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**ORIGINAL PAPER**

# Advanced methods for antitumoral screening applied on pharmaceutical product development

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**Abstract**

Although the advances in the forms of treatment have allowed small benefits regarding survival, results are still far from being optimal and cancer remains as a significant health problem of the world. The discovery and screening of components that are capable of regulating cell proliferation, cell cycle progression, and/or apoptosis from a dietary or/and natural source is an important aspect of developing new agents that are used in cancer prevention and treatment that possess low toxicity or side effects.

The aim of this paper is to present **one of the most important steps in pharmaceutical product development, the "in vitro" screening on specific tumor cell lines** (prostate, cervix and breast), in order to set the cellular target action of active principles. In respect with the increased demand for new drugs that could raise the efficacy of tumor treatment, we applied our experimental model on the development of therapeutic solutions based on naturally occurring substances: herbal extracts having chemical configuration similar with steroid hormones and entomological preparation containing a mucopolisaccharidic complex.

The screening was performed on human tumours standardized cell line with epithelial morphology: HeLa (cervix adenocarcinoma - ATCC nr. CCL-2), Hep-2 (Human Negroid Cervix carcinoma, HeLa derivative - ECACC nr. 86030501), MCF – 7 (breast adenocarcinoma - ATCC nr. HTB-22) and DU 145 ( human prostate carcinoma - ATCC - HTB-81) and explore the natural complexes effects on apoptosis, DNA synthesis and cell proliferation.

The method of analyse is flow cytometry, based on different fluorescent staining of phosphatidilserine externalisation and nuclear DNA (for early and late events of apoptosis detection), PE for cell cycle sequentiation and CFSE (carboxy fluorescein diacetat succin imidil ester) for cell proliferation.

The antiproliferative and apoptotic screening prove to be a relevant "in vitro" model, a starting point for the drug-design of a potential antitumoral agent. The application we performed (the antitumoral effect evaluation of two types of active compounds from two different natural sources: plant and animal) reveals that this "in vitro"screening is a valuable tool, a promissing checking point for the start of advanced genomic and proteomic investigations of a wide range of natural compounds.

This study demonstrates the potential use of *Hedera helix* compound GSO1 in the prevention or treatment of malignant diseases, especially breast and cervix cancers and the

antitumoral potential for prostate diseases of the mucopolysaccharidic components with entomological origin.

**Key words:** cancer, cell proliferation, cell cycle, apoptosis, HeLa, Hep-2, MCF-7, DU 145.

## Introduction

Although the advances in the forms of treatment have allowed small benefits regarding survival, results are still far from being optimal and cancer remains as a significant health problem of the world. Chemotherapy together with surgery and radiotherapy are still the fundamental pillar of treatment, the majority of cancer patients needing this form of therapy. Even if chemotherapy is individually tailored, side effects can often cause more pain and discomfort than the cancer itself. Drug resistance is one of the main reasons limiting the efficacy of chemotherapy in cancer treatment, while the low selectivity of the active ingredients can cause other important side effects. Prevention has recently been suggested as an attractive approach for cancer, especially exploiting the benefits of natural products, food additives, vitamin and mineral supplements. Thus, the progress of therapeutical approaches is directed to find new chemical agents that could differentiate between normal and cancerous cells, destroying the last ones, but having low toxicity to the first one .

In tumoral biology area, the malign profile is accomplish by a series of disturbancies including the proliferation pattern and apoptosis. DNA changes cause the accumulation of abnormal cells and the progression of malignancy [Zheng X., 2006].

One of" critical events" in cancer development is deregulated cell proliferation, which, together with the compensatory suppression of apoptosis needed to support it, providing a minimal "platform" necessary to support further neoplastic progression [Evan and, 2001; Devarajan et al, 2002]. The role of apoptosis in cancers has been studied in detail, apoptosis being a highly regulated cellular response with crucial checkpoints regulating the fate of cells. These checkpoints are processing centres sensing extracellular signals, amplifying localised signals, integrating information from these cells and directing them towards death cascade [Thompson, 1995]. In response to diverse intracellular damage signals, including those evoked by cancer therapy, the cell's decision to undergo apoptosis have potent and specific therapeutic consequences [Adams and Cory, 2007]. Using apoptosis effectors as targets for new drug development, several new compounds with different chemical entities have been identified from medicinal plants. A wide variety of natural substances have been recognised to induce apoptosis in various tumor cells of human origin [Giridharan et al, 2002].

Cell proliferation is critical to repair processes following damage caused by toxic and pathogenic agents and in carcinogenesis. The control of cell division and proliferation is used as a tool in detecting the proliferative activity of tumors

or inhibitory impacts by drugs, as prognostic markers in neoplasia, and as indicators of carcinogenic potential. Cell proliferation has also been used as a biomarker of toxic exposure.

Cell cycle progression is another important biological event having controlled regulation in normal cells, which almost universally becomes aberrant or deregulated in transformed and neoplastic cells. In this regard, targeting deregulated cell cycle progression and its modulation by various natural and synthetic agents are gaining widespread attention in recent years to control the unchecked growth and proliferation in cancer cells [Singh and Agarwal, 2006]. Although DNA synthesis can occur without cell division, the quantity of DNA being synthesized reflects the rate of proliferation. A cell in its replicative phase determines susceptibility to chemicals that damage DNA. In studies of the cell cycle, the most frequently measured attribute is DNA content, whereby this marker indicates cell maturity in the cycle. Subsequent analysis by flowcytometry yields histograms that will allow the determination of the fraction of cells in the G0/G1 (nonreplicating diploid), S (DNA synthesis) and G2/M (mitosis) phases of the cell cycle.

In order to solve the selectivity and specificity problem for anti-tumor agents, molecularly targeted therapies were developed, drugs were rationally designed or selected to inhibit the activity of specific molecules [Allgayer and Fulda, 2008]. If used in the right patient population - on tumors with specific molecular lesions - these therapies promised significant tumor selectivity. Of course, although targeted therapies are heralded as a considerable advance from standard chemotherapies, they are still affected by resistance and toxicity effects [Lord and Ashworth, 2010]. Cancer cell lines are standardized tools useful for the study of the corresponding pathology and also for developing new therapeutic solutions. There are currently several types of well characterized cell lines that reflect the tumor phenotypes diversity, representing appropriate models for the study of cancer heterogeneity. They kept the basic properties of the expression and secretion of antigens, neuroendocrine features, gene expression and amplification, chemo sensitivity profile to cytotoxic agents. This creates the premises for reproducible “in vitro” studies, reconstructing a largely conditions of "in vivo" disease evolution. As an example, monocultures of MCF-7 cells appear to be well suited for high-throughput testing of pharmacological agents against breast cancer that has advanced beyond the basement membrane, as clinically presenting tumours are likely to be no longer under the paracrine control of the my epithelium [Barbara et al, 2006].

In contrast to combinatorial chemistry, natural product provide a wealth of small molecules with drug-like properties and with incredible structural diversity.

A comparison of published databases of natural products and synthetic chemicals has revealed that almost half of the chemical skeletons found in natural libraries are not present in libraries of synthetic chemicals. This is despite the fact that natural products have been the source of many of the best-known pharmaceuticals currently on the market – for example, aspirin, morphine, quinine, digitoxin and ephedrin. Indeed, approximatively 85% of the curent treatment regimens of 80% of the world's population are based on natural products.

Efforts to exploit natural products in drug discovery have largely been dominated by plants, microbes and marine organisms. However, plants and microorganisms represent less than a quarter of all known species on earth, whereas insects and arthropods represent more than two thirds of all species. The added diversity of insects is clear. Whilst plants have generally been employed because they are more readily available, insects offers a huge resource of potentially useful and chemically diverse compounds. The discovery and screening of components that are capable of regulating cell proliferation, cell cycle progression, and/or apoptosis from a dietary or/and natural source is an important aspect of developing new agents that are used in cancer prevention and treatment that possess low toxicity or side effects. Previous studies reveal the effects of compounds isolated from fruits on cervix carcinoma (Hela) cell viability arrest and apoptosis through a mechanism similar to vitamin C [Zhang et al, 2011]. Vitamin C is involved in normal physiological processes and exerts beneficial effects in combined therapy of certain tumors, and could be a promising additional drug for the treatment of tumors resistant to chemotherapy [Osmak et al, 1997]. Studies on purified phytocompounds (alpha-hederin, chlorophyllin and ascorbic acid) showed that the effective antimutagenic concentrations had no clastogenic or aneugenic effects in human lymphocytes [Amara-Mokrane et al, 1996; Villani et al, 2001].

The antioxidant activities of alpha-hederin and hederasaponin-C from *Hedera helix*, and hederacolchisides-E and -F from *Hedera colchica* were investigated, were proved using different antioxidant tests [Gulcin et al, 2004].

Three saponins isolated from *Hedera helix L. (Araliaceae)*, inhibit tumor cell growth through a caspase-3-mediated apoptosis mechanism [Sparg et al, 2004]. The separate effects of alpha-hederin and thymoquinone, the two principal bioactive constituents of *Nigella sativa*, on four human cancer cell lines [A549 (lung carcinoma), HEp-2 (larynx epidermoid carcinoma), HT-29 (colon adenocarcinoma) and MIA PaCa-2 (pancreas carcinoma)] were proved. [Ronney and Ryan, 2005]. *In vitro* treatment with the monodesmosidic, triterpene saponin alpha-hederin (extracted from *Hedera helix*), inhibits proliferation of mouse B16 melanoma cells and non-cancer mouse 3T3 fibroblasts [Danloy et al, 1994], and

also induces apoptosis, intracellular glutathione depletion and reactive oxygen species generation in P388 murine leukaemia cells [Swamy and Huat, 2003].

There is also concerning about isolation of biologically active substances from insects biomass and their pharmacological evaluation. It was confirmed the presence of various terpenoids, carbohydrates, organic alcohols, saponins, glycosides polyphenols, quinones and cyanogen substances, alkaloids, lipids, hormones from adult insects, larvae, eggs, exuviae, cocoons and secretions of various insects (Ramos-Elorduy E. and Monte-Florac E., 2000; Costa-Neto E. and Eraldo M.,2002). These substances have shown antimicrobial, immune-correcting, anti-inflammatory and antioxidant activity. Several directions in developing insect products as human therapeutic drugs are possible solutions for the treatment of some disorders. As insect immune molecules have been specifically designed and evolved over millions of years to carry out their functions, they have several advantages over other potential lead compounds. In this respect there are some studies that highlight substances with pharmacological effect e.g. tumoricidal (particularly mentioned prostate adenoma), cell protective, anticoagulant, antimicrobial.

