PINUS BRUTIA TEN. AND CEDRUS BREVIFOLIA (HOOK. F.)
A. HENRY – CHEMICAL CHARACTERIZATION AND
POSSIBILITIES OF VALORIZATION IN THERAPEUTICS

PhD THESIS ABSTRACT

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IASI, 2013
PhD thesis contains:

- 210 pages,
- 31 tables,
- 161 figures,
- 292 references,
- papers published in the thesis topics.

In this abstract the numbers used to identify the contents, tables and figures are the same with the ones used in PhD thesis.

Key words: *Pinus brutia*, *Cedrus brevifolia*, polyphenols, proanthocyanidins, antioxidant activity, antiproliferative activity.

The financial support provided by POSDRU/88/1.5/S/58965 is greatly acknowledged.
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MOTIVATION OF THE STUDY/AIM AND OBJECTIVES

Until the end of the 19th century medicinal plants were the most commonly used medicines; today medicinal plants, as extracts and pure vegetal metabolites, are a viable alternative to synthetic drugs.

Vegetal extracts are complex mixtures of substances that can act synergically and/or complementary. Therefore, their pharmacological profile is often more complex than that of the synthetic drugs. As most illnesses have a multifactor etiopathogenicity, vegetal extracts offer a combination of active compounds that can simultaneously regulate the different mechanisms involved in triggering and evolution of an illness. Generally, vegetal extracts have a better tolerability and they generate less adverse reactions compared to synthetic drugs.

EGb 761 is a standardized extract (22-27% flavonoid glycosides, 5-7% terpene lactones) isolated from the leaves of Ginkgo biloba L. (Ginkgoaceae). The main constituents of the extract are kaempferol and quercetin glycosides and their derivatives, proanthocyanidins, sesquiterpenes (α-bilobalid), diterpenes (ginkgolides A, B, C, J, M). EGb 761 is effective in the prevention and treatment of vascular and cerebral diseases. The polyphenolic components of the extract (flavonoids, proanthocyanidins) inactivate reactive oxygen species (ROS), which increase the level of TXA2, a vasoconstrictor and platelet aggregrant. Ginkgolide B antagonizes the platelet aggregation induced by the platelet activation factor (PAF). Certain components of the extract (terpenes, flavonoid aglycones released from the glycosylated forms in the gastrointestinal tract) cross the blood-brain barrier and stimulate α-secretase activity, an enzyme responsible for the non-amyloidogenic cleavage of the β-amyloid precursor protein. The stimulation of α-secretase reduces the level of β-amyloid, a protein found in the senile plaques of patients suffering from Alzheimer’s disease.

LI 132 (2,25% flavonoids and 11,3% proanthocyanidins) and WS 1442 (18,75% proanthocyanidin oligomers) are standardized extracts isolated from the leaves and flowers of hawthorn (Crataegus monogyna, Crataegus laevigata, Rosaceae). Numerous clinical studies have proven the effectiveness of these two extracts in cardiac insufficiency, stages II and III (NYHA). Both
flavonoids and proanthocyanidin oligomers act as positive inotrope agents by increasing the intracellular Ca\(^{2+}\) level necessary in the contractile process via inhibition of Na\(^{+}\), K\(^{-}\)-ATP-ase. Similar to cardiotonic heterosides, hawthorn extracts increase the contraction strength of the myocardium. Unlike the cardiotonic heterosides, they extend refractory period and, as a result, they stabilize the cardiac rhythm. The cardiotonic heterosides decrease the refractory period, thus generating cardiac arrhythmias. For this reason, in certain forms of cardiac insufficiency (class II - NYHA), LI 132 and WS 1442 extracts are recommended.

Numerous other vegetal extracts and extractive fractions (silymarin of Cardui mariae fructus, LI 160 and WS 5572 extracts of Hyperici herba, EPs 7630 extract of Pelargonii radix) prove the fact that very often, a total extract is more active than its constituents in pure state.

Another example is Pycnogenol\(^{®}\), obtained from the bark of Pinus maritima Mill. Pycnogenol\(^{®}\) contains simple phenolic compounds (catechin, epicatechin, taxifolin, phenolic acids) and condensed flavonoids (proanthocyanidins). Its constituents act on several levels by different mechanisms (inhibition of lipid peroxidation, inhibition of some proinflammatory cytokines: IL-6, IL-2, TNF-\(\alpha\), as well as COX-2 and 5-LOX, activation of NF-\(\kappa\)B reduction, stimulation of eNOS expression), making Pycnogenol\(^{®}\) efficient as a food supplement in cardiovascular disorders, diabetes, asthma, allergies.

Numerous studies have highlighted the fact that the barks of the coniferous species represent an important source of active compounds, especially of polyphenols and terpenes. This fact has justified the present research subject which aimed to study the possibilities for therapeutical valorization of the barks from two species of conifers native to Cyprus: Pinus brutia Ten., a less studied species from a chemical and biological perspective and Cedrus brevifolia (Hook. f.) A. Henry, for which the literature mentions only studies regarding the genetic diversity.

In this context, the main aims of this research were:

− isolation of some polyphenolic extracts from the barks of the two coniferous species, and fractionation of the extracts using a fast and convenient method;
– characterization of the polyphenolic profile of the extracts/extractive fractions;
– evaluation of the antioxidant effects of the extracts/extractive fractions using different methods;
– isolation of some other types of extracts (hexane, dichloromethane and methanol extracts) from the barks of the two coniferous species, and their chemical characterization;
– assessment of some biological effects of these extracts (vasorelaxant activity, ferrous ion chelating activity, antiproliferative effects on HeLa cells);
– fractionation of the extracts for isolation of fractions/constituents and their chemical characterization;
– assessment of some biological effects of the fractions/isolated constituents (antiproliferative effects on HeLa cells).
PERSONAL RESEARCH

CHAPTER 6. STUDY OF THE HYDROMETHANOL EXTRACT ISOLATED FROM PINUS BRUTIA TEN. BARK

6.1. Isolation of 80% methanol extract and its extractive fractions

The barks of coniferous species contain polyphenols with different molecular weights and structures (monoarylic phenols, flavanones, lignans, stilbenes, catechins, proanthocyanidin oligomers and polymers). For this reason, identification of methods for the quantitative extraction of polyphenols from coniferous barks was a real challenge for phytochemists. The extractibility of polyphenols is influenced by numerous factors: the vegetal material’s degree of grounding, the nature of the solvent, the extraction time and temperature, the pH of the extraction medium, the number of extractions, the stability of the polyphenols under the extraction conditions. The extraction solvent is the most important factor because it influences not only the quantity of polyphenols, but also the type of extracted polyphenols and, consequently, the biological activity of the extract. The literature mentions a large number of solvents used in the extraction of polyphenols from various vegetal sources (methanol, ethanol, ethyl acetate, water, alcohol-water mixtures or acetone-water mixtures). Most studies have revealed the fact that 80% methanol and 75% acetone are the most effective to extract the simple polyphenolic compounds, as well as the condensed ones.

In the present study the aim was to isolate a total polyphenolic extract containing simple as well as condensed polyphenolic constituents, (proanthocyanidins). Therefore, the vegetal material was extracted with 80% methanol (v/v) thus yielding the raw extract PbE (20.07 g; η = 13.38%)\(^1\). According to the nature of the constituents and the interactions between them, the fractionation of a raw extract can lead to more active extractive fractions or, on the contrary, it can reduce the biological activity, the raw extract being more active than the fractions containing certain constituents. In

\(^1\) Expressed to the vegetal product
order to study how the fractionation influences the biological effects of PbE extract, a fast fractionation method was chosen, based on successive extractions with solvents of different polarities (diethyl ether, ethyl acetate, n-butanol). Finally, four extractive fractions were obtained: PbE1, containing constituents extractible in diethyl ether (2.18 g; $\eta = 11.87\%$); PbE2, containing constituents extractible in ethyl acetate (1.84 g; $\eta = 10.02\%$); PbE3, containing constituents extractible in n-butanol (8.59 g; $\eta = 46.78\%$); PbE4 (the remaining aqueous phase), containing water soluble constituents (5.74 g; $\eta = 31.26\%$) (fig. 6.1).

6.2. Chemical study

The chemical study consisted in the quantification of total polyphenols and proanthocyanidins in the extract and extractive fractions, as well as the analysis of their polyphenolic profile using RP-HPLC-DAD-ESI-MS.

6.2.1. Quantification of total polyphenols

The analysis revealed a high polyphenolic content in the extract, as well as in the extractive fractions, the richest one being PbE2 fraction (448.90 ± 1.38 mg/g), followed by PbE raw extract (412.41 ± 7.55 mg/g) and PbE4 (393.49 ± 5.39 mg/g), PbE1 (366.71 ± 5.63 mg/g) and PbE3 (303.79 ± 7.34 mg/g) fractions (fig. 6.3).

