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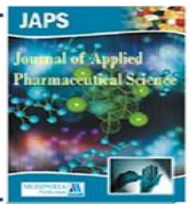

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Evaluation of Dimethylthiazol Diphenyl Tetrazolium Bromide and Propidium Iodide Inclusion Assays for the Evaluation of Cell Viability by Flow Cytometry

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ABSTRACT

The natural products are widely used in many fields as pharmaceutical, flavor, industrial, and additive compounds. In many cases, there is a need to evaluate the cytotoxic effect of these products and to determine the non cytotoxic concentration at which they can be used in a safe manner. The objective of the present study was to compare two methods of evaluation the cytotoxic effect of a plant extract. The MTT assay and the PI dye-inclusion assay were used to carry out the experiments. The results were then evaluated by calorimetric and flow cytometry assays. The obtained results showed that a clear difference between the two methods and the cell death using the MTT assay seem to be overestimated. To better evaluate the cytotoxic effect of natural product, we need to carry out several *in vitro* and *in vivo* tests before the judgment that a compound is safe to use and at which concentration.

Keywords: MTT assay, Cytotoxicity, Propodium Iodide, Flow cytometry, natural extract.

INTRODUCTION

Cell death can occur by either of two distinct (Schwartzman *et al.*, 1993; Vermes *et al.*, 1994) mechanisms, necrosis or apoptosis. In addition, certain compounds are reported to be cytotoxic to the cell, that is, to cause its death. Necrosis ("accidental" cell death) is the pathological process which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis ("normal" or "programmed" cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Cytotoxicity is the cell-killing property of a chemical compound. In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism. There are many observable morphological and biochemical differences between necrosis and apoptosis (Vermes *et al.*, 1994).

Necrosis occurs when cells are exposed to extreme variance from physiological conditions which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like toxic products and lytic viruses. Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response. Live cells with intact membranes are distinguished by their ability to exclude dyes that easily penetrate dead or damaged cells. The MTT assay is widely used for measuring cell viability, proliferation of living cells, and cytotoxicity of new drug candidates in the 96−well plate format (Liu *et al.*, 1997). In addition, staining of non-viable cells with propidium iodide (PI) has been performed on most cell types. Its broad application is most likely due to ease of use: the procedure is very simple, and the stained cells are bright red and easy to identify. The aim of the present study was to compare and evaluate two dye-inclusion methods for the assessment of cell viability after treatment of Vero cells with plant extract.

MATERIALS AND METHODS

Plant extract and Cell culture

Salvia officinalis (common sage) was used in the present study. It is one of the most known and used aromatic and medicinal plant, to which several ethnopharmaceutical properties are attributed. It is a popular herb commonly used as a culinary spice for flavouring and seasoning that has also been used for centuries in folk medicine for the treatment of a variety of ailments.

The Vero cell line was maintained in RPMI 1640 supplemented with fetal bovine serum (10% v/v), L-Glutamin (2mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

IN VITRO CELL VIABILITY/CYTOTOXICITY STUDIES

Cell treatment

Briefly, cells were maintained, as previously described, and then seeded into 48-well plates at a density that would allow the formation of a 90% monolayer in 24 hours. Once a confluent cell monolayer was observed, media from the wells was removed. Each extract was diluted in medium supplemented with 2% serum. Three concentrations of plant extract, including 500, 250, and 125 µg/ml, were tested. Controls of non-treated cells were also included. Then, 1mL/well of diluted extracts was added to the plates, and then the plates were incubated for 48 hours. Cell morphology was assessed with a phase-contrast microscope. For cell visualization, medium was aspirated and Vero cells were then fixed with cold acetone for 10 min. The acetone was aspirated and the fixed cells were incubated with 20% Giemsa solution followed by extensive washing with distilled water.

Mitochondrial function

Mitochondrial function was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). The evaluation is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells, to give a blue formazan product which can be measured spectrophotometrically (Figure 1). Fourty eight hours after seeding the cells, different concentrations (1250, 2500, 5000 µg/ml) of extracts were added to the microtiter wells. The cultures were further incubated for 48 h, and then 100µl of MTT solution (5 mg/ml) was directly added to the culture wells for 4 h at 37°C. After incubation, the absorbance at 540 nm was measured with a standard microplate reader (BIO-TEK® ELx800™ Universal Microplate Reader, USA). The quantity of formazan product as measured by the amount of 540 nm absorbance is directly proportional to the number of living cells in culture. Each experiment was done in triplicate. The relative cell viability (%) related to control wells containing cell culture medium without extracts was calculated by [A]test/[A] control x 100. Where: [A]test is the absorbance of the test sample and [A]control is the absorbance of control sample.

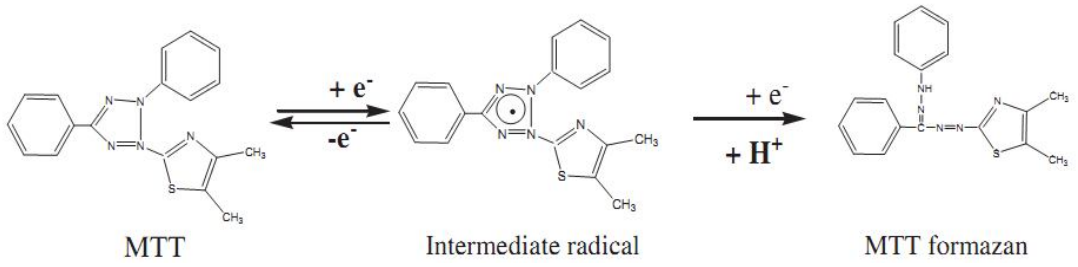

Fig 1. Reduction of MTT and the formation of formazan.

