Romanian Biotechnological Letters *Vol. 16, No. 2, 2011*
Copyright © 2011 University of Bucharest Printed in Romania. All rights reserved
ORIGINAL PAPER

Cytostatic Activity of *Viscum album L.* Extract Processed by Microfiltration and Ultrafiltration

Received for publication, June 28, 2010
Accepted, March 4, 2011

G. PAUN[1], P. ROTINBERG[2], C. MIHAI [2], E. NEAGU[1] AND G.L. RADU[3]
[1]*National Institute for Research-Development of Biological Sciences, Centre of Bioanalysis, 296 Spl. Independentei, PO Box 17-16, 060031, Bucharest 6, Romania;*
tel./fax:+4 021-2200900; e-mail corresponding author: gabrielaroman2000@yahoo.com
[2]*Biological Research Institute – National Institute of Research-Development, Lascăr Catargi Street, No. 47, Iassy, Romania*
[3]*Faculty of Applied Chemistry and Materials Science, POLITEHNICA University of Bucharest, 313 Splaiul Independentei, Romania*

Abstract

The aim of this paper consists in 'non-stressing' process' use that, with respect to traditional plants operates under milder temperature conditions. Particular attention was devoted to investigate the possibility to purify and concentrate Viscum album extracts with a microfiltration/ultrafiltration unit, allowing the preservation of thermolabile compounds from the extracts and their antineoplastic properties examination. In vitro investigation of the HeLa cancerous cells interaction with biopreparations (extracted, separated and purified from Viscum album through successive ultrafiltrations on membranes) highlighted and quantified their cytostatic and cytotoxic impact, expressed by the inhibition of protein synthesis and cellular proliferation, by cell viability decrease and by the inhibition of cell cultures development.

Keywords*:* *Viscum album L*. extract, microfiltration, ultrafiltration, cytostatic activity.

Introduction

The interest for *Viscum album L.* (Loranthaceae) is justified by its large utilization in the traditional medicine. Today, parts of the pharmacodynamic effects are biomedicaly capitalized in curative and preventive, single or associated treatments [1].

A part of the biologically active compounds is mainly represented by phenolic acids (caffeic acid, p-coumaric acid, galic acid, syringic acid), alkaloids, flavonoids (catechine, myrecitin, quercitin), lignans, polyalcohols, lectins, polysaccharides, terpenoids, toxins etc. [2]. The interest for these compounds is due to their biologic properties revealed as hypotensive, anti-oxidative [3], cardiac-depressant [4], cellular agglutinating [5], antidiabetical [6], diuretic, antimicrobial [7], inflammatory [8], sedative, antispasmoic, tonic, vasohypotonic, immunomodulatory [10] and antitumoral actions [9-13].

The antineoplastic chemotherapy is characterized by a relatively low toxicity [14, 15]. This explains the major importance given to the oncobiologic research, focused on its efficiency optimization, by discovering new oncochemotherapeutic agents with directed activity on the cancerous cells [16-18].

This study is focused on the researches performed on the *Viscum album L*. [19], since this is one of the most frequently analyzed, yet a major controversial subject of the medicinal plants, due to numerous experimental data and to their heterogeneous character (only to remind about the controversy between American and European pharmacy schools about the therapeutic use of different mistletoe bioproducts).

This paper proposes the utilization of a "non-stressing" extract process, which may occur in milder temperature conditions. A particular attention was provided to investigate the possibility to purify and concentrate mistletoe extracts by means of a microfiltration/ ultrafiltration unit. Previous studies revealed the ultrafiltration performances for the concentration of some medicinal plant extracts [20, 21]. Ultrafiltration processes provide many advantages over conventional technologies. Their application in the phytopharmaceutical industry is particularly interesting, as membrane processes can be developed at mild temperatures, avoiding damages caused by thermal processes, thus, maintaining the original characteristics of the processed products.

Materials and methods

Plant material: Mistletoe biomass (leaves and stems) was collected at Bod, Brasov County, Romania, in July 2006 and was identified by lect. dr. Paulina Anastasiu from Botanics and Microbiology Department, University of Bucharest.