6.2.2. Quantification of proanthocyanidins

Sun assay revealed an approximately equal content of proanthocyanidins in PbE extract (225.79 ± 3.93 mg/g) and PbE2, PbE3 and PbE4 fractions (231.41 ± 1.23 mg/g, 229.10 ± 1.12 mg/g and 250.39 ± 1.44 mg/g, respectively). In PbE1, a non-polar fraction, the content of

---

2 Expressed to the hydromethanol extract PbE
Proanthocyanidins reached only 10.05 ± 0.22 mg/g; this low level can be explained by their lack of solubility in non-polar organic solvents (fig. 6.3).
6.2.3. Study of polyphenolic profile by RP-HPLC-DAD-ESI-MS

PbE and its fractions PbE1-PbE4 were analyzed by RP-HPLC-DAD-ESI-MS (negative ionisation mode) (fig. 6.7-6.11).

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**Fig. 6.3.** The content in total polyphenols and proanthocyanidins

**Fig. 6.7.** RP-HPLC-DAD trace (280 nm) of the raw extract (PbE)
Fig. 6.8. RP-HPLC-DAD trace (280 nm) of the diethyl ether fraction (PbE1)

Fig. 6.9. RP-HPLC-DAD trace (280 nm) of the ethyl acetate fraction (PbE2) (1-catechin, 2-procyanidin dimer, 3- procyanidin trimer)
**Fig. 6.10.** RP-HPLC-DAD trace (280 nm) of the n-butanol fraction (PbE3)

**Fig. 6.11.** RP-HPLC-DAD trace (280 nm) of the aqueous fraction (PbE4)

PbE1 contains a major constituent (RT=16.92 min.) identified as taxifolin (dihydroquercetin) by comparing its UV and MS spectra and retention time with those of the standard compound analyzed under the same experimental conditions. The mass spectrum of taxifolin is characterized by the molecular ions [M–H]− at m/z 302.9 and [2M–H]− at m/z 607.3, together with an ion fragment [M–H2O]− at m/z 284.8 (fig. 6.13).
A *O*-taxifolin hexoside, catechin and proanthocyanidin oligomers (two dimers, two trimers) were identified in **PbE2** (fig. 6.9). Catechin (RT=13.53 min.) was identified by comparing its UV and MS spectra and retention time with those of the standard compound.

The mass spectrum of catechin presents the molecular ions \([M–H]^–\) at \(m/z\) 289.0 and \([2M–H]^–\) at \(m/z\) 579.1 and an ion fragment at \(m/z\) 244.7 formed by the elimination of a hydroxyethylene unit. The presence of two ions at \(m/z\) 465.2 and \(m/z\) 302.7 is noticed in the mass spectrum of catechin, belonging to a compound that co-elutes (fig. 6.16 and 6.17).
Fig. 6.16. The mass spectrum of catechin
(co-elution with an unidentified compound having ions at $m/z$ 465.2 and $m/z$ 302.7)

Taxifolin $O$-hexoside and procyanidin oligomers were identified by comparing the UV and MS spectra with those mentioned in literature.

The mass spectrum of taxifolin $O$-hexoside presents the molecular ions $[M–H]^{-}$ at $m/z$ 465.1 and $[2M–H]^{-}$ at $m/z$ 931.4, together with two ion fragments: one at $m/z$ 303.2 corresponding to the aglycone, formed by the loss of a unit of hexose (162 amu) and the second one at $m/z$ 284.6, formed from aglycone by the loss of water (fig. 6.20). The mentioned spectral characteristics (UV, MS) are identical with those reported in the literature for taxifolin $O$-glucosides.
Based on the mass spectra, in PbE2 there were identified two dimers and two trimers. The mass spectrum of the dimer with RT=11.78 min. (fig. 6.21) is characterized by the molecular ions [M–H] at m/z 577.3 and [2M–H] at m/z 1155.7, together with five other ion fragments at m/z 286.7 and 289.2 formed by cleavage of the interflavonoid bond, m/z 407.6 formed by the elimination of water from the ion fragment with m/z 425.3 which, in turn, is generated by the retro-Diels-Alder type fragmentation, m/z 451.2 formed by the elimination of a molecule of phloroglucinol, a process also known as the heterocyclic ring fission. By the same fragmentation mechanisms, the dimer with RT=12.10 min. generated the molecular ions [M–H] at m/z 577.3 and [2M–H] at m/z 1155.4, together with the ion fragments at m/z 286.8, m/z 289.2, m/z 407.2, m/z 425.2 and m/z 451.2 (fig. 6.22 and 6.23).
The trimer fragmentation occurs through similar mechanisms. The mass spectrum of the trimer with RT=12.95 min. (fig. 6.24) is characterized by the presence of the molecular ion [M–H]− at m/z 865.5 and several ion fragments at m/z 286.0 and m/z 577.4, m/z 288.6 and m/z 575.5 formed by the cleavage of the interflavonoid bond, m/z 713.9 formed by the retro-Diels-Alder type fragmentation, m/z 739.2 formed by the heterocyclic ring fission. Due to the
small amount, in the mass spectrum of the trimer with RT=15.89 min. only the molecular ion [M–H]$^-$ at $m/z$ 865.1 was detected (fig. 6.25 and 6.26).

**Fig. 6.24.** The mass spectrum of the procyanidin trimer with RT=12.95 min.

**Fig. 6.25.** The mass spectrum of the procyanidin trimer with RT=15.89 min.
6.3. Study of the antioxidant activity

Due to the fact that the 80% methanol extract and the extractive fractions isolated from the bark of *Pinus brutia* have a high content of total polyphenols (303.79 – 448.90 mg/g), several methods were used to study their antioxidant ability:

- DPPH radical scavenging assay,
- ABTS radical cation scavenging assay,
- reducing power assay,
- superoxide anion radical scavenging assay (method based on the inhibition of the NBT reduction and method based on inhibition of pyrogallol autoxidation),
- hydroxyl radical scavenging assay,
- nitric oxide scavenging assay,
- inhibition of lipid peroxidation assay,
- inhibition of 15-lipoxygenase assay.

6.3.1. DPPH radical scavenging assay

Evaluation of the DPPH radical scavenging capacity is a simple, fast method, often used as preliminary *screening* in the assessment of the antioxidant ability.

The low EC$_{50}$ values (11.80-21.10 µg/mL) prove a good scavenging activity towards DPPH radical for the extract and its extractive fractions. **PbE2** and **PbE3**, the most active ones, have EC$_{50}$ values comparable to BHA and approx. twice and four times higher than those of catechin and quercetin, respectively (tab. 6.1).
Table 6.1. The EC$_{50}$ values in DPPH radical scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>14.8 ± 0.2</td>
</tr>
<tr>
<td>PbE1</td>
<td>21.1 ± 0.0</td>
</tr>
<tr>
<td>PbE2</td>
<td>11.8 ± 0.1</td>
</tr>
<tr>
<td>PbE3</td>
<td>12.6 ± 0.1</td>
</tr>
<tr>
<td>PbE4</td>
<td>18.36 ± 0.35</td>
</tr>
<tr>
<td>Catechin</td>
<td>6.36 ± 0.05</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.13 ± 0.05</td>
</tr>
<tr>
<td>BHA</td>
<td>12.6 ± 0.1</td>
</tr>
</tbody>
</table>

6.3.2. ABTS radical cation scavenging assay

Taking into account the EC$_{50}$ values after 6 min. reaction time, the most active as ABTS cation radical scavenger was quercetin, followed by catechin, BHA, PbE2, PbE, PbE4, PbE3 and PbE1 (fig. 6.31). The low EC$_{50}$ values (4.50-8.60 µg/mL) prove a good scavenger ability towards ABTS radical cation for the raw extract and extractive fractions. PbE2 and PbE, the most active, showed EC$_{50}$ values that were almost twice higher than BHA value and almost four times higher than the values of catechin and quercetin (tab. 6.3).

According to the TEAC values (mM Trolox equivalent to 1 mg/mL extract/extractive fraction/positive control), PbE4 showed the best scavenging activity towards ABTS radical cation (1.53 ± 0.00). PbE (1.46 ± 0.00) and PbE2 (1.42 ± 0.00) showed a slightly reduced activity compared to that of PbE4 (tab. 6.3).