Literature survey with reference to animal sources used to extract functional peptides for the treatment of prostate diseases, including benign prostatic hyperplasia, showed that totally lacks research using insect biomass to obtain these valuable compounds. Is known that insect biomass is a priceless treasure of polyfunctional substances (Costa-Neto E., 2005; Feng Y. et al., 2009; Sun L et al., 2009). Besides, in history of insect biomass use for therapeutic purposes are registered some effects of urinary diseases. Thus, for several hundred years locust biomass is used in Chinese medicine and Brazil to treat bladder. The entomological components are intensively studied and used as antiproliferative and antitumor remedies, their diversity assuring a high degree of therapeutic efficacy (Pemberton R., 1999; He T.P., 2005, Shi Q. et al., 2005).

Substances with evident anticancer properties as dicostatina and isoxantopterina were isolated from wings of *Catopsilia crunchy* and limbs of *Allomyrina dichotomus*. In the 70s of last century was carried out a screening of the extracts obtained from over 800 species of arthropods, of which approximately 4% have shown anticancer properties (Oldfield, 1989).

In respect with the increased demand for new drugs that could raise the efficacy of tumor treatment, we focused our studies on the development of therapeutic solutions based on naturally occurring substances: herbal extracts having chemical configuration similar with steroid hormones and entomological preparation containing a mucopolisaccharidic complex. Biotehnos Laboratories

developed original technologies in order to designed structured groups – derivatives from Hedera helix (*Araliacae* family) with biological activity in gynaecological cancers and derivatives from entomological sources active in proliferative diseases . The aim of this paper is to present **one of the most important steps in pharmaceutical products development, the ”in vitro” screening on specific tumor cell lines** (prostate, cervix and breast), defining for setting the target of active principles’ cellular action.

We choose an experimental model based on human tumours standardized cell line with epithelial morphology: HeLa (cervix adenocarcinoma - ATCC nr. CCL-2), Hep-2 (Human Negroid Cervix carcinoma, HeLa derivative - ECACC nr. 86030501), MCF – 7 (breast adenocarcinoma - ATCC nr. HTB-22) and DU 145 ( human prostate carcinoma - ATCC ¯ HTB-81) and explore the natural complexes effects on apoptosis, DNA synthesis and cell proliferation.

## Materials and Methods

**Cell cultures**: Following human cell lines were used for the in vitro experiments:

**MCF-7** - (Human Caucasian breast adenocarcinoma, originating from ECACC- 86012803). Cells were cultured in EMEM with 10% fetal bovine serum, 2mM Glutamine, under standard culture conditions (37°C, 95% humidified air and 5% $CO_2$), harvested 48h before treatment, and 48 h with tested substances.

**HeLa** - (Human Negroid Cervix Carcinoma originating from ECACC-93021013) Cells were cultured in EMEM with 10% fetal bovine serum, 2mM Glutamine, 1% Non Essential Aminoacids (NEAA) under standard culture conditions (37°C, 95% humidified air and 5% $CO_2$), harvested 24h before treatment, 48 h with tested substances.

**Hep2** (HeLa derivatives) – (Human Negroid Cervix Carcinoma originating from ECACC- 86030501) Cells were cultured in EMEM with 10% fetal bovine serum, 2mM Glutamine, 1% Non Essential Aminoacids (NEAA) under standard culture conditions (37°C, 95% humidified air and 5% $CO_2$), harvested 24h before treatment, 48 h with tested substances. Sub-confluent cells (70 – 80%) were split 1:3 to 1:10, seeding 1-3x10 000 cells / $cm^2$ using 0.25% trypsin or trypsin/EDTA.

**DU145** – Human prostate carcinoma; derived from brain as metastatic site, originating from ATCC ¯ HTB-81). Cells were cultured in EMEM with 10% fetal bovine serum, 2mM Glutamine, 1% Non Essential Aminoacids (NEAA) under standard culture conditions (37°C, 95% humidified air and 5% $CO_2$), harvested 24h before treatment, 72 h with tested substances. Sub-confluent cells (70 – 80%)

were split 1:3 to 1:10, seeding 1-3x10 000 cells / $cm^2$ using 0.25% trypsin or trypsin/EDTA.

**Chemicals and reagents:**
Cell Trace CFSE Cell Proliferation Kit (Invitrogen)
Cycle TEST PLUS DNA Reagent (BD PHARMINGEN)
ANNEXIN V - FITC apoptosis detection Kit (BD PHARMINGEN)

**Equipments:**

- Flow cytometer FACS CANTO II with DIVA 6.1 and FCS Express softwares.

**Methods:**

**a)Apoptosis evaluation through phosphatidil serine (PS) translocation:**
In apoptotic cells, phosphatidilserine (PS) - normally found on the internal part of the membrane- becomes translocated to the external portion of the membrane. Annexine V is a 35-36 kDa protein binding phospholipids in a $Ca^{2+}$ dependent process, with high affinity for PS. The procedure consist of the binding of Annexin V-FITC to PS in the membrane of cells which are beginning the apoptotic process and the binding of propidium iodide to the cellular DNA in cells where the cell membrane has been totally compromised. – Annexin V-FITC is detected through f*low cytometry* as green fluorescence (FITC-A); PI is detected as red fluorescence (PE-A).

**b) DNA staining for flow cytometry cell cycle analysis:**

DNA amount measurements by flow cytometry are possible due to the specific label of DNA with propidium iodide (PI) fluorochrome. PI is stoichiometrically bound to the base pairs of nucleic acids double bonds and has no specificity for nitrogenous bases. Fluorescence intensity is proportional to cell dye quantity, corresponding with the DNA quantity to which it had been bound. By fluorescence measurement, a distribution histogram is obtained for DNA quantity distributed in the whole cell population. In the distribution histogram for DNA quantity/normal population, the following are plotted: a first peak corresponding to the cell fraction which is found in the phase Go/G1, having a diploid DNA quantity (2N); a second peak, found at a double distance from the first one, which represents the cell fraction in phases G2+M, having a tetraploid DNA quantity (4N); area included between the two peaks, representing the cells in the synthesis phase S, having an intermediate content of nucleic acids. The isolation and label of the nuclei in cell suspensions is done using Cycle TEST PLUS DNA Reagent (BD PHARMINGEN), data were acquired with FACS CANTO II flow cytometer, and the cell cycle analysis is performed with FCS Express software – DNA cell cycle module.

**c) Cell proliferation assay – flow cytometric analysis of cell division by dye dilution (CFSE staining):** CFSE (carboxy fluorescein diacetat succin imidil ester) is a cell permeant fluorescein-based dye which covalently attaches to cytoplasm components of cells, resulting in uniform bright fluorescence. Upon cell division, the dye is distributed equally between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry [Lyons, 2000].CFSE staining technique is based on 10µM CFSE incubation of adherent cells (24h. after seeding) for 15 min. at $37^{o}$ C, followed by quenching with culture medium with 10% fetal bovine serum and 2 washing steps [Lyons and Doherty, 1998]. The stained cells are then harvested in the appropriate culture medium containing GSO1 doses or solvent controls. After harvesting time (ex. 48h.), cells are analysed through flow cytometry, in terms of quantify the green fluorescence emission of CFSE labelled cell generations on FITC-area scale. Results are presented by calculating the proliferative index - (PI) - the sum of the cells in all generations divided by the calculated number of original parent cells. It is useful for comparing quantity of cell division between cultures of the same cells undergoing different treatments.

**Statistics:** Data were analyzed for statistical significance using a one-way ANOVA followed by Dunnett's t-test to compare treated groups to the appropriate solvent control group. The level of statistical significance used was $P < 0.05$.

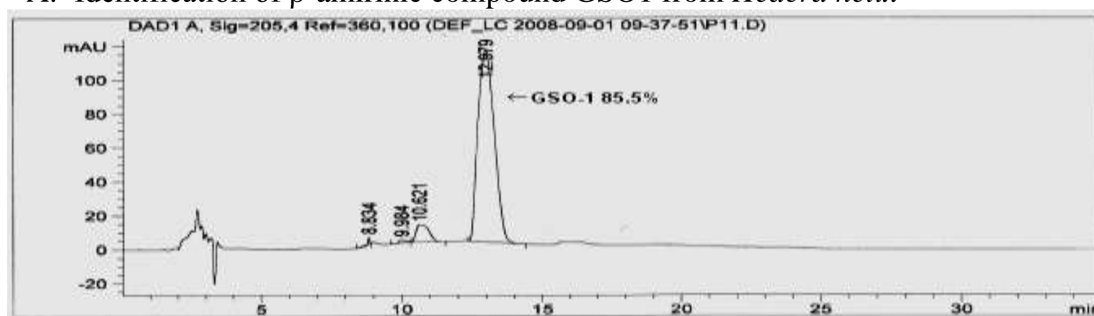
**Isolation and purification of active compounds:**

**Vegetal extract**: The β-amirinic compound was isolated in Biotehnos laboratories from *Hedera helix* through 3 steps dynamic percolation followed by successive liquid / liquid extractions, selective separation of bi-glycosidase derivative in organic phase and vacuum concentration. The final product is obtained after ethylic alcohol recristalisation, the purity being min.80 - max.90%. The HPLC chromatogram for GSO1 is presented in figure 1A.

