Investigation of Pro-apoptotic Activity of *Equisetum arvense* L. Water Extract on Human Leukemia U 937 Cells

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Abstract

It is now widely accepted a role for apoptosis in tumorigenesis. An effective compound which can kill tumor via apoptotic pathway appears to be an appealing strategy to suppress various human tumors. We are showing in the present study that the water extract from sterile stems of Equisetum arvense L. has a dose dependent cytotoxic effects on human leukemic U 937 cells. DNA fragmentation, externalisation of phosphatidilserine, the colapse of mithocondrial transmembrane potential, were all observed in cells cultured for 48 h with the herb extract. Taken together these results suggest that the cytotoxicity of Equisetum arvense L. water extract against U 937 cells is due to apoptosis.

Keywords: Equisetum arvense, water extract, cytotoxicity, apoptosis, cell cycle

Introduction

Apoptosis or programmed cell death is a highly organized physiological process to eliminate damaged or abnormal cells. Also it plays a major role in embryogenesis where apparently normal cell undergo apoptosis. It also is involved in maintaining homeostasis in multicellular organisms. The apoptotic cell destroys itself in a manner which will neither harm neighboring cells nor induce an inflammatory response [1]. This physiological process is distinct from necrotic cell death, which occurs as a result of severe cell injury and results in swelling and lysis.

Many works have clearly established that apoptosis could be involved in hyperplasia, neoplastic transformation, tumor expansion, metastasis and regresion [2]. The accepted modality for cancer treatment involves surgery, radiation and drugs, singly or in combination. A succesful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. One potential source of apoptosis-inducing compounds is the divers area of natural products. It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. Evidence has emerged from various studies that suggest that products derived from plants are useful in the treatment as well as in the prevention of cancer.

In our search for apoptosis-inducing constituents from folk medicinal plants, the water extract of *Equisetum arvense* L.(WEE), which has been used as traditional medicine for curing many health problems in Romania, was found to induce in human leukemia U937 cells changes characteristic of apoptosis.

Materials and Methods

Plant material and extraction

Non cultivate *Equisetum arvense L. (*horsetail) was collected from Neamt County, located at the North of Romania. Collection was made near Piatra Neamt city. The sample was identified and authenticated by Prof. Dr. Nicolae Stefan from the Botany Departement, Faculty of Biology, Alexandru Ioan Cuza University, Iasi**.** The voucher specimen was deposited at "Stejarul" Research Center, Piatra-Neamt, Romania.

For the preparation of the water extract a traditional method was used. Dried sterile stems of horsetail were cut in small pieces, suspended and extracted in the ratio 1:20 with distilled water for eight hours at room temperature. For in vitro assays the extract was clarified and sterilised by filtration through Millipore syringe

filters with 0.45 and 0.22 μm porosity. In the final extract the dry matter was 5mg /ml. The extract was stored at 4˚C for maximum 24 h. A fresh sterile solution was used for each experiment.

Cell culture conditions and treatment

The human leukemia cell line U 937 (human leukemia cell line, was obtained from the University of Pittsburg Cancer Institute) was cultured in RPMI 1640 medium (Sigma Aldrich, St.Louis, Missouri, USA) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100μg/ml streptomycin, 2 mM L-glutamine and incubated at 37˚C in a humidified atmosphere containing 6% CO_2. Cells were treated for fourty eight hours with different doses (0-496 μg dry matter/ml) of herb extract. As control were used cells cultured in the same conditions but in the absence of extract. The total number of cells and viability, as judged by trypan blue exclusion, were determined by counting in a hemocitometric chamber.

Flow cytometric analyses for apoptosis:

Cell Cycle Determination

The cells were harvested and washed in PBS. The pellet was fixed in cold 70% ethanol at 4˚C overnight, washed in phosphate-citrate buffer (0.2M HPO_4, 0.1M citric acid pH 7.8) and after centrifugation the cell pellet was resuspended in 1ml DNA staining solution (200μg propidium iodide plus 2mg RNase A/10 ml PBS) (Boehringer Corp., Manheim, Germany) and incubated for 30 min in the dark at room temperature. Samples were analysed by a FACScan flow-cytometer (Becton Dickinson, Franklin Lakes, NJ. USA) [3], investigating 40,000 gated events with CellFIT program (RFIT model).

