After determining the total polyphenolic content (TPC) in each sample, they were tested for antioxidant and enzymatic activity, while taking note of the influence of the varying altitudes and different harvesting regions on the intensity of each activity. The results from the TPC analysis show that rhytidome samples collected from the highest altitude (2429 m) displayed the highest content of polyphenols, with a general tendency for the amount of polyphenolic content to be directly correlated to altitude, this finding being further supported by the antioxidant activity also growing directly proportionally with the altitude. Enzymatic inhibition activity was found to be moderate for α-glucosidase and low for α-amylase. Following our findings, we can safely say that *Pinus cembra* L. extracts can be a great source of compounds with antioxidant activity; however, further studies are warranted to best determine the true potential of this species.

Keywords: *Pinus cembra* L.; polyphenols; antioxidant activity; enzymatic activity; environmental factors; histo-anatomical analysis

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1. Introduction

Pinus cembra L., otherwise known as Swiss pine, Swiss stone pine, stone pine, arolla pine or Austrian stone pine, is a coniferous species native to the Central European Alps

and Carpathian Mountains [1]. This species grows at high altitudes, ranging from 700 m to 2500 m, which exposes the plant to extreme conditions, such as high concentrations of ozone and UV irradiation. Both heightened ozone levels and UV radiations cause the formation of reactive oxygen species (ROS) in the plant, therefore leading to tissue degradation and cell death. Due to the presence of these stress factors in its environment, this species has adapted by increasing its production of antioxidants [2,3]. In a study performed by Shashikumar et al. on the effect of ozone on pine forests in south-eastern France it was determined that out of all the studied species, Pinus cembra is one of the most sensitive species to ozone [4]. The phytochemicals that have been most studied in *Pinus cembra* L. are polyphenols, secondary metabolites in plants that have the role of protecting them against UV rays and other types of aggression caused by various pathogens since these compounds are able to reduce ROS levels by donating electrons or hydrogen atoms to free radicals, neutralizing them, and thus stopping chain reactions. On top of that, polyphenols are also capable of chelating Fe^{2+} ions, therefore reducing the rate of ROS formation. Countless studies and meta-analyses have demonstrated that antioxidants offer protection against developing several chronic illnesses caused by oxidative stress (atherosclerosis, rheumatoid arthritis, muscular dystrophy, cancer, etc.). *Pinus cembra* L. bark and needle extracts have been studied before; however, extracts from other parts of the plant and the influence of environmental factors on the concentration of polyphenols in this species have not been fully studied [5].

Polyphenols present in the bark and needle extracts of *Pinus cembra* L. have several pharmacological actions, such as antimicrobial activity on various pathogens, with the bark extract having inhibitory activity similar to that of ampicillin and higher than chloramphenicol on *Sarcina lutea* and *Escherichia coli* [6].

There are data from the literature that demonstrate the cytotoxicity of some extracts obtained from the bark of different pine species [7–9]. Cytotoxic activity was also observed in hydromethanolic extracts obtained from *Pinus cembra* L. needles when testing their inhibition level on the HeLa cell line (human cervix cancer cell line). Extracts were found to be capable of slowing down the protein synthesis, viability, proliferation, and apoptosis of HeLa cells [10].

Moreover, polyphenols have been found to possess antidiabetic properties by inhibiting α-glucosidase and α-amylase and having a beneficial effect on β-pancreatic cells survival and insulin sensitivity [11–13].

Seeing as the literature has limited data available on the chemical composition and potential biological activity of *Pinus cembra* L., the objective of this research paper is to assess whether extracts obtained from the rhytidome and periderm of *Pinus cembra* L. possess chemical compounds with a beneficial action on the human body, and if yes, how environmental factors, such as altitude and being exposed to a certain amount of irradiation, can influence the concentration of phytochemicals in the plant, in order to be able to deduce which areas are the best for harvesting this species and extracting its beneficial substances in hopes of using them for medical purposes.

2. Results and Discussions

2.1. Histo-Anatomical Examination of Pinus cembra L. Stem

Regarding the stem structure of Swiss pine, it was observed that its characteristics are specific to those of dicotyledonous plants.

The external outline of the stem was observed to be intensely green colored, corresponding to the periderm that is constituted from several layers of suberum, and in succession to that the phellogen and pheloderm can be observed, both having a slightly collenchymatic aspect (Figure 1a-A,c). The cortex is of a parenchymatic nature and contains numerous resin ducts (Figure 1a-C) organized in the same manner as the ones present in the leaves, however, they are much larger. The phloem is situated immediately after the cortex (Figure 1a-D), the cambium is interposed between the secondary phloem and secondary xylem and has a circular aspect, composed of 2–3 strongly bound cell layers.

