Comparative Analysis upon Action of Aqueous and Alcoholic *Trigonella sp.* Extracts on Fibroblasts Culture

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Flow cytometry with its numerous advantages, has become indispensable for modern biochemistry research, being a highly efficient and sensitive in many areas, particularly in the fundamental or applied cellular biology. Flow cytometry has greatly contributed to the exploration of the cellular structure and functions, combining analytical and preparative aspects. In combination with the use of the probes (fluorochromes, fluorogenic substrates, monoclonal antibodies and lectins), the FCM enables multiple investigations for the characterization of cellular populations, the analysis of the cellular components (nucleic acids, proteins, intercellular and surface antigens, enzyme activities, membrane potential, mitochondrial activity, intracellular pH, ion flux and membrane fluidity). We chose to study the behavior of fibroblasts using flow cytometry, in terms of effects induced by the presence of extracts from Trigonella foenum graecum L.

Keywords: aqueous and alcoholic extracts, fibroblasts, cellular viability, flow cytometry, Trigonella foenum graecum L.

The cell viability estimation via flow cytometry (FCM), can be done by measuring the absorption and diffusion of light in the absence of any dye, or by using fluorescent and non-fluorescent dyes.

The cellular parameters measurable via flow cytometry are of an extrinsical or intrinsical nature. The essential intrinsical parameters are morphological in nature, such as the size, cellular refringence, granularity, density, etc. and are at the basis of cytometric analysis. In contrast, the analysis of extrinsical parameters requires the use of fluorescent products (fluorochromes or probes connected to fluorescent molecules) and constitutes the cytofluorimetric analysis. The informatic system of the device treats these signals and allows their interpretation according to the utilized analysis programs.

Flow cytometry (FCM) is an accurate and comprehensive method of analyzing cells in an isolated state, entrained in a fluid stream [1-4]. The basic principle of all flow cytometers is to quantify the diffusion and fluorescent light emitted by the cells whilst passing by a laser beam, used as the excitation light source [5-6]. The analysis speed can reach 5-10000 cells per second and allows a multiparametric analysis and simultaneous recording for each cell [7-9].

The cells subject to analysis are dispersed in an isotonic medium as slurry and subjected to a pressure which allows them to advance and be injected into a liquid stream which drives them. The minimum volume of sample is 100 L cell suspension and the concentration must be between $5x10^4$ and 10^7 cells/mL [10-12].

The most recent methods used in the *in situ* detection of cellular death, in light of the latest morphological and biochemical alterations highlighted in this field, aim to identify and discriminate between an apoptotic cell and a necrotic one, by emphasizing the cellular morphology alterations, the loss of the membrane asymmetry with the externalization on the cellular surface of the phosphatidylserine debris, the reduction of the mitochondrial membrane potential, as an early event in the development of cellular death by apoptosis and the activation of the caspases, especially the highlighting of the 3 effector caspase [13-17].

All these methods can be complemented with optical microscopy analysis techniques [18-21].

Fluorescent dyes or fluorochromes, are able to absorb the photonic energy of the incident beam and transform it, transmitting at a higher wavelength, with an emission maximum, inferior to the absorption one. They are very specific markers for certain cellular constituents and are both exclusionary, as well as supravital stains. They are easy to use, but one has to remember that they provide information on membrane integrity and that, for a cell which is considered living, it does not necessarily mean that it has also retained its ability to proliferate [9, 22-24].

The most used fluorochromes are fluorescein-diacetate (FDA) or other calcein-AM fluorescein derivatives, products that serve as a substrate for the cellular esterases [7-8, 25]. These products penetrate the membrane of the adherent and non-adherent cells and serve as measuring markers for the enzymatic activity necessary for the activation of their fluorescence; they also serve as integrity markers for the cellular membrane, which is necessary for the retention within the cell of these fluorescent products. In contrast, both the non-hydrolyzed substrates as well as their cleavage products, will be rapidly eliminated from the dead or altered cells that have compromised cell membranes [3, 4, 7].

