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ORIGINAL ARTICLE

Cytostatic activity of *Geranium robertianum* L. extracts processed by membrane procedures

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HEp-2p cells;
Potential antitumor agents

Abstract In the present study the antioxidant and cytostatic capacities of some 8% *Geranium robertianum* (geranium) aqueous extracts processed by membrane procedures (micro- and ultrafiltration) were determined. The extracts were purified by microfiltration and then concentrated by successive ultrafiltrations using Millipore membranes with 10,000 and 1000 Da cut-off. Two methods were used to establish the extracts' antioxidant activity: DPPH and ABTS; the cytostatic capacity was evaluated on HEp-2p cell lines, by a qualitative method (cytochemical stain with Giemsa solution) and quantitative one (MTT test). Four concentrations of *Geranium* aqueous extracts were used to test cell viability: 100, 500, 1000 and 2000 μg/mL, 24 and 48 h, against the control culture (100% viability). The concentrated samples had the highest content of total polyphenols and the strongest antioxidant effect, having also the biggest impact upon cell viability.

1. Introduction

The plant natural compounds represent some of the most important sources for research in the pharmaceutic industry. In the last years several studies were directed toward traditional medicines in order to identify natural compounds with therapeutic properties (Hamburger and Hostetmann, 1991; Weisburger et al., 1996).

Pharmaceutic and pharmacodynamic researches demonstrated that the drugs of vegetal origin are biological products that are more accessible to human metabolism than the synthetic ones which, sometimes, cause, in addition, poisonous side effects.

Geranium robertianum L. (*Geraniaceae* family) has its origin in Asia, Central and Meridional Europe and it is frequently used in traditional medicine to treat inflammatory diseases and cancer. The *Geraniaceae* family consists in about 11 genera with almost 800 species spread in temperate and subtropical areas of both hemispheres. Some species belonging to *Gera-*

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nium genus are used in traditional medicine, while others are cultivated in ornamental scope or for their aromatic oils (Camacho-Luis et al., 2008).

The active principles identified in geranium are polyphenolic compounds of glycosylated flavonoid type (quercetin, kaempferol, rhamnetin) and phenolic acids (Miliauskas et al., 2004a,b). As it has already been reported, the flavonoids exercise multiple biological effects due to their antioxidant capacity and ability to neutralize free radicals (Papuc et al., 2009, 2007).

The flavonoids have a significant role in the plant biochemistry and, at physiological level, they have hepatoprotective, antithrombotic, anti-inflammatory, antiviral, antiallergic, antiproliferative, anticancer and immunostimulant actions (Harborne, 1988; Saija et al., 1995). A series of studies reported the antibacterial, antifungal, antiallergic actions of flavonoids, as well as, their protective role in heart diseases, cancer and different pathological states (Martinez et al., 2002; Matsuda et al., 2003).

The plant polyphenols' role in cancer chemoprevention led to the development of an interesting research domain. Some polyphenols and their derivates present selective cytotoxicity against various tumoral cells, much greater than upon normal cells (Salucci et al., 2002; Serrano et al., 1998). Such compounds could serve as antitumor agents or as a basis for the synthesis of new drugs by targeted chemical modification (Pellegrinaa et al., 2005; Yang et al., 2000).

In the present study the membrane procedures, micro- and ultrafiltration, were used to process some aqueous *G. robertianum* extracts, in order to purify and concentrate them. Previous studies evidenced the ultrafiltration performances to concentrate some extracts of medicinal plants (Neagu et al., 2011, 2010; Paun et al., 2011; Paun-Roman et al., 2009).

The membrane procedures of separation and concentration might have an improved efficiency and lower operation costs in comparison to the traditional concentration processes used in the pharmaceutical industry (Shi et al., 2006).

2. Materials and methods

2.1. Extract obtain

The *G. robertianum* extract was prepared through maceration, using the bidistilled water as solvent. The plant leaves were, previously, finely grinded by means of a GRINDOMIX GM200 grind mill; the maceration lasted 24 h, at 20 °C, mechanical stirring was sporadically performed; the plant mass concentration was of 8%.

