The Purification, Physico-chemical Characterization and Bioactivity of Polysaccharides from Viscum album

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The aim of this paper was the isolation and purification of medical interest polysaccharides from the European mistletoe (Viscum album L.) and their chemical and biological characterization in view of the usefulness for the regeneration of dermal tissue wounds. Four fractions (A1-A4) were obtained by ethanol precipitation of the polysaccharide aqueous total extract (PZT). The fraction A2 was the richest in hexoses (42.12 %) and was purified by ion-exchange chromatography, resulting six fractions (B1-B6). The chemical composition of the purified fractions showed that B3 had the highest content in hexoses (30.06 %) and uronic acids (23.74 %). The gelatin-zymography analysis of the proteinases secreted by the dermal fibroblasts in the presence of A2 and B3 samples has demonstrated that, at a concentration of 100 µg/ml, they didn't affect the cellular metabolism. The spectrophotometric method used to study the influence of purified polysaccharides on cell viability showed that the tested fractions were not toxic. In conclusion, the selected polysaccharide fractions, A2 and B3, can be used as active compounds in the composition of bioproducts for the regeneration of injured dermal tissue.

Keywords: polysaccharides, Viscum album L., hexoses, uronic acids, cell viability

Polysaccharides (Pz) play an important role in various biological activities, such as inflammation, fertilization, cell adhesion, etc [1]. Pz from mistletoe (Viscum album) have been less studied regarding their possible role in dermal wound healing. Mistletoe was used to cure headaches, epilepsy, hypertension, infertility, arthritis and rheumatism. This plant having a positive influence on the entire glandular system and metabolism [2]. Due to this, mistletoe is considered a modifier of the biological response. Currently, it is used as adjuvant in the treatment of different cancer types because it stimulates the immune system and destroys the tumor cells [3]. The most studied mistletoe compounds, alkaloids, viscotoxins and lectins were used destroys the tumor cells [3]. The most studied mistletoe cancer types because it stimulates the immune system and destroys the tumor cells [3]. The most studied mistletoe compounds, alkaloids, viscotoxins and lectins were used destroys the tumor cells [3]

**Experimental part**

Pz extraction. Leaves of Viscum album L. bought from Romplant S.A. (Bucharest, Romania) were authenticated by Dr. Elvira Gille from "Stejarul" Research Center, Piatra Neamþ, Romania. 10 g of dried and minced plant have been extracted 3 times successively with 50 mL acetone to remove chlorophyll, and with 50 mL methanol at room temperature, to remove polyphenols. After filtration, the vegetal residue was extracted with hot water at 100 °C for 1 h in a Soxhlet apparatus. The procedure was repeated, obtaining the total extract of Pz soluble in water (PZT) (fig. 1).

Pz purification. PZT was successively precipitated with ethanol of increasing concentrations (30, 50, 75 and 90% ethanol), obtaining 4 fractions, named A1-A4. The richest extract in Pz (A2) was purified by ion-exchange chromatography on a 250 x 20 mm column filled with DEAE-Spheroex LD (IBF Biotechnics, France), having particle sizes between 100-300 µm. The column was then eluted with stepwise linear gradient of NaCl solution (0; 0.15; 0.25; 0.5; 1.0 and 2.0 M), resulting 6 fractions, named B1-B6 (fig. 1). Each collected fraction was dialyzed against distilled water for 2 days and concentrated using an evaporator (Heidolph, V Micro, CE).

Chemical analysis. The chemical compositions of all the Pz fractions were analyzed. Their total hexose content was determined by the phenol-sulfuric acid method [12]. Briefly, the sample (200µL) and D-glucose used as a standard, respectively, were mixed under stirring with a 5% phenol solution (200µL) and 2 mL of concentrated sulfuric acid, at room temperature, for 30 min. The absorbance was measured at 490 nm using a) asco V-650 spectrophotometer. The uronic acid content was determined with the orcinol method [13] based on the conversion of uronic acids to furfural derivatives by boiling the samples with orcinol reactive in concentrated hydrochloric acid. The absorbance was registered at 665 nm and transformed in concentration values from the standard curve plotted for glucuronic acid. The protein content was determined by Bradford method, using

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Coomassie Brilliant Blue reagent (Biorad) [14]. Bovine serum albumin was used as standard.