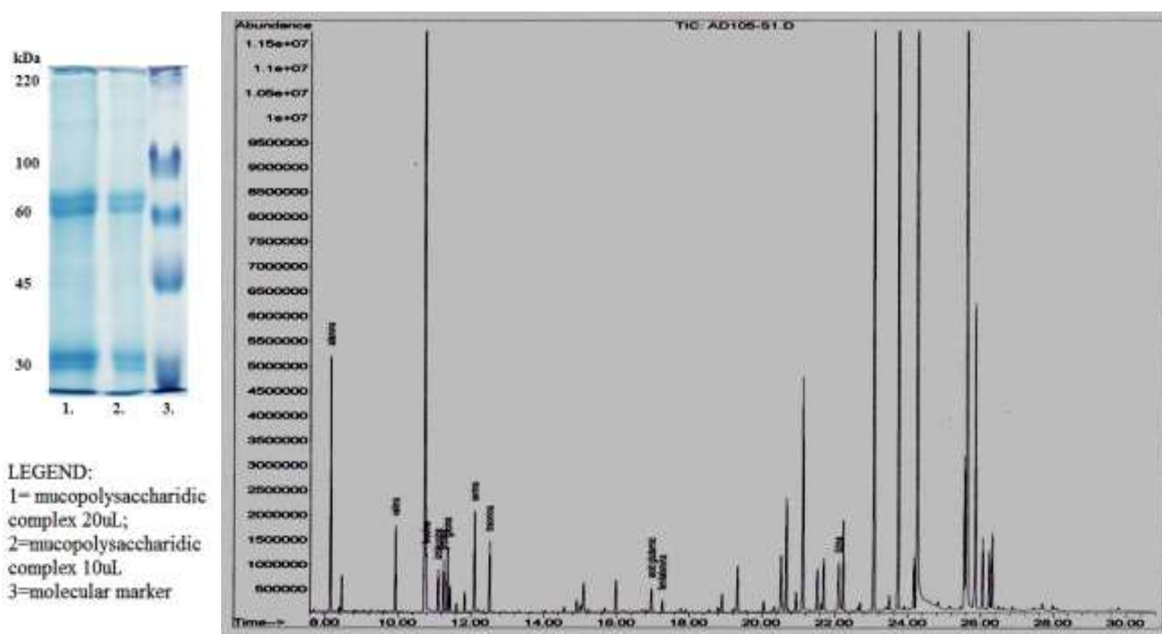
**Entomological extract**: The mucopolysaccharidic complex was obtained by original extractive methods from entomological sources. The analitical characterisation emphasized that composition of biological active substances is mainly based on protein, amino acids, polysaccharides (Figure 1 B).

**Figure 1.** Structured compounds from the vegetal and entomological extract

A. Identification of β-amirinic compound GSO1 from *Hedera helix*




B. Proteic pattern (gel of polyacrylamide) and aminoacid (GC-MS) configuration of mucopolysaccharidic complex from entomologic origin.



## Results and Discussion

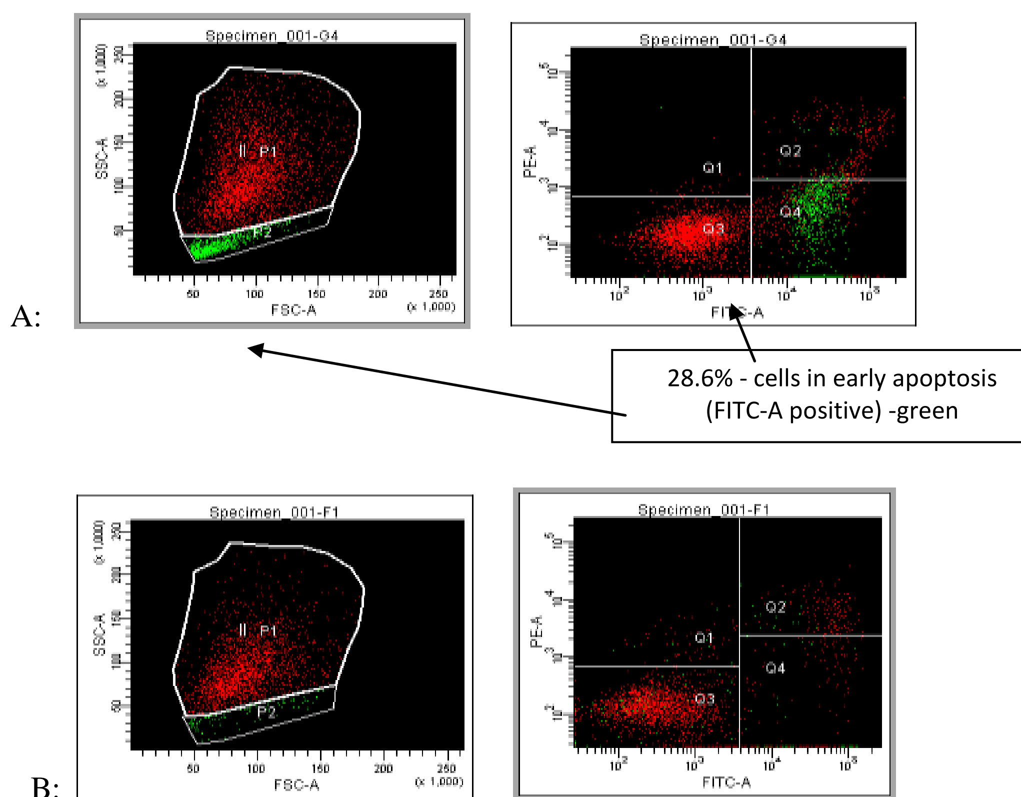
### *A.* Vegetal extract from *Hedera helix*

Our previous "in vitro" studies concerning the action on breast cancer cell line (MCF 7) of GSO1 and its derivative GSO2, both isolated from Hedera helix, [Olariu et al, 2011] were completed in order to define the anti-tumour action for GSO1, the most efficient modulator for apoptosis induction and as well as for inhibition of cell proliferation. This paper presents comparative results for GSO1 action on MCF7, Hep2 and HeLa cell lines.

Tumoral cells from MCF-7, HeLa and Hep2 lines were harvested in the same conditions (as described in chapter Matherials and Methods*)* and received three doses of GSO1 treatment (previous citotoxicity specific studies established GSO1 - IC50 between 6.8µM and 14.5 µM depending on cell type). Cellular action of these compounds was evaluated for each dose of GSO1 comparing with the appropriate solvent controls.

Apoptosis evaluation through flow cytometry provide correlative informations about phosphatidil serine externalisation and cellular membrane disruption (on FITC-A / PE-A coordinates) as well as dimensions and granularity (on FSC-A / SSC-A coordinates) of specific selected apoptotic subpopulation (figure 2). Flow – cytometric quadran analysis using Diva 6.2 software shows cellular percent of living cells (FITC-A and PE-A negative), early apoptotic (FITC-A positive and PE-A negative), late apoptotic (FITC-A and PE-A positive), or necrotic (FITC-A negative and PE-A positive).

**Figure 2.** Pro-apoptotic effect of GSO1 8μM on cervix tumors type Hep2. P2 (green) is an apoptotic sub-population defined by small dimensions and granularity on FSC-A / SSC-A axes and positive staining for Annexin V-FITC **(A);**
It is absent in control population **(B)**

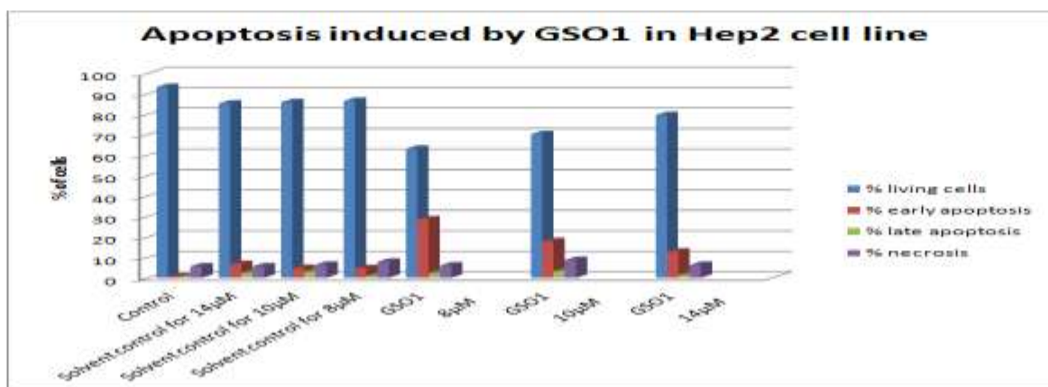


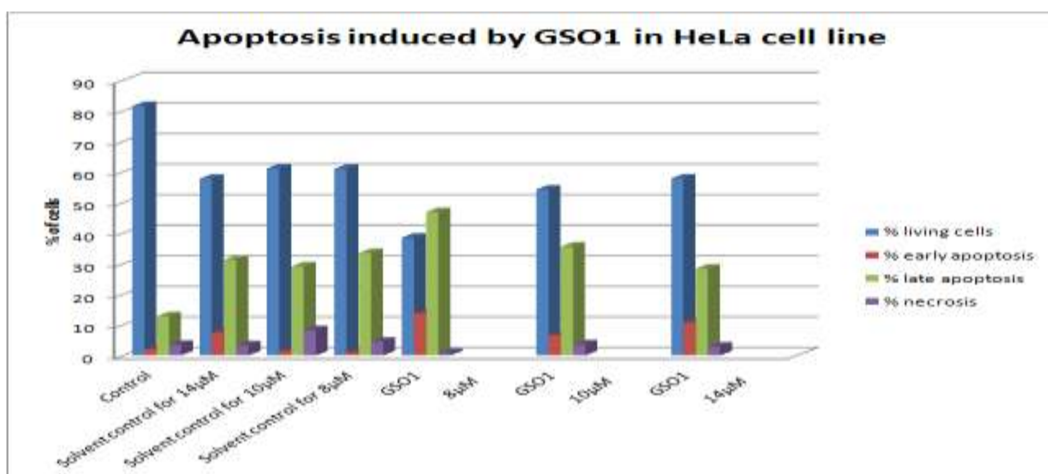

Cellular percent of early/late apoptosis induced by progressive doses of GSO1 are presented in table 1 and figure 3.