The AUC values prove, as well as TEAC values, a good activity for the extract and its fractions. (tab. 6.3).
Table 6.3.
EC₅₀, AUC and TEAC values in ABTS radical cation scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC₅₀ (µg/mL)</th>
<th>AUC</th>
<th>TEAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>4.7 ± 0.1</td>
<td>1.28 ± 0.00</td>
<td>1.46 ± 0.02</td>
</tr>
<tr>
<td>PbE1</td>
<td>8.6 ± 0.2</td>
<td>0.91 ± 0.02</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td>PbE2</td>
<td>4.5 ± 0.0</td>
<td>1.28 ± 0.03</td>
<td>1.42 ± 0.00</td>
</tr>
<tr>
<td>PbE3</td>
<td>5.33 ± 0.05</td>
<td>1.21 ± 0.01</td>
<td>0.88 ± 0.00</td>
</tr>
<tr>
<td>PbE4</td>
<td>4.86 ± 0.05</td>
<td>1.39 ± 0.01</td>
<td>1.53 ± 0.00</td>
</tr>
<tr>
<td>Catechin</td>
<td>1.2 ± 0.0</td>
<td>4.16 ± 0.02</td>
<td>4.53 ± 0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.03 ± 0.05</td>
<td>4.39 ± 0.02</td>
<td>5.62 ± 0.04</td>
</tr>
<tr>
<td>BHA</td>
<td>1.76 ± 0.05</td>
<td>2.06 ± 0.02</td>
<td>2.43 ± 0.03</td>
</tr>
</tbody>
</table>

6.3.3. Reducing power assay

Regarding the reducing capacity, there were no significant differences between PbE extract and its fractions PbE1-PbE4 (tab. 6.4). The EC₅₀ values of PbE and PbE1-PbE4 indicate a very good reducing capacity (tab. 6.4).

Table 6.4.
The EC₅₀ values in the reducing power assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>9.16 ± 0.12</td>
</tr>
<tr>
<td>PbE1</td>
<td>10.26 ± 0.11</td>
</tr>
<tr>
<td>PbE2</td>
<td>9.48 ± 0.01</td>
</tr>
<tr>
<td>PbE3</td>
<td>11.37 ± 0.23</td>
</tr>
<tr>
<td>PbE4</td>
<td>9.54 ± 0.17</td>
</tr>
<tr>
<td>Catechin</td>
<td>3.74 ± 0.03</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.19 ± 0.45</td>
</tr>
<tr>
<td>BHA</td>
<td>4.30 ± 0.06</td>
</tr>
</tbody>
</table>
6.3.4. Superoxide anion radical scavenging assay

6.3.4.1. Method based on inhibition of NBT reduction

According to the EC$_{50}$ values, PbE4 and PbE were more active than catechin, and the effects of PbE2 and PbE3 were comparable to those of catechin (tab. 6.5).

Table 6.5. The EC$_{50}$ values in superoxide anion radical scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>CE$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>39,36 ± 0,85</td>
</tr>
<tr>
<td>PbE1</td>
<td>70,33 ± 2,65</td>
</tr>
<tr>
<td>PbE2</td>
<td>51,10 ± 2,66</td>
</tr>
<tr>
<td>PbE3</td>
<td>54,93 ± 2,85</td>
</tr>
<tr>
<td>PbE4</td>
<td>33,5 ± 1,1</td>
</tr>
<tr>
<td>Catechin</td>
<td>52,60 ± 1,65</td>
</tr>
<tr>
<td>Quercetin</td>
<td>26,63 ± 2,75</td>
</tr>
<tr>
<td>BHA</td>
<td>undetermined</td>
</tr>
</tbody>
</table>

6.3.5. Hydroxyl radical scavenging assay

The EC$_{50}$ values indicate PbE4 fraction to be the most active, followed by PbE, PbE2 and PbE3; PbE1 showed the weakest activity. Regarding the positive controls, the most active was quercetin, followed by catechin and BHA (tab. 6.7).
Table 6.7.
The EC$_{50}$ values in hydroxyl radical scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>513.26 ± 4.90</td>
</tr>
<tr>
<td>PbE1</td>
<td>1076.40 ± 9.66</td>
</tr>
<tr>
<td>PbE2</td>
<td>543.43 ± 7.70</td>
</tr>
<tr>
<td>PbE3</td>
<td>627.8 ± 4.8</td>
</tr>
<tr>
<td>PbE4</td>
<td>452.03 ± 7.35</td>
</tr>
<tr>
<td>Catechin</td>
<td>318.40 ± 4.45</td>
</tr>
<tr>
<td>Quercetin</td>
<td>172.80 ± 2.20</td>
</tr>
<tr>
<td>BHA</td>
<td>426.73 ± 3.95</td>
</tr>
</tbody>
</table>

6.3.6. Nitric oxide scavenging assay

For all tested concentrations, PbE, PbE2 and PbE4 were more active than catechin (fig. 6.43). According to the EC$_{50}$ values, PbE, PbE2 and PbE4 were more active than catechin and as active as quercetin (tab. 6.8).

Table 6.8.
The EC$_{50}$ values in nitric oxide scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>160.63 ± 0.85</td>
</tr>
<tr>
<td>PbE1</td>
<td>Absent</td>
</tr>
<tr>
<td>PbE2</td>
<td>162.96 ± 2.65</td>
</tr>
<tr>
<td>PbE3</td>
<td>216.26 ± 5.00</td>
</tr>
<tr>
<td>PbE4</td>
<td>160.23 ± 2.35</td>
</tr>
<tr>
<td>Catechin</td>
<td>242.66 ± 7.65</td>
</tr>
<tr>
<td>Quercetin</td>
<td>156.76 ± 5.05</td>
</tr>
<tr>
<td>BHA</td>
<td>absent</td>
</tr>
</tbody>
</table>
6.3.7. Inhibition of lipid peroxidation assay

The low EC$_{50}$ value of PbE2, comparable to that of quercetin, proves that this fraction has a good ability to inhibit lipid peroxidation (tab. 6.9).

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>CE$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>182.63 ± 5.45</td>
</tr>
<tr>
<td>PbE1</td>
<td>361.2 ± 10.1</td>
</tr>
<tr>
<td>PbE2</td>
<td>128.06 ± 5.05</td>
</tr>
<tr>
<td>PbE3</td>
<td>438.83 ± 23.97</td>
</tr>
<tr>
<td>PbE4</td>
<td>194.93 ± 4.05</td>
</tr>
<tr>
<td>Catechin</td>
<td>170.76 ± 0.95</td>
</tr>
<tr>
<td>Quercetin</td>
<td>134.23 ± 1.55</td>
</tr>
<tr>
<td>BHA</td>
<td>92.0 ± 0.9</td>
</tr>
</tbody>
</table>

6.3.8. Inhibition of 15-lipoxygenase assay

The low EC$_{50}$ values of PbE, PbE2, PbE3 and PbE4 (22.50-24.90 µg/mL), slightly more elevated than that of quercetin (18.73 ± 0.85 µg/mL) and inferior to those determined for BHA and catechin, prove a very good 15-LOX inhibition activity (tab. 6.10)
Table 6.10.
The EC$_{50}$ values in inhibition of 15-lipoxygenase assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbE</td>
<td>22.5 ± 0.8</td>
</tr>
<tr>
<td>PbE1</td>
<td>34.46 ± 2.20</td>
</tr>
<tr>
<td>PbE2</td>
<td>24.3 ± 0.4</td>
</tr>
<tr>
<td>PbE3</td>
<td>24.9 ± 0.5</td>
</tr>
<tr>
<td>PbE4</td>
<td>22.93 ± 0.55</td>
</tr>
<tr>
<td>Catechin</td>
<td>29.43 ± 0.55</td>
</tr>
<tr>
<td>Quercetin</td>
<td>18.73 ± 0.85</td>
</tr>
<tr>
<td>BHA</td>
<td>78.66 ± 0.45</td>
</tr>
</tbody>
</table>
CHAPTER 7. STUDY OF THE HEXANE, DICHLOROMETHANE AND METHANOL EXTRACTS FROM *PINUS BRUTIA TEN.* BARK

7.1. Isolation of hexane, dichloromethane and methanol extracts

Considering the biological effects of the non-polar extracts isolated from other coniferous barks and some of their constituents, *Pinus brutia* bark was extracted successively with non-polar solvents (hexane, dichloromethane); afterwards, the vegetal residue was extracted with methanol thus yielding three extracts: **PbEH** (hexane extract) (4.92 g; η = 1.23%), **PbED** (dichloromethane extract) (5.93 g; η = 1.48%), **PbEM** (methanol extract) (76.97 g; η = 19.24%) (fig. 7.1).

7.2. Chemical study

7.2.1. Quantification of total polyphenols

The study revealed a high polyphenolic content in **PbEM** extract (313.31 ± 0.55 mg/g), followed by **PbED** (56.77 ± 0.65 mg/g) and **PbEH** extracts (35.81 ± 0.29 mg/g) (fig. 7.2).