Cell lines, chemicals and equipments: Mycoplasm-negative negroid human cervix epithelium carcinoma cells (HeLa ACC 57, DSMZ, Germany), DMEM medium (Biochrom AG, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany). Streptomycin, penicillin and amphotericin B were purchased from Biochrom AG, Germany. Folin-Ciocalteu's Reagent, sodium carbonate, acetic acid glacial, caffeic acid, sodium acetate, were purchased from Sigma Aldrich (Germany), Albumin from bovine serum was purchased from Fluka (Switzerland) and acetonitrile was purchased from Merk (Germany).

Microfiltration membrane and ultrafiltration membrane from regenerated cellulose were purchased from Millipore (SUA).

Preparation and concentration of extracts: Extracts were prepared by maceration, using the distilled water as solvent, paying attention to the following factors: the herbal was crushed with a GRINDOMIX GM200 mill (Retsch – Germany), the contact time between the herbal and the solvent was of 24 hours, sporadic, mechanically stirring, operating room temperature (22°C±2°C). The herbal's mass concentration in the solvent was of 10% [22]. After filtering the extract through filter paper, each of the filtrates was processed by microfiltration (MF) (Millipore filters with 0.45 □m) at 2 bar, followed by ultrafiltration (UF), processed at 4 bar pressure. All extracts were sterilized by passage through microfiltration membrane.

Ultrafiltration, last step of the extract processing, has given permeate (clear extract) and retentate (containing large molecular weight components e.g. proteins, polysaccharides). The concentration ratio (expressed as a volumetric ratio between the permeate and concentrate) was 2:1. The used installation for micro- and ultrafiltration was the KMS Laboratory Cell CF-1 (acquired from Koch Membrane – Germany). Two flat sheets of polyvinyliden fluoride with MWCO 50,000 Da (UF1) and regenerated cellulose with MWCO 5,000Da (UF2) membranes were used having an effective area of 0.0028 m^2. The membrane processes were performed in a tangential flow regime, at room temperature (~22°C) and in duplicate.

Determination of total protein and total phenols content in extracts: The initial extracts and samples of permeate and retentate obtained from extract ultrafiltration have been characterized by determination of the total protein content, using UV-VIS spectrometry, through the Lowry method [23]. Total proteins contents were obtained from the regression equation of the Bovine Serum Albumin (BSA) calibration curve ($y = 0.0034x + 0.0351$, $R^2=0.9974$) and expressed as BSA equivalent.

The total phenolic content was determined by the Folin–Ciocalteu method [24]. Caffeic acid (0–100 □g/mL) was used to calibrate the standard curve; total polyphenols contents were obtained from the regression equation of the calibration curve of caffeic acid ($y = 0.0063x + 0.019$, $R^2 = 0.9927$) and expressed as caffeic acid equivalent.

Cell culture, treatments and analysis: The *in vitro* comparative pre-screening of the cytostatic and cytotoxic activities of the *Viscum album* crude extract, micro- and ultrafiltration concentrates and permeates has been performed on HeLa cell line. Mycoplasm-negative negroid human cervix epithelium carcinoma (HeLa ACC 57, DSMZ, Germany) cells were cultured in DMEM medium (Biochrom AG, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany), 100 µg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany) and 50 µg/mL amphotericin B (Biochrom AG, Germany), at a density of $5x10^5$ cells in 75 cm^3 flasks, in a CO_2 atmosphere, with 5% RH at 37°C. When the cells reached confluence they were taken off the flask using a mixture of 0.25% trypsin + 0.02% EDTA (Biochrom AG, Germany), in the normal medium and then centrifuged at 1800 rpm, for 2 minutes. The cells were seeded at a density of $1x10^5$ cells/mL in the experimental tubes containing 2mL of DMEM medium. The medium of the 24 hours cell cultures was changed either with a normal one (control cultures) or with one containing the vegetal extracts (treated cultures), in dose of 1.5 mg/mL.