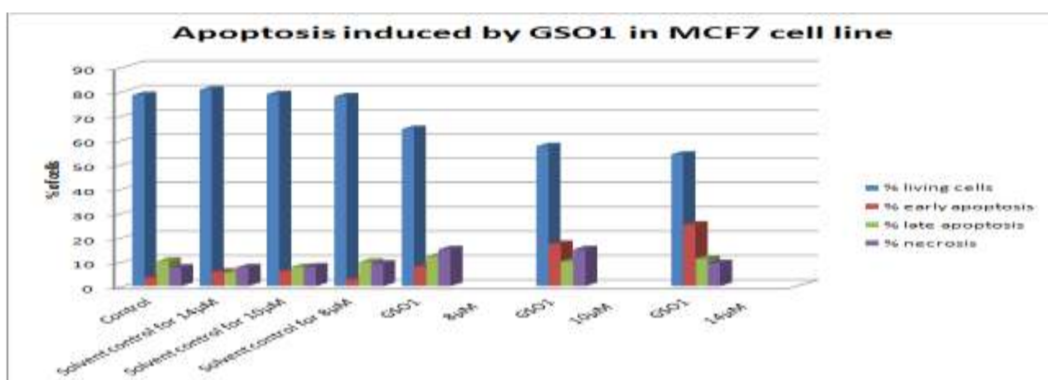
**Table 1.** Cellular percent of early/late apoptosis induced by progressive doses of GSO1

| Sample (μM) | Hep 2 cells | | | | HeLa cells | | | | MCF 7 cells | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *% living cells* | *% early apopt osis* | *% late apopt osis* | *% necro sis* | *% living cells* | *% early apopt osis* | *% late apopt osis* | *% necro sis* | *% living cells* | *% early apopt osis* | *% late apopt osis* | *% necro sis* |
| Control | 93,3 | 0,6 | 0,4 | 5,6 | 81,6 | 2,1 | 12,8 | 3,5 | 78,5 | 3,59 | 10,3 | 7,57 |
| Solvent control for 14μM | 85,2 | 6,8 | 2,4 | 5,6 | 57,8 | 7,6 | 31,3 | 3,3 | 80,77 | 5,82 | 5,68 | 7,73 |
| Solvent control for 10μM | 85,9 | 4,8 | 2,9 | 6,4 | 61,2 | 1,4 | 29,1 | 8,3 | 78,9 | 6,2 | 7,9 | 8 |
| Solvent control for 8μM | 86,5 | 4,7 | 0,9 | 7,9 | 61 | 1 | 33,5 | 4,5 | 77,98 | 2,88 | 9,94 | 9,2 |
| GSO1 8μM | 63,1 | 28,6 | 2,3 | 6 | 38,5 | 13,8 | 46,9 | 0,8 | 64,56 | 8,01 | 12,29 | 15,14 |
| GSO1 10μM | 70,2 | 17,9 | 3,2 | 8,7 | 54,3 | 6,6 | 35,6 | 3,6 | 57,5 | 17,3 | 10,2 | 15,1 |
| GSO1 14μM | 79,6 | 12,8 | 1,3 | 6,3 | 57,9 | 10,8 | 28,4 | 2,9 | 54,05 | 25,27 | 11,28 | 9,4 |

**Figure 3.** Apoptosis induced by progressive doses of GSO1 in Hep 2 cells, MCF cells, HeLA cells



After receiving apoptotic signals, such as those provided by GSO1, MCF 7 and Hep2 cells undergo early apoptosis, which is followed by late apoptosis / secondary necrosis. GSO1 induce in HeLa cell line apoptotic cell death through DNA fragmentation caused by endonucleolytic cleavage of genomic DNA (a significant percent of late apoptosis cells with disrupted cellular membrane).

The rising percent of apoptotic cells is accompaigned by a similar number of necrotic ones in GSO1 treated samples compared to control , showing an appropriate toxically effect of this compound.

The significant dose of GSO1 in respect of its biological action is different for each type of tumor (8μM for cervical cancers: Hep2 and HeLa cell lines and 14 μM for breast adenocarcinoma: MCF7 cell line)

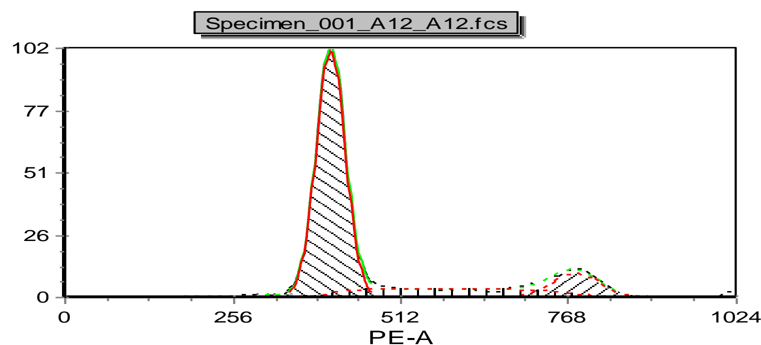
Regarding the cell cycle sequentiation, flow cytometry could be the first rapid identification method of phases of the cell cycle : G0 / G1, S si G2 / M. This analysis is based on the ability to stain the cellular DNA in a stoichiometric manner with a fluorescent reagent PE (the amount of stain is directly proportional to the amount of DNA within the cell).

When diploid cells which have been stained with a dye that stochiometrically binds to DNA (propidium iodide) are analyzed by flow cytometry, a “narrow” distribution of fluorescent intensities is obtained. This is displayed as a histogram of fluorescence intensity (X-axis) vs. number of cells .

Usually, the diploid cells consist of lymphocytes, endothelial cells, fibroblasts and other stromal elements. When a malignancy is distinguishable as DNA-aneuploid by flow cytometry, histogram analysis almost invariably shows a mixture of aneuploid and diploid cells in the tumor.

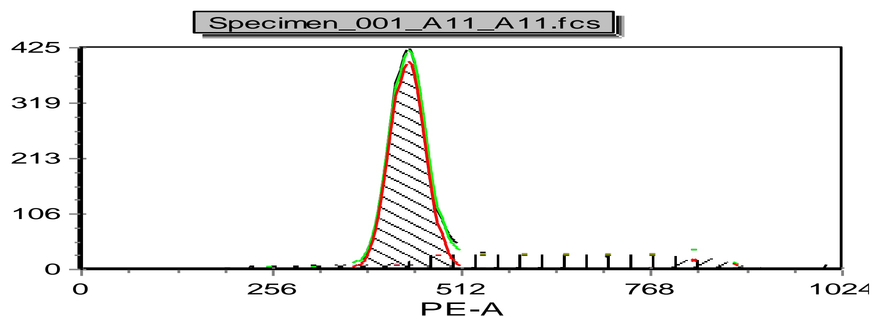
Both the malignant and normal cells have some subset of cells proceeding through the progressive G0/G1 → S → G2 → M stages, but the stromal S and G2 phases are usually much smaller than those of malignant cells. We can distinguish the cellular response to a cytostatic agent measuring the percent of rising distribution in Go/G1 phase simultaneously with the decrease of S (DNA synthesis) phase. Specific software (FCS Express V3) has been designed to deal with cell cycle analysis in order to a proper quantification of percents of distribution through G0/G1 → S → G2 → M stages (Figure 4).

**Figure 4**. Flow cytometry DNA histogram showing the cellular response to a cytostatic agent: the percent of rising distribution in Go/G1 phase simultaneously with the decrease of S (DNA synthesis) phase.




**Interpretation**
MultiCycle suggestions (a guideline only):
No abnormal DNA content is observed.
The diploid %S=15.7, %G2=11.7
The S Phase confidence is good

**Experiment Statistics**
Chi sq: 0.08
BAD: 1.45
Number of cells: 1941.00
Number of cycles: 1.00
Cycle fit model: 1 Cycle




**Interpretation**
MultiCycle suggestions (a guideline only):
No abnormal DNA content is observed.
The diploid %S=30.4, %G2=4.52
The S Phase confidence is good

**Experiment Statistics**
Chi sq: 0.31
BAD: 3.02
Number of cells: 9863.00
Number of cycles: 1.00
Cycle fit model: 1 Cycle

Our data show the responsiveness of breast and cervix tumoral cells to GSO1 action on DNA synthesis mechanisms in order to induce a significant inhibition of replicative cells (table 2). Cell cycle sequentiation is modulated by GSO1: the cellular growth is blocked in interphase with cell cycle arrest in Go (table 3). Extremely significant effects were obtained for MCF-7 and Hep2 cell lines (Figure 5).

**Table 2.** The responsiveness of breast and cervix tumoral cells to GSO1 action on DNA synthesis mechanisms in order to induce a significant inhibition of replicative cells.

| Cell line / Biocomplex | MCF 7% | HeLa | Hep |
|---|---|---|---|
| | DNA Synthesis S phase | | |
| **Culture control** | 70.20±1.507 | 29.43±1.737 | 12.88±1.455 |
| **Solvent control for 8.5μM** | 86,56±1,018 | 35,57±1,741 | 30,10±1,484 |
| **Solvent control for 10.6μM** | 83,25±1,009 | 31,54±1,091 | 33,08±0,878 |
| **Solvent control for 14.1μM** | 85,25±1,097 | 24,08±1,568 | 34,09±0,358 |
| **GSO1 [8.5μM]** | 56,37±0,815$^{**a}$ | 24,46±0,279$^{**a}$ | 7,820±0,716$^{***a}$ |
| **GSO1 [10.6μM]** | 53,08±0,215$^{**b}$ | 24,44±0,859$^{*b}$ | 12,00±0,732$^{***b}$ |
| **GSO1 [14.1μM]** | 33,72±0,317$^{***c,**d}$ | 23,33±0,930$^{nsc}$ | 15,09±0,273$^{***b}$ |