The xylem is situated in the central part of the section, taking the form of concentric rings, also containing multiple resin ducts that are smaller than the ones found in the outer parts of this section (Figure 1a-E,b). The central part of the section is composed of the pith, which can be observed to be of a parenchymatic nature (Figure 1b).

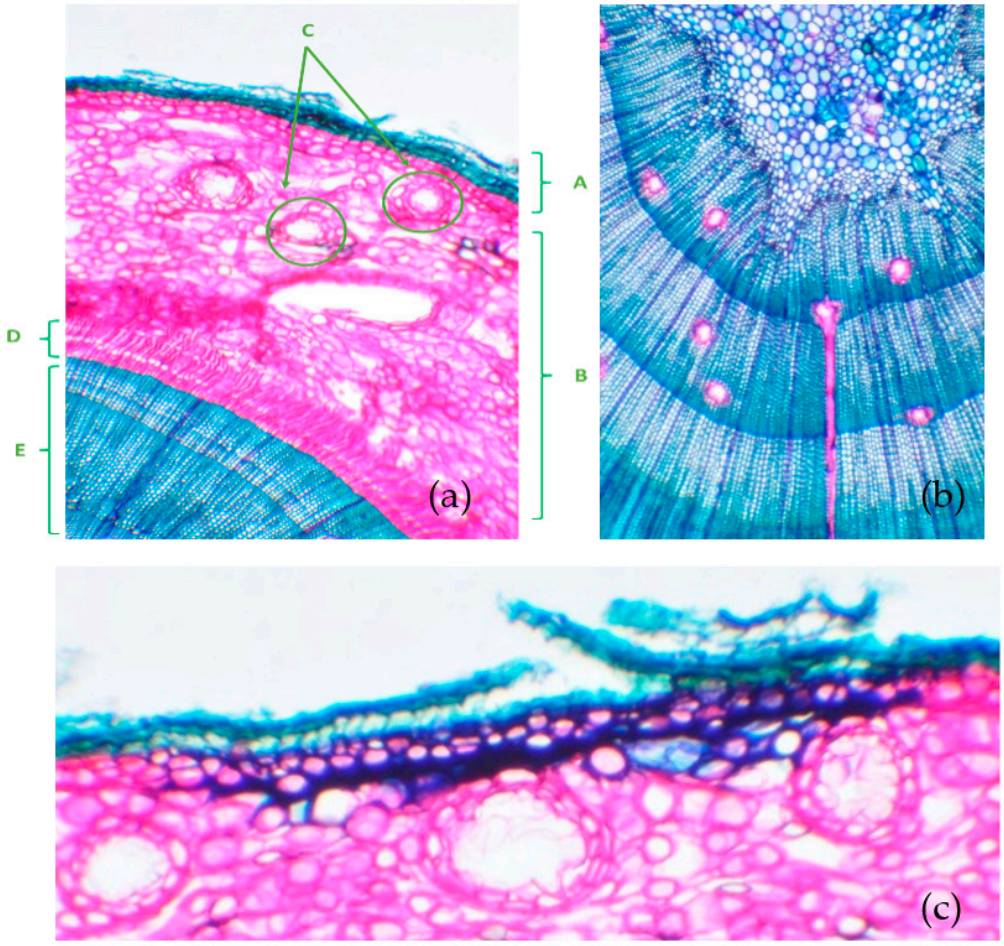


Figure 1. The histo-anatomical aspects of the *Pinus cembra* L. stem: (**a**) *Pinus cembra* L. stem cross-section; A—periderm, B—cortex, C—resin ducts, D—phloem, and E—secondary xylem; (**b**) central area of the *Pinus cembra* L. stem cross-section; (**c**) periderm of *Pinus cembra* L. in detail.

2.2. Total Polyphenolic Content of Pinus cembra L. Extracts in Relation to Altitude

The results summarized in Table 1 show that the quantitative distribution of the total polyphenolic content (TPC) varies depending on the altitude and location of the sample from which the extracts have been obtained. Moreover, there is a significant difference between the TPC in rhytidome extracts and periderm extracts, with the latter possessing a significantly lower concentration of polyphenols.

Our findings suggest that altitude plays a decisive role in the difference in polyphenol content in the analyzed extracts, most evidently observed in the rhytidome extracts where the samples collected from the altitude of 2429 m had a higher concentration of polyphenols, but not statistically significant compared to those collected from an altitude of 1620 m. According to Figure 2, a significant difference ($p < 0.0001$) was observed in the content of polyphenols between the periderm and the rhytidome within the species *Pinus cembra* L. at the highest altitudes, more precisely at 2429 m. The rhytidome extracts showed the highest content of polyphenols at this altitude, while the periderm extracts recorded the lowest content of polyphenols. These results suggest that altitude can influence the content of polyphenols in different parts of the plant and that there is a significant variation depending on the altitude and the parts analyzed.