2.2. Extract concentration

The extract was firstly filtrated through a filter paper, and then purified by microfiltration using a Millipore membrane of regenerated cellulose with 0.45 μm pores. The extract concentration was realized by successive ultrafiltrations using Millipore membranes with 10,000 and 1,000 Da cut-offs. The concentration ratio (expressed as volumes' ratio between permeate and concentrate) was of 3:1. A KMS Laboratory Cell CF-1 installation, purchased from Koch Membrane firm – Germany, was used for both microfiltration and ultrafiltration.

2.3. Determination of polyphenol content

Determination of polyphenol content was done by the Folin–Ciocalteu method (Folin and Ciocalteu, 1927). The polyphenol concentration in the sample was calculated based on an etalon curve of 10–100 μg/mL gallic acid (GAE).

2.4. Determination of antioxidant activity

Determination of antioxidant activity was done with a spectrophotometer, by evaluation of Trolox Equivalent Antioxidant Capacity (TEAC) with two methods:

- One based on the decrease of the DPPH maximal absorbance, at 519 nm in the presence of antioxidants (Bondet et al., 1997; Litescu et al., 2000).
- Other based on the decrease of the ABTS maximal absorbance, at 731 nm in the presence of antioxidants (Rice-Evans and Miller, 1994).

2.5. HPLC analysis of the obtained extracts

The chromatographic analyses of *G. robertianum* aqueous extracts were performed by the HPLC–DAD method. The chromatographic measurements were carried out using a HPLC SHIMADZU system with following components: LC-20AD sp Pump, the Kromasil C18 column for polyphenol–polycarboxylic acids, DGU-20As Degasser, CTO-20AC thermostat column, detector SPD-M20A diode array Shimadzu, LCMS – Shimadzu solution software.

The used standards were: elagic acid, rutin, luteolin, quercetin, kaempferol, gallic acid, cafeic acid, coumaric acid, and ferulic acid. The used mobile phase had two components: (A) water, acidulated with 1% formic acid and (B) acetonitril with 1% formic acid.

2.6. Testing the cytostatic activity of extracts

The cytostatic activity of *G. robertianum* extracts processed by micro- and ultrafiltration was tested on the Hep-2 (human epidermoid carcinoma of larynges) cell line. The neoplastic cells were maintained in MEM (Sigma–Aldrich) culture medium supplemented with 10% fetal bovine serum (Biochrom), 1% PSN (penicillin, streptomycin, neomycin; Sigma–Aldrich) in an incubator at 37 °C, in wet atmosphere with 5% CO_2. The extracts' antitumor activity was evaluated both by qualitative (cytochemical stain with Giemsa solution) and quantitative (MTT test) methods.

2.6.1. MTT test

The cell viability was determined by the MTT test. The spectrophotometer method is based on the conversion of dimetiltiazol-2-difeniltetrazolium bromide (MTT) in purple crystals of formazan under the action of mitochondrial dehydrogenases in live cells. The formed formazan amount is proportional to the number of live cells and is appraised with a spectrophotometer after the crystals' dissolution into isopropylic alcohol.

Table 1 Polyphenol content in *Geranium robertianum* extracts.

Type of membrane	Polyphenols (μg/mL)				
	Millipore 10,000 Da		Millipore 1000 Da		
Sample	Permeate	Concentrate (UF1)	Permeate	Concentrate (UF2)	Concentrate UF1 + UF2 (50:50)
6% aqueous extract	675.04 ± 6.3	745.61 ± 9.1	536.89 ± 5.3	763.89 ± 9.3	695.55 ± 6.2

Table 2 Antioxidant activity of analyzed extracts.