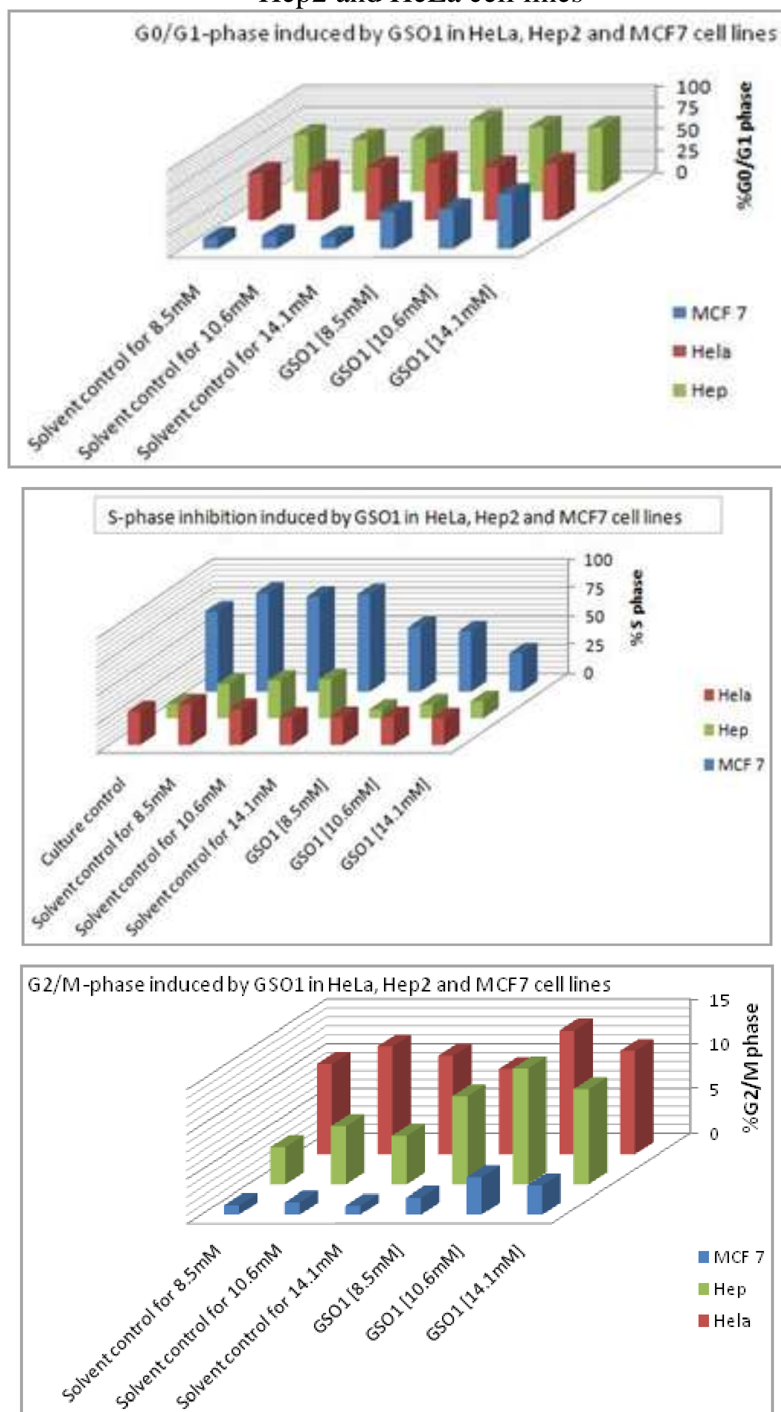
Statistically significant differences in proliferative index (P < 0.05) are indicated by:
*** - extremely significant P< 0.001
** - very significant 0.001 < P< 0.01
* - significant 0.01 < P< 0.05
ns - not significant P > 0.05
a - vs DMSO 8.5μM; b - vs DMSO 14.1μM; c - vs cellular control; d - vs DMSO 10.6μM

**Table 3**. Cell cycle sequentiation is modulated by GSO1: the cellular growth is blocked in interphase with cell cycle arrest in Go.

| **Cell line** / **Biocomplex** | **MCF 7%** | **HeLa** | **Hep** | **MCF 7%** | **HeLa** | **Hep** |
|---|---|---|---|---|---|---|
| | **G0/G1 phase** | | | **G2/M phase** | | |
| Cellular Control [8.5μM] | 12.41±0,364 | 54.46±0,148 | 65.75±0,245 | 1.03±0,106 | 10.06±0,317 | 4.15±0,554 |
| Solvent Control [10.6μM] | 15.21±0,354 | 56.36±0,465 | 60.39±0,843 | 1.36±0,222 | 12.05±0,614 | 6.53±0,34 |
| Solvent Control [14.1μM] | 13.22±0,225 | 60.54±0,786 | 62.4±0,124 | 0.98±0,144 | 11±0,247 | 5.43±0,443 |
| GSO1 [8.5μM] | 42.75±0,412 ***a, **d | 66.2±0,317**a | 82.33±0,348* **a | 1.88±0,096 | 9.5±0,156 | 9.85±0,113** *a |
| GSO1 [10.6μM] | 45.44±0,365 ***b, **d | 61.74±0,235* *b | 75.04±0,454* **b | 4.17±0,088** *b | 13.82±0,456 | 12.95±0,765* **b |
| GSO1 [14.1μM] | 63.02±0,554 ***c | 65.3±0,287 | 74.27±0,113* **c | 3.26±0,012** *c | 11.54±0,273 | 10.65±0,336* **c |

a – vs Solvent control for 8.5μM, b – vs Solvent control for 10.6μM, c – vs Solvent control for 14.1 μM, d - vs GSO1 14.1 μM

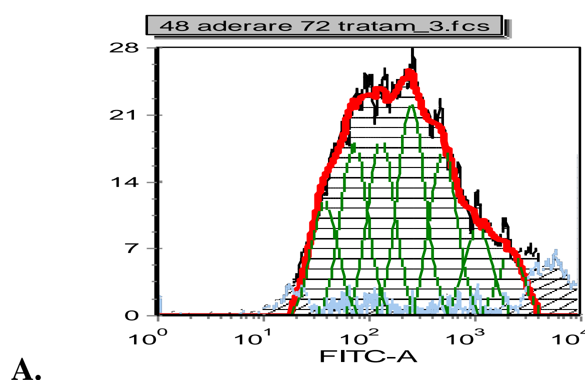
**Figure 5**. Effects of GSO1 on cell cycle sequentiation of MCF-7, Hep2 and HeLa cell lines



## Cell proliferation

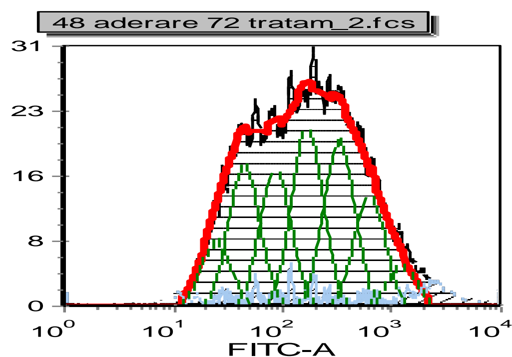
Cell proliferation is an essential feature for the antitumoral response. The cellular staining with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) has made possible to monitor the number of cell divisions during proliferation and to examine the relationship between proliferation and differentiation. Although qualitative examination of CFSE data may be useful, substantially more information about division and death rates can be extracted from quantitative CFSE experiments and statistics approach with specific software. (figure 6). Quantitative methods can reveal in detail how proliferation is regulated and altered by signals such as those received from drugs. In the following data we present *Hedera helix* extract-GSO1 action on MCF-7, Hep2 and HeLa cell lines. (table 4).

**Figure 6**. Basal proliferation of MCF7 cells : solvent control (A), GSO1 12µM (B), positive control Methotrexat 5nM (C)



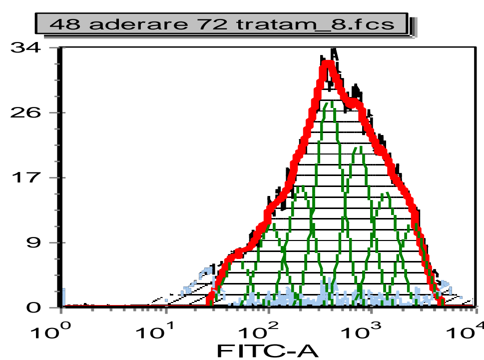

**A.**

| Generation | Generation # | Peak Channel | Peak Value | Log Std Dev | Log CV | % of Orig Cells |
|---|---|---|---|---|---|---|
| Undivided Cells | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Generation 1 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Generation 2 | 2.00 | 1860.08 | 6.00 | 47.94 | 5.61 | 5.75 |
| Generation 3 | 3.00 | 938.98 | 9.00 | 47.94 | 6.20 | 8.64 |
| Generation 4 | 4.00 | 461.38 | 17.00 | 47.94 | 6.95 | 16.41 |
| Generation 5 | 5.00 | 235.01 | 22.00 | 47.94 | 7.83 | 21.18 |
| Generation 6 | 6.00 | 118.64 | 18.00 | 47.94 | 8.88 | 17.49 |
| Generation 7 | 7.00 | 69.16 | 18.00 | 47.94 | 10.11 | 16.94 |
| Generation 8 | 8.00 | 36.19 | 12.00 | 47.94 | 11.91 | 10.95 |




**B.**

| Generation | Generation # | Peak Channel | Peak Value | Log Std Dev | Log CV | % of Orig Cells |
|---|---|---|---|---|---|---|
| Undivided Cells | 0.00 | 1104.00 | 5.00 | 52.42 | 6.64 | 4.30 |
| Generation 1 | 1.00 | 562.34 | 13.00 | 52.42 | 7.33 | 12.78 |
| Generation 2 | 2.00 | 319.08 | 20.00 | 52.42 | 8.15 | 19.34 |
| Generation 3 | 3.00 | 153.99 | 21.00 | 52.42 | 9.21 | 21.08 |
| Generation 4 | 4.00 | 77.04 | 16.00 | 52.42 | 10.61 | 16.02 |
| Generation 5 | 5.00 | 42.17 | 17.00 | 52.42 | 12.46 | 16.40 |
| Generation 6 | 6.00 | 23.08 | 8.00 | 52.42 | 14.79 | 7.18 |




**C.**

| Generation | Generation # | Peak Channel | Peak Value | Log Std Dev | Log CV | % of Orig Cells |
|---|---|---|---|---|---|---|
| Undivided Cells | 0.00 | 2186.97 | 11.00 | 47.23 | 5.47 | 9.76 |
| Generation 1 | 1.00 | 1207.90 | 15.00 | 47.23 | 5.92 | 13.58 |
| Generation 2 | 2.00 | 667.14 | 21.00 | 47.23 | 6.46 | 19.33 |
| Generation 3 | 3.00 | 352.27 | 27.00 | 47.23 | 7.16 | 25.22 |
| Generation 4 | 4.00 | 192.82 | 16.00 | 47.23 | 7.99 | 14.32 |
| Generation 5 | 5.00 | 97.34 | 11.00 | 47.23 | 9.14 | 9.68 |
| Generation 6 | 6.00 | 42.55 | 6.00 | 47.23 | 10.91 | 5.37 |