Detection of cells with fractional (sub G_1) DNA content using propidium iodide

The cells were washed in PBS and centrifuged at 200g for 5min. The pellet was resuspended in 1.5 ml fluorochrome solution (propidium iodide 50mg/l in 0.1% sodium citrate plus 0.1% Triton X-100), incubated for 60min in the dark at 4˚C prior to analysis by flow cytometry..

Detection of early and late apoptotic cells after staining with Annexin V-FITC and propidium iodide (PI)

It was shown [4] that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose phosphatidylserine (PS), upon translocation to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. The exposure of PS is detected with the protein Annexin V which preferentially binds to negativelly charged phospholipids like PS in the presence of Ca^{++}. By conjugating FITC to Annexin V it is possible to identify and quantitate apoptotic cells on a single cell basis by flow cytometry [5,6,7]. For detection of PS externalisation U 937 cells were washed 2x with PBS and treated first with FITC-AnnexinV (40 μg/ml) (Sigma Aldrich, St.Louis, Missouri, USA) in binding buffer (HEPES buffered saline solution supplemented with 25 mM $CaCl_2$), incubated in the dark for 10 min at RT, then was added PI solution (50 μg/ml) prior to analysis by FACS. Simultaneous staining of cells with FITC-Annexin V (green fluorescence) and with the non-vital dye propidium iodide (red fluorescence) allowed the discrimination of intact cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$) and late apoptotic cells ($FITC^+PI^+$).

Detection of the collapse of mitochondrial electrochemical potential using the aggregate dye, JC-1

The loss of mitochondrial membrane potential ($\Delta\varphi_m$) is a hallmark for apoptosis. Changes in mitochondria membrane potential was assesed using JC-1(5,5',6,6'–tetrachloro–1,1',3,3'–tetraethylbezimidazolcarbocyanineiodide) (Molecular Probes, Inc. Eugene, Oregon, USA), a lipophiilic cation that accumulates and retained in mitochondria [8,9].

U 937 cells were treated with 2.5μg/ml JC-1, kept in the dark at RT for 15-20 min, washed twice with PBS and anlysed immediately with the flow cytometer.

Statistical Analysis

Data are expressed as mean values (± SD) of at least three observations. Means and standard deviations were calculated with a software of Excel for Windows.

Results and Discussion

Effect of E.arvense extract on cell cycle distribution and growth rate proliferation

To find out whether *E.arvense* extract posseses growth-arresting features, cells were harvested and sujected to cell cycle analysis after 48h of incubation with or without herb extract. The results are shown in **Table 1** and indicate the percentage of cells in the specific phases of the cell cycle. These show that the low dose

(124 µg/ml) WEE induces the accumulation of cells in the G_0-G_1 phase and a decrease of the cell population of cells in phase S as compared to control values, indicating the arrest of the cycle in G_1, probably due to inactivation of G_1 checkpoint.

Table 1. Cell cycle distribution of U 937 cells

Extract concentration µg dry matter/ml	G_0 - G_1	S - phase	G_2 - M
0	38.2	47.6	14.2
124	44.4	41.2	14.4
248	43.3	51,1	5.6
496	40.0	50.0	4.6

Cells were seeded in culture media at 2 x 10^5cells/ml and incubated at 37˚C with and without herb extracts. On 48h cells were explored for cell cycle distribution by FACS analysis. Values show percentage of viable cells (cells with sub-G_1 DNA content are excluded). One representative experiment of three is shown.

At higher doses (248 and 496µg/ml) the results are shown that there appears an increase of cells population in phase S and a decrease of cell population in phase G_2-M as compared to control, indicating the arrest of cell cycle due to the inactivation of G_2 checkpoint.

Because WEE has been shown in the first experiment to posses growth arresting features, we asked whether the treated cells show a decrease proliferation rate. Treated and untreated cells after 48h of incubation were counted, and viability assessed by trypan blue exclusion. A differentiation in proliferation rate was seen between control and the treated cells, indicating that the herb extract influences the proliferation rate of the cells (**Figure 1**). The increased concentration of the extract decreased the growth rate of the cells in a concentration dependent manner.