7.2.2. Quantification of total saponins

The study revealed a high saponin content in all extracts, the richest being **PbEM** (336.74 ± 1.12 mg/g), followed by **PbEH** (270.79 ± 0.86 mg/g), and **PbED** extracts (174.16 ± 0.06 mg/g) (fig. 7.4).
7.3. Biological study

The biological study of the hexane, dichloromethane and methanol extracts from *Pinus brutia* bark aimed:
- the evaluation of the endothelium-dependent vasorelaxant activity,
- the evaluation of the ferrous ion chelating activity,
- the evaluation of the antiproliferative activity towards HeLa tumor cells.
7.3.2. Ferrous ion chelating assay

PbED and PbEM extracts and the positive controls catechin, quercetin and BHA did not show ferrous ion chelating activity. The EC₅₀ value of PbEH proves a low chelation ability compared to EDTA (tab. 7.2).

Table 7.2.
The CE₅₀ values in ferrous ion chelating assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbEH</td>
<td>249.6 ± 2.0</td>
</tr>
<tr>
<td>PbED</td>
<td>absent</td>
</tr>
<tr>
<td>PbEM</td>
<td>absent</td>
</tr>
<tr>
<td>Catechin</td>
<td>absent</td>
</tr>
<tr>
<td>Quercetin</td>
<td>absent</td>
</tr>
<tr>
<td>BHA</td>
<td>absent</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.30 ± 0.00</td>
</tr>
</tbody>
</table>

7.3.3. Antiproliferative assays

The antiproliferative activity of PbEH, PbED and PbEM extracts was assessed by studying the effects on protein synthesis, viability and apoptosis in HeLa cells.

7.3.3.1. Study of the effect on protein synthesis

There were no significant differences in activity between the three extracts, PbEH, PbED and PbEM, for the same tested concentration.

At 100 µg/mL, PbEH was the most active, causing the most significant decrease in the protein content to 12.46 ± 0.71%, whereas PbEM and PbED extracts reduced the protein content to 15.28 ± 0.83% and 20.37 ± 0.84%, respectively. For the same concentration, etoposide reduced the cellular protein content to 21.92 ± 0.12%, having an activity comparable to that of PbED, but lower than that of PbEH and PbEM (fig. 7.9).
Fig. 7.9. The effects of PbEH, PbED and PbEM on the protein content in HeLa cells after 48 h treatment (*p < 0.05; **p < 0.01; ***p < 0.001)

7.3.3.2. Study of the effect on cell viability (MTT assay)

At 100 µg/mL, PbEH, PbED and PbEM extracts reduced cell viability to 34.65 ± 8.93%, 31.45 ± 4.16 and 34.56 ± 8.31%, respectively. Etoposide, at the same concentration, reduced cell viability to 15.89 ± 4.36%. At 200 µg/mL, the percentage of living cells was reduced to 10.95 ± 2.36% (PbEH), 5.56 ± 2.57% (PbED) and 6.76 ± 2.99% (PbEM) (fig. 7.10).
7.3.3.3. Study of the effect on cell viability and apoptosis (flow cytometry)

Starting with 50 µg/mL, after 48 h treatment, all extracts increased the percentages of dead and apoptotic cells. **PbED** increased the percentage of dead cells to 17.23 ± 2.76% vs. 5.62 ± 1.34% for the control, followed by **PbEH** (13.54 ± 1.78%). **PbEM** showed the weakest activity (10.15 ± 1.99%). At 200 µg/mL, the most active was **PbEH** which increased the percentage of dead cells to 67.89 ± 2.08% and the percentage of apoptotic cells to 19.92 ±0.93% (fig. 7.20-7.22).

Etoposide (60 µg/mL) increased the percentage of dead and apoptotic cells to 20.91 ± 9.61% and 41.03 ± 10.78%, respectively (fig. 7.23).
**Fig. 7.20.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with PbEH (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)

**Fig. 7.21.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with PbED (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)
**Fig. 7.22.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with PbEM (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)

**Fig. 7.23.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with etoposide (*p<0.05; **p<0.01; ***<0.001)
7.4. Isolation and characterization of some extractive fractions from PbEH and PbED extracts

7.4.1. Isolation of some extractive fractions from PbEH extract

The fractionation of PbEH extract is shown in fig. 7.24.

7.4.2. Isolation of some extractive fractions from PbED extract

The fractionation of PbED extract is shown in fig. 7.25.
Fig. 7.24. Isolation of extractive fractions from PbEH
(CC-column chromatography, GF-gel filtration, FC-flash chromatography, PTLC-preparative thin layer chromatography)
Fig. 7.25. Isolation of extractive fractions from PbED
(CC-column chromatography, FC-flash chromatography,
PTLC-preparative thin layer chromatography)
PH9.4.2, PH9.6.4.1 and PD10.2.3.2 fractions were chemically characterized and studied regarding the antiproliferative effects on HeLa tumor cells.

7.4.4. Assessment of the antiproliferative activity of isolated extractive fractions

The antiproliferative activity of PH9.4.2, PH9.6.4.1 and PD10.2.3.2 was assessed by studying the effects on protein synthesis, viability and apoptosis in HeLa cells.

7.4.4.1. Study of the effect on protein synthesis

At 100 µg/mL, PH9.4.2, PH9.6.4.1 and PD10.2.3.2 caused a decrease in cell protein content to 7.59 ± 0.90%, 5.35 ± 0.59% and 3.40 ± 0.25% respectively, being more active than PbEH and PbED extracts they were isolated from and etoposide (12.76 ± 0.71%, 20.37 ± 0.84% and 21.92 ± 0.12%, respectively).

![Fig. 7.32. The effects of PH9.4.2, PH9.6.4.1 and PD10.2.3.2 on the protein content in HeLa cells after 48 h treatment (*p < 0.05; **p < 0.01; ***p < 0.001)](image-url)
7.4.4.2. Study of the effect on cell viability (MTT assay)

At 100 µg/mL, PH9.6.4.1 was the most active, reducing cell viability to 8.80 ± 0.78%, an effect which is superior to that induced by etoposide at the same concentration (15.89 ± 4.36%). The activity of PD10.2.3.2 (16.29 ± 1.31%) was comparable to that of etoposide. At 100 µg/mL, PH9.4.2, PH9.6.4.1 and PD10.2.3.2 caused a significant decrease in cell viability, being more active than the extracts they were isolated from (34.65 ± 8.93% and 31.45 ± 4.16%, respectively) (fig. 7.33).

Fig. 7.33. The effects of PH9.4.2, PH9.6.4.1 and PD10.2.3.2 on HeLa cells viability after 48 h treatment (*p < 0.05; **p < 0.01; ***p < 0.001)
7.4.4.3. Study of the effect on cell viability and apoptosis (flow cytometry)

After 48 h treatment, the percentage of dead cells increased significantly for PH9.6.4.1 and PD10.2.3.2 at 100 µg/mL (79.34 ± 6.43% and 78.07 ± 2.94%, respectively). At 100 µg/mL and 200 µg/mL, all fractions caused significant increases in the number of apoptotic cells (fig. 7.41-7.43).

![Graph showing cell distribution](image)

**Fig. 7.41.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with PH9.4.2 (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)
Fig. 7.42. The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with **PH9.6.4.1** (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)

Fig. 7.43. The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with **PD10.2.3.2** (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)
CHAPTER 8. STUDY OF THE HYDROMETHANOL EXTRACT ISOLATED FROM 
CEDRUS BREVIFOLIA (HOOK. F.) A. HENRY BARK

8.1. Isolation of 80% methanol extract and its extractive fractions

The vegetal material was extracted with 80% methanol (v/v) thus yielding \textbf{CbE} raw extract (18.96 g; \(\eta = 12.64\%\))^3.

The successive fractionation with solvents of different polarities led to four extractive fractions: \textbf{CbE1}, containing constituents extractible in diethyl ether (6.72 g; \(\eta = 38.62\%\))^4; \textbf{CbE2}, containing constituents extractible in ethyl acetate (1.54 g; \(\eta = 8.85\%\))^4; \textbf{CbE3}, containing constituents extractible in n-butanol (6.13 g; \(\eta = 35.22\%\))^4; \textbf{CbE4} (the remaining aqueous phase) containing water soluble constituents (2.30 g; \(\eta = 13.21\%\))^4.

8.2.1. Quantification of total polyphenols

The study revealed a high polyphenolic content in the extract, as well as in the extractive fractions, the richest being \textbf{CbE2} fraction (415.00 ± 2.15 mg/g), followed by \textbf{CbE} raw extract (248.92 ± 8.43 mg/g), and \textbf{CbE3} (241.33 ± 2.16 mg/g), \textbf{CbE1} (171.79 ± 10.03 mg/g) and \textbf{CbE4} fractions (168.43 ± 6.29 mg/g) (fig. 8.2).