The medium was removed from the test tubes, after 24 and 48 h of *in vitro* treatment and the layers of cells were washed with PBS (saline phosphate buffer). The cytostatic study was performed for:

a) evaluation of the total protein content (Lowry method modified by Oyama) [25] and tracing of the protein dynamics;

b) the cytometrical assessment of the total cellular number with Türk haemocytometer using $N = n \times d \times 10^4$ formula, where: N= total cellular number; n = number cells from a square of 1/25; d = dilution (= 2);

c) the mathematical estimation of the cell proliferation inhibition: % mitoinhibitory impact = Nt / Nm x 100, where: Nt = treated sample cells number; Nm = control sample cells number; the cytometrical assessment of the living and dead cells number by trypan blue exclusion test [26];

d) the mathematical estimation of the % viability = N_{lcn} / N_{tcn} x 100, where: N_{lcn} = living cells number of the treated sample; N_{tcn} = total cells number of the treated sample.

The cytostatic properties of the studied extracts was appreciated according to American prescreening program criteria, which imposed an induced 50% minimum inhibitory impact for the potential antitumoral agents *in vitro* selection [27]. Five culture tubes were used and the results were evaluated statistically by Student's "t" test, for each culture type and time interval [28].

The total extract from *Viscum album* and three fractions obtained by ultrafiltration were used in the *in vitro* pre-screening, as follows: V1 (aqueous extract), V2 (the aqueous extract being pre-concentrate – UF1), V3 (permeate UF2) and V4 (concentrate UF2).

Results and discussions

Table 1 shows the protein and total polyphenol concentration in *Viscum album* initial aqueous extracts and after concentration by micro- and ultrafiltration.

Variation of permeate flux under operating constant pressure (4 bar) during the ultrafiltration processes is shown in Fig.1.

Table 1. Concentration of protein and total polyphenol in aqueous extract from *V. Album*

Process	Protein concentration, mg/mL		Polyphenols concentration, □g/mL		Permeate flow, L/m^{2}h
	permeate	concentrate	permeate	concentrate	
Aqueous extract of *V. album*	3.79	-	168.41	-	-
Microfiltration (MF)	3.26	-	139.53	-	128.5
Pre-concentration (UF1)	3.07	-	168.27	-	61.7
Concentration (UF2)	1.72	5.51	94.56	179.32	53.8

The experimental values of the flux show the decrease tendency in time, specific to all of the pressure driven membrane separation processes. The loss of permeate flux in cross-flow ultrafiltration is caused by concentration polarization and membrane fouling, but by using flow reversal the formation of stable hydrodynamic and concentration boundary layers at the membrane surface was prevented.

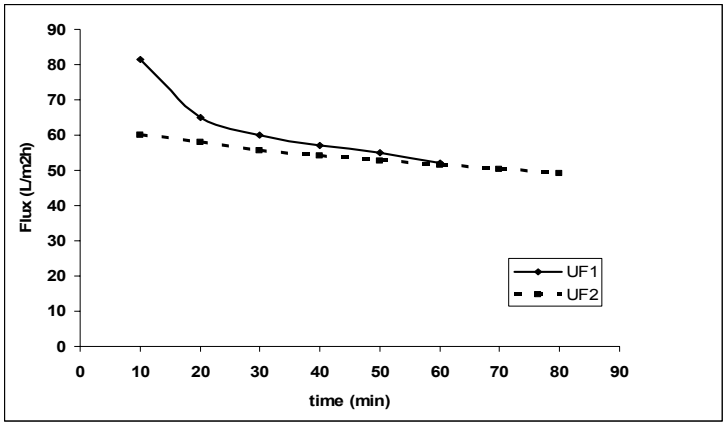

Fig. 1 Permeate flux decline vs. time during ultrafiltration of mistletoe extract

The protein synthesis process of the HeLa cells cultures was investigated, expressed by the total proteins concentration and by the protein dynamics for cytostatic activity estimation. The aim was to highlight and appraise the protein synthesis inhibitory impact induced by the V1, V2, V3, and respectively by the V4 extracts (Fig.2).