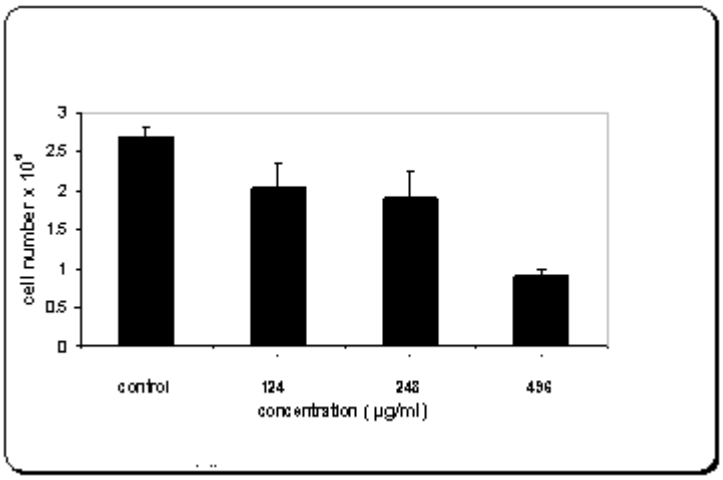

Figure 1. The effects of different doses of WEE on growth rate of U 937 cells.

U 937 cells at an initial concentration of 0.2 x 10^6 cells/ml were incubated at 37˚C with and without herb extract. The total number of cells and viability, as judged by trypan blue exclusion was determined after 48h. The bars in the figure indicate the means ± SD (n = 3).

Effect of E.arvense extract on the viability of U 937cells

The WEE showed a notable cytotoxicity against human leukemia U 937 cells. WEE-mediated cytotoxicity was increased in a dose-dependent manner (**Figure 2**). However, whether the cytotoxicity of this extract is due to apoptosis or necrosis has not been proven, and hence remains to be established.

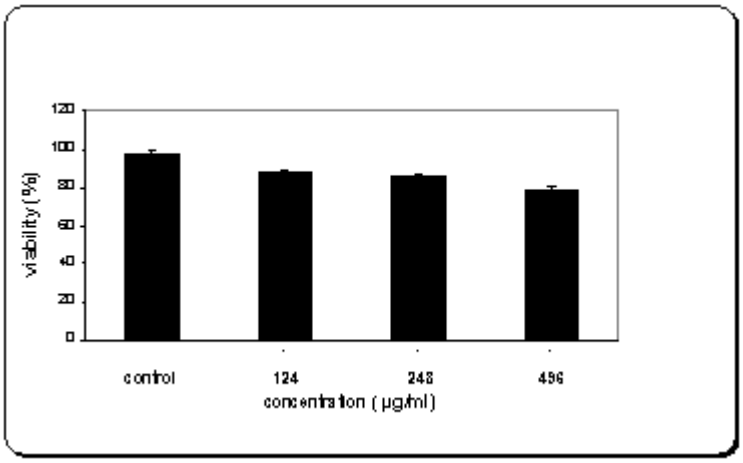

Figure 2. The effects of WEE on viability of U 937 cells. Cells were seeded in culture media at an initial concentration of 0,2 x 10^6 cells/ml and incubated with and without herb extracts at 37˚C. Viability as juged by trypan blue exclusion was determined after 48h. Data are presented as the means ± S.D. (n = 3)

Induction of apoptosis in U 937 cells

To determine whether the cell death was attributable to apoptosis, the cells were analysed for expression of Annexin V by FACS analysis concomitently with propidium iodide staining (PI) which make it possible to exclude necrotic cells, providing a selective method for detecting apoptosis. The cells become reactive with Annexin V prior to their loss of plasma membrane ability to exclude cationic dyes such PI. The results indicated that the low concentrations (124 and 248µg/ml) do not influence the apoptotic process while the highest concentration (496µg/ml) induced early and late apoptosis as compared to the control. As shown in **Figure 3**, WEE induced apoptosis of U 937 cells in a dose-dependent manner.

