Bioactive Compounds (Vascotoxins) from *Viscum Album L.* Extracts Characterization

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Abstract

Viscotoxins are bioactive compounds that were discovered in a plant named Viscum album L. Viscotoxins A2 and B are together with viscotoxin A3, among the most abundant viscotoxin I isoforms that occur in mistletoe-derived medicines used in anti-cancer therapy. This paper presents studies of characterization proteins like viscotoxins by assay electrophoresis. Plant extracts had been separated and concentrated by using the membrane processes. Our results shown that the extracts tested structure was reproducible and same major fragments repeated to be dominated.

Keywords: *Viscum album* L; Viscotoxins; Bioactive compounds; Electrophoresis.

Introduction

Viscotoxins are toxic proteins isolated from mistletoe (*Viscum album* L) with anticancer activity [1]. They are low molecular mass (approx. 5 kDa) basic polypeptides and their amino acid sequence, disulphide bridge arrangement and distribution in plant tissues have been recently reviewed [2]. Six isoforms, A1, A2, A3, B, 1-PS and U-PS, have been described [3, 4] and correlated, on the basis of sequence similarity, to the family of a and b thionins, a group of cysteine-rich proteins found in the seeds and other tissues of several *Graminae* [4]. Thionins are best known for their toxicity to bacteria [5] and fungi [6], as well as to animal and plant cells [7], and this toxicity is believed to be important for the plant defense mechanism. Evidence for a role in the constitutive defense of plants against pathogen attack has been provided by the expression of thionins in transgenic plants [8, 9].The determination of high-resolution three-dimensional structures of these proteins, and, more generally, of thionins is therefore crucial to the understanding of their function [10]. Study on the NMR structural determination of viscotoxin A3, has been shown that it is the most abundant and cytotoxic isoform from *Viscum album* L [10]. In view of the fact that extracts from *Viscum album* L. are widely used as an adjuvant in complementary cancer therapy and that they have been shown to possess both immunomodulating and cytotoxic properties [11], studied of viscotoxins, at a molecular level, might be of both biological and clinical relevance.

A comparative study on aqueous extracts of *Viscum album* L, separated and concentrated by ultrafiltration on specific membranes, are reported and discussed. Our studies were performed with the object of characterization plant extracts as specific compounds that are bioactive principles with anticancer activity.

Materials and methods

Extract from mistletoe

The extract from mistletoe has been obtained from maceration of twig of mistletoe leaves, with distilled water, for 24 hours, at room temperature, with intermittent stirring. Further, the extract was concentrated by ultrafiltration, in 2 variants, on module KMS Laboratory Cell CF-1 (Koch Membrane - Germany), with membranes ultrafiltration Millipore „cut-off" 50 kDa (UF1), and 20 kDa (UF2). The investigated extracts were produced by a research group from National Institute of Research and Development for Biological Sciences, Bucharest.

Micro-electrophoresis

Characterization of mistletoe extracts from *Viscum album* L has been achieved by on chip micro-electrophoresis (Agilent 2100 Bioanalyzer), (similar to SDS-PAGE), in non-reducing denaturing conditions. This is a new method of molecular investigation which allows study molecular mass of unknown compounds and investigation composition of some proteins mixture, offering an image of nature bioactive compounds studies.

For these studies, we used Agilent Protein 230 Kit on the Agilent 2100 Bioanalyzer. We used the Agilent 2100 expert software to have the separation process dates and the final obtain. Reagents and samples preparations, chip preparation, loading and separation were made according to the protocol recommended in the Agilent Protein 230 Kit Guide (Manual Part Number G2938-90054 Edition 08/2006)

For determinations we combine 4 µl of protein extract sample and 2 µl of denaturing solution in a 0.5 ml micro centrifuge tube and mix well for 10 minutes. Then, the sample tub is kept for 5 minutes in a heating block at 95 – 100^0C. You add add 84µl of deionized water and vortex. It is not recommended to change the dilution ratio, because errors fo determination can appear.

The method permits the determination of proteins with molecular weights ranging 14-230 kDa, with molecular weight accuracy of 3%, detection limit 6ng/µl, and quantization range 6-2000ng/ µl, to determination range of molecular mass of 14-230 kDa±3%.

Protein measurement

Protein concentration was determined by Lowry method.

Results and discussions

Our caracterization studies were performed on aqueous mistletoe extract (V1), concentrated mistletoe extract from ultrafiltration-UF1 (V2), permeate resulting from ultrafiltration-UF1 of mistletoe extract (V3), permeate resulting from ultrafiltration-UF2 of mistletoe extract (V4) and concentrated mistletoe extract resulting ultrafiltration-UF2 (V5).