Table 1. Total polyphenolic content (TPC) and antioxidant activity of *Pinus cembra* L.

Sample Code	TPC (mg GAE/g Extract)	DPPH (IC_{50}—µg/mL)	ABTS (IC_{50}—µg/mL)
IR1919	235.01 ± 5.57 ^{a,d,e}	6.58 ± 1.62 ^{d,e}	0.49 ± 0.03 ^{b,c}
IIIR1620	205.97 ± 3.13 ^{a,b,d,e}	2.26 ± 0.02 ^{b,c}	0.35 ± 0.01 ^{b}
IVR1790	140.33 ± 40.64 ^{a}	3.72 ± 0.92 ^{b,c}	0.71 ± 0.01 ^{c}
VR2429	306.17 ± 0.95 ^{a,d,e}	2.20 ± 0.70 ^{b,c}	0.40 ± 0.00 ^{b}
IP1919	137.07 ± 39.79 ^{a}	3.83 ± 0.48 ^{b,c,d}	1.39 ± 0.15 ^{d,f}
IIIP1620	86.39 ± 23.23 ^{a,b,c,d}	6.55 ± 0.22 ^{c,d,e}	1.04 ± 0.12 ^{d,e}
IVP1790	91.74 ± 1.64 ^{a,b,c,d}	8.24 ± 0.82 ^{d}	1.19 ± 0.02 ^{d,e,f}
VP2429	79.73 ± 1.04 ^{a,b,c}	15.36 ± 0.14 ^{f}	1.22 ± 0.11 ^{d,e,f}

Note: dw—dry weight. Statistical analyses were performed in triplicate. Different superscript letters (b–f) in the same column mean statistically significant differences at $p < 0.05$ and the same alphabetical superscript in a column indicates no statistically significant difference and the letter "a" does not interact with anyone. ± standard deviation. TPC—total polyphenolic content; GAE—gallic acid equivalent; DPPH—2,2-diphenyl-1-picrylhydrazyl; ABTS—2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid; IR1919—rhytidome dry extract harvested at 1919 m from the Rodnei mountains; IIIR1620—rhytidome dry extract harvested at 1620 m from the Rodnei mountains; IVR1790—rhytidome dry extract harvested at 1790 m from the Maramures mountains; VR2429—rhytidome dry extract harvested at 2429 m from the Rhaetian Alps; IP1919—periderm dry extract harvested at 1919 m from the Rodnei mountains; IIIP1620—periderm dry extract harvested at 1620 m from the Rodnei mountains; IVP1790—periderm dry extract harvested at 1790 m from the Maramures mountains; VP2429—periderm dry extract harvested at 2429 m from the Rhaetian Alps.