Sample	$TEAC_{DPPH}$ (μmol Trolox/g)	$TEAC_{ABTS}$ (μmol Trolox/g)
6% *Geranium robertianum* aqueous extract_MF	169.02 ± 6.86	539.56 ± 11.21
6% *Geranium robertianum* extract perm_UF_1	151.12 ± 7.85	390.00 ± 4.29
6% *Geranium robertianum* extract conc_UF_1	180.91 ± 6.04	573.20 ± 9.29
6% *Geranium robertianum* extract perm_UF_2	176.87 ± 1.71	452.54 ± 9.76
6% *Geranium robertianum* extract conc_UF_2	216.39 ± 6.64	1286.96 ± 3.89
6% *Geranium robertianum* extract conc_UF_1 + UF_2 (50:50)	182.57 ± 6.38	1123.85 ± 13.04

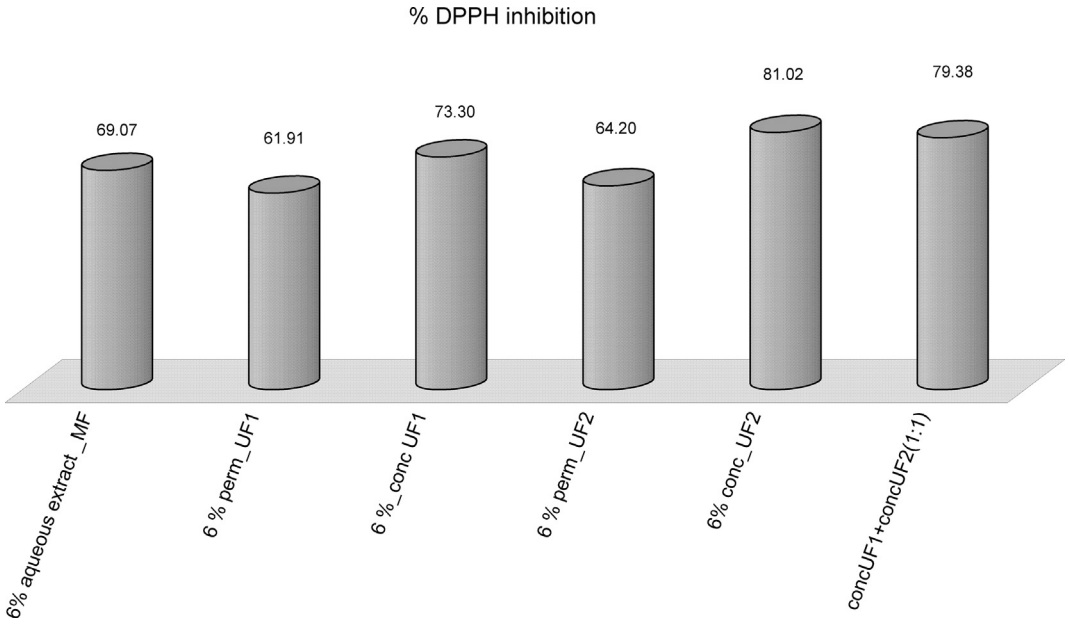

Figure 1 DPPH inhibition.

The cells were inoculated at 3.5×10^4 cells/ml density on plates with 24 wells and incubated at 3700 BAC in wet atmosphere with 5% CO_2, 24 h, to allow cell adherence. After incubation, the medium was carefully removed and replaced with fresh culture medium supplemented with different extract concentrations. The culture medium was removed 24 and 48 h after the addition of the extract, and the MTT solution (0.25 mg/ml) was added. The culture was incubated at 37 °C, 3 h. Then, the obtained formazan crystals were solubilized with isopropanol and the colorant absorbance was measured at 570 nm by means of a Tecan Microplate Reader.

2.6.2. Cell morphology

In order to observe their morphology, the cells were inoculated on plates with 24 wells at a density of 3.5×10^4 cells/ml. 24 and 48 h after the addition of the extract, they were washed with PBS, fixed with cold methanol (−20 °C), stained with Giemsa solution (15 min) and photographed with a Zeiss Axio Observer optic microscope.

3. Results and discussions

3.1. Total polyphenol content

In the fractions obtained during the ultrafiltrations, respectively the permeates and concentrates are presented in Table 1.

The highest polyphenol content was found in the concentrated extracts, especially in extract UF2 processed by Millipore membrane with 1000 Da cut-off.