Apoptosis/Necrosis assay

Staining of nonviable cells with PI has been performed on most cell types. Its broad application is most likely due to ease of use: the procedure is very simple, and the stained cells are bright red and easy to identify. Cells were cultured in 24-well plates with different concentrations of plant extracts for 48 h. They were then washed with PBS and incubated with trypsin. The numbers of necrotic cells were immediately evaluated with Flow Cytometry. FACSCalibur (BD Biosciences, San Jose, CA) was used in the present study, which provides two excitation sources (488-nm air-cooled argon-ion laser and red diode laser He-Ne source with 635 nm). Population distributions of PI-labeled cells were detected by plotting the fluorescence F12 at 585 ± 21 nm against the forward scattering using the first excitation option. About 10 000 events/sample were acquired. The raw data obtained from flow cytometry are then subjected to analysis after suitable transfer of fluorescence-activated cell sorting (FACS) files.

RESULTS AND DISCUSSION

The Cytotoxic effect of the plant extract was evaluated by MTT reduction assay. The cell response to plant extract was by dose-dependent manner. Great cytotoxic effect was not shown for the tested concentrations. The percent of viability was ranged between 66% (at concentration 10 mg/mL) and 73% (at concentration 5 mg/mL). The CC_{50} was 22.7 mg/mL. The cytopathic effect of plant extract at concentration of 10 mg/mL was shown in cell culture using Giemsa staining method (Figure 2).

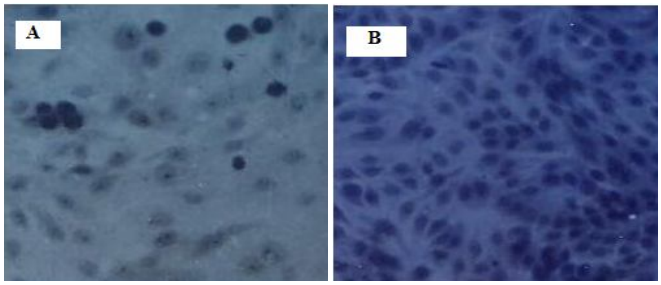

Fig. 2: Cytotoxic effect of plant extract on Vero cell culture. (A): Vero cells treated with 10 mg/mL of plant extract after 48 h. (B): Uninfected Vero cells.

In order to better evaluate the cytotoxic effect of the tested extracts, we have carried out an experiment using PI inclusion assay. Three non cytotoxic concentrations were used to evaluate the degree of their cytotoxic effect; 5 mg/mL, 2.5 mg/mL, and 1.25 mg/mL. The percent of viability for all these concentrations was 73%. Cytotoxicity of plant extract was evaluated by measuring the cellular uptake of PI after treatment with the plant extract at different concentrations for 48 hours. Normally live cells are impermeable to PI, and PI uptake was used to quantify the population of cells in which membrane integrity was lost. The obtained results showed significant difference between the MTT method and the PI assay. The assessment of cell viability by flow cytometry showed a quantitative change in the number of dead cells after treatment. Compared to untreated cells (Fig. 3 A and B, dead cells = 2.3%), the most cytotoxic effect was shown for the plant extract at a concentration of 5mg/mL (Figure 3C and D, 19%), followed by 2.5 mg/mL (Figure 3E and F, 14.7%), and 1.25 mg/mL (Figure 3G and H, 5.4%) (Figure 3). The reduction of MTT by viable cells in culture is a method widely recommended for examining the cytotoxic effect of xenobiotics, assessing proliferation rates, and analyzing cell activity (Denizot *et al.*, 1986; Mosmann, 1983). MTT is reduced inside viable cells to water-insoluble purple-colored formazan by mitochondrial dehydrogenases, although in various cell lines over 50% of the dye penetrating the cell membrane can be reduced by non mitochondrial, cytosolic, and microsomal enzymes (Collier *et al.*, 2003; Gonzalez *et al.*, 2001).

MTT reduction is attributed to mitochondrial activity, although it is related both to non−mitochondrial enzymes and to lysosomes and endosomes (Liu *et al.*, 1997). However, Tested compounds can directly interact with MTT (Ulukaya *et al.*, 2008). For example, several plant extracts, such as polyphenols (resveratrol) and flavonoids (quercetin, luteolin, kaempferol), strongly reduced MTT in the absence of living cells (Peng *et al.*, 2005; Weyermann *et al.*, 2005; Bruggisser *et al.*, 2002). For the above discussed reasons, many studies have reported that MTT reduction appears to be an inadequate test of viable cell number, yielding false results which are a source of misinterpretation. The main reasons for such pitfalls and limitations of the MTT assay are: (!) Tested compounds can directly interact with MTT (Ulukaya *et al.*, 2008). Many reports have revealed that several plant extracts, such as polyphenols (resveratrol) and flavonoids (quercetin, luteolin, kaempferol), strongly