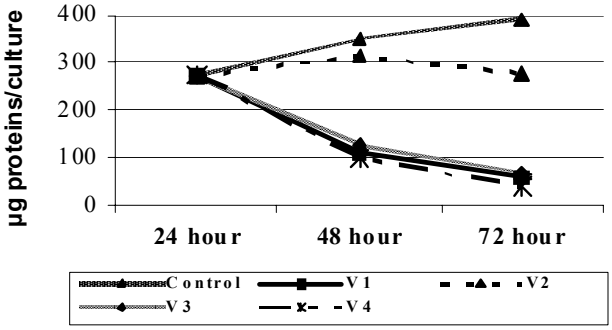

Fig. 2 The protein dynamics of the HeLa cellular cultures, treated with different *Viscum album* extracts, as compared with a control cultures.

The HeLa control cultures evolution is analyzed at 24, 48 and 72 hrs intervals, by the mean values of total proteins concentration: 273.8, 348.6 and 390.2 µg protein/culture. Progressively increasing the protein concentration leads to the protein synthesis

intensification, also correlated to the cellular replication rate increase. The results of the HeLa cultures treated with mistletoe biopreparations are given bellow (see fig.2.):

- protein content was successively of 110.7 (V1), 125.4 (V2), 98.6 (V3) and 313.7 (V4) µg protein/culture at culture age of 48 hours;
- protein content was of 61.3 (V1), 68.5 (V2), 42.3 (V3) and 278.7 (V4) µg protein/culture at the culture age of 72 hours.

The evolution of the protein average concentrations, estimated at various stages of the HeLa - control and mistletoe extracts treated cultures - leaded to the registration of some protein dynamics specific to each experiment. Thus, if the untreated tumoral cell cultures protein dynamics present an ascendant trend with progressively increased amplitude, the protein dynamics of the treated cell cultures reveals a descendent trend of decreased amplitude. These data emphasize the protein synthesis inhibitory impact induced by the mistletoe extracts, and the effect intensity depended on extract type. The maximum inhibitory potential, which reaches the level of the cytostatic and statistic significance, characterizes the ultrafiltration concentrate (V4), followed by V1 crude hydrous extract, V3 and V2 ultrafiltration concentrates. The cytostatic effect could be directly correlated to the mistletoe lectins content (complex proteins of high molecular weight [MW=30-120 kDa]), being of about 3 times greater in retentate than in permeate and about 1.4 times greater in retentate than in aqueous extract.

Another evaluation parameter of the mistletoe extracts cytostatic effect was the total cellular number (the cellular proliferation degree), as a result of the bioactive agents interference with the cancerous cells mitosis. These results are summarized in table 2 with living and dead cells number.

Table 2. The living, dead and total cells number variation ($x10^4$) under the condition of the HeLa cell incubation with a dose of 1.5 mg/mL of the biopreparations from *Viscum album*

Experimental group	Cytostatic activity		Cytotoxic impact					
	Total cells no.*		Living cells no.*			Dead cells no.*		
	$X*10^4$±ES	p	$X*10^4$±ES	% Viability	p	$X*10^4$±ES	% Cytotoxicity	p
Control 48 h	98.5±6.42(5)	–	94.6±5.34(5)	96.0	–	3.9±1.08(5)	4.0	–
V1	36.2±2.53 (5)	<0.002	13.3±8.9 (5)	36.8	<0.001	22.9±8.62 (5)	63.2	<0.001
V2	16.9±0.67 (5)	<0.001	2.54±0.52 (5)	15.0	<0.001	14.36±2.7(5)	85.0	<0.001
V3	29.2±10.04(5)	<0.001	8.68±2.50(5)	29.7	<0.001	20.5±9.65(5)	70.3	<0.05
V4	28.9±2.67 (5)	<0.001	1.5±0.67 (5)	5.2	<0.001	27.4±2.0(5)	94.8	<0.001

* corresponding to the same control or treated cultures, the last aspect being requested to assess their *in vitro* cytotoxic impact

The action of the vegetable mistletoe extracts on cell viability has been analyzed in vitro, in the next experimental model. This impact was expressed by numerical differences between living and dead cells, as they were traced with a trypan blue stain (see table 2).

