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 viabillity 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 in cancer treatment [4]. Besides these, the mistletoe extract has a high percent of Pz with possible antineoplasic and dermatologic activities. The immunostimulating and cytotoxic effects are more obvious when applying total extracts in treatments instead of using purified extracts containing only lectins or viscotoxins [5]. The effects of the mistletoe extract are assumed to appear because of a synergic action between its compounds that interact during complex reactions [6]. Studies have shown that the administration of unpurified extract determined crisis, bradycardia, and even death [7]. This is due to lectins responsible for inhibiting the protein synthesis at ribosomal level [8, 9]. The chemical composition of the mistletoe extract varies according to the tree species on which the plant developed and the harvesting period [10, 11].

The aim of this paper was the isolation and purification of medical interest Pz 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.

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 ethanolic fraction in Pz (A2) was purified by ion-exchange chromatography on a 250 x 20 mm column filled with DEAE-Spherodex LS (IBF Biotechnics, France), having particle sizes between 100-300 µm. The column was then eluted with stepwise 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, VV 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 Jasco V-650 spectrophotometer. The uronic acid content was determined with the orcinol method [13] based on the conversion of uronic acids to furfural derivates 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% CO_2), 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, *p*H 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-thiazolyl-diphenyl 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 50μL 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 $p < 0.05$.

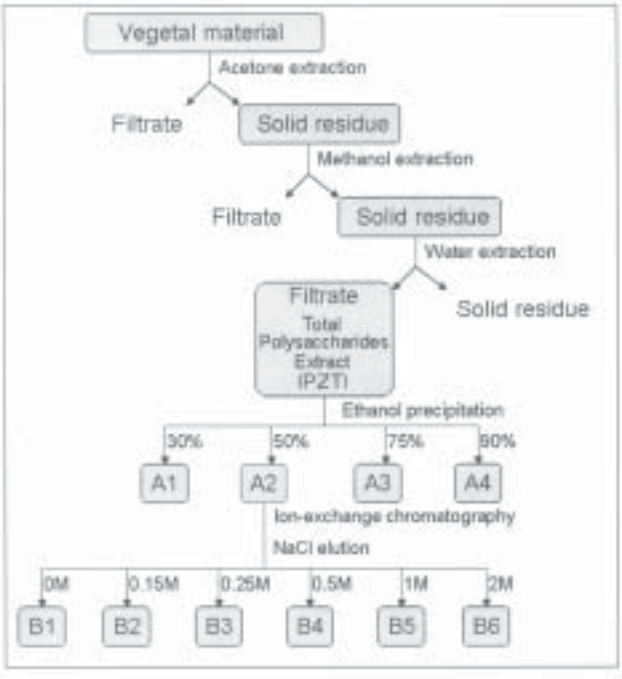

Fig. 1. Isolation and purification of Pz from *Viscum album*

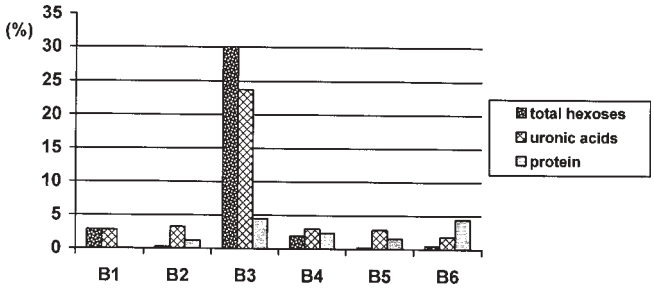

Fig. 2. Variation of the chemical composition of Pz fractions, purified by ion-exchange chromatography

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

Table 1
ANALYTICAL DETERMINATION FOR MISTLETOE Pz ETHANOLLIC SAMPLES

Sample	Total hexoses (%)	Uronic acids (%)	Protein (%)
A1	16.66	20.83	0.99
A2	42.12	36.68	0.78
A3	16.07	22.82	0.75
A4	10.11	19.08	0.66

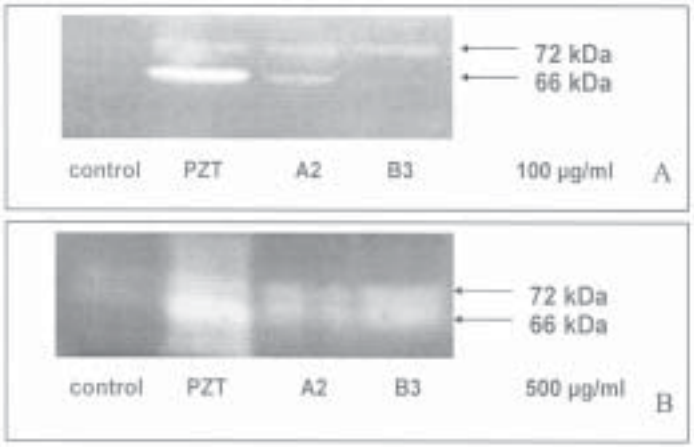

Fig. 3. Gelatin-zymography of the culture medium from dermal fibroblasts cultivated in the presence of 100 (A) and 500 (B)µg/mL mistletoe Pz, respectively. Two bands corresponding to MMP-2 latent form (72 kDa) and MMP-2 active form (66 kDa), respectively, were noticed

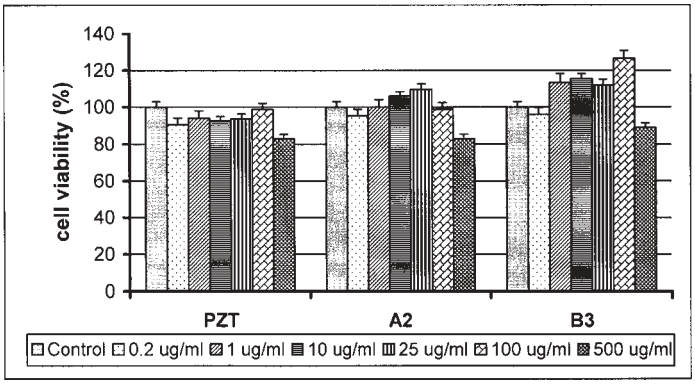

Fig. 4. Human dermal fibroblast viability at 48 h after cultivation in the presence of mistletoe Pz, assessed by the MTT assay. Values are expressed as mean of three determinations ± S.D

metalloproteinases, based on their molecular weight and their relative abundance from the band intensity. Pro-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 (500µg/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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