For the caracterization of mistletoe extract was necesary to determine the amount of protein concentration (Lowry method), values are listed in the table below:

Sample	The Designated Sample	Protein Concentration mg/ml
V1	Aqueous mistletoe extract	3.793
V2	Concentrated mistletoe extract from ultrafiltration -UF1	5.514

V3	Permeate resulting from ultrafiltration-UF1 of mistletoe extract	1.711
V4	Permeate resulting from ultrafiltration-UF2 of mistletoe extract	1.724
V5	Concentrated mistletoe extract from ultrafiltration-UF2	5.343

The extract compositions studied was made by determine the relative concentrations of proteins with detectable molecular weight and of percentages in which the analyzed protein exists in every sample (see tables 1- 5).

A plot electrophoregram designed Bioanalyzer Agilent 2100 shows us the proteins present in the studied extracts and by measuring the peak areas it's determined their own molecular weight.(see figures 1-5).

Table 1. Composition of aqueous mistletoe extract (*Viscum album* L) (V1)

Size [kDa]	Observations	Aligned Migration Time [s]	Area	Rel. Conc. [ng/µl]	Total %
4.5	Lower Marker	17	11.4	0	0
14.8		20.87	1.1	239.1	16
23.6		22.5	3.7	697	46.7
76.7		28.97	4.4	557.5	37.3
240	Upper Marker	41	0.8	60	0

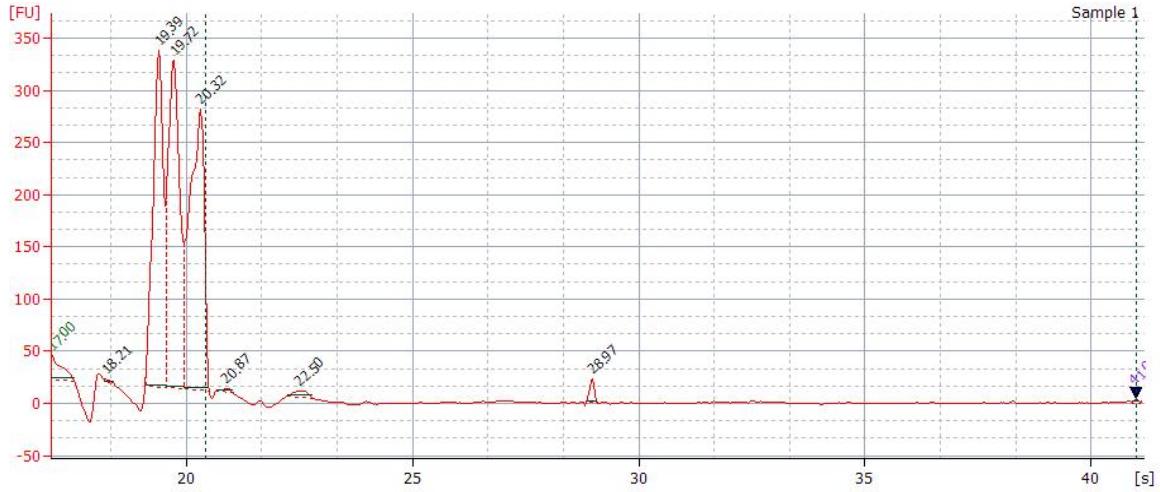

Figure 1. The protein electrophoregram made on the sample V1

Analysing the results obtained from the sample V1, aqueous mistletoe extract, with 3.793mg/ml protein concentration, bioactive compounds with protein characteristics have the following molecular weight: 14.8kDa, 23.6kDa and 76.7kDa.

By ultrafiltrating on Millipore membranes with "cut-off" procedure, 50 kDa (UF1) of aqueous mistletoe extract results a concentrate (the sample V2) and a permeate (the sample V3). Both samples were analyzed to characterize from the point of view of proteic compounds (viscotoxins and / or viscolectins) with anticancer activity.