The comparative analysis of the living and dead cells number for the HeLa cell cultures, control and respectively for 48 hrs treated cell cultures with mistletoe extracts, in a dose of 1.5 mg/mL, reveals a different behavior of those two cell culture types. Thus, the *Viscum album* products diminished the neoplastic cells viability, as it is separated from the

initial, crude extract by successive ultrafiltrations. Therefore, they are also characterized by a high cytotoxic effect, while the maximum potential was registered in the case of V4 fraction (94.8%), followed by the V2 and V3 extracts (85% and 70.3%).

An influence of the mistletoe preparation on the HeLa cancerous cell culture evolution was noticed, its degree depending on the bioactive compound effect on the protein synthesis process, on the replication process and on cell viability (Fig.3).

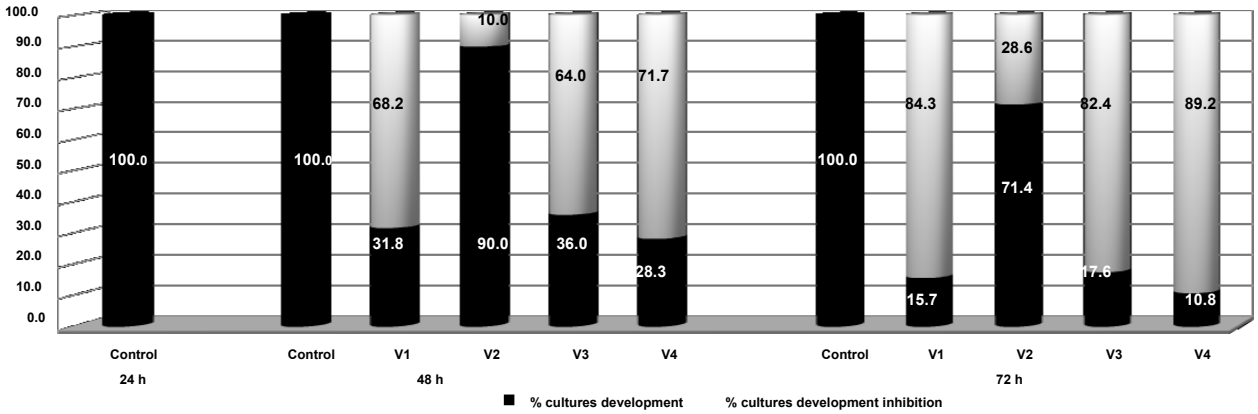

Fig. 3. The effect of the *Viscum album* based extracts on the HeLa tumoral cell cultures development

The neoplastic cell cultures incubated with the tested mistletoe extracts were compared with those assigned to the control cell cultures, considered to be 100%. The bioactive agents inhibitory potential upon HeLa cultures development depends on the used preparation, the most active being V4 (71.7%, at 48h and 89.2%, at 72h) successively followed by V1, V3 and V2.

The comparative analysis on the *Viscum album* extracts cytostatic and cytotoxic potential with the standard value (at least 50%) - stipulated *in vitro* prescreening program [14, 26] for discovery of new antineoplastic agents – led us to the appreciation that V1, V3 and V4 products, in this experimental model, have significant cytostatic and cytotoxic effects.

The slight difference between the i*n vitro* cytostatic and cytotoxic efficiency of our crude and ultrafiltrated mistletoe extracts (V1, V3 and V4), could be assigned to the presence of various active biomolecules in the biopreparations final composition.