---

^3 Expressed to the vegetal product
^4 Expressed to the \textbf{CbE} hydromethanol extract
8.2.2. Quantification of proanthocyanidins

The highest content in proanthocyanidins was determined in CbE2 (272.07 ± 2.86 mg/g). The content in proanthocyanidins of CbE1 was only
7.41 ± 0.19 mg/g. In fractions CbE3, CbE and CbE4, the total amount of proanthocyanidins was 147.15 ± 1.09 mg/g, 109.47 ± 0.59 mg/g and 60.52 ± 1.06 mg/g, respectively (fig. 8.2).

Fig. 8.2. The content in total polyphenols and proanthocyanidins

8.2.3. Study of polyphenolic profile uby RP-HPLC-DAD-ESI-MS

CbE raw extract and CbE1-CbE4 fractions isolated from Cedrus brevifolia bark were studied using RP-HPLC-DAD-ESI-MS (negative ionisation mode) (fig. 8.3-8.7).

RP-HPLC-DAD-ESI-MS analysis (negative ionisation mode) allowed the identification of the main constituents by comparing the UV, MS spectra and retention times with those of the standard substances analyzed under the same conditions (taxifolin, catechin, epicatechin) and with those mentioned in the literature (proanthocyanidin dimers and trimers) (fig. 6.12, 6.15, 6.19). Taxifolin was identified in CbE1. A taxifolin-O-hexoside, catechin, epicatechin, three procyanidin dimers and two procyanidin trimers were identified in CbE2 (fig. 8.9-8.16). In CbE3 and CbE4 there were identified polymers with a high degree of condensation which eluted as an unresolved peak (fig. 8.6 and 8.7).
Fig. 8.3. RP-HPLC-DAD trace (280 nm) of the raw extract (CbE)

Fig. 8.4. RP-HPLC-DAD trace (280 nm) of the diethyl ether fraction (CbE1)
Fig. 8.5. RP-HPLC-DAD trace (280 nm) of the ethyl acetate fraction (PbE2) (1-catechin, 1’-epicatechin, 2- procyanidin dimer, 3- procyanidin trimer)

Fig. 8.6. RP-HPLC-DAD trace (280 nm) of the n-butanol fraction (CbE3)
8.3. Study of the antioxidant activity

8.3.1. DPPH radical scavenging assay

According to the EC\textsubscript{50} values, the most active fraction was CbE2, its EC\textsubscript{50} value being comparable to that of BHA (tab. 8.1).

Table 8.1.

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC\textsubscript{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>24.03 ± 0.15</td>
</tr>
<tr>
<td>CbE1</td>
<td>94.3 ± 1.2</td>
</tr>
<tr>
<td>CbE2</td>
<td>13.9 ± 0.3</td>
</tr>
<tr>
<td>CbE3</td>
<td>18.43 ± 0.25</td>
</tr>
<tr>
<td>CbE4</td>
<td>33.93 ± 0.15</td>
</tr>
<tr>
<td>Catechin</td>
<td>6.36 ± 0.05</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.13 ± 0.05</td>
</tr>
<tr>
<td>BHA</td>
<td>12.6 ± 0.1</td>
</tr>
</tbody>
</table>
8.3.2. ABTS radical cation scavenging assay

The EC$_{50}$ values varied between 2.3 ± 0.0 µg/mL (CbE2) and 11.9 ± 0.1 µg/mL (CbE4) (tab. 8.3). According to the TEAC values (mM Trolox equivalent to 1 mg/mL extract/extractive fraction/positive control), CbE2 showed the best scavenging ability towards ABTS radical cation (2.69 ± 0.04) (tab. 8.3). According to the TEAC and AUC values, CbE2 was the most active (tab. 8.3).

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
<th>AUC</th>
<th>TEAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>5.03 ± 0.05</td>
<td>1.36 ± 0.01</td>
<td>1.48 ± 0.00</td>
</tr>
<tr>
<td>CbE1</td>
<td>11.83 ± 0.15</td>
<td>0.52 ± 0.01</td>
<td>0.57 ± 0.00</td>
</tr>
<tr>
<td>CbE2</td>
<td>2.3 ± 0.00</td>
<td>2.53 ± 0.01</td>
<td>2.69 ± 0.04</td>
</tr>
<tr>
<td>CbE3</td>
<td>8.6 ± 0.2</td>
<td>0.65 ± 0.00</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>CbE4</td>
<td>11.9 ± 0.1</td>
<td>0.72 ± 0.02</td>
<td>0.81 ± 0.03</td>
</tr>
<tr>
<td>Catechin</td>
<td>1.2 ± 0.0</td>
<td>4.16 ± 0.02</td>
<td>4.53 ± 0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.03 ± 0.05</td>
<td>4.39 ± 0.02</td>
<td>5.62 ± 0.04</td>
</tr>
<tr>
<td>BHA</td>
<td>1.76 ± 0.05</td>
<td>2.06 ± 0.02</td>
<td>2.43 ± 0.03</td>
</tr>
</tbody>
</table>

8.3.3. Reducing power assay

The EC$_{50}$ values for the extract and extractive fractions varied between 9.13 ± 0.13 µg/mL (CbE2) and 25.32 ± 0.62 µg/mL (CbE4). The EC$_{50}$ value of CbE2, approx. two-three times higher than that of the controls, indicates a very good reducing ability (tab. 8.4).
Table 8.4.
The EC\textsubscript{50} values in reducing power assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC\textsubscript{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>15.26 ± 0.30</td>
</tr>
<tr>
<td>CbE1</td>
<td>24.90 ± 0.36</td>
</tr>
<tr>
<td>CbE2</td>
<td>9.13 ± 0.13</td>
</tr>
<tr>
<td>CbE3</td>
<td>14.56 ± 0.18</td>
</tr>
<tr>
<td>CbE4</td>
<td>25.32 ± 0.62</td>
</tr>
<tr>
<td>Catechin</td>
<td>3.74 ± 0.03</td>
</tr>
<tr>
<td>Quercetol</td>
<td>3.19 ± 0.45</td>
</tr>
<tr>
<td>BHA</td>
<td>4.30 ± 0.06</td>
</tr>
</tbody>
</table>

8.3.4. Superoxide anion radical scavenging assay

8.3.4.1. Method based on inhibition of NBT reduction

According to the EC\textsubscript{50} values, the activity of the extract and extractive fractions varied between 52.73 ± 1.25 µg/mL (CbE) and 91.09 ± 4.45 µg/mL (CbE4), the extract activity being comparable to that of catechin (52.60 ± 1.65 µg/mL) (tab. 8.5).
Table 8.5.
The CE$_{50}$ values in superoxide anion radical scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>57.73 ± 1.25</td>
</tr>
<tr>
<td>CbE1</td>
<td>84.93 ± 1.35</td>
</tr>
<tr>
<td>CbE2</td>
<td>76.33 ± 3.50</td>
</tr>
<tr>
<td>CbE3</td>
<td>87.20 ± 1.95</td>
</tr>
<tr>
<td>CbE4</td>
<td>91.06 ± 4.45</td>
</tr>
<tr>
<td>Catechin</td>
<td>52.60 ± 1.65</td>
</tr>
<tr>
<td>Quercetin</td>
<td>26.63 ± 2.75</td>
</tr>
<tr>
<td>BHA</td>
<td>undetermined</td>
</tr>
</tbody>
</table>

8.3.5. Hydroxyl radical scavenging assay

Fraction CbE2 showed the best scavenging activity towards hydroxyl radical, its EC$_{50}$ value being comparable to that of BHA and only 1.8 times higher than the value of catechin (tab. 8.7).

Table 8.7.
The EC$_{50}$ values in hydroxyl radical scavenging assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>822.9 ± 13.9</td>
</tr>
<tr>
<td>CbE1</td>
<td>absent</td>
</tr>
<tr>
<td>CbE2</td>
<td>580.20 ± 18.72</td>
</tr>
<tr>
<td>CbE3</td>
<td>792.10 ± 15.36</td>
</tr>
<tr>
<td>CbE4</td>
<td>1278.73 ± 15.36</td>
</tr>
<tr>
<td>Catechin</td>
<td>318.40 ± 4.45</td>
</tr>
<tr>
<td>Quercetin</td>
<td>172.8 ± 2.2</td>
</tr>
<tr>
<td>BHA</td>
<td>426.73 ± 3.95</td>
</tr>
</tbody>
</table>
8.3.6. Nitric oxide scavenging assay

According to the EC$_{50}$ values, CbE4 showed effects comparable to that of catechin, whereas CbE and CbE2 were more active than catechin (tab. 8.8).