Table 2. Composition of mistletoe extract (*Viscum album* L), concentrated by ultrafiltration (V2), on membrane with cut-off, 50 kDa

Size [kDa]	Observations	Aligned Migration Time [s]	Area	Rel. Conc. [ng/µl]	Total %
4.5	Lower Marker	17	1.3	0	0
14.6		20.8	154.3	53,984.20	38
15.8		21.09	130.6	44,397.80	31.2
19		21.67	129	41,466.60	29.2
22.2		22.24	2.3	714.8	0.5
30.8		23.68	1.7	464.6	0.3
105.4		31.38	1.9	321.1	0.2
121.8		32.85	1.1	174	0.1
134.6		34	3.5	526.5	0.4
142.6		34.72	0.7	99	0.1
240	Upper Marker	41	0.5	60	0

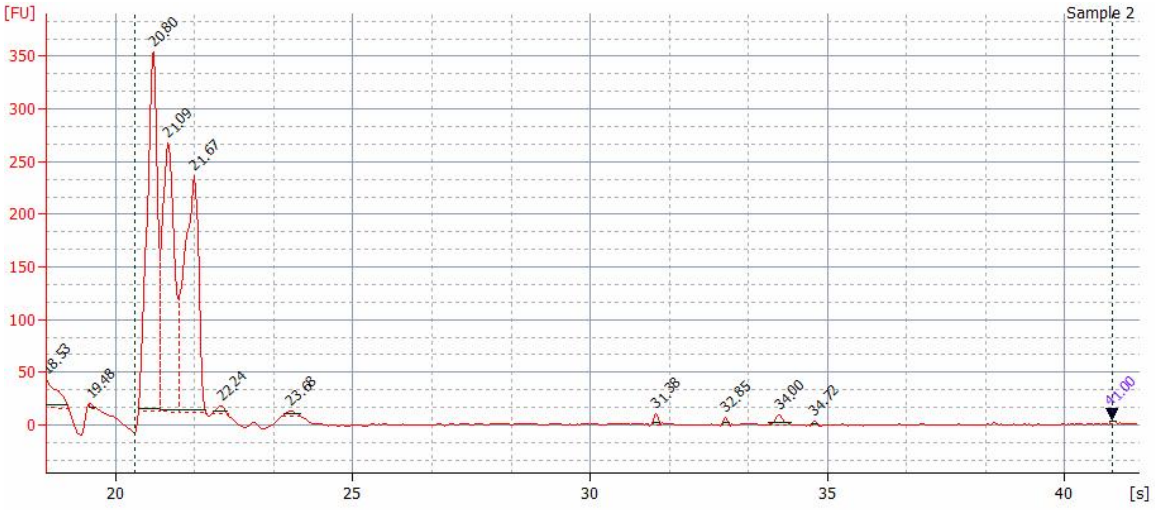

Figure 2. The protein electrophoregram made on the sample V2

The examination results obtained from the concentrate V2, with 5.514mg/ml protein concentration, bioactive compounds have the next molecular masses: 14.6kDa, 15.8kDa, 19kDa, 22.2kDa, 30.8kDa, 105.4kDa, 121.8kDa, 134.6kDa and 142.6kDa.

By electrophoresis of the sample V3, with 1.711mg/ml protein concentration, we observed that the permeate resulted have a protein composition with next molecular masses: 14.6kDa, 15.7kDa, 18.4kDa, 21.6kDa, 28.7kDa, 106.9kDa and 135.6 kDa.

Table 3. Composition of the permeate (V3) from ultrafiltration on membrane with cut-off, 50kDa

Size [kDa]	Observations	Aligned Migration Time [s]	Area	Rel. Conc. [ng/µl]	Total %
4.5	Lower Marker	17	1.3	0	0
14.6		20.78	135.1	39,068.50	49
15.7		21.07	52.8	14,816.60	18.6
18.4		21.56	86.9	23,270.50	29.2

21.6		22.13	3	763.2	1
28.7		23.39	4.9	1,105.60	1.4
106.9		31.51	0.7	101.9	0.1
135.6		34.09	4.8	578	0.7
240	Upper Marker	41	0.6	60	0