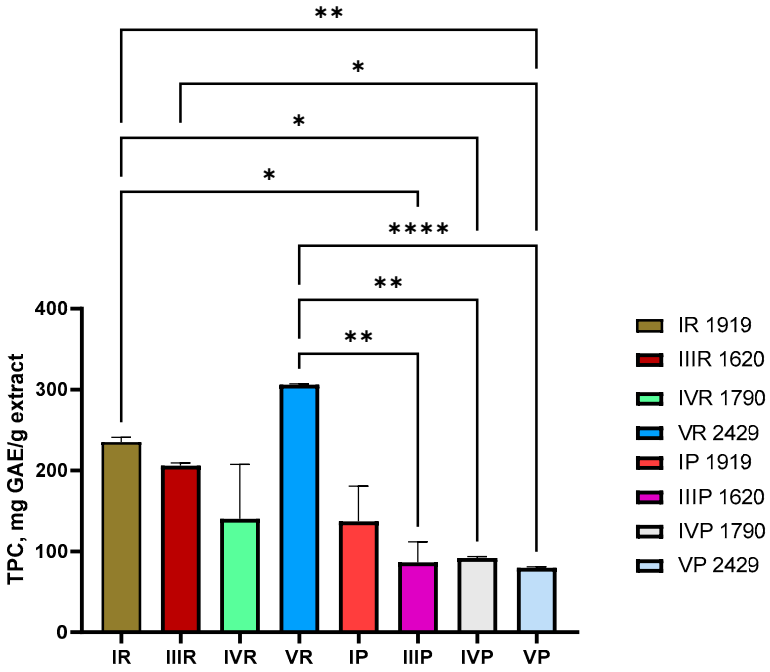


Figure 2. Analysis of TPC in *Pinus cembra* extracts and evaluation of significant differences used Dunn's post hoc test, symbols "*" (for $p < 0.05$), "**" (for $p < 0.01$) and "****" (for $p < 0.0001$). TPC—total polyphenolic content; GAE—gallic acid equivalent; IR1919—rhytidome dry extract harvested at 1919 m from the Rodnei mountains; IIIR1620—rhytidome dry extract harvested at 1620 m from the Rodnei mountains; IVR1790—rhytidome dry extract harvested at 1790 m from the Maramures mountains; VR2429—rhytidome dry extract harvested at 2429 m from the Rhaetian Alps; IP1919—periderm dry extract harvested at 1919 m from the Rodnei mountains; IIIP1620—periderm dry extract harvested at 1620 m from the Rodnei mountains; IVP1790—periderm dry extract harvested at 1790 m from the Maramures mountains; VP2429—periderm dry extract harvested at 2429 m from the Rhaetian Alps.

Quantitatively speaking, our results are also supported by the reports of other studies performed on the polyphenolic content in the bark of *Pinus cembra* L.; in a study performed

by Lungu-Apetrei et al., it was found that the TPC in extracts obtained from the bark of this species has a value of 299.3 ± 1.4 mg GAE/g [6].

Moreover, the variation in the TPC in relation to altitude has also been previously studied on other species of *Pinus*, one study by Emrah-Dönmez et al. evaluated the impact of altitude on the production of suberin in the periderm of *Pinus sylvestris*, reporting that the content of suberin grew proportionally with the altitude [14]. In another study, performed by Delgado-Alvarado et al. on the TPC of various *Pinus* species, the results obtained were higher than the present findings, with the exception of rhytidome extracts collected from the highest altitude of 2429 m, which yielded comparable results to the TPC of the *Pinus* species assessed, despite the study reporting that *Pinus cembra* L. has a lower concentration of polyphenols in comparison to other species of *Pinus* [15]. This further proves that altitude and other particularities of climate change need to be taken into account when determining the phytocompounds in plants, especially when they play a major role in the protection of the plant against environmental stress factors.

It was noticed that the literature lacks similar data on describing the variation in total polyphenolic content in relation to altitude for the *Pinus cembra* L. species, which constituted another motive behind this study. Further studies should also focus on the effect of climate change, different environmental factors, and environmental stress factors on the phytocharacteristics of plants.

2.3. Antioxidant Activity of Pinus cembra L. Extracts

The findings of the TPC examination prove that Swiss pine extracts possess an antioxidant effect, the evidence is further supported by the results obtained through the DPPH and ABTS methods, summarized in Table 1 and in the inhibition percentage graphs found in Figure 3.

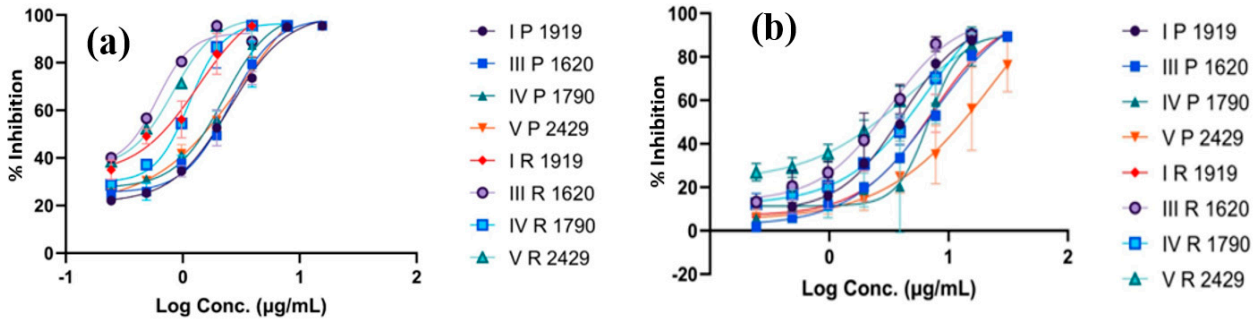


Figure 3. Inhibition percentage graphical representation for the ABTS (**a**) and DPPH (**b**) free radical scouting capabilities of rhytidome and periderm extracts of *Pinus cembra* L. DPPH—2,2−diphenyl−1−picrylhydrazyl; ABTS—2,2−azino−bis(3-ethylbenzothiazoline)−6−sulfonic acid; IR1919—rhytidome dry extract harvested at 1919 m from the Rodnei mountains; IIIR1620—rhytidome dry extract harvested at 1620 m from the Rodnei mountains; IVR1790—rhytidome dry extract harvested at 1790 m from the Maramures mountains; VR2429—rhytidome dry extract harvested at 2429 m from the Rhaetian Alps; IP1919—periderm dry extract harvested at 1919 m from the Rodnei mountains; IIIP1620—periderm dry extract harvested at 1620 m from the Rodnei mountains; IVP1790—periderm dry extract harvested at 1790 m from the Maramures mountains; VP2429—periderm dry extract harvested at 2429 m from the Rhaetian Alps.