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC$_{50}$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>203.43 ± 3.15</td>
</tr>
<tr>
<td>CbE1</td>
<td>absent</td>
</tr>
<tr>
<td>CbE2</td>
<td>170.30 ± 2.70</td>
</tr>
<tr>
<td>CbE3</td>
<td>268.36 ± 8.25</td>
</tr>
<tr>
<td>CbE4</td>
<td>226.83 ± 2.75</td>
</tr>
<tr>
<td>Catechin</td>
<td>242.66 ± 7.65</td>
</tr>
<tr>
<td>Quercetin</td>
<td>156.76 ± 5.05</td>
</tr>
<tr>
<td>BHA</td>
<td>absent</td>
</tr>
</tbody>
</table>

8.3.7. Inhibition of lipid peroxidation assay

The EC$_{50}$ value of CbE2, higher than that of BHA, but lower than that of catechin and quercetin, proves a good lipid peroxidation inhibition (tab. 8.9).
Table 8.9.
The EC\textsubscript{50} values in inhibition of lipid peroxidation assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC\textsubscript{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>385.80 ± 17.30</td>
</tr>
<tr>
<td>CbE1</td>
<td>absent</td>
</tr>
<tr>
<td>CbE2</td>
<td>125.93 ± 2.05</td>
</tr>
<tr>
<td>CbE3</td>
<td>245.80 ± 12.10</td>
</tr>
<tr>
<td>CbE4</td>
<td>479.10 ± 9.60</td>
</tr>
<tr>
<td>Catechin</td>
<td>170.76 ± 0.95</td>
</tr>
<tr>
<td>Quercetin</td>
<td>134.23 ± 1.55</td>
</tr>
<tr>
<td>BHA</td>
<td>92.00 ± 0.90</td>
</tr>
</tbody>
</table>

8.3.8. Inhibition of 15-lipoxygenase assay

The EC\textsubscript{50} value of CbE\textsubscript{2}, almost twice higher than that of quercetin and comparable to that of catechin, proves a good 15-LOX inhibition activity (tab. 8.10).

Table 8.10.
The CE\textsubscript{50} values in inhibition of 15-lipoxigenase assay

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>EC\textsubscript{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbE</td>
<td>undetermined</td>
</tr>
<tr>
<td>CbE1</td>
<td>63.60 ± 0.60</td>
</tr>
<tr>
<td>CbE2</td>
<td>34.00 ± 0.90</td>
</tr>
<tr>
<td>CbE3</td>
<td>40.96 ± 0.45</td>
</tr>
<tr>
<td>CbE4</td>
<td>undetermined</td>
</tr>
<tr>
<td>Catechin</td>
<td>29.43 ± 0.55</td>
</tr>
<tr>
<td>Quercetin</td>
<td>18.73 ± 0.85</td>
</tr>
<tr>
<td>BHA</td>
<td>78.66 ± 0.45</td>
</tr>
</tbody>
</table>
CHAPTER 9. STUDY OF THE HEXANE, DICHLOROMETHANE AND METHANOL EXTRACTS FROM CEDRUS BREVIFOLIA (HOOK. F) A. HENRY BARK

9.1. Isolation of the hexane, dichloromethane and methanol extracts

*Cedrus brevifolia* bark was extracted successively with non-polar solvents (hexane, dichloromethane), and afterwards, the vegetal residue was extracted with methanol; thus three extracts were obtained: **CbEH** (hexane extract) (11.95 g; $\eta = 2.43\%$), **CbED** (dichloromethane extract) (32.14 g; $\eta = 6.55\%$), **CbEM** (methanol extract) (40.19 g; $\eta = 8.20\%$) (fig. 9.1).
9.2. Chemical study

9.2.1. Quantification of total polyphenols

The study revealed a high polyphenolic content in **CbEM** (230.97 ± 1.85 mg/g), followed by **CbEH** (71.65 ± 0.96 mg/g) and **CbED** (37.11 ± 0.95 mg/g) (fig. 9.2).

9.2.2. Quantification of total saponins

The study revealed a high content of saponins in all extracts, the richest being **CbEM** (363.53 ± 1.13 mg/g), followed by **CbEH** (315.85 ± 0.84 mg/g), and **CbED** (247.83 ± 1.26 mg/g) (fig. 9.3).

9.3. Biological study

9.3.2. Ferrous ion chelating assay

Only **CbEH** showed the ability to chelate ferrous ions (EC_{50} = 183.93 ± 9.70%); the other extracts proved to be inactive.

<table>
<thead>
<tr>
<th>Extract/Positive control</th>
<th>CE_{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CbEH</td>
<td>183.93 ± 9.70</td>
</tr>
<tr>
<td>CbED</td>
<td>absent</td>
</tr>
<tr>
<td>CbEM</td>
<td>absent</td>
</tr>
<tr>
<td>Catechin</td>
<td>absent</td>
</tr>
<tr>
<td>Quercetin</td>
<td>absent</td>
</tr>
<tr>
<td>BHA</td>
<td>absent</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.3 ± 0.0</td>
</tr>
</tbody>
</table>

Table 9.2. The EC_{50} values in ferrous ion chelating assay
9.3.3. Antiproliferative assays

9.3.3.1. Study of the effect on protein synthesis

At 100 µg/mL, CbEH had an activity comparable to that of etoposide (21.92 ± 0.12%), causing a decrease in the protein content to 21.33 ± 0.43%. For the same concentration, CbEM and CbED reduced the protein content to 15.70 ± 1.12% and 12.12 ± 0.13%, respectively, the activity of these two extracts being superior to that of etoposide (fig. 9.8).

![Fig. 9.8. The effects of CbEH, CbED and CbEM on the protein content in HeLa cells after 48 h treatment (*p < 0.05; **p < 0.01; ***p < 0.001)](image)

9.3.3.2. Study of the effect on cell viability (MTT assay)

At 100 µg/mL, CbEH reduced cell viability to 27.99 ± 3.07% being more active than etoposide (15.89 ± 4.36%) (fig. 9.9).
Fig. 9.9. The effects of CbEH, CbED and CbEM on HeLa cells viability after 48 h treatment (*p < 0.05; **p < 0.01; ***p < 0.001)

9.3.3.3. Study of the effect on cell viability and apoptosis (flow cytometry)

Marking HeLa cells with both annexin V – FITC and 7-AAD revealed, in case of CbED and CbEH an increase of the apoptotic cells which was concentration-dependent. CbED and CbEH increased the percentage of apoptotic cells from 7.64 ± 1.37% and 4.53 ± 0.61%, respectively (50 µg/mL) to 39.29 ± 2.98% and 37.71 ± 2.78%, respectively (200 µg/mL). Both extracts also increased the percentage of dead cells, the most effective concentrations in this case being the concentration of 100 µg/mL for CbEH (60.96 ± 2.44%) and 200 µg/mL for CbED (49.78 ± 7.16%) (fig. 7.17 and 7.18).
Fig. 9.17. The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with **CbEH** (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)

Fig. 9.18. The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with **CbED** (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)
**Fig. 9.19.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with CbEM (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)

9.4. Isolation and characterization of some extractive fractions from CbEH extract

9.4.1. Isolation of some extractive fractions from CbEH extract

The fractionation of CbEH extract is shown in fig. 9.20.
CH4.1 fraction was chemically characterized and studied regarding the antiproliferative effect on HeLa tumor cells.
9.4.3. Assessment of the antiproliferative activity of the isolated extractive fraction

9.4.3.1. Study of the effect on protein synthesis

CH4.1 fraction showed a remarkable inhibitory effect on protein synthesis at all tested concentrations. At 50, 100 and 200 µg/mL, it reduced the protein content to 9.63 ± 1.23%, 3.06 ± 0.43% and 1.88 ± 0.05%, respectively, being more active than etoposide (21.92 ± 0.12% protein content) (fig. 9.22).

![Fig. 9.22. The effects of CH4.1 on the protein content in HeLa cells after 48 h treatment (*p < 0.05; **p < 0.01; ***p < 0.001)](image)

9.4.3.2. Study of the effect on cell viability (MTT assay)

In the MTT test as well, at 100 µg/mL, CH4.1 considerably reduced cell viability to 7.29 ± 0.72%, being more active than etoposide (15.89 ± 4.36%). A significant decrease in cell viability (23.35 ± 1.67%) was also determined at 50 µg/mL. For all tested concentrations, fraction CH4.1 was more active than the extract it was isolated from (fig. 9.23).
9.4.3.3. Study of the effect on cell viability and apoptosis (flow cytometry)

After 48 h treatment, CH4.1 significantly increased the percentage of dead cells to 13.64 ± 3.94% (50 µg/mL), 31.78 ± 8.22% (100 µg/mL) and 79.51 ± 8.36% (200 µg/mL). At 100 µg/mL the percentage of apoptotic cells was of 15.61 ± 5.09% (fig. 9.27). Etoposide (60 µg/mL) increased the percentage of dead and apoptotic cells to 20.91 ± 9.61% and 41.03 ± 10.78%, respectively (fig. 7.23).
**Fig. 9.27.** The percentage distribution of living, dead, apoptotic and preapoptotic HeLa cells after treatment with CH4.1 (50-200 µg/mL) (*p<0.05; **p<0.01; ***<0.001)
CHAPTER 10. GENERAL CONCLUSIONS. ORIGINAL CONTRIBUTIONS. RESEARCH PERSPECTIVES

10.1. General conclusions

Several extracts were isolated from the barks of *Pinus brutia* and *Cedrus brevifolia* and they were chemically and biologically characterized.