In the *G. robertianum* extracts obtained and processed by micro- and ultrafiltration the antioxidant activity was determined by two (with DPPH and ABTS) spectrophotometric

Table 3 Content in active substances of interest, expressed in μg/g vegetal mass in *Geranium robertianum* extracts.

Sample	Elagic acid	Rutin	Luteolin	Quercetin	Kaemfoerol	Gallic acid	Cafeic acid	Ferulic acid	Cumaric acid
	(μg/g vegetal mass)								
6% Aqueous extract_MF	744.15	1.73	3.56	0	1.86	978.86	0	0	0
Conc_UF1 (Millipore 10,000 Da)	779.63	3.83	4.03	0	0	1056.79	0	0	0
Conc_UF2 (Millipore 1,000 Da)	900.13	4.935	8.16	0	0.964	1070.78	0	0	0
Conc_UF1 + Conc_UF2 (1:1)	729.13	5.41	5.01	0	0	958.43	0	0	0

Table 4 Cell viability in Hep-2p cultures under the action of 8% *Geranium robertianum* aqueous concentrated extracts.

Sample	% Cell viability							
	24 h				48 h			
	100 μg/mL	500 μg/mL	1000 μg/mL	2000 μg/mL	100 μg/mL	500 μg/mL	1000 μg/mL	2000 μg/mL
P1-MF	90.7	91.1	38.7	6.8	89.7	70.0	23.5	2.8
P2-Conc UF1	60.7	54.0	36.2	5.9	71.5	50.3	20.9	3.1
P3-Conc UF2	60.0	60.9	36.0	5.9	70.4	56.0	21.6	2.8
P4 = P2 + P3 (50:50)	61.2	56.4	39.0	10.9	70.5	56.3	30.2	7.6

methods, and also appraised the DPPH inhibition percent. The results are shown in Table 2 and Fig. 1.

A correlation between the polyphenol amount of studied extracts and their antioxidant capacity could be observed, the highest antioxidant capacity being found in the concentrated extracts, especially in the UF2 extract, in which the highest amount of total polyphenols was also recorded, in correlation with literature data (Andarwulan et al., 2010; Velioglu et al., 1998).

It was revealed that the highest value (81.02%) of DPPH inhibition was found in the same UF2 concentrated extract.

3.2. HPLC analysis of obtained extracts

The optimum analysis conditions were established using the standards specific to the compounds of interest.

The samples of plant extracts were analyzed by the interpolation of the areas obtained for each compound. The results were expressed in μg/g dry matter and presented in Table 3.

The main polyphenols identified in the analyzed extracts were gallic and elagic acids – the highest amounts, while rutin and luteolin – lower amounts; after the extract concentration, the gallic and elagic acid amounts were preponderant in the UF2 concentrate.

3.3. Testing the extracts' cytostatic activity

The number of viable cells was calculated by reference to control (cells cultivated in the absence of extracts) considered as having 100% viability (Table 4).

It was observed that:

- Cell viability decreased as the extracted dose increased and it at the same time decreased with the exposure duration (preponderantly from the second concentration – 500 μg/mL).
- P2 and P3 concentrated samples similarly affected the viability of Hep-2 cell cultures, having the strongest effect.

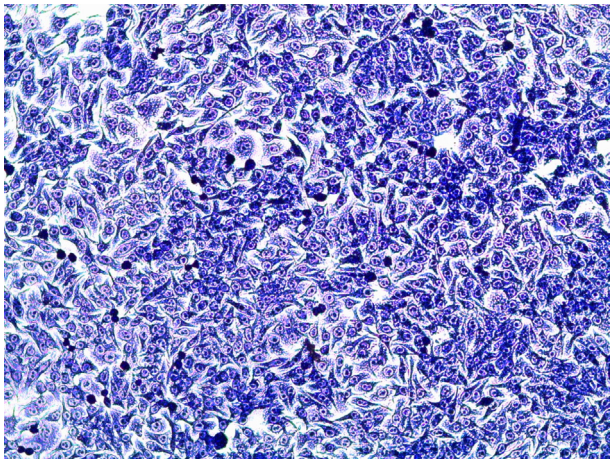

Figure 2 Control cells – 24 h.