It can be observed that all the extracts possess a neutralizing effect on both the ABTS and DPPH radicals, growing directly proportionally with the TPC examined in Section 2.2. In the case of DPPH, it was noticed that there was no major difference between the neutralizing power of the rhytidome and periderm extracts, however in the case of ABTS it was observed that the antioxidant capabilities of the rhytidome extracts are higher in comparison to those of the periderm extracts, with the lowest inhibition power being found in the rhytidome sample obtained from the Rodnei Mountains at the lowest altitude (1620 m). All

of these findings further prove that the higher the altitude, the more polyphenols the plant will produce, and therefore have a possible higher antioxidant potential.

However, it has to be noted that these findings are based only on one of the mechanisms through which plants act as antioxidant agent and tests that are closer to biological mechanisms, such as the lipid peroxidation inhibition test, reduced glutathione level tests, and malondialdehyde level tests should also be applied since they could offer even more information on the true potential of these extracts to function as antioxidant agents. In one study performed by Willför et al., the in vitro lipid peroxidation inhibition assay of *Pinus cembra* L. stilbenes showed a low antioxidant property, however when the same extracts were tested for their scavenging capabilities on BHA and Trolox, it was noted that *Pinus cembra* L. had high potency in neutralizing the free radicals in the tested reagents [16]. Another study demonstrated the antioxidant activity of the extract obtained from the *P. cembra* bark, by determining the EC 50 values. The results obtained by the authors are comparable to those presented above.

2.4. Enzyme Inhibitory Activity of Pinus cembra L. Extracts

The periderm and rhytidome extracts of Swiss pine were tested for their potential to inhibit α-glucosidase and α-amylase, so as to determine whether they would have potential use in diabetes therapy.

The results obtained from the one-way ANOVA test (Table 2) show a moderate, statistically significant inhibitory of both rhytidome and periderm extracts on α-glucosidase, with inconsistent inhibitory activity across the samples, probably due to the degradation of the enzyme caused by different phytochemicals in the plant extracts (e.g., tannins, that are known to degrade proteins in high concentrations). Among the rhytidome samples, the highest activity was noticed in the sample collected at the altitude of 2429 m from the Rhaetian Alps, and among the periderm samples, the highest activity was observed in the sample harvested from the Retezat mountains at an altitude of 1919 m. In rhytidome extracts, altitude also seems to not have played an important role in the intensity of the enzyme inhibition activity, since the obtained IC_{50} values are close to each other.

Table 2. IC_{50} values of the *Pinus cembra* L. extracts obtained from the α-glucosidase and α-amylase inhibition tests.

Sample Code	IC_{50} (μg/mL) α-Glucosidase	IC_{50} (μg/mL) α-Amylase
IR1919	$29.60 \pm 0.66^{c,e}$	459.66 ± 30.51^{c}
IIIR1620	29.15 ± 0.92^{c}	$662.22 \pm 21.64^{d,e}$
IVR1790	34.85 ± 0.54^{e}	1686.84 ± 120.46^{g}
VR2429	$25.77 \pm 0.97^{c,d}$	$625.24 \pm 28.05^{c,d,e}$
IP1919	16.62 ± 0.74^{b}	108.07 ± 1.80^{b}
IIIP1620	$32.54 \pm 0.89^{e,f}$	$713.33 \pm 28.17^{d,e}$
IVP1790	$26.26 \pm 1.30^{c,d}$	1399.81 ± 97.41^{f}
VP2429	$31.12 \pm 1.13^{c,e}$	$813.28 \pm 16.79^{d,e}$

dw—dry weight. Statistical analyses were performed in triplicate. Different superscript letters (b–g) in the same column mean statistically significant differences at $p < 0.05$ and the same alphabetical superscript in a column indicates no statistically significant difference; ±standard deviation. IR1919—rhytidome dry extract harvested at 1919 m from the Rodnei mountains; IIIR1620—rhytidome dry extract harvested at 1620 m from the Rodnei mountains; IVR1790—rhytidome dry extract harvested at 1790 m from the Maramures mountains; VR2429—rhytidome dry extract harvested at 2429 m from the Rhaetian Alps; IP1919—periderm dry extract harvested at 1919 m from the Rodnei mountains; IIIP1620—periderm dry extract harvested at 1620 m from the Rodnei mountains; IVP1790—periderm dry extract harvested at 1790 m from the Maramures mountains; VP2429—periderm dry extract harvested at 2429 m from the Rhaetian Alps.