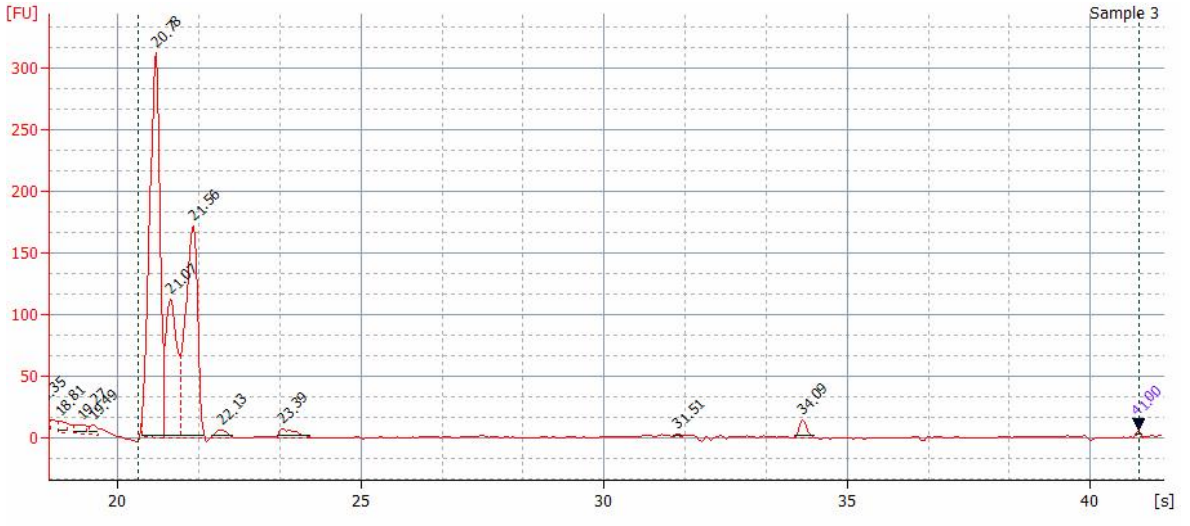

Figure 3. The protein electrophoregram made on the sample V3

By ultrafiltrating on Millipore membranes with „cut-off" procedure, 20 kDa (UF2) of aqueous mistletoe extract, results a concentrate (the sample V5) and permeate (the sample V4). Both samples were also analyzed to characterize from the point of view of bioactive compounds.

Table 4. Composition permeate (V4) from ultrafiltration on membrane with cut-off, 20 kDa

Size [kDa]	Observations	Aligned Migration Time [s]	Area	Rel. Conc. [ng/µl]	Total %
4.5	Lower Marker	17	2.6	0	0
16.8		21.26	0.8	379.1	0.4
21.3		22.08	119.2	49,887.70	46.8
24		22.58	51.7	20,843.20	19.6
27.5		23.2	83.3	32,087.50	30.1
46.3		25.8	1.3	434.7	0.4
48.1		26.03	1.6	513.2	0.5
56.3		27.04	2.2	680	0.6
59.1		27.39	2.4	709	0.7
152.4		35.53	0.9	185	0.2
226.3		40.15	4.8	838.2	0.8
240	Upper Marker	41	0.4	60	0

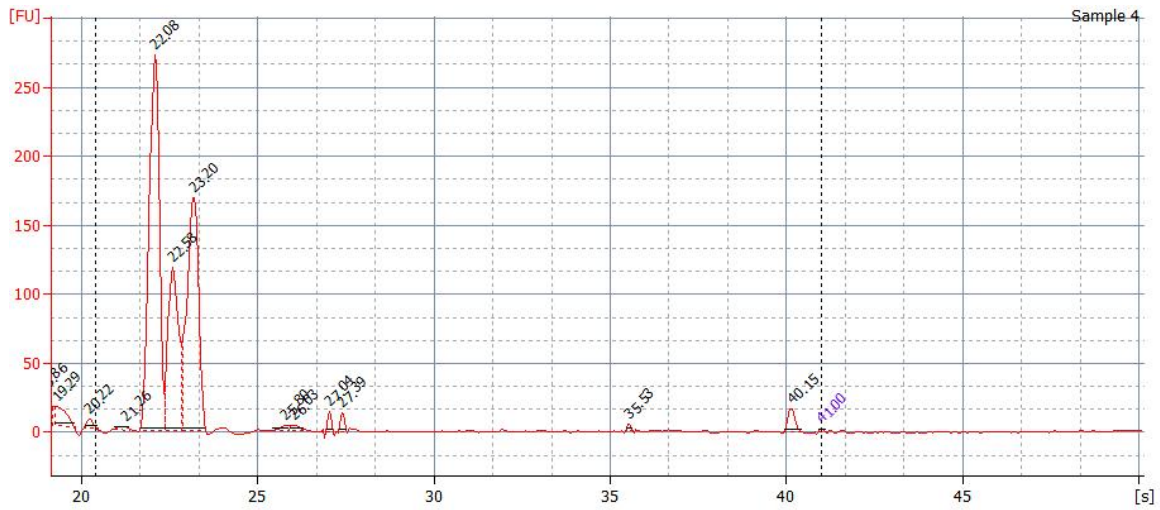

Figure 4. The protein electrophoregram made on the sample V4

By electrophoresis assay on the sample V4 (1.724mg/ml protein concentration), we observed that permeate resulted has a protein composition with the next molecular weight: 16.8kDa, 21.3kDa, 24kDa, 27.5kDa, 46.3kDa, 48.1kDa, 56.3kDa, 59.1kDa, 152.4kDa and 226.3kDa.