Calcein-AM is the best cellular viability marker due to its superior retention in the cell and its relative insensitivity of fluorescence, emitted at *pH* in the physiological limits [13].

Calcein-AM passes through the membrane of the viable cells and will be hydrolyzed by the cytosolic esterases in calcein, a green fluorescent product, which will be retained in the cells with an intact membrane. The calcein loss, which is similar to the cells viability loss, is determined via flow cytometry [10-12].

Trigonella foenum graecum L., (Fabaceae family), fenugreek, is an annual herbaceous plant. The literature records a variety of therapeutic actions, including hypocholesterolaemia, hypoglycaemia, antibacterial, antiviral, anti-inflammatory, antioxidant activities [26-30].

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Oxidative stress is well-known to cause many diseases and scientists, in many different disciplines, have become more interested in natural sources which could provide active components to prevent or reduce its impact on cells. Antioxidants can inhibit or delay the oxidation of an oxidative substrate in a chain reaction and therefore, appear to be very important in the prevention of many diseases [31-35].

Experimental part

We chose to study the behavior of fibroblasts, in terms of effects induced by the presence of *Trigonella foenum graecum L.* plant extract. We used a standardized preparation of powdered *Trigonella sp.L.* seeds, which was obtained by maceration in alcoholic and aqueous solution. *Trigonella sp.L.* seeds (100 g) were powdered down and mixed with 80% methanol. The resulted mass was kept at room temperature for 5 days. After 5 days the solution was filtered and the solvent was evaporated. The residue was the base of development of the prepared aqueous and alcoholic solution for the experiment.

Trigonella sp.L. was chosen because it's multiple active metabolic effects and most of all, because it's anti apoptotic inducing effects.

The cellular viability was determined according to the Bratosin protocol, after the model of Chung et al., 2000 [36].

A 10 mM calcein-AM in DMSO (1 mg in 100 mL DMSO) stock solution was prepared, preserved at -20°C and a work substance of 100 µM prepared extemporaneously, in a *p*H = 7.4, PBS buffer [8- 9].

The fibroblast cells (4 x 10^5 in 200 µM of PBS buffer) were incubated with 10 mL of calcein-AM working solution, the final concentration of the calcein being 5 µM, for 45 min in the dark. Before the analysis via flow cytometry 0.5 mL of PBS buffer were added. The acquisition and analysis of the cells was carried out in a linear system for FSS/SSC and log system for FL1. All analyses were performed in triplicate.

Results and discussions

Fibroblasts cultured in the presence of aqueous and alcoholic extracts of *Trigonella sp.L.*, according to the described protocol were counted and analyzed for cellular viability with the automatic analyzer Countess™, via a 0.4% trypan blue coloration [18-20, 23-24].

As shown in the figures below, the aqueous *Trigonella sp.L.* extract did not influence the fibroblasts division, they scaled between 3 x 10^5 and 3.9 x 10^5 compared to the control batch. The presence of the alcoholic extract reduces the number of cells in the culture of 1.5 x 10^5 cells/mL in 5% alcoholic extract, 8 x 10^4 cells/mL for a 10% alcoholic extract and for 4 x 10^4 cells/mL for the concentration of 30% alcoholic extract. In all cases the viability has decreased, demonstrating that, for a cell culture, the concentrations chosen were very high (fig. 1-3).

A living cell exhibits certain characteristics that will form the basis for assessing cellular viability in flow cytometry, namely size, shape (FSC) and refractive (SSC), which are conditional on the moisture level of the cell, the state of the cytoskeleton and organelles. When a cell is altered, it begins to lose these characteristics [8-9, 22-24].

The Dot-plot analysis demonstrates the existence of two zones. Zone A of viable platelets and zone B of aged cells, with an apoptotic phenotype, with morphologically changed parameters (decrease of size and cellular content).