The most active V4 extract potential would probably result from the high viscolectins and alkaloids content (retained on membrane with MWCO of 5,000 Da). The viscotoxines increased presence in the V2 extract (retained on the membrane with MWCO 50,000 Da) explains its significant cytostatic and cytotoxic impact upon HeLa cells cultures. The presence of all types of the active chemical structures in the V1 crude extract is correlated to its high cytostatic and cytotoxic effectiveness *in vitro*, the amplitude of its impact being close to the one of V4 product, the most active concentrated extract.

Conclusion

These results revealed that the *Viscum album* extract could be purified and concentrated successfully by micro- and ultrafiltration.

As a conclusion, *in vitro* investigation of the interaction of some preparations – extracted, purified and concentrated from *Viscum album* through successive micro- and ultrafiltrations on membranes (UF1 membrane with MWCO 50,000 Da and UF2 membrane with MWCO of 5,000 Da) – on the HeLa cancerous cells has highlighted and monitored their cytostatic and cytotoxic impact. The cytostatic effect could be directly correlated to the

mistletoe lectins content, which is about 3 times greater in retentate than in permeate and about 1.4 times greater in retentate than aqueous extract.

The bioactive agents inhibitory potential upon HeLa cultures development depends on the used preparation, the most active being V4 (71.7%, at 48 h and 89.2%, at 72 h) successively followed by V1, V3 and V2. In the same time the highest cytotoxic effect was registered on V4 fraction (94.8%).

The biopreparations extracted, purified and concentrated from *Viscum album,* by the membrane processes, turn them into the perfect candidates for their characterization as autochthonous, original oncochemotherapeutic agents.

Acknowledgments

This work has been financed from BIODIV national project no.106/2009

References

1. L. KANNER, Mistletoe, magic and medicine, *Bull. Hist. Medicine,* **7**, 875–883, (1939)
2. U. PFÜLLER, *Mistletoe. The Genus Viscum,* Büssing A, editor*.,* Harwood Academic Publishers, Amsterdam, 101–22 (2000)
3. O.I. ARUOMA, Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods, *Mutat. Res.*, 523-524, 9-20 (2003)
4. T. FUKUNAGA, I. KAJIKAWA, K. NISHIYA, Y. WATANABE, K. TAKEYA, H. ITOKAWA, Studies on the constituents of the European mistletoe, Viscum album L, *Chem Pharm Bull*., **35,** 8, 3292-3297 (1987)
5. R. SAMTLEBEN, M. KIEFER, P. LUTHER, *Lectin,* In Bog Hansen TC and Breborowicz J (Eds.). Berlin Walter de Gruyter & Co. Vol. IV, 617–626 (1985)
6. A.M. GRAY, P.R. FLATT, Insulin-secreting activity of the traditional antidiabetic plant *Viscum album* [mistletoe], *J. Endocrinol.*, **160,** 3, 409-414 (1999).
7. J. DOUGHARI, A. ELMAHMOOD, S. MANZARA, Studies on the antibacterial activity of root extracts of Carica papaya L*., Afr. J. Microbiol. Res*, **1,** 3, 37–41 (2007)
8. A. BÜSSING, *Mistletoe - The Genus Viscum*, Harwood Academic Publishers, Arndt Büssing [Ed.] (2004)
9. M. CAZACU, T. ONIU, C. LUNGOCI, A. MIHAILOV, A. CIPAK, R. KLINGER, T. WEISS, N. ZARKOVIC, The influence of Isorel on the advanced colorectal cancer, *Cancer Biother. Radio.*, **18,** 1, 27-34 (2003)
10. T. HAJITO, Immunomodulatory effects of iscador: a Viscum album preparation, *Oncology.,* **43,** Suppl.1**,** 51-65 (1986)
11. W.B. PARK, S.Y. LYU, J.H. KIM, S.H. CHOI, H.K. CHUNG, S.H. AHN, S.Y. HONG, T.J. YOON, M.J. CHOI , Inhibition of tumor growth and metastasis by Korean mistletoe lectin is associated with apoptosis and antiangiogenesis, *Cancer Biother. Radio.*, **16,** 5, 439-447 (2001)
12. G. KELTER, H.H. FIEBIG, Absence of tumor growth stimulation in a panel of 26 human tumor cell lines by mistletoe [*Viscum album L.*] extracts Iscador in vitro*, Arzneimittel-Forsch*, **56,** 6A, 435-440 (2006)
13. M. AUGUSTIN, P.R. BOCK, J. HANISCH, M. KARASMANN, B. SCHNEIDER, Safety and efficacy of the long-term adjuvant treatment of primary intermediate- to high-risk malignant melanoma [UICC/AJCC stage II and III] with a standardized fermented European mistletoe [*Viscum album L.*] extract. Results from a multicenter, comparative, epidemiological cohort study in Germany and Switzerland, *Arzneimittel-Forsch*, **55,** 1, 38-49 (2005)
14. V. STROESCU, *Pharmacological basis of medical practice*, Ed. Medicală Bucureşti, 48-63, 207-225, 1050-1102 (1998)
15. V.T. DE VITA, S. HELLMAN, S.A. ROSENBERG, *Cancer: Principles and Practice of Oncology*, Lippincott, Williams &Wilkins Publishers, 276-300 (2001)
16. L. MIRON, *Oncologie generala*, Ed. Egal, Bacau, 9-131 (2000)
17. J. OWENS, Something old and something new: taking cancer therapy forward, *Drug Discov. Today*, **6,** 23, 1203 – 1205 (2001)
18. W.R. RUDDON, *Cancer biology*, 4th Ed, Oxford University Press, 3-14, 117-236 (2007).
19. P.J. MANSKY, Mistletoe and cancer: controversies and perspectives, *Semin. Oncol.*, **29,** 6, 589-594 (2002)