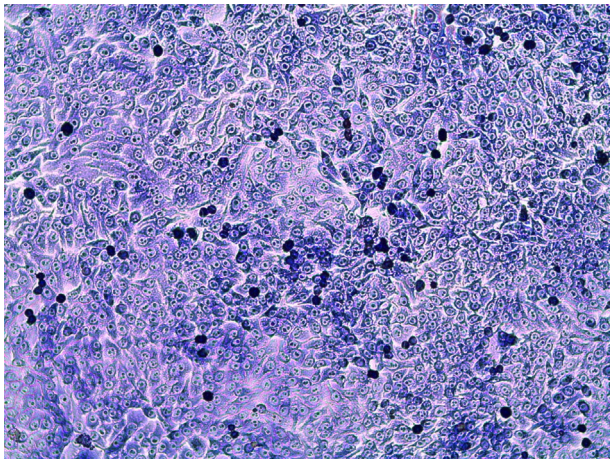

Figure 3 Control cells – 48 h.

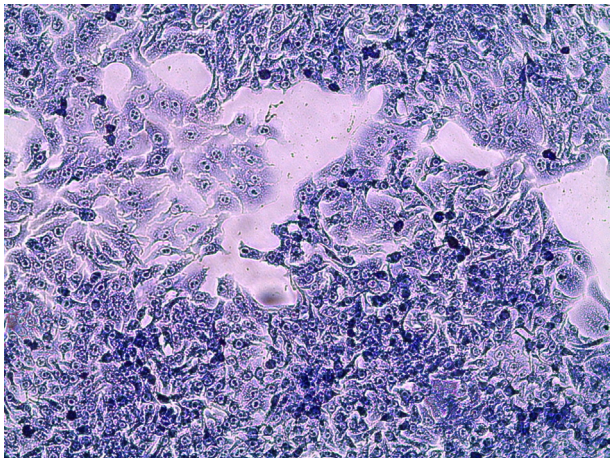

Figure 4 P1–500 μg – 48 h.

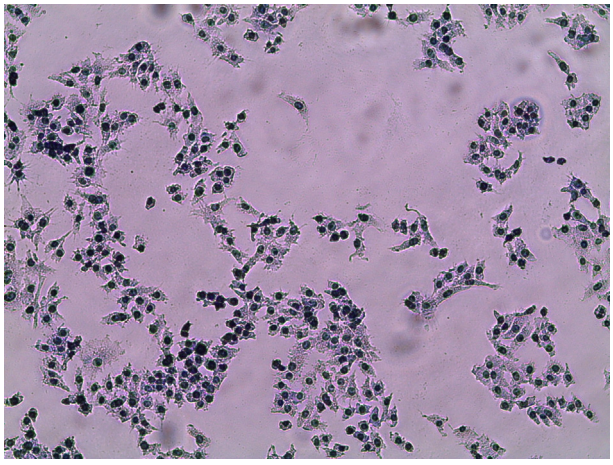

Figure 5 P1–1000 μg – 48 h.

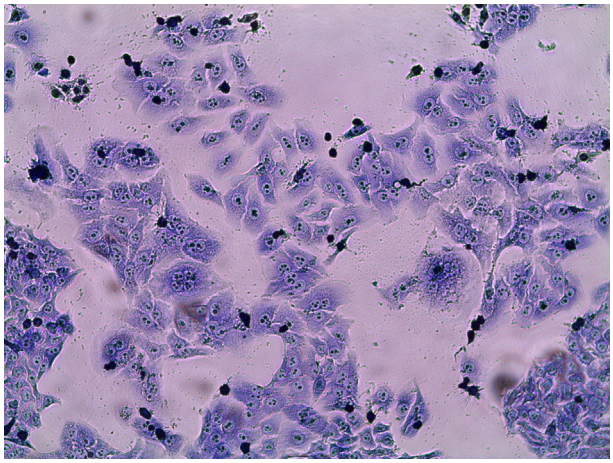

Figure 6 P2–500 μg – 48 h.