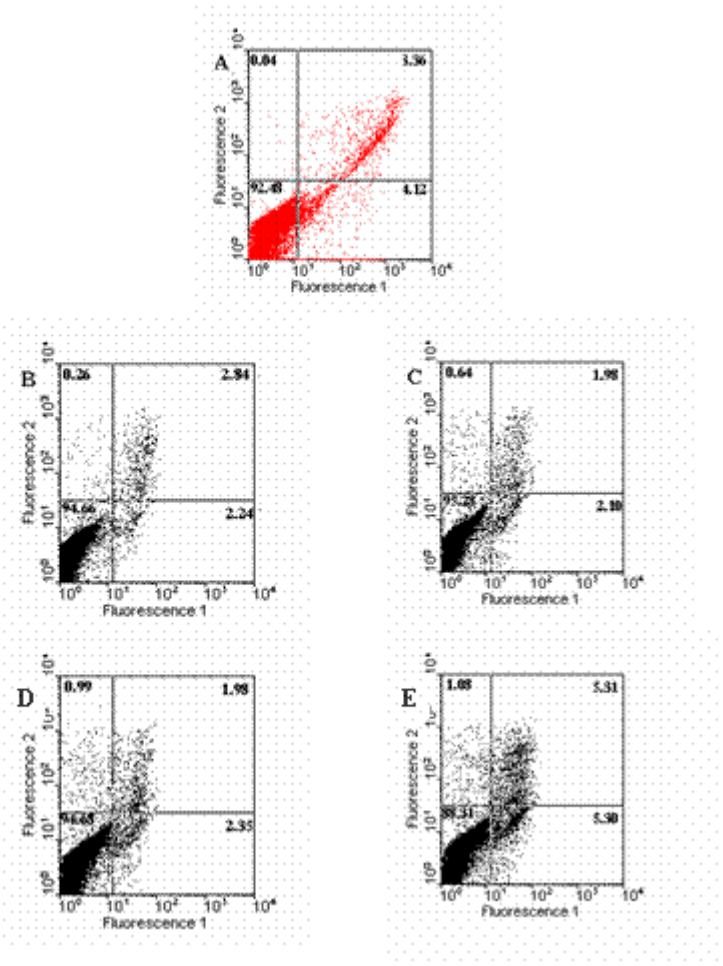

Figure 3. Expression of the apoptosis-related cell surface antigen Annexin V in U 937 cells. Cells were seeded in cultured medium at an initial concentration of 2x10^5 cells/ml and incubated without and with different doses of herb extracts at 37˚ C. After 48h, cells were subjected to analysis of Annexin V by flow cytometry. **A** - untreated control cells, **B, C, D, E** – treated cells with 124, 248, 496 µg/ml WEE. Live cells (intact cells) are confined to the lower left quadrant. Early apoptotic cells appear in the lower right

quadrant due to the externalization of PS. Late apoptotic cells appear in the upper right quadrant as a result of loss of cell membrane integrity. One representative experiment of three is shown.

To confirm the above findings we next conducted a DNA-fragmentation assay based on the detection of cells with fractional (sub G_1) DNA content using PI. **Table 2** shows the dose dependency of WEE in inducing chromatin fragmentation of U 937 cells, suggesting the involvement of apoptosis in WEE-induced cell death. The appearance of sub diploid DNA peak is a specific marker of apoptosis; necrosis induced by metabolic poisons or lysis produced by complement did not induce any G1 peak in the DNA fluorescence histogram [3].

Table 2. Percentage of U 937 cells present in the sub-G1 phase of the cell cycle after treatment with different doses of WEE.

Extract concentration µg dry matter/ml	Percentage of cells in sub-G1 phase ± S.D. (n = 3)
0	40 ± 4.3
124	50 ± 1.4
248	55 ± 1.5
496	60 ±1.2

Cells were seeded at 2 x 10^5 cells/ml in culture medium and incubated at 37˚C with and without herb extracts. After 48h cells were subjected to cell cycle analysis by flow cytometry as described in "Materials and Methods". Mean values from three experiments are shown.

In many systems, apoptosis is associated with the loss of mitochondrial inner membrane potential, which may be regarded as a limiting factor in the apoptotic pathway [10]. To observe the change in $\Delta\varphi_m$ in cells exposed to herb extract, JC-1(5,5',6,6'–tetrachloro–1,1',3,3'–tetraethylbezimidazolcarbocyanineiodide), a mitochondria – specific and voltage- dependent dye, was employed. Its uptake by charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence from green, which is caracteristic of its monomeric form, to orange, which reflects its aggregation in mitochondria [11]. In non-apoptotic cells JC-1 accumulates as aggregates in mitochondria, resulting orange fluorescence. The brightness of orange fluorescence is proportional to $\Delta\varphi_m$. In apoptotic and necrotic cells, JC-1 exists in monomeric form and stains cells green [12]. The results showed that the lower dose of the extract (124 µg/ml) did not enhance the loss of mitochondrial membrane potential ($\Delta\varphi_m$). The higher doses (248 and 486 µg/ml) showed signs of enhancing the loss of mitochondrial membrane potential (Δφm), especially the higest concentration (486 µg/ml), as compared to control. **Figure 4** shows the dose dependency of WEE in inducing the collapse of mitochondrial transmembrane potential, suggesting the involvement of apoptosis in WEE-inducing cell death.