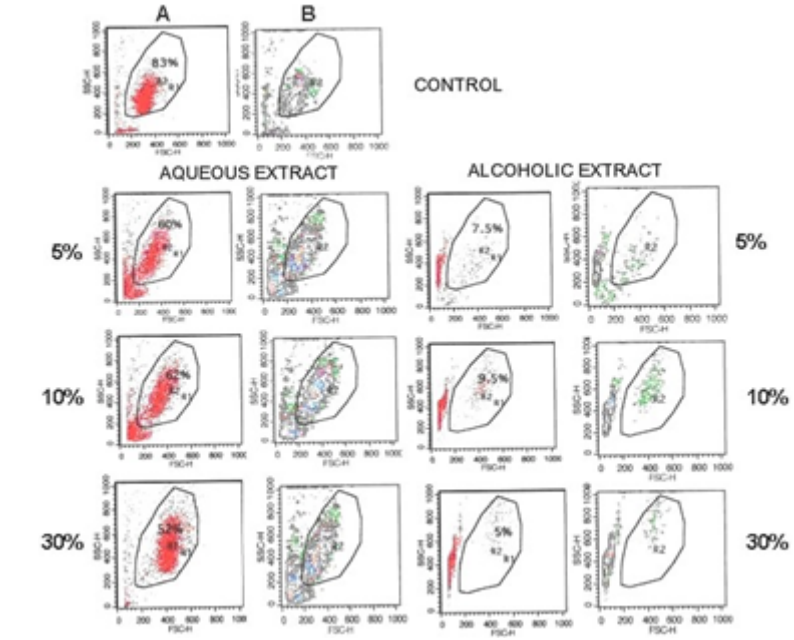

Fig. 1. The flow cytometry analysis in a FSC/SSC system on fibroblasts and the morphological changes induced by culturing for 24 h in the presence of the aqueous or alcoholic extract of *Trigonella sp.L.*, in various concentrations, A - density - plot; B - contour-plot

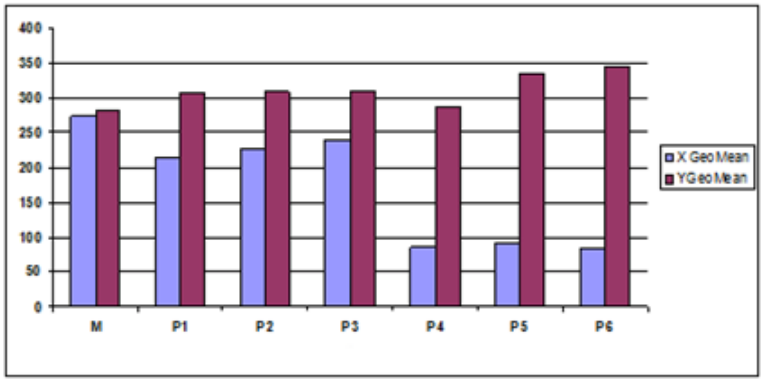

Fig. 2. The comparative histogram of the X Geo Mean and Y Geo Mean values for the fibroblasts cultured for 24 h in the presence of the aqueous (P1-P3) and alcoholic extract (P4-P6) of *Trigonella sp.L.* compared to control culture (M)