Metalloproteinase (MMP) analysis. A primary culture of human dermal fibroblasts [15] was cultured in the presence of different Pz concentrations, ranging between 0.2 - 500 µg/mL, in an incubator (air containing 5% CO₂), at 37°C, for 48 h. The conditioned culture medium was analyzed by gelatin-zymography. According to the modified Manicourt and Lefebvre method (1993) [16], gelatin type A from porcine skin (Sigma) was added, until a final concentration of 0.5 mg/mL, to the standard mixture of acrylamide used for electrophoresis (Laemmli). The samples were migrated in a 7.5% SDS-polyacrylamide gel, for 2 h. After electrophoresis, the gels were washed twice in buffer solution, pH 7.6, for 30 min. The gel was stained with a 0.1% Coomassie Brilliant Blue R-250 solution, for 1 h. The enzymatic activity was visualized as colourless bands on a blue background. The samples were co-migrated together with a molecular weight standard of 6,500 - 205,000 Da (Sigma).

MTT test. This spectrophotometric method is based on the conversion of dimethyl-2-thiazolyldiphenyl-tetrazolium bromide (MTT) to formazan blue insoluble crystals by the mitochondrial dehydrogenases from live cells [17]. In our experiment, the cells cultivated in the presence of Pz for 24 and 48 h, respectively, were washed and 50 µL MTT was added in the culture medium. The culture plates were incubated at 37°C, for 3 h, and then 1 mL of isopropanol was added to each well to render the formazan crystals soluble. Absorbance was measured at 570 nm using a Jasco V-650 spectrophotometer (Japan). The number of viable cells was calculated by referring to the control (cells cultured in the absence of vegetal extract), considered to have 100 % cell viability.

Statistics. The results were expressed as mean of 3 values ± standard deviation (S.D.). Student's t-test was used to make a statistical analysis. Significant differences were considered at values of p < 0.05.

Results and discussion

Viscum album Pz purification

In this study, we used an extraction method based on boiling water as extraction solvent in order to obtain the Pz total extract (PZT) (fig. 1). Before this extraction, preliminary chemical treatments were carried out in order to remove chlorophyll and polyphenols (flavonoids, phenolic acids).

The Pz extraction parameters can vary depending on the studied vegetal material. Previous studies showed that water is the best suited solvent for extraction of both small and large molecular weight glucides [18]. Another solvent suited for extraction is a solution of alcohol in water. The type of alcohol, the temperature and the procedure varies considerably in literature. In some cases these solvents led to an incomplete Pz extraction from vegetal tissues.

However, recent studies demonstrated that there were no differences regarding the glucide extraction yield when using methods with 80 % alcohol or water as solvents [18]. Four Pz fractions were obtained after ethanol precipitation. According to table 1, fraction A2 was the richest in glucides and uronic acids.

After the preparative chromatography of the A2 fraction, only the Pz fraction eluted with 0.25 M NaCl solution (B3 fraction) presented a high content of hexoses and uronic acids (fig. 2). The protein content of B3 fraction was very low (< 5 %).

Mistletoe Pz effect on synthesis of proteolytic enzymes in dermal fibroblast culture

Gelatin-zymography is an electrophoretic technique allowing simultaneous detection of several types of proteolytic enzymes. However, this technique requires a large amount of protein from the sample and is time-consuming. In order to overcome these limitations, we used the MTT test, which is a rapid and sensitive method for determining cell viability.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total hexoses (%)</th>
<th>Uronic acids (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>16.66</td>
<td>20.83</td>
<td>0.99</td>
</tr>
<tr>
<td>A2</td>
<td>42.12</td>
<td>36.68</td>
<td>0.78</td>
</tr>
<tr>
<td>A3</td>
<td>16.07</td>
<td>22.82</td>
<td>0.75</td>
</tr>
<tr>
<td>A4</td>
<td>10.11</td>
<td>19.08</td>
<td>0.66</td>
</tr>
</tbody>
</table>
metalloproteinases, based on their molecular weight and their relative abundance from the band intensity. Prote- 
enzymes are activated by a conformational modification 
induced by SDS, so that both the latent and the active forms 
of an enzyme could be visualized on the gel.