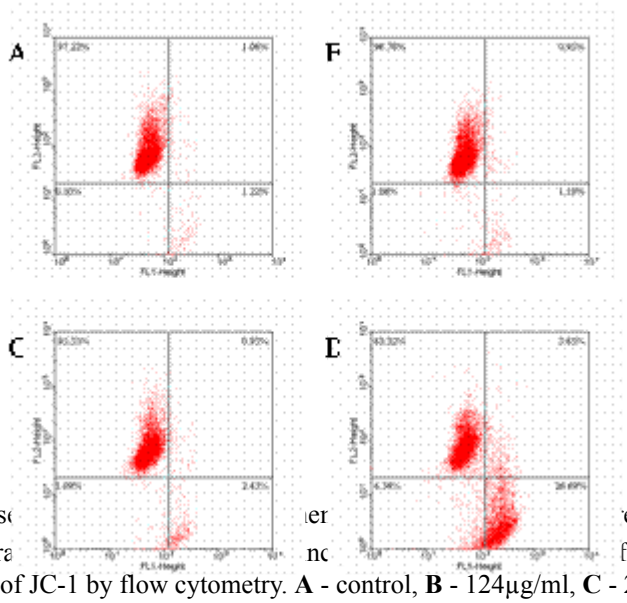

Figure 4. Detection of the collapse of mitochondrial membrane potential by the aggregate dye, JC-1. Cells were seeded in culture medium at an initial concentration of 2 x 10^5 cells/ml and incubated with different doses of WEE at 37˚C. After 48h, cells were subjected to analysis of JC-1 by flow cytometry. **A** - control, **B** - 124µg/ml, **C** - 248µg/ml, **D** - 486µg/ml. The green emission 530 nm (monomeric form JC -1, apoptotic or necrotic cells) was analysed in fluorescence Channel 1 (FL1) and orange emission 590 nm (J aggregate, nonapoptotic cells) in Channel 2 (FL2).

Modulating apoptosis may be useful in manegement of cancer prevention or therapy. The apoptotic death involves an active participation of the affected cells in their self destruction cascades that culminate in DNA degradation via endonuclease activation, nuclear desintegration, and the formation of "apoptotic bodies" which

involve the cell remnants [13]. These apoptotic bodies are rapidly cleaned from the local tissue by macrophage [14,15]. Several anti-tumor agents including ionizing radiation [16], alkylating agents such as cisplatin [17], topoisomerase inhibitor etoposide [18], cytokine tumor necrosis factor (TNF)[19], taxol [20], metoclopramide an 3- chloroprocainamide [21], hypertermia, UV radiation [22] have been reported to induce apoptosis. It is possible to postulate that naturally occuring compounds, which are specially used as a folk medicine for curing cancer, may regulate apoptosis in cancer cells. However, little is known about the regulation of apoptosis in cancer cells by natural compounds.

E.arvense is a herbaceous non flowering plant distributed throughout the temperate climate of Northern Hemisphere including Asia, North America, and Europe (23). In Europe its therapeutic uses date back to the Ancient Romans and Greeks.

E.arvense is a unique plant with two distinctive type stems. One variety of stem (fertile) grows early in spring, reaching a height of 4-7 inches, and bearing on top a cornelike spike which contains spores. The mature stem (sterile) of the herb, appears in summer and grows up to 18 inches high and features whorls of small branches.

The sterile green stems fresh or dried are used as folk remedy in Romania for curing many and diverse health problems: cardiac, kidney, bladder and skin trouble, arthritis, bleeding ulcers, tuberculosis and some forms of cancer [24,25,26].

In recent years some works demonstrated the antioxidative [27,28], antinociceptive and anti-inflammatory [29], hepatoprotective [30], antimicrobial [31], platelet anti-aggregant [32], sedative and anticonvulsant [33] activities of horsetail which justifies partialy its medicinal use. The aerial parts of this herb, are rich in silicic acid and silicates, which provide approximately 2-3% elemental silicon. Potassium, aluminium, and manganase, along with fifteen different types of flavonoids, are also found in this herb. However, there is no experimental evidence of the anti cancer action of its compounds or of an extract obtained from the stems. Therfore, in the present study, we attempt to investigate the anti-cancer activity of *E.arvense.*

Using the assay of plasma membrane integrity (exclusion of PI) and Annexin V binding, DNA fragmentation assay, the analysis of the dissipation of mitochondrial transmembrane potential and the analysis of the cell cycle in U 937 cells treated with WEE, we have provided experimental evidence that this herb shows an anti-cancer activity against leukemia via apoptosis.