**Table 4**. Proliferation index (IP) of MCF-7, Hep2 and HeLa cells harvested with GSO1 / solvent control

| Cell line | Proliferation Index (IP) | | | Number of generations | | | %of arrested cells in parent generation | | |
|---|---|---|---|---|---|---|---|---|---|
| Biocom-plex | MCF 7 | HeLa | Hep2 | MCF 7 | HeLa | Hep2 | MCF 7 | HeLa | Hep2 |
| Cellular Control | 7,76 ±0,42 | 1,470 ±0,06 | 4,150 ±0,213 | 7 | 2 | 4 | 0 | 42.03 | 0 |
| DMSO 10.6µM | 17.6 ±0,57 | 1.52 ±0,41 | 1.68 ±0,12 | 8 | 2 | 1 | 0 | 31.1 | 32.2 |
| DMSO 12µM | 20.93 ±0.213 | 1.64 ±0,23 | 1.43 ±0,02 | 8 | 2 | 1 | 0 | 27.3 | 37.7 |
| DMSO 14.1µM | 26.3 ±0,61 | 1.780 ±0.087*a | 1.380 ±0.1 | 8 | 2 | 1 | 0 | 19.66 | 43.56 |
| DMSO 16µM | 38.81 ±1 | 1.96 ±0,72 | 1.22 ±0,032 | 9 | 2 | 1 | 0 | 18.3 | 46.3 |
| GSO1 [10.6µM] | 5,02 ±0.116 | 1,673 ±0,034 | 1,97 ±0,029***a | 6 | 2 | 1 | 3.2 | 20.25 | 24.2 |
| GSO1 [12µM] | 4,91 ±0,231**a | 1,026 ±0,018*a | 1,83 ±0,102 ***a | 6 | 2 | 1 | 4.3 | 24.05 | 28.8 |
| GSO1 [14.1µM] | 3,56 ±0.198 ***a | 1,00 ±0,008*b | 1,4±0,2 ***a | 7 | 2 | 1 | 1.3 | 30.05 | 36.6 |
| GSO1 [16µM] | 14.54 ±0,242 ***a; ***b | 0,876 ±0,054*a | 1±0,078 ***a, **b | 9 | 2 | 1 | 0 | 38.12 | 42.3 |
| MTX [5nM] | 3,77 ±0,046 ***a | 0,398 ±0,008*a | 1,675 ±0,067***a | 6 | 1 | 1 | 9.76 | 63.2 | 58.3 |

Statistically significant differences in proliferative index ($P < 0.05$) are indicated by:

*** - extremely significant $P< 0.001$

** - very significant $0.001 < P< 0.01$

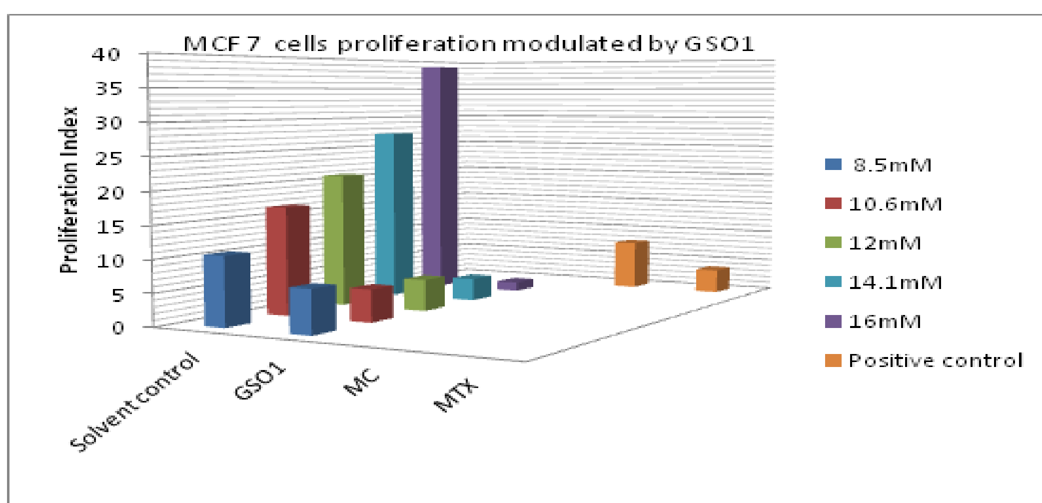
* - significant $0.01 < P< 0.05$

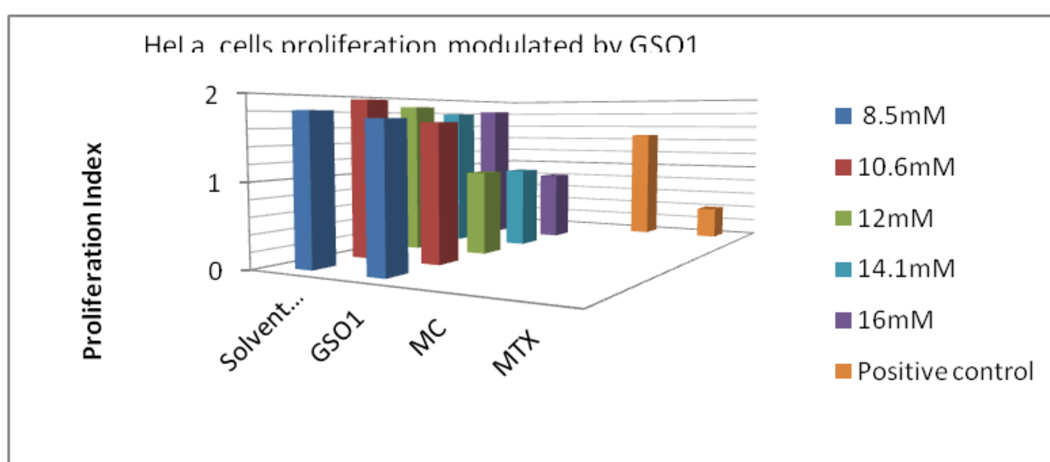
ns - not significant $P > 0.05$

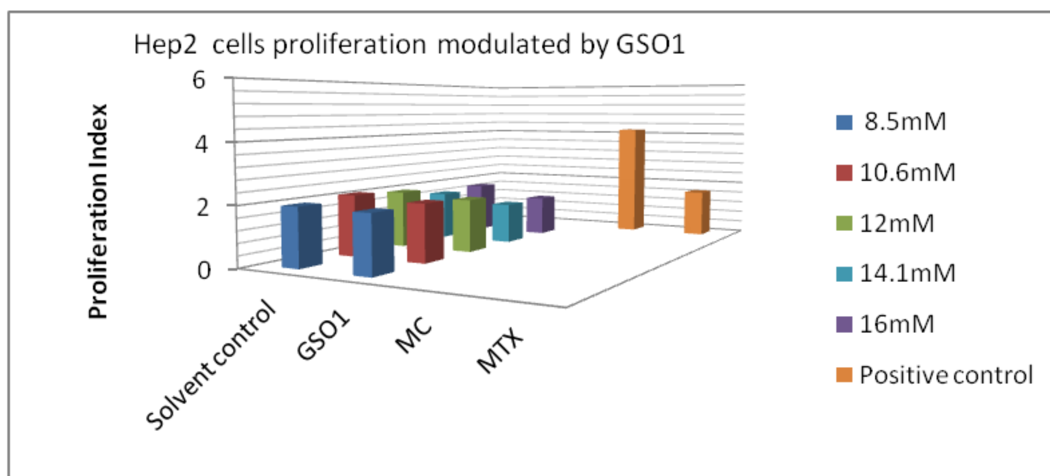
a - vs DMSO 8.5µM; b - vs DMSO 14.1µM; c - vs control; d – vs MTX 5nM; e - GSO2 32µM; f - vs DMSO 16µM; g - vs DMSO 12µM

Experiments shows a significant inhibition of proliferation capacity of breast and cervix tumoral cells, especially for Hep2 and MCF7 cells (Figure 7). GSO1 decrease the number of generations in proliferative processes, having a similar effect with the citostatic drug methotrexat and produce the accumulation of non-dividing cells in parent generation (Figure 7).

**Figure 7**. Proliferation capacity of breast and cervix tumoral cells modulated by GSO1, compared with the positive control methotrexate (MTX).





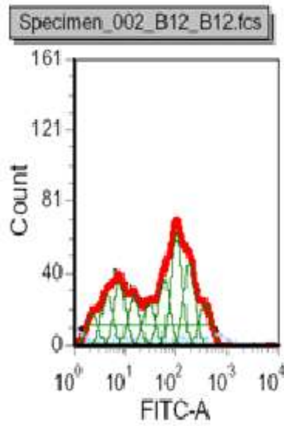

**B. Mucopolysaccharidic complex from entomological sources**

Another aspect of our screening was to explore the **mucopolysaccharidic complex** action on a prostate carcinoma cell line (standardized cell line DU 145), compared with a positive control, the cytostatic methotrexate (10µM), in order to define the antiproliferative action.

Tumoral cells from DU 145 standardised line were harvested in the same conditions (as described in chapter Matherials and Methods*)* and treated for 72h. with the entomological complex (previous citotoxicity specific studies established the appropiate interval of concentration: 0.5-5 µg/ml). Cellular action was evaluated for succesive doses of entomological complex, comparing with the methotrexate, a well known cytostatic.

**The proliferative capacity of the tumoral cells** was investigated through a method based on Cell Trace CFSE Cell Proliferation Kit (Invitrogen) presented in our previous sessions (the test on vegetal extract from *Hedera helix*). The tests were done on cell culture after 48h. of treatment with the bioactive complex. Flow cytometry aquisition and statistic interpretation of FACS Express software allowed a precise calculation of proliferative index (figure 8 and 9 and table 5).

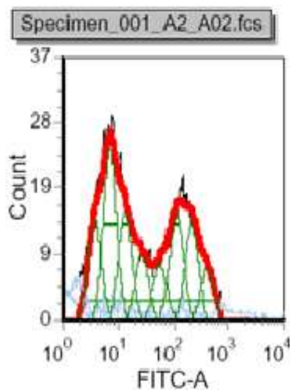
**Figure 8**. Proliferative capacity of prostate tumoral cells (DU 145 cell line) modulated by the entomologic polysaccharidic complex




| Generation | Peak Channel | Peak Value | Log Std Dev | Log CV | Peak Ratio | # of Cells |
|---|---|---|---|---|---|---|
| Undivided Cells | 345.99 | 22.00 | 40.59 | 6.18 | 0.00 | 1555.00 |
| Generation 1 | 179.43 | 46.00 | 40.59 | 7.00 | 0.50 | 3241.00 |
| Generation 2 | 98.22 | 63.00 | 40.59 | 7.89 | 0.55 | 4503.00 |
| Generation 3 | 54.74 | 36.00 | 40.59 | 9.00 | 0.57 | 2558.00 |
| Generation 4 | 28.64 | 24.00 | 40.59 | 10.79 | 0.51 | 1649.00 |
| Generation 5 | 14.33 | 28.00 | 40.59 | 13.54 | 0.50 | 1943.00 |
| Generation 6 | 7.37 | 35.00 | 40.59 | 17.86 | 0.52 | 2475.00 |
| Generation 7 | 4.14 | 28.00 | 40.59 | 24.80 | 0.56 | 1976.00 |
| Generation 8 | 2.17 | 18.00 | 40.59 | 44.40 | 0.52 | 1234.00 |