In order to exemplify the geranium extracts' cytotoxic effect upon cell cultures we present also some microscopic images of control and treated (500 and 1000 μg/mL, 48 h, P1–P4 Geranium extracts) cell cultures (Figs. 2–11).

The untreated cells revealed morphology typical of a culture of tumoral cells: they were polygonal or fusiform, with big nuclei,

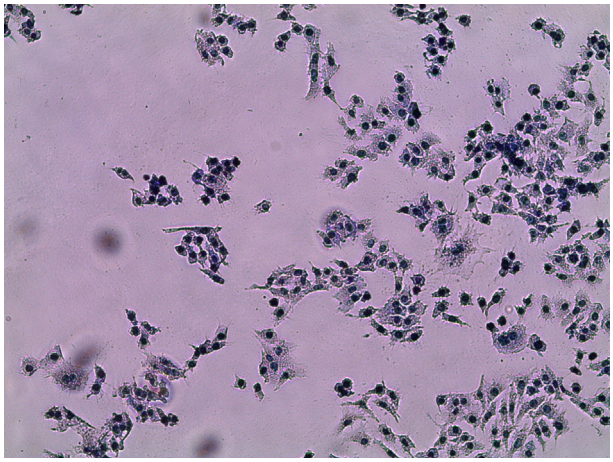

Figure 7 P2–1000 μg – 48 h.

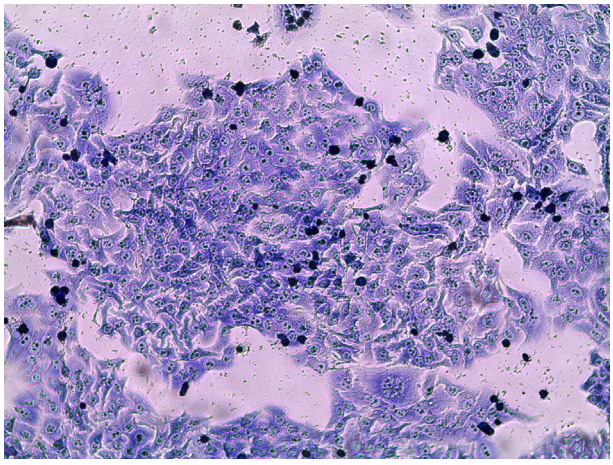

Figure 8 P3–500 μg – 48 h.

covered by 1–3 nucleoli, and had fine cytoplasm. Here and there some dividing cells were observed. The culture was dense; the cells covered more than 95% of the well surface (Figs. 2 and 3).

The treated cultures, starting with the 500 μg/mL dose, showed a substantially modified morphology, the cells were smaller, the nuclei were condensed, the cytoplasm was granular and here and there vacuoles were observed. The cells were rare, covering about 40% of the well surface. The dividing cells were not observed (Figs. 4–11).

4. Conclusions

- In the present work, purified and concentrated *G. robertianum* extracts were obtained and analyzed from the point of view of polyphenolic compounds;
- The antioxidant capacity of the studied extracts showed the highest TEAC values in case of extracts concentrated especially through the ultrafiltration membrane with 1000 Da cut-off (UF2). The same extract had the highest percentage of DPPH inhibition;
- The complex *in vitro* evaluation of the cytostatic activity revealed the significant potential of *G. robertianum* concentrated extracts;

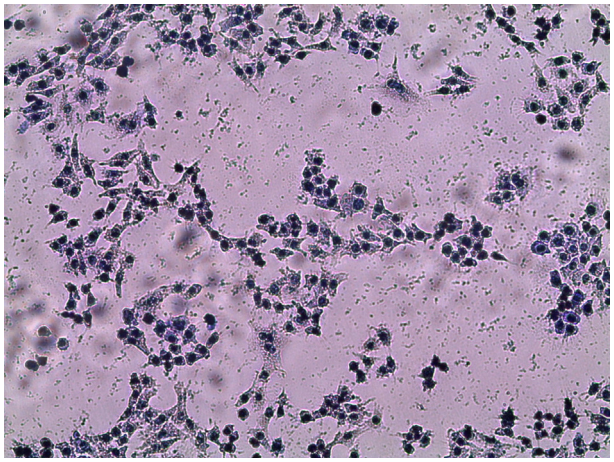

Figure 9 P3–1000 μg – 48 h.