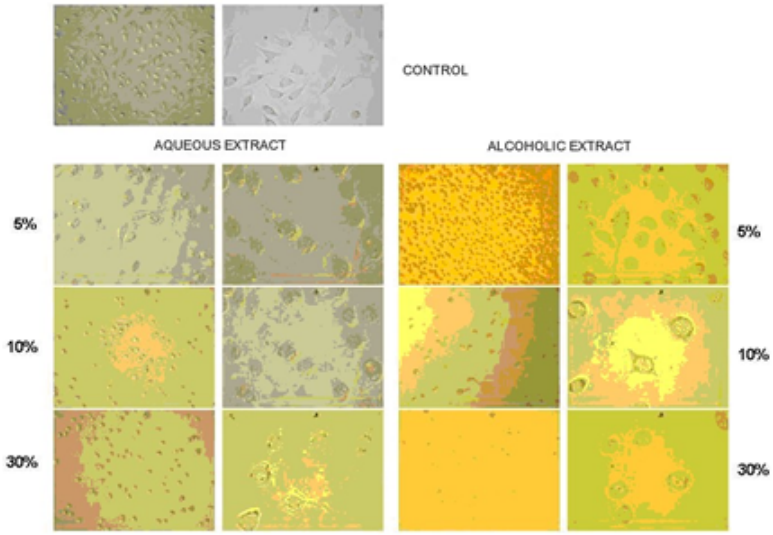

Fig. 3. The optical microscopic analysis of the fibroblasts culture, in the presence of the *Trigonella sp.L.* aqueous or alcoholic extract, compared to control sample

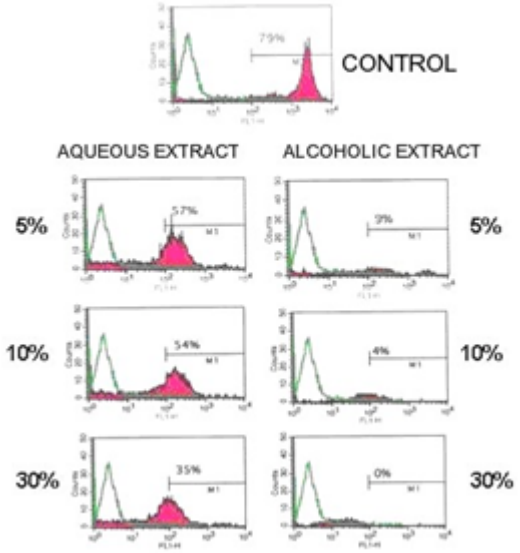

Fig. 4. The comparative analysis of the viability of the fibroblasts cultured in an environment supplemented with a *Trigonella sp.L.* aqueous or alcoholic extract, by determining the activity of the intercellular esterases with calcein - AM.

The comparative analysis of the fluorescence histograms in FL1 (the calceins fluorescence intensity) for the M1 area (area of viable cells) and M2 area (area of dead cells) both as percentages as well as a MFI (Mean Fluorescence Intensity), are shown in figure 4.

The viability of the cultured fibroblasts in the presence of the aqueous *Trigonella sp.L.* extracts diminishes progressively with the increase of concentration from the culture environment, ranging between 57 - 35%, in comparison to control, where viability was 79%.

The viability of the cells cultured in an environment supplemented with alcoholic extract induces the death of the fibroblasts, which is consistent with the results obtained via the dot-plot FSC/SSC analysis.

Subjecting the fibroblast to a low-frequency laser treatment, leads to the spectacular growth of the fibroblast cells viability, as reflected in the assessment based on the measurement of the intracellular esterases activity with calcein-AM.

Conclusions

The comparative analysis of the two types of cytograms, density dot-plot (A) and contour dot-plot (B) for the fibroblasts cultured for 24 h (control) with the fibroblasts cultured in the presence of aqueous or alcoholic extracts of *Trigonella sp. L.*, for 24 h, at concentrations of 5%, 10% and 30%, has led, as can be seen from the results, to the following conclusions.

The aqueous extract of *Trigonella sp.L.* provide less morphological (density and contour) changes induced in cultured fibroblasts, compared with the alcoholic extract (alcohol provide lot of cellular membrane changes).

There is a significant difference between the comparative histogram of the X Geo Mean and Y Geo Mean values for the fibroblasts cultured for 24 h in the presence of *Trigonella sp.L.* aqueous extract (P1-P3) and alcoholic extraxt (P4-P6) compared to the control culture (M).

By determining the activity of the intercellular esterases with calcein-AM we observe an increase in the viability of the fibroblasts cultured in an environment supplemented with a *Trigonella sp.L.* aqueous and alcoholic extract.

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