References

1. J.F.R. KERR, A.H. WYLLIE, A.R. CURRIE, Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics, *Br. J.Cancer,* **26**, 239-257, (1972).
2. B. GRASL-KRAUP, W. ROSSMANITH, B. RUTTKAY-NEDECKY, L. MULLAUER, B. KAMMERER, W. BURSCH, R.SCHULTE-HERMANN, Levels of transforming growth factor beta and transforming growth factor beta receptors in rat liver during growth, regression by apoptosis and neoplasia, *Hepatology*, **28**, 717-726, (1998).
3. I. NICOLETTI, G. MIGLIORATI, M.C PAGLIACCI, F. GRIGNANI, C. RICCARDI, A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry, J. *Immunol. Methods,***139**, 271-279, (1991).
4. V. FADOK, D.L. BRATTON, D.M. ROSE, A. PEARSON, R.A EZEKKEWITZ, P.M. HENSON, A receptor for phosphatidylserine-specific clearance of apoptotic cells, *Nature*, **105**, 85-90,(2000).
5. I. VERMES, C. HAANEN, H. STEFFENS-NAKKEN, C.P. REUTELINGSPERGER, A novel assay for apoptosis – flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, *J. Immunol.*, **184**, 39-51, (1995).
6. G. KOOPMAN, C.P. REUTELINGSPERGER, G.A.M. KUIJTEN, R.M.J. KEEHNEN, S.T. PALS, M.H.J. VAN OERS, Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, *Blood*, **84***,*1415-1420, (1994).
7. C.H. HOMBURG, M. DE HAAS, A.E. VON DEM BORNE, A.J. VERHOEVEN, C.P. REUTELINSPERGER, D. ROOS, Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro , *Blood*, **85**, 532-540, (1995).
8. A. COSSARIZZA, M. BACCARANI CONTRI, G. KALASHNICOVA, C. FRANCESCHI, A new method for the cytofluorimetric analysis of mitochondrial membrane potential using lipofilic cation

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), *Biochem. Biophys. Res. Commun.*, **197**, 40-45, (1993).