Regarding the inhibition activity on α-amylase, a lower activity was observed across all samples, with the lowest being among the rhytidome and periderm samples collected from the Maramures mountains at an altitude of 1790 m. The highest inhibition activity

on α-amylase was observed among the samples collected in the Retezat mountains at the altitude of 1919 m.

Antidiabetic potentials of pine species have been assessed formerly, the findings from a study performed by Dziedziński M. et al. suggest that the ethanolic bark extract of *Pinus roxburghii* showed a significant decrease in blood glucose levels after administering the extracts in rats induced with diabetes through alloxan injections [17]. Another study performed by Delgado-Alvarado E.A. et al. tested the inhibition activity on α-glucosidase and α-amylase of a series of *Pinus* species: *Pinus engelmannii*, *Pinus arizonica*, *Pinus durangensis*, and *Pinus cembroides*. It was found that all foliar extracts analyzed were more active on α-glucosidase inhibition than acarbose (an antidiabetic drug), but less active than acarbose on inhibiting α-amylase, underlining the fact that there is an important antidiabetic potential in *Pinus* species [15].

Pinus cembra L. has not been formally assessed in order to determine its enzyme inhibitory activity and therefore antidiabetic properties, which constitutes an element of novelty in the present study.

In Figure 4, we analyzed Spearman's correlation coefficients between the periderm and the rhytidome of *Pinus cembra* L. extracts, for TPC, DPPH, ABTS, α-glucosidase, and α-amylase. Our results reveal significant differences in the relationships between these components between periderm and rhytidome. For example, we observed a statistically significant strong correlation ($p < 0.0001$) between DPPH-ABTS in the periderm extracts, whereas, in the rhytidome extracts, we observed a moderately significant correlation ($p = 0.001$). The same thing was observed between DPPH- α-glucosidase. On the other hand, between ABTS-α-glucosidase for periderm extracts, a significant strong correlation was observed ($p < 0.0001$), and for rhytidome extracts, a very weak statistically significant negative correlation was observed ($p = 0.042$). Between DPPH-α-amylase and ABTS-α-amylase, a very weak statistically insignificant correlation was observed for both rhytidome extracts and periderm extracts. According to the obtained results, TPC was found to show a weak/very weak correlation with all other measured variables, including DPPH, ABTS, α-glucosidase, and α-amylase. This means that there is no strong or significant association between TPC and these variables. This finding suggests that the total content of phenolic compounds, represented by TPC, does not significantly influence the antioxidant activity measured by tests such as DPPH and ABTS or the enzymatic activity of α-glucosidase and α-amylase.

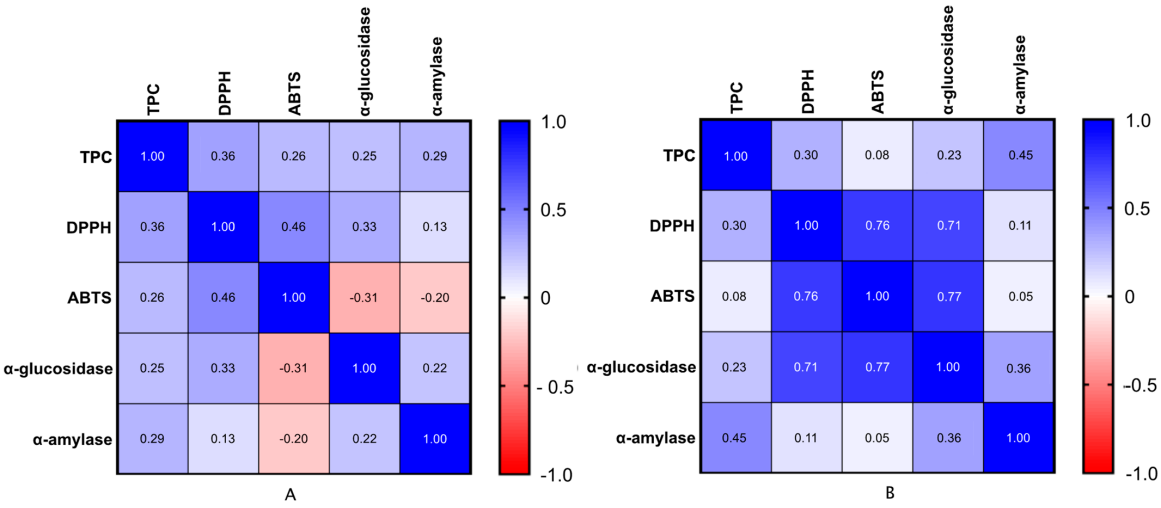


Figure 4. Spearman's correlation coefficient between TPC, DPPH, ABTS, α-glucosidase and α-amylase calculated for the extracts obtained from rhytidome (**A**) and periderm (**B**) of *Pinus cembra* L. TPC—total polyphenolic content; DPPH—2,2-diphenyl−1−picrylhydrazyl; ABTS—2,2-azino−bis(3−ethylbenzothiazoline) −6−sulfonic acid.