Analazing the results obtained by electrophoresis of concentrated mistletoe extract by ultrafiltration-UF2 (sample V5), with 5.343mg/ml protein concentration, we observed a content in the protein compounds with the following molecular masses: 22.6kDa, 25.3kDa, 30.1kDa, 36.4kDa and 54.2kDa.

Table 5. Composition of mistletoe extract (*Viscum album* L), concentrated by ultrafiltration (V5), on membrane with cut-off, 20 kDa

Size [kDa]	Observations	Aligned Migration Time [s]	Area	Rel. Conc. [ng/µl]	Total %
4.5	Lower Marker	17	0.9	0	0
22.6		22.32	203.1	4,563.70	54.3
25.3		22.82	87.8	1,905.00	22.6
30		23.59	91.1	1,874.40	22.3
36.4		24.45	0.6	11.5	0.1
54.2		26.79	3.3	56.4	0.7
240	Upper Marker	41	6.3	60	0

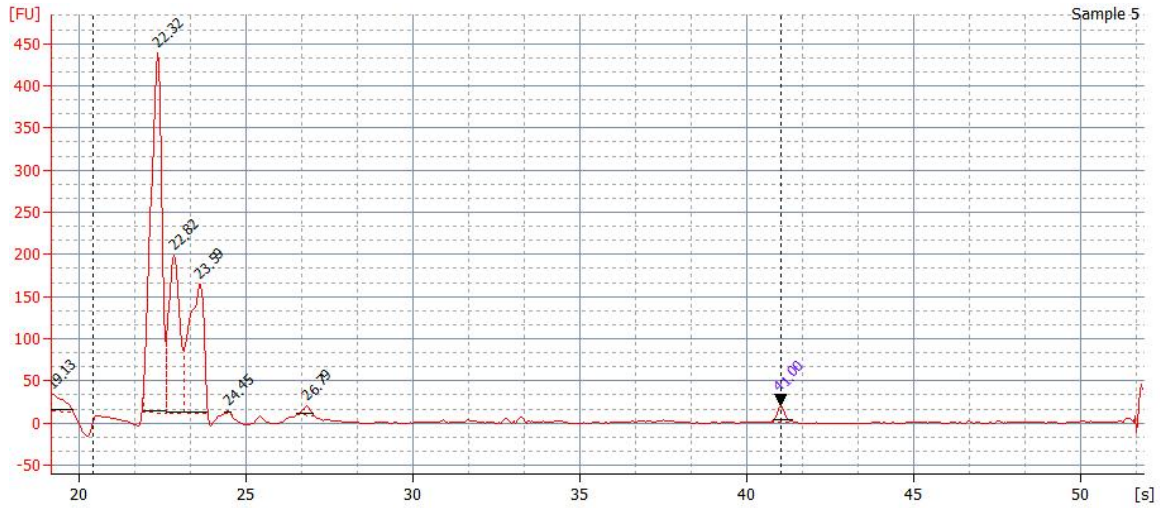

Figure 5. The protein electrophoregram made on the sample V5

The examination of electrophoresis data, we can conclude that samples have similar compositions, with 3 major protein peaks, with molecular weights of 22, 25 and 30kDa.

The protein composition has various aspects depending on extract processing, so that a variable number of minor peaks are detected.

The ratio of concentration into the three major peaks it change modifications in conformity with method of preparation, but 95% from protein content of study extracts represent about constant over 95% from proteins total.

Conclusions

In this work we had in view the characterization of specific proteins (viscotoxins and / or viscolectins) from aqueous mistletoe extract (*Viscum album*), separated by ultrafiltration. We realized characterization of specific proteins content from spectrophotometer determinations (method Lowry) and protein electrophoresis with Agilent 2100 Bioanalyzer.

The samples put to the test has a proteins content among 1,7 and 5,5 mg/ml; observing in permeate a small concentration and significant great in the concentrates obtained by ultrafiltration from initial aqueous mistletoe extract, that what demonstrate capacity of ultrafiltration membranes used in division into fractions, separation, and concentration of proteins from studies extracts.

From the data gathered by electrophoresis we concluded that a reproductibile compozition exists in all the extracts; there are a few major fractions that repetes them selves, and dominates. Other products that are found separatly, exists because of the work method that can influence the coumpound separation.

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