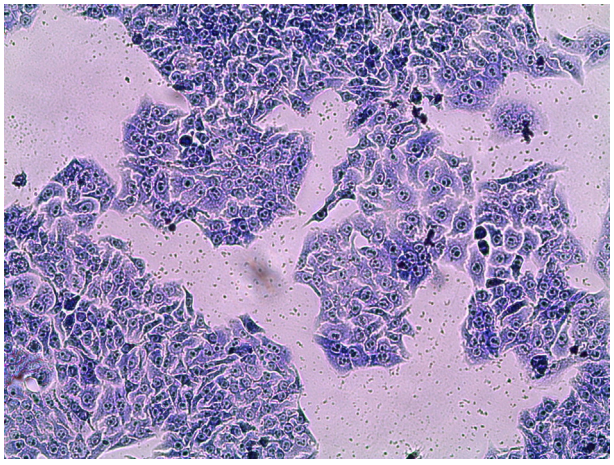

Figure 10 P4–500 μg – 48 h.

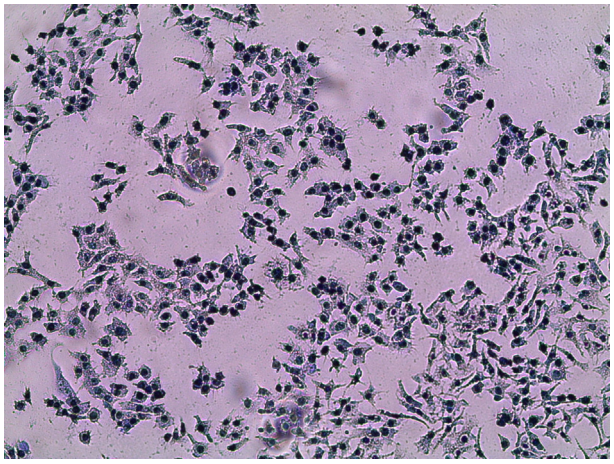

Figure 11 P4–1000 μg – 48 h.

- The purified and concentrated *G. robertianum* extracts, due to their antioxidant and cytostatic properties could be considered potential chemotherapeutic agents.

References

Andarwulan, N., Batari, R., Sandrasari, D.A., Bolling, B., Wijaya, H., 2010. Flavonoid content and antioxidant activity of vegetables from Indonesia. Food Chem. 121, 1231–1235.

Bondet, V., Brand-Williams, W., Berset, C., 1997. Lebensm. Wiss. U. Technol. 30, 609.

Camacho-Luis, A., Gayosso-De-Lucio, J.A., Torres-Valencia Muñoz-Sánchez, J.L., Alarcón-Hernández, E., López, R., Barrón, B.L., 2008. Antioxidant constituents of geranium bellum rose. J. Mex. Chem. Soc. 52 (2), 103–107.

Folin, O., Ciocalteu, V., 1927. J. Biol. Chem. 27, 627–650.

Hamburger, M., Hostetmann, K., 1991. Bioactivity in plants: the link between phytochemistry and medicine. Phytochemistry 30, 3864–3884.

Harborne, J.B., 1988. Flavonoids in the environment: structure activity relationships. Prog. Clin. Biol. Res. 280, 17–27.

Litescu, S., Radu, G.L., 2000. Eur. Food Res. Technol. Part A 211, 218.

Martinez, S., Gonzalez, J., Culebras, J., Munon, M., 2002. Los flavonoides: propiedades y acciones antioxidants. Nutr. Hosp. 17, 271–278.