3. Materials and Methods

3.1. Plant Samples

Plant samples were collected from three different mountainous regions: Rodnei mountains, Maramures mountains and the Rhaetian Alps. The goal was to collect three rhytidome and periderm samples, each from a different plant situated at varying altitudes, however this was only possible for the samples collected in the Rodnei mountains. Rhytidome and periderm samples were collected from 1- to 2-year-old stems using a knife; the same method was applied for each specimen.

Samples from the Rodnei mountains were collected on the Ineu massif in October 2021 from the altitude of 1919 m (47°31′32.05′′ N, 24°54′28.26′′ E; exp. 303° NW), 1735 m (47°31′59′′ N, 24°55′22′′ E; exp. SE) and 1620 m (47°91′55.15′′ N, 24°55′51.66′′ E; exp. 320° NW).

From the Maramures mountains, sample collecting was only performed from one specimen situated at an altitude of 1790 m (47°38′47.15′′ N, 24°51′50.99′′ E; exp. 170° S) in October 2021.

In January 2023, samples were collected from a single specimen in the Rhaetian Alps in the Italian Lombardian region, that was situated at an altitude of 2429 m (46°30′51.42′′ N, 10°06′12.69′′ E; exp. 84° E).

3.2. Extraction

Plant samples were processed by removing remnants of pine needles and removing the husk using a knife. Afterwards, samples were dried at room temperature and stored intactly in paper or linen bags to avoid rotting until extraction.

Prior to extraction, plant samples were pulverized using a grinder. The extraction was performed three times for each sample, and was assisted by ultrasounds (Hielscher Ultrasonics, UP 200St, 200 W, 26 kHz, Teltow, Germany), using a formerly optimized method for the extraction of the total polyphenols from the bark of *Thuja occidentalis*. The experimental design was generated, modeled, and experimentally and statistically confirmed by the *Modde*® software (version 13.0.2, Sartorius Stedim, Umeå, Sweden). The exact extraction parameters can be found in Table 3 below.

Table 3. Extraction parameters applied for obtaining rhytidome extracts.

Parameter (Measurement Unit)	Value
Sample:solvent ratio (m:v; g/mL)	1:20
Alcoholic solvent concentration (%EtOH)	44
Extraction time (min)	15
Ultrasound amplitude (%)	40

The resulted extracts were then vacuum filtered, concentrated in a rotary evaporator until the ethanol was fully evaporated, and finally freeze dried through lyophilization.

3.3. Total Polyphenol Content

TPC was determined using the Folin–Ciocâlteu method [18]. Extracts were dissolved to obtain a concentration of 2 mg/mL and then diluted at 1:5 prior to the testing. From the diluted solution, 400 µL were mixed with another 400 µL of Folin–Ciocâlteu reagent and 3200 µL Na_2CO_3 5% solution. The obtained solution was mixed well and incubated for 1 h at room temperature. Afterwards, the absorbance of each sample was measured spectrophotometrically at a wavelength of 750 nm. The final TPC was expressed in relation to gallic acid (mg GAE/g extract).

3.4. Antioxidant Activity

Two in vitro assays were used to evaluate the antioxidant potential of the samples using DPPH and ABTS reagents [18,19]. For the radical neutralization activity on DPPH,

50 µL of each extract were dissolved in methanol 50% and diluted in a series on 96-well plates, to which 200 µL of DPPH 0,1 mM methanolic solution were added. The plates were incubated in darkness for 30 min, after which their absorbance was determined at a wavelength of 517 nm. The results were calculated using the following inhibition percentage equation (I%):

$$inhibition(\%) = \frac{Ac - As}{Ac} \times 100, \quad (1)$$

where *Ac* is the absorbance of the control solution and *As* is the absorbance of the sample being tested. Results were then expressed through IC_{50}, which is the nonlinear regression plot of the I% against the logarithm of the concentrations.

The process for testing the neutralizing activity of the samples on the ABTS reagent followed the same instructions as the DPPH method, with the difference that plates were incubated for 10 min at room temperature and their absorbance was determined at a wavelength of 734 nm. The equation used for the ABTS method was as follows:

$$inhibition(\%) = \frac{Ac - As}{Ac} \times 100, \quad (2)$$

where *Ac* is the absorbance of the control solution and *As* is the absorbance of the sample being tested. Results were then expressed through IC_{50}, similarly to the DPPH method.