Mucopolisaccharidic complex 5uM

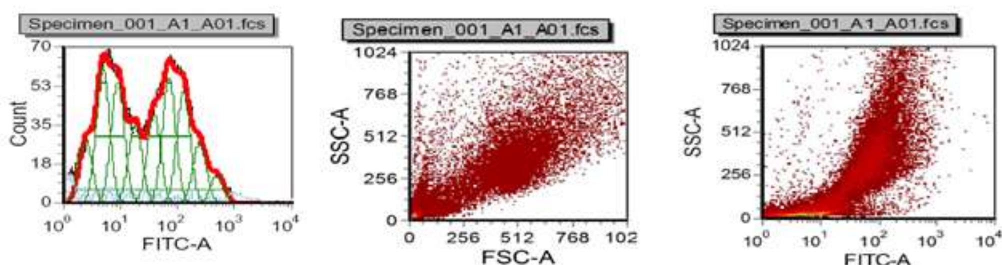
| Division Index | Proliferation Index | % Divided | Peak Ratio | # of Fitted Cells | # of Original Cells | RMS Error | R-Chi Square |
|---|---|---|---|---|---|---|---|
| 5.95 | 4.36 | 67.90 | 0.53 | 21442.00 | 4843.71 | 11.40 | 26.65 |




| Generation | Peak Channel | Peak Value | Log Std Dev | Log CV | Peak Ratio | # of Cells |
|---|---|---|---|---|---|---|
| Undivided Cells | 378.55 | 7.00 | 45.02 | 6.71 | 0.00 | 511.00 |
| Generation 1 | 205.35 | 14.00 | 45.02 | 7.54 | 0.51 | 1037.00 |
| Generation 2 | 106.50 | 15.00 | 45.02 | 8.56 | 0.53 | 1142.00 |
| Generation 3 | 52.80 | 8.00 | 45.02 | 10.01 | 0.50 | 581.00 |
| Generation 4 | 23.93 | 9.00 | 45.02 | 12.28 | 0.47 | 694.00 |
| Generation 5 | 12.30 | 15.00 | 45.02 | 15.63 | 0.49 | 1155.00 |
| Generation 6 | 6.49 | 24.00 | 45.02 | 20.95 | 0.52 | 1864.00 |
| Generation 7 | 3.49 | 14.00 | 45.02 | 31.52 | 0.52 | 1033.00 |

Methotrexate 10uM

| Division Index | Proliferation Index | % Divided | Peak Ratio | # of Fitted Cells | # of Original Cells | RMS Error | R-Chi Square |
|---|---|---|---|---|---|---|---|
| 7.56 | 5.33 | 66.03 | 0.51 | 8227.00 | 1504.29 | 5.31 | 16.26 |
| 5.95 | 4.36 | 67.90 | 0.53 | 21442.00 | 4843.71 | 11.40 | 26.65 |




Cellular control

| Division Index | Proliferation Index | % Divided | Peak Ratio | # of Fitted Cells | # of Original Cells | RMS Error | R-Chi Square |
|---|---|---|---|---|---|---|---|
| 9.23 | 7.27 | 76.14 | 0.52 | 26974.00 | 3663.41 | 14.62 | 43.69 |

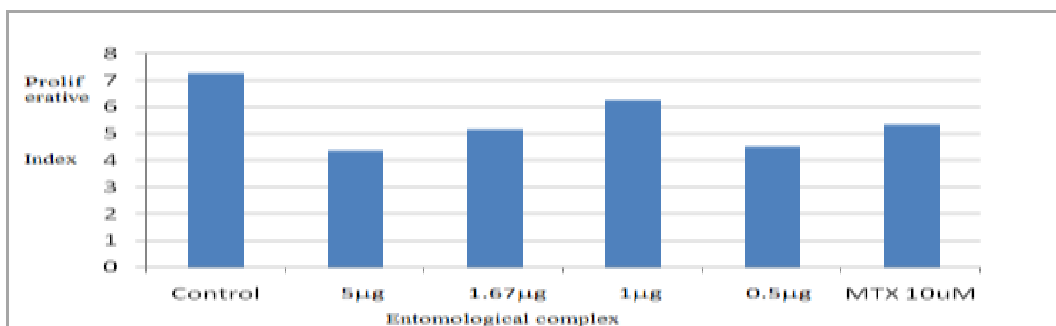
| Generation | Generation # | Peak Channel | Peak Value | Log Std Dev | Log CV | Peak Ratio | # of Cells | # of Orig cells | % of Orig Cells |
|---|---|---|---|---|---|---|---|---|---|
| Undivided Cells | 0.00 | 445.08 | 12.00 | 41.95 | 6.09 | 0.00 | 874.00 | 874.00 | 3.24 |
| Generation 1 | 1.00 | 226.71 | 27.00 | 41.95 | 6.88 | 0.49 | 1973.00 | 986.50 | 7.31 |
| Generation 2 | 2.00 | 119.71 | 50.00 | 41.95 | 7.80 | 0.52 | 3684.00 | 921.00 | 13.66 |
| Generation 3 | 3.00 | 67.32 | 56.00 | 41.95 | 8.88 | 0.56 | 4120.00 | 515.00 | 15.27 |
| Generation 4 | 4.00 | 36.52 | 40.00 | 41.95 | 10.38 | 0.54 | 2914.00 | 182.13 | 10.80 |
| Generation 5 | 5.00 | 17.31 | 35.00 | 41.95 | 13.04 | 0.48 | 2545.00 | 79.53 | 9.44 |
| Generation 6 | 6.00 | 8.58 | 54.00 | 41.95 | 17.19 | 0.50 | 3982.00 | 62.22 | 14.76 |
| Generation 7 | 7.00 | 4.74 | 61.00 | 41.95 | 23.72 | 0.55 | 4486.00 | 35.05 | 16.63 |
| Generation 8 | 8.00 | 2.31 | 28.00 | 41.95 | 42.07 | 0.50 | 2045.00 | 7.99 | 7.58 |

.

| **Tested Substances** | | **Proliferative Index** |
|---|---|---|
| Control | | $7,270\pm0,0016$ |
| Entomologic polysaccharidic complex | 5 µg/ml | $4,360\pm0,0012^{***a}$ |
| | 1.67 µg/ml | $5,160\pm0,0015^{***a, **b}$ |
| | 1 µg/ml | $6,260\pm0,0005^{***a, **b}$ |
| | 0.5 µg/ml | $4,520\pm0,0010^{***a}$ |
| Methotrexate (MTX) 10µM | | $5,33\pm0,0012^{***a}$ |

Statistically significant differences in proliferative index (P < 0.05) are indicated by:
*** - extremely significant P< 0.001
** - very significant 0.001 < P< 0.01
* - significant 0.01 < P< 0.05
ns - not significant P > 0.05
a – vs Control, b – vs 5µg mucopolysaccharidic complex

**Figure 9.** Proliferative capacity of DU145 cells modulated by the entomological complex



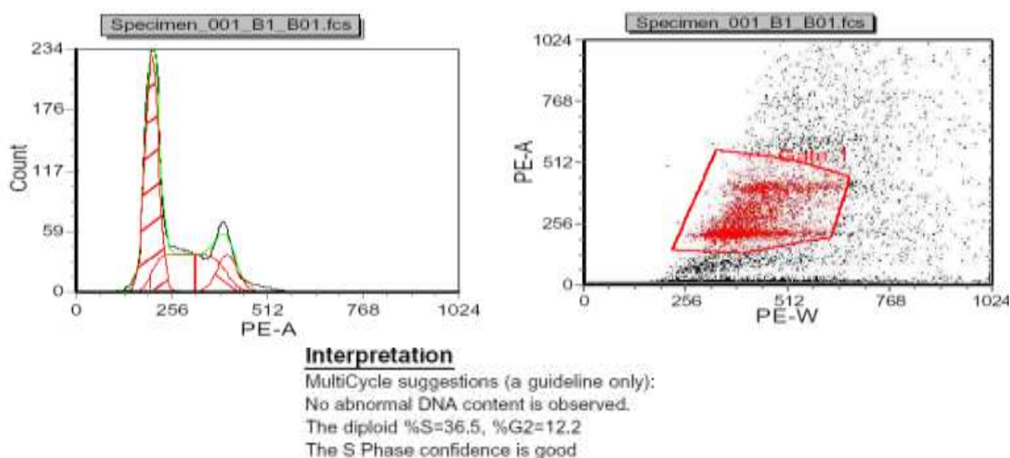

The proliferative rate of DU 145 cells treated with the entomological complex decrease, similar with the positive control, methotrexate, sustaining the significant anti-proliferative action on prostate carcinoma.

**Cell cycle analysis** was performed in conjunction with cell proliferation data, in order to explain the mechanism involved in cell division stopping.

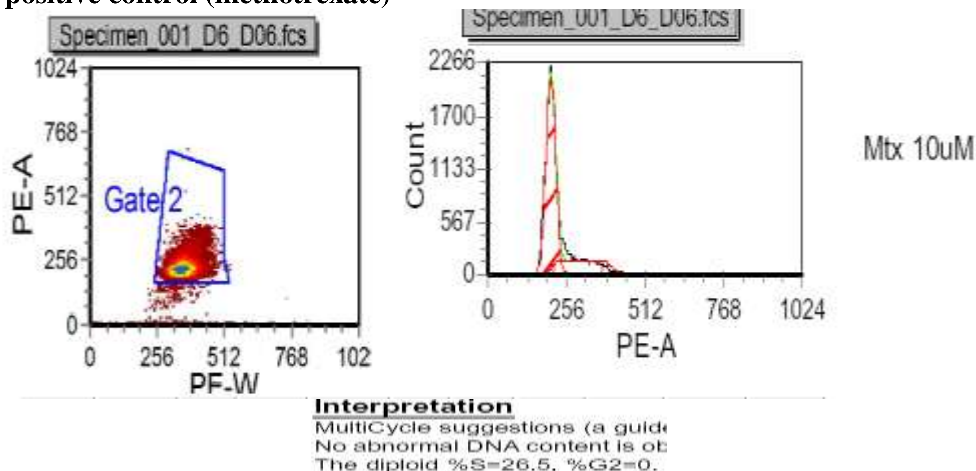
Cell cycle sequentiation results are based on data aquired with FACS Canto II flow cytometer and processed with FCS Express –DNA cell cycle module in respect with the protocol previously describe (figure 10).