Three types of Viscum album Pz extracts were selected 
(PZT, A2 and B3 fractions) and added into the culture 
medium, in different concentrations. Proteinase activities 
in the medium harvested after 48 h were observed on SDS-
polyacrylamide gels copolymerized with gelatin, in non-
reducing conditions (fig. 3).

The PZT extract induced an increase in MMP-2 synthesis, 
detected as two intense bands corresponding to the latent 
(72 kDa) and active (66 kDa) forms of the enzyme, unlike 
A2 and B3 fractions which presented similar bands but 
having lower intensity. When B3 was present at a 
concentration of 100 µg/mL in the culture medium of 
dermal fibroblasts, similar bands as in the control were 
observed, proving that B3 sample is biocompatible. At 
higher Pz concentration (500 µg/mL), all samples presented 
more intense bands corresponding to the latent and the 
active MMP-2 form. The obtained results showed that MMP 
synthesis is influenced by high concentrations of mistletoe 
Pz.

MMP-2 (gelatinase A) is reactive towards denatured 
collagens, fibronectin, components of the basal membrane 
and elastin. The presence of MMP-2 in both pro-enzymatic 
and active forms can be correlated with its involvement in 
tissue remodeling and increased collagen turn-over.

Gelatin-zymography did not reveal the presence of other 
enzymes in the culture medium, such as MMP-3 or 
MMP-13 which are associated with chronic wounds in 
dermal pathology [19]. Our results indicated that a normal 
process of cell metabolism took place as a reaction to the 
tested exogenous substances. It is known that in vivo, the 
presence of MMP-2, MMP-9 and MMP-1 active forms is 
correlated to a normal wound healing process [20].

When variations of MMP expression and activity are 
registered, useful information about cell metabolism can 
be gathered. Thus, gelatin-zymography studies allow the 
development of a diagnostic method regarding the process 
of wound healing.

Mistletoe Pz effect on cell viability
The viability of the cells treated with Pz was analyzed 
by measuring the mitochondrial succinate dehydrogenase 
activity, after 24 and 48 h respectively, using the MTT 
spectrophotometric assay, similar to other biocompatibility 
studies [21].

The results indicated that all three tested Pz extracts, in 
the concentration range of 0.2-100 g/mL were not cytotoxic 
and they allowed cell proliferation (fig. 4). The values of 
cell viability, calculated as percentage from control (100%) 
were higher than 80 %. Moreover, Pz purified by ion-
exchange chromatography (B3 fraction) induced a 
significant increase of the cell viability in the dermal 
fibroblast culture (p<0.05). The maximum value was 126.5 
± 3.4 %, at a concentration of 100µg/mL B3.

Conclusions
The present paper proposes the obtaining of Pz from 
Viscum album L. in view of their utilization in dermal tissue 
regenerative medicine. The polysaccharides obtained by 
ethanol precipitation and ion-exchange chromatography 
were physico-chemically analyzed by the determination 
of total hexoses, uronic acids and protein contents. Two 
polysaccharide fractions were selected and their biological 
activity was assessed in contact with a dermal fibroblast 
culture. The biotests (MTT assay and gelatin-zymography) 
showed that the selected polysaccharide fractions had no 
cytotoxic effect and didn’t affect the metabolism of cultured 
cells. In conclusion, our results demonstrated that the 
selected Pz fractions can be used as active compounds in
bioproducts intended for the regeneration of injured dermal tissue.

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