20. G. PAUN-ROMAN, E. NEAGU, V. MOROEANU, GH. NECHIFOR, G.L.RADU, The ultrafiltration performance of composite membranes for the concentration of plant extracts, *Roum. Biotechnol. Lett.*, 14, 5, 4620-4624 (2009)
21. E. NEAGU, G. PAUN ROMAN, G. L. RADU, Gh. NECHIFOR, Concentration of the bioactive principles in *Geranium robertianum* extracts through membranare procedures (ultrafiltration), *Roum. Biotechnol. Lett.*, 15 (1), 5042-5048 (2010)
22. G. PAUN-ROMAN, E. NEAGU, G.L. RADU, Antiradical activities of *Salvia officinalis* and *Viscum album L.* extracts concentrated by ultrafiltration process*, Acta Sci.Pol., Technol. Aliment. 8 (3), 47*-58 (2009)
23. O. H. LOWRY, N. J. ROSEBROUGH, A.L. FARR, R. J. RANDALL, Protein measurement with the Folin phenol reagent *, J. Biol. Chem.*, 193, 265-275 (1951)
24. V.L. SINGLETON, R. ORTHOFER, R.M. LAMUELA-RAVENTOS, Analysis of total phenols and other oxidation substrates and antioxidant by means of Folin-Ciocalteu reagent, *Methods Enzymol.,* **299**, 152-178 (1999)
25. V. OYAMA , H. EAGLE*,* Measurement of Cell Growth in Tissue Culture with a Phenol Reagent [Folin-Ciocalteau]*, Proc. Soc. Exp. Biol. Med.,* **91,** 2, 305-307 (1956)
26. A. DOYLE, J. GRIFFITHS, *Cell and Tissue Culture: Laboratory Procedures in Biotechnology,* John Wiley & Sons Ltd., West Sussex, 57-58 (1998)
27. J. LEITER, B.J. ABBOTT, S.A. SCHEPARTZ*, S*creening data from the cancer chemotherapy national service center screening laboratories. XXII *, Cancer Res.*, **25**, 1-195 (1965)
28. G..W. SNEDECOR, *Metode statistice aplicate în agricultura si biologie [Statistical methods applied to agricultural and biological research]*, Ed. Didactica si Pedagogica, Bucuresti, 37-38 (1968)