Matsuda, H., Morikawa, T., Ando, S., Toguchida, I., Yoshikawa, M., 2003. Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. Bioorgan. Med. Chem. 11, 1995–2000.

Miliauskas, G., Van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H., De Waard, P., 2004a. Antioxidative activity of Geranium macrorrhizum. Eur. Food Res. Technol. 218, 253–261.

Miliauskas, G., Van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H., De Waard, P., Sudholter, E.J.R., 2004b. Antioxidant activity of Potentilla fruticosa. J. Sci. Food Agric. 84, 1997–2009.

Neagu, E., Paun, G. Roman, Radu, G.L., Nechifor, Gh., 2010. Concentration of the bioactive principles in *Geranium robertianum* extracts through membranare procedures (ultrafiltration). Roum. Biotechnol. Lett. 15 (1), 5042–5048.

Neagu, E., Paun, G. Roman, Radu, G.L., 2011. Antioxidant capacity of some *Salvia officinalis* concentrated extracts. Rev. Roum. Chem. 56 (7), 777–782.

Papuc, C., Diaconescu, C., Nicorescu, V., Crivineanu, C., 2007. Antioxidant properties of aromatic plant alcoholic extracts. Roum. Biotechnol. Lett. 12 (6), 3533–3537.

Papuc, C., Nicorescu, V., Crivineanu, D.C., Goran, G., 2009. Phytochemical constituents and free radicals scavenging activity of extracts from sea buckthorn fruits (Hippophae rhamnoides). Acta Hort. 806 (1), 187–192.

Paun, G., Neagu, E., Tache, A., Radu, G.L., Parvulescu, V., 2011. Application of the nanofiltration process for concentration of polyphenolic compounds from *Geranium robertainum* and *Salvia officinalis* extracts. Chem. Biochem. Eng. Q. 25 (4), 453–460.

Paun-Roman, G., Neagu, E., Radu, G.L., 2009. Antiradical activities of Salvia officinalis and Viscum album L. extracts concentrated by ultrafiltration process. Acta Sci. Pol. Technol. Aliment 8 (3), 47–58.

Pellegrinaa, C.D., Padovanib, G., Mainentea, F., Zoccatellia, G., Bissolia, G., Mosconia, S., Veneria, G., Peruffoa, A., Andrighettob, G., Rizzia, C., Chignolaa, R., 2005. Anti-tumour potential of a gallic acid-containing phenolic fraction from Oenothera biennis. Cancer Lett. 226, 17–25.

Rice-Evans, C., Miller, N.J., 1994. Meth. Enzymol. 234, 279.

Saija, A., Scalase, M., Lanza, M., Marzulla, D., Bonina, F., Castelli, F., 1995. Free Radical Biol. Med. 19, 481–486.

Salucci, M., Stivala, L.A., Maiani, G., Bugianesi, R., Vannini, V., 2002. Flavonoids uptake and their effect on cell cycle of human

colon adenocarcinoma cells ($CaCo_2$). Br. J. Cancer 86, 1645–1651.

Serrano, A.C., Palacios, G., Cespon, C., Villar, M.L., Nocito, M., Gonzales-Porque, P., 1998. Derivatives of gallic acid induce apoptosis in tumoural cell lines and inhibit lymphocyte proliferation. Arch. Biochem. Biophys. 350, 49–54.

Shi, D., Kong, Y., Yu, J., Wang, Y., Yang, J., 2006. Separation performance of polyimide nanofiltration membranes for concentrating spiramycin extract. Desalination 191, 309–317.

Velioglu, Y.S., Mazza, G., Gao, L., Oomah, B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. 46, 4113–4117.

Weisburger, J.H., Hara, Y., Solan, L., Luo, F.Q., Pittman, B., Zang, E., 1996. Tea polyphenols as inhibitors of mutagenicity of major classes of carcinogens. Mutat. Res. 371, 57–63.

Yang, L.L., Lee, C.Y., Yen, K.Y., 2000. Induction of apoptosis by hydrolysable tannins from *Eugenia jambos* L. on human leukaemia cells. Cancer Lett. 157, 65–75.