9. A. COSSARIZZA, D. CECCARELLI, A. MASINI, Functional heterogeneity of isolated mitochondrial population revealed by cytofluorimetric analysis at the single organelle level, *Exp.Cell Res.*, **222**, 84-94 (1996).
10. E. BOSSY-WETZEL, D.R. GREEN, Apoptosis: checkpoint at the mitochondrial frontier, *Mut. Res.*, **434**, 243-251, (1999).
11. A. COSSARIZZA, S. SALVIOLI, Analysis of mitochondria during cell death. In Z.Darzynkiewicz., H.A.Crissman, J.P. Robinson,eds, *Methods in Cell Biology*, "Cytometry, Part A". Academic Press, San Diego (CA), 2001, pp. 467-486.
12. M. REERS, T. W. SMITH, L.B. CHEN, J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential, *Biochemistry*, **30**, 4480-4486, (1991).
13. C.J. SUNDERSON, *Mechanisms of Cell mediated Cytotoxicity*, Plenum, New York, 1982, pp. 3-21.
14. J. SAVILL, V. FADOK, Corpse clearance defines the meaning of cell death, *Nature*, **407**, 784-788, (2000).
15. E. DUVALL, A.H. WYLLIE, R.G. MORRIS, Macrophage recognition of cells undergoing cell death (apoptosis*)*, *Immunology*, **56**, 351-358, (1985).
16. A.Y. LEUNG, S. FOSTER, *Encyclopedia of Common Natural Ingredients Used in Food*, *Drugs, and Cosmetics*, 2nd ed., John Wiley& Sons, New York, 1996, pp. 306-308.
17. I. R. RADFORD, T. K. MURPHY, J. M. RADLEY, S.L. ELLIS, Radiation response of mouse lymphoid and myeloid cell lines. Part III. Different signals can lead to apoptosis and may influence sensitivity to killing by DNA double-strand breakage, *Int. J. Radiat. Biol.*, **65**, 217–227, (1994).
18. A.V. D'AMICO, W. G. MCKENNA, Apoptosis and a re-investigation of biologic basis for cancer therapy, *Radiother. Oncol.*, **33**, 3–10, (1994).
19. S. H. KAUFMAN, S. DESNOYERS, Y. OTTAVIANO, N.E. DAVIDSON, G.G. POIRIER, Specific proteolytic cleavage of poly (ADP-ribose) polymerase an early marker of chemotherapy-induced apoptosis, *Cancer Res.*, **53**, 3976–3985, (1993).
20. S.C. SHIH, O. STUTMAN, Cell cycle-dependent tumor necrosis factor apoptosis**,** *Cancer Res.*, **56**, 1591–1598, (1996).
21. R. K. GIBB, D.D. TAYLOR, T. WAN, D.M. O'CONNOR, D.L. DOERING, C. GERCEL-TAYLOR, Apoptosis as a measure of chemosensitivity to cisplatin and taxol in ovarian cancer cell lines, *Gynecol. Oncol.*, **65**, 13–22, (1997).
22. R.W. PERO, A. OLSSON, M. SIMANAITIS, A. AMIRI, I. ANDERSON, Pharmacokinetics, toxicity, side-effects, receptor affinities and in vitro radiosensitizing effect of the novel metoclopramide formulations, sensamide and new sensamide, *Pharm. Toxicol.*, **80**, 231–239, (1997).
23. M. FUJITA**,** T. TSUCHIDA, K. HIGASHINO, Effect of vesnarinone in combination with anti-cancer drugs on lung cancer cell lines, *Anticancer Drugs*, **10**, 119-127, (1999).
24. F. CRACIUN, O. BOJOR, M. ALEXAN, *Farmacia naturii*, Ceres, Bucuresti, 1976, pp. 53-55.
25. M. ALEXAN, O. BOJOR, F. CRACIUN, *Flora medicinala a Romaniei*, Ceres, Bucuresti, (1992).
26. D. DRAGOS, M.M. BRATU, A. CICORTAS*, Plante de leac pentru tamaduirea sufletului*, Tritonic, Bucuresti, 2002, pp.239-240.
27. G.J. JR DOS SANTOS, H.M.F. DO MONTE, M.M. BLANCO, V.M. LANZIOTTI, D.F. MAIA, K. L. LEAL, Cognitive enhancement in aged rats after chronic administration of *Equisetum arvense* L. with demonstrated antioxidant properties in vitro"*, Pharmacol. Biochem. Behav.*, **81**, 593-600, (2005).
28. T. NAGAI, T. MYODA, T. NAGASHIMA, Antioxidative activities of water extract and ethanol extract from field horsetail (tsukushi) *Equisetum arvense* L., *Food Chemistry*, **91**, 2275-2281, (2005).
29. F.H. DO MONTE, J.G. Jr DOS SANTOS, M. RUSSI, V.M. LANZIOTTI, L.K. LEAL, G.M.CUNHHA, Antinociceptive and anti-inflammatory properties of the hydroalcoholic extract of stems from *Equisetum arvense* L. in mice. *Pharmacol. Res.*, **49**, 239-243, (2004).
30. H.OH, D.H. KIM, J.H. CHO, Y.C. KIM, Hepatoprotective and free radical scavenging activities of phenolic petrosins and flavonoids isolated from *Equisetum arvense*, *J. Ethnopharmacol.*, **95**, 421-424, (2004).
31. N. RADULOVIC, G. STOJANOVIC, R. PALIC, Composition and antimicrobial activity of *Equisetum arvense* L. essential oil, *Phytother Res.*, 20, 85-88, (2006).

32. H. MEKHFI, M.E. HAOURI, A. LEGSSYER, M. BNOUHAM, M. AZIZ, F. ATMANI, A. REMMNAL, A. ZIYYAT, Platelet anti-aggregant property of some Moroccan medicinal plants, *J. Ethnopharmacol.*, **94**, 317-322, (2004).
33. J.G. JR. DOS SANTOS, M.M. BLANCO, F.H. DO MONTE, M. RUSSI, V.M. LANZIOTTI, L.K. LEAL, G.M. CUNHA, Sedative and anticonvulsant effects of hydroalcoholic extract of *Equisetum arvense*, *Fitoterapia*, **76**, 508-513, (2005).