**Figure 10** : Flow cytometry analysis of DU145 cell cycle modulation by the entomologic mucopolysaccharidic complex

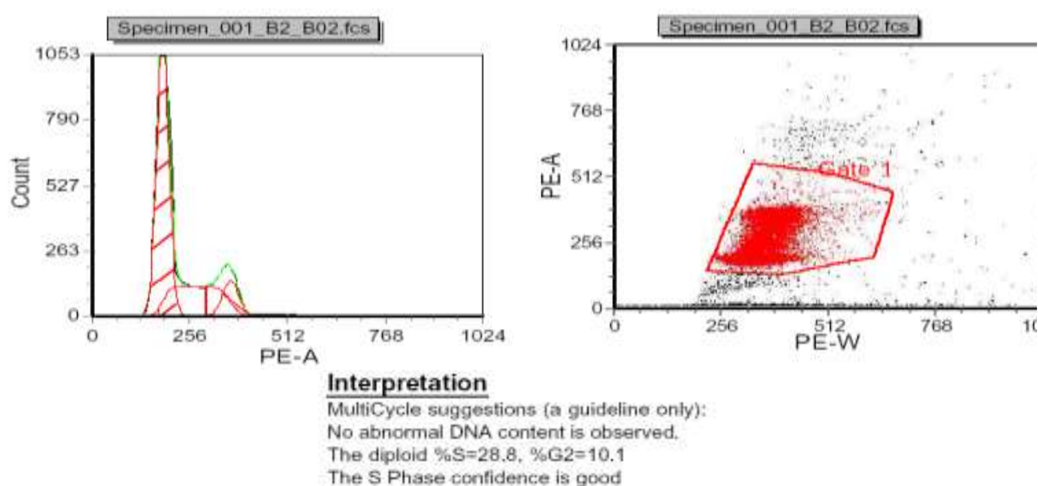
**a) cellular control**



**b) positive control (methotrexate)**




**c)mucopolysaccharidic complex**

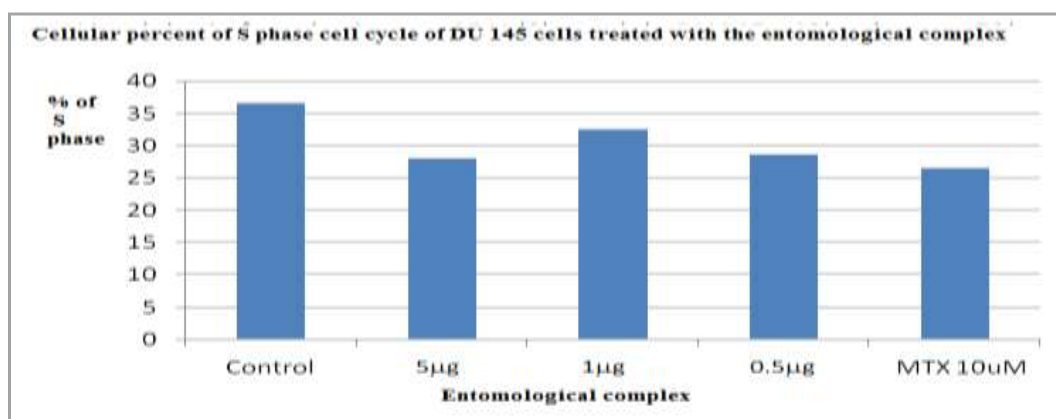



Results from three succesive experiments (figure 11) proved the stop of the mithotic process, highlighted by the decrease of DNA synthesis phase and accumulation in G0/G1, similar with the cytostatic methotrexate. The inhibition of aberrant DNA replication, in conjunction with the stop of proliferative process, confirm that the entomologic structured group could be a part of the therapy of prostate aberrant proliferative disorders.

| Tested substances (µg/ml) | | Cellular % in G0/G1 phase | Cellular % in S phase | Cellular % in G2/M phase |
|---|---|---|---|---|
| **Cellular control** | | 51.22 | 36.53±0.427 | 12.2 |
| **Mucopolysaccharidic complex** | **5 µg/ml** | 61.08 | 28.8±0.5715[***a] | 10.1 |
| | **1 µg/ml** | 52.34 | 32.5±0.623[**a, ***b] | 15.2 |
| | **0.5 µg/ml** | 61.42 | 28.59±0.532[***a,ns b] | 9.99 |
| **Methotrexat 10µM** | | 73.27 | 26.5±0.473[***a,ns b] | 0.2 |

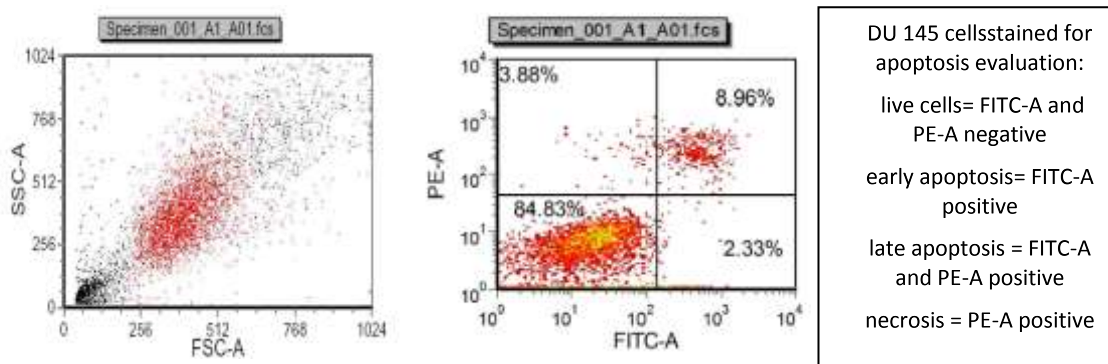
Statistically significant differences in proliferative index ($P < 0.05$) are indicated by:
*** - extremely significant $P< 0.001$
** - very significant $0.001 < P< 0.01$
* - significant $0.01 < P< 0.05$
ns - not significant $P > 0.05$
a – vs Control, b – vs 5µg mucopolysaccharidic complex

**Figure 11.** DU 145 Cell cycle S phase modulation by entomological complex, compared with methotrexate (MTX 10µM)



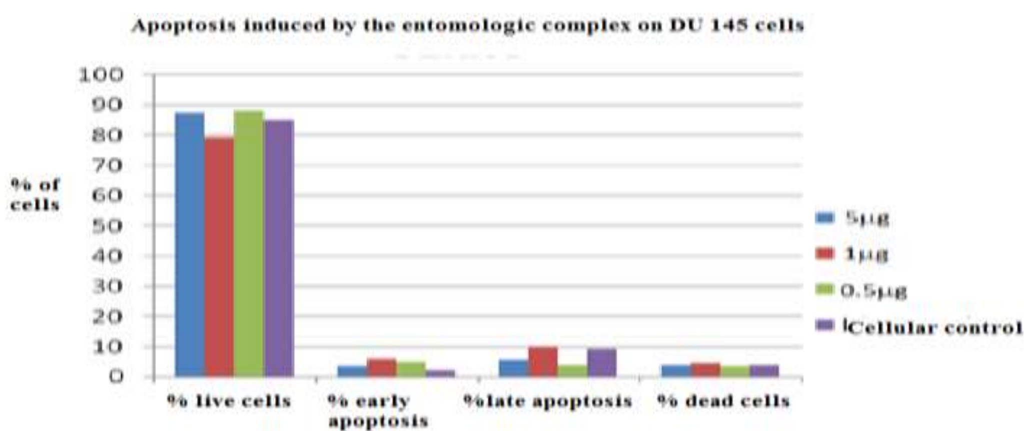

Another important aspect **in antitumoral screening is the apoptosis triggering**, essential mechanism for a potent therapeutic agent. We evaluate this effect through Anexina V / Propidium iodide doble staining method, previously described (figure 12).

**Figure 12.** Flow cytometry apoptosis evaluation of DU 145 cells




<table>
<tr><th colspan="2">Sample</th><th>% live cells</th><th>% early apoptosis</th><th>% late apoptosis</th><th>% dead cells</th></tr>
<tr><td colspan="2">Cellular control</td><td>87.83</td><td>2.33</td><td>5.96</td><td>3.88</td></tr>
<tr><td rowspan="3">Mucopolysaccharidic complex</td><td>0.5 µg/ml</td><td>87.5</td><td>3.39</td><td>5.5</td><td>3.62</td></tr>
<tr><td>1 µg/ml</td><td>79.33</td><td>6.09</td><td>10.10</td><td>4.49</td></tr>
<tr><td>5 µg/ml</td><td>88.03</td><td>4.73</td><td>3.85</td><td>3.4</td></tr>
</table>

**Figure 13**. Apoptosis stages induced by the entomological compound on DU 145 cells



DU 145 cells respond at mucopolysaccharidic complex treatment through apoptotic signals, inducing early and late apoptosis especially at 1 μg/ml. (figure 13). This effect accomplish the anti-proliferative features previously demonstated, proving complementary mechanisms of antitumoral action for the entomological structures selected.

## Conclusions

The antiproliferative and apoptotic screening is a relevant "in vitro" model, a starting point for the drug-design of a potential antitumoral agent. The application we performed (the antitumoral effect evaluation of two types of active compounds from two different natural sources: plant and animal) reveals that this “in vitro”screening is a valuable tool, a promissing checking point for the start of advanced genomic and proteomic investigations of a wide range of natural compounds.

The *Hedera helix* compound GSO1 possess multiple bioactivities including inhibition of tumor growth, proliferation and pro-apoptotic properties on epithelial carcinoma cell lines, in a cell type and dose dependent manner. GSO1 has cellular specificity as an anti-tumor agent, acting on MCF-7 and Hep2 (a derivative of HeLa), but not significantly on HeLa cells, suggesting the different cellular target for this compound. GSO1 acts similar to methotrexate, a well known anticancer drug, by mechanisms of apoptosis induction, and cell cycle arrest, suggesting a safety alternative to synthesis cytostatics.

The entomologic group, with ***mucopolysaccharidic components,*** acts on DU 145 cell line, stopping the cell proliferation as well as DNA synthesis phase, but is a week apoptosis inductor.

This study demonstrates the potential use of *Hedera helix* compound GSO1 in the prevention or treatment of malignant diseases, especially breast and cervix cancers and the antitumoral potential for prostate diseases of the mucopolysaccharidic components with entomological origin.

All the screening methods we used in the antitumoral effect monitoring proved accurate confirmatory results for cellular mechanisms defining and could be an important step in drug development processes.

## Acknowledgements

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