**PbE** and **CbE** raw extracts were isolated by extracting the barks with 80% methanol. They were fractioned by successive extractions with solvents of different polarities (diethyl ether, ethyl acetate, n-butanol). Thus, from each extract there were isolated four extractive fractions: **PbE1/CbE1** containing constituents extractible in diethyl ether, **PbE2/CbE2** containing constituents extractible in ethyl acetate, **PbE3/CbE3** containing constituents extractible in n-butanol and **PbE4/CbE4** – the remaining aqueous phases.

The quantitative chemical study showed the fact that the ethyl acetate fractions have the highest content of total polyphenols (448.90 ± 1.38 mg/g in **PbE2** and 415.00 ± 2.15 mg/g in **CbE2**). The content of total polyphenols and proanthocyanidins was higher in the raw extract and extractive fractions isolated from *Pinus brutia* bark.

RP-HPLC-DAD-ESI-MS analysis showed the major polyphenolic constituents in each extractive fraction:

- taxifolin in **PbE1** and **CbE1**;
- a taxifolin-**O**-hexoside, catechin, two procyanidin dimers and two procyanidin trimers in **PbE2**;
- a taxifolin-**O**-hexoside, catechin, epicatechin, three procyanidin dimers and two procyanidin dimers in **CbE2**;
- procyanidin polymers in **PbE3**, **PbE4**, **CbE3**, **CbE4**.

The study of the scavenging ability towards DPPH radical showed that, in the case of both species, the ethyl acetate fractions, **PbE2** (EC$_{50}$ = 11.8 ± 0.1 µg/mL) and **CbE2** (EC$_{50}$ = 13.9 ± 0.3 µg/mL) were the most active. According
to the EC$_{50}$ values, their effects are comparable to those of BHA (EC$_{50}$ = 12.6 ± 0.1 µg/mL) and approx. twice and four times lower than those of catechin and quercetin, respectively, substances known for their antioxidant effects.

The capacity of raw extracts and extractive fractions to inactivate free radicals was also confirmed by ABTS radical cation scavenging assay. PbE extract (EC$_{50}$ = 4.7 ± 0.1 µg/mL, TEAC = 1.46 ± 0.02, AUC = 1.28 ± 0.0), PbE2 (EC$_{50}$ = 4.5 ± 0.0 µg/mL, TEAC = 1.42 ± 0.00, AUC = 1.39 ± 0.01), PbE4 (EC$_{50}$ = 4.86 ± 0.05 µg/mL, TEAC = 1.53 ± 0.00, AUC = 1.28 ± 0.03) and CbE2 (EC$_{50}$ = 2.3 ± 0.0 µg/mL, TEAC = 2.69 ± 0.04, AUC = 2.53 ± 0.01) showed high activity.

The study of the reducing ability has not showed significant differences between the extract and extractive fractions isolated from Pinus brutia bark. The most active were PbE (EC$_{50}$ = 9.16 ± 0.12 µg/mL), PbE2 (EC$_{50}$ = 9.48 ± 0.01 µg/mL) and PbE4 (EC$_{50}$ = 9.54 ± 0.17 µg/mL). A good reducing ability was also showed for CbE2 (EC$_{50}$ = 9.13 ± 0.13 µg/mL). The reducing power of PbE, PbE2, PbE4 and CbE2 was approx. two-three times weaker than that of the positive controls: BHA (EC$_{50}$ = 4.30 ± 0.06 µg/mL), catechin (EC$_{50}$ = 3.74 ± 0.03 µg/mL) and quercetin (EC$_{50}$ = 3.19 ± 0.45 µg/mL).

The scavenging ability towards superoxide anion radical (O$_2^-$), a reactive species involved in inflammation and microvascular hyperpermeability, was assessed by two methods.

In the first method (O$_2^-$ was generated by NADH-phenazine methosulfate system), PbE (EC$_{50}$ = 39.36 ± 0.85 µg/mL) and PbE4 (EC$_{50}$ = 33.5 ± 1.1 µg/mL) were more active than catechin (EC$_{50}$ = 52.60 ± 1.65 µg/mL). The scavenging effects of PbE2 (EC$_{50}$ = 51.10 ± 2.66 µg/mL) and PbE3 (EC$_{50}$ = 54.93 ± 2.85 µg/mL), as well as of CbE (EC$_{50}$ = 57.73 ± 1.25 µg/mL), were comparable to the effects of catechin.

In the second method (O$_2^-$ was generated by pyrogallol autoxidation), PbE1 (EC$_{50}$ = 115.43 ± 0.25 µg/mL) and CbE1 (EC$_{50}$ = 339.23 ± 3.20 µg/mL) proved to be the most active. The generation of other ROS (for example, ^1$O_2$) during the pyrogallol autoxidation, as well as the prooxidant effects of polyphenols, but also of other constituents, under certain experimental
conditions, could explain the lack of consistency between the results obtained in these two methods.

The hydroxyl radical (HO•) is responsible for the oxidative lesions of the purine and pyrimidine bases in the DNA, which are known to be involved in mutagenesis and carcinogenesis. The most active was PbE4 (EC₅₀ = 452.03 ± 7.35 µg/mL) with an activity comparable to that of BHA (EC₅₀ = 426.73 ± 3.95 µg/mL), followed by PbE (EC₅₀ = 513.26 ± 4.90 µg/mL), PbE2 (EC₅₀ = 543.43 ± 7.70 µg/mL) and CbE2 (EC₅₀ = 580.20 ± 18.72 µg/mL). A higher scavenging ability was determined for the extract and extractive fractions isolated from Pinus brutia bark.

The excessive production of nitric oxide (NO•), especially under conditions of oxidative stress, is responsible for the occurrence of some DNA mutagenic lesions, the promotion of lipid peroxidation, the inactivation of some cell enzymatic systems (caspases, calpains). In this case there was also noticed a higher scavenging activity for the extract and extractive fractions from Pinus brutia bark. PbE (EC₅₀ = 160.63 ± 0.85 µg/mL), PbE2 (EC₅₀ = 162.96 ± 2.65 µg/mL), PbE4 (EC₅₀ = 160.23 ± 2.35 µg/mL) and CbE2 (EC₅₀ = 170.3 ± 2.7 µg/mL) had an activity comparable to that of quercetin (EC₅₀ = 156.76 ± 5.05 µg/mL) and superior to that of catechin (EC₅₀ = 242.66 ± 7.65 µg/mL). The effects of PbE3 (EC₅₀ = 216.26 ± 5.00 µg/mL), CbE4 (EC₅₀ = 226.83 ± 2.75 µg/mL) and CbE (EC₅₀ = 203.43 ± 3.15 µg/mL) were also comparable to those of catechin.

Numerous disorders (Alzheimer’s disease, Parkinson’s disease, diabetes, renal failure, chronic hepatic illnesses, colon cancer) are characterized by significant increases in the level of lipid peroxidation products. PbE2 (EC₅₀ = 128.06 ± 5.05 µg/mL) and CbE2 (EC₅₀ = 125.93 ± 2.05 µg/mL) were the most active ones, with an activity comparable to that of quercetin (EC₅₀ = 134.23 ± 1.55 µg/mL) and superior to that of catechin (EC₅₀ = 170.76 ± 0.95 µg/mL). PbE (EC₅₀ = 182.63 ± 5.45 µg/mL) and PbE4 (EC₅₀ = 194.93 ± 4.05 µg/mL) also showed a good ability to inhibit lipid peroxidation.
15-Lipoxygenase plays an important role in the pathogenesis of bronchial asthma, Alzheimer’s disease, Parkinson’s disease, stroke, prostate cancer. PbE (EC$_{50}$ = 22.5 ± 0.8 µg/mL), PbE2 (EC$_{50}$ = 24.3 ± 0.4 µg/mL), PbE3 (EC$_{50}$ = 24.9 ± 0.5 µg/mL) and PbE4 (EC$_{50}$ = 22.93 ± 0.55 µg/mL) have inactivated 15-LOX more effectively than catechin; the activity of PbE and PbE4 was similar to that of quercetin (EC$_{50}$ = 18.73 ± 0.85 µg/mL). The extract and extractive fractions isolated from Cedrus brevifolia bark were less active; the best activity was determined for CbE2 (EC$_{50}$ = 34.0 ± 0.9 µg/mL).