3.5. α-Glucosidase and α-Amylase Inhibitory Assay

In order to determine the samples' capabilities of inhibiting α-glucosidase, dry extracts were dissolved in phosphate buffer (100 mM, pH = 6,8) supplemented with 5% dimethyl sulfoxide (DMSO). Afterward, 50 µL of sample extract were mixed with 50 µL of enzymatic solution (0.75 U/mL in phosphate buffer, pH = 6,8) and 50 µL of substrate solution (pNPG), and then were incubated for 15 min, at 37 °C. The absorbance was measured at a wavelength of 405 nm, with the control being acarbose (3.33–333.33 µg/mL). Results were first calculated using the I% Equation (3) below and afterward were expressed through the IC_{50} value (µg/mL).

$$inhibition(\%) = \frac{(B - A) - (D - C)}{(B - A)} \times 100 \quad (3)$$

where *A* is the absorbance of the control, *B* is the absorbance of the blank control, *C* is the absorbance of the sample, and *D* is the absorbance of the blank sample.

When testing the extracts capabilities of inhibiting α-amylase, the Caraway-Somogyi method was used, and the protocol was described in detail by Babotă et al. [19]. A volume of 50 µL of the sample were mixed with 50 µL of enzymatic solution (0.05 mg/mL phosphate buffer), and then incubated for 10 min at 37 °C, in the dark. Afterwards, 50 µL of 0.05% starch solution was added, and the plates were incubated for another 10 min, at 37 °C, in the dark. Following that, the catalytic reaction was stopped by adding 25 µL of hydrochloric acid 100 mM and 100 µL IKI 5 mM solution. The absorbance of the samples was determined at the wavelength of 615 nm using acarbose (3.12–200 µg/mL) as the control. The inhibition percentage was calculated using Equation (3), the final results being expressed through the IC_{50} value (µg/mL).

3.6. Histo-Anatomical Analysis

In order to analyze the histo-anatomical aspects of the stem of *Pinus cembra* L., the protocol described by Tanase et al. was used [18]. The plant material was immersed in ethanol 70% (*v*/*v*) and then was sectioned with the help of a knife and microtome blade, the sections being collected in a container with water. To be able to observe each histo-anatomical detail, the sections were dyed using iodine green by immersing them in the solution for 10 min and afterward washing away the excess with water. The dyed sections were then fixed on microscope blades using glycerinated albumin and were analyzed

with the help of a Motic B Series microscope equipped with a digital camera (Nikon, Tokyo, Japan).

3.7. Statistical Analysis

The data obtained from our evaluations were presented as the mean ± standard deviation (SD). To assess whether our data follow a Gaussian distribution, we used two tests of normality: the Shapiro–Wilk test. For DPPH, ABTS, α-glucosidase, and α-amylase a one-way analysis of variance (ANOVA) was performed, and the differences between means were assessed using the post hoc Tukey test. For the TPC analysis, we employed nonparametric Kruskal–Wallis tests. To identify differences between groups, we conducted multiple pairwise comparisons using the Dunn procedure. To investigate the relationship between TPC, ABTS, DPPH, α-glucosidase, and α-amylase, we used Spearman's nonparametric correlation.

All statistical analyses were performed in triplicate, and a p-value < 0.05 was considered significant for interpretation of results. GraphPad Prism 8 software was used to perform statistical analyses.

4. Conclusions

Being a lesser known and harder to obtain species of pine that grows in the spontaneous flora of mountainous regions in Romania, *Pinus cembra* L. is less studied, despite the literature pointing out that it could have great potential as a source of active compounds with health benefits, which constituted the motive behind this study.

Following the analyses, we can conclude that extracts obtained from the rhytidome of *Pinus cembra* L. had a higher content of polyphenols compared to the periderm extracts. Therefore, their antioxidant activity also varied directly proportional to their total phenolic content, with an overall better efficiency in neutralizing the ABTS free radicals compared to the DPPH free radicals.

The antidiabetic property of *Pinus cembra* L. was also determined using enzyme inhibition tests on α-glucosidase and α-amylase, observing a moderate inhibition on α-glucosidase, and a much more diminished inhibition effect on α-amylase, with the activity being more pronounced in the rhytidome extracts rather than the periderm extracts, the intensity once again being correlated to the total phenolic content of each sample.

Since the literature data do not present in vivo studies that evaluate the cytotoxicity of the extracts obtained from the rhytidome/periderm of *Pinus cembra*, future studies are needed to clarify these aspects.

Finally, to address the impact of ecological factors, such as altitude on the chemical and biological profile of the samples collected from *Pinus cembra* L., we have concluded that the total polyphenolic content, the antioxidant and enzymatic activity were all directly correlated with the altitude from which the samples were harvested, further proving that with the growing altitude and higher stress factors, the plant adapts itself by producing phytochemicals with a protective effect against oxidative stress.

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