The antioxidant studies, except inhibition of pyrogallol autoxidation assay, have indicated PbE, PbE2, PbE4 and CbE2 to be the most active. They contain the largest quantities of total polyphenols. It is obvious that the antioxidant effects are strongly influenced by the polyphenolic content.

In the case of CbE, its fractionation led to CbE2 with an antioxidant activity superior to the extract it was isolated from. However, in the case of Pinus brutia bark, in some antioxidant tests (reducing power assay, hydroxyl radical scavenging assay, superoxide anion scavenging assay, nitric oxide scavenging assay and inhibition of 15-lipoxygenase assay), the total extract was as active or more active than PbE4 and PbE2. In this case, the fractionation did not lead to a significant increase in the antioxidant activity, which proves the fact that the phytocomplex in a total extract can be just as active as or sometimes even more active than the fractions containing parts of its constituents.

PbE2 and CbE2 fractions contain low molecular weight polyphenols (taxifolin-hexoside, catechin, proanthocyanidin dimers and trimers) which are absorbed in the gut and therefore they can act systemically.

PbE4 fraction contains proanthocyanidin polymers which are not absorbed, acting only locally in the gastrointestinal tract.

Concerning the antioxidant effects, PbE2 is important if a systemic activity is desired, whereas PbE4 is of interest only for the local effects in the gastrointestinal tract.
PbEH/CbEH, PbED/CbED and PbEM/CbEM extracts were isolated by the successive extraction of the barks with hexane, dichloromethane and methanol.

The quantitative chemical study showed a high content of total saponins and polyphenols in PbEM (336.74 ± 1.12 mg/g and 313.31 ± 0.55 mg/g, respectively) and CbEM extracts (336.53 ± 1.13 mg/g and 230.97 ± 1.85 mg/g, respectively). Large quantities of total saponins were also determined in the other extracts (270.79 ± 0.86 mg/g in PbEH, 174.16 ± 0.06 mg/g in PbED, 315.85 ± 0.84 mg/g in CbEH and 247.83 ± 1.26 mg/g in CbED).

The study of possible vasorelaxant effects on aortic rings precontracted with phenylephrine showed PbEH extract to be the most active (pEC\textsubscript{50} = 5.20 ± 0.44). The extracts isolated from Cedrus brevifolia bark were less active (pEC\textsubscript{50} = 2.26L4.55).

Ferrous ions are involved in carcinogenesis due to the capacity to generate ROS (hydroxyl radical) which cause DNA mutagenic lesions. Only PbEH (EC\textsubscript{50} = 249.6 ± 2.0 µg/mL) and CbEH extracts (EC\textsubscript{50} = 183.93 ± 9.70 µg/mL) presented the ability to chelate the ferrous ions; their effects were lower in comparison to that of EDTA (EC\textsubscript{50} = 1.3± 0.0 µg/mL).

All extracts significantly reduced the protein synthesis in HeLa cells. At 100 µg/mL, CbEH reduced the protein content to 21.32 ± 0.43%, having an activity comparable to that of etoposide (21.92 ± 0.12%). At the same concentration, PbEH, PbEM, CbED and CbEM reduced the protein content to values between 12.12 and 15.70%, being more active than etoposide.

The extracts ability to reduce the viability of HeLa cells was initially assessed using the MTT assay. At 100 µg/mL the extracts isolated from Pinus brutia bark reduced the viability to 31-34%; in the case of CbEH, it decreased the viability to 27.99 ± 3.07%. Etoposide was more active, reducing the cell viability to 15.89 ± 4.36%.
The effects on the viability and apoptosis of HeLa cells were studied by flow cytometry. At 100 µg/mL, the extracts caused significant increases in the percentage of dead cells: PbEH (56.35 ± 2.14%), PbED (56.23 ± 2.46%), PbEM (53.69 ± 2.32%), CbEH (60.98 ± 2.44%), CbED (38.56 ± 2.99%), CbEM (36.29 ± 2.37%). At the same concentration, except for CbEM, all other extracts increased apoptosis in HeLa cells by approx. 10-12% compared to the control, CbED (20.32 ± 1.62%) being very active. At 200 µg/mL, the extracts caused significant increases in the percentage of apoptotic cells. PbED had the strongest influence on apoptosis, significantly increasing the percentage of apoptotic cells at all tested concentrations (50 µg/mL: 12.39 ± 0.61%, 100 µg/mL: 13.39 ± 1.46%, 200 µg/mL: 61.27 ± 3.56%). CbEM had a weak activity; at 200 µg/mL it determined an increase of the percentage of dead cells of only 44.89% and it did not affect apoptosis.

The fractionation of the extracts by different chromatographic methods (column chromatography, gel filtration, flash chromatography, preparative thin-layer chromatography) led to the isolation of four fractions: PH9.4.2 and PH9.6.4.1 from PbEH extract, PD10.2.3.2 from PbED extract and CH4.1 from CbEH extract.

All isolated fractions caused a considerable decrease of protein synthesis in HeLa cells. Thus, at 100 µg/mL, PH9.4.2, PH9.6.4.1, PD10.2.3.2 and CH4.1 decreased the protein content to 7.56 ± 0.90%, 5.35 ± 0.59%, 3.40 ± 0.25% and 3.06 ± 0.43%, respectively, being more active than the extracts they were isolated from.

In MTT assay, PH9.4.2, PH9.6.4.1, PD10.2.3.2 and CH4.1 reduced the cell viability to 7-24% (100 µg/mL); CH4.1 (7.29 ± 0.72%) was the most active. The effects of these fractions on cell viability were higher than those determined for the extracts they were isolated from.

The effects on HeLa cells viability, studied by flow cytometry, were superior to those induced by the extracts they were isolated from. Thus, at 100 µg/mL, PH9.4.2, PH9.6.4.1, PD10.2.3.2 and CH4.1 caused major increases in the percentage of dead cells: 43.58 ± 7.60%, 79.34 ± 6.43%, 78.07 ± 2.94%
and 31.78 ± 8.22%, respectively. CH4.1 had the strongest influence on apoptosis, increasing the percentage of apoptotic cells to 15.61 ± 5.09% (100 µg/mL). In this assay, the antitumor activity of PH9.4.2, PH9.6.4.1, PD10.2.3.2 and CH4.1 proved to be superior to that of the extracts they were isolated from (PbEH, PbED, CbEH).

10.2. Original contributions

The originality of the doctoral thesis consists in:
- the chemical characterization of some polyphenolic extracts/extractive fractions from Pinus brutia and Cedrus brevifolia bark by spectrophotometric methods and RP-HPLC-DAD-ESI-MS;
- in vitro assessment of the antioxidant potential of the polyphenolic extracts/extractive fractions by different methods (DPPH radical scavenging assay, ABTS radical cation scavenging assay, reducing power assay, superoxide anion radical scavenging assay, hydroxyl radical scavenging assay, nitric oxid scavenging assay, inhibition of lipid peroxidation assay, inhibition of 15-lipoxygenase assay);
- the assessment of possible biological effects of the hexane, dichloromethane and methanol extracts isolated from Pinus brutia and Cedrus brevifolia barks: the vasorelaxant activity, the ferrous ions (pro-oxidants and cancerigens) chelation activity, the antitumor activity assessed by studying the effects on protein synthesis, viability and apoptosis in HeLa cells;
- isolation of some extractive fractions from the hexane and dichloromethane extracts, their chemical characterization by NMR;
- the study of antitumor activity of the isolated fractions on HeLa cells (evaluation of the effects on protein synthesis, viability and apoptosis).

10.3. Research perspectives

The results of this research justify further studies in the following directions:
- *in vivo* evaluation of the antioxidant activity of the polyphenolic extracts/extractive fractions which proved to be active in *in vitro* tests;
- evaluation of other possible biological effects, related to antioxidant activity;
- isolation of larger amounts of antitumor extractive fractions, their further purification for isolation of the major constituents in a pure state, the elucidation of the mechanism of antitumor activity;
- study of the antitumor potential of other extractive fractions, purification of the most active fractions, isolation of the constituents responsible for the antitumor activity, elucidation of the mechanism of activity.
LITERATURE REVIEW


SCIENTIFIC PAPERS

Papers in extenso

1. Elena Crețu, Maarit Karonen, Juha-Pekka Salminen, Cornelia Mircea, Adriana Trifan, Christiana Charalambous, Andreas I. Constantinou, Anca Miron. Antioxidant and 15-lipoxygenase inhibitory effects of a polyphenol-rich extract from Pinus brutia bark and its fractions. J Med Food (FI=1,6) 2013; accepted for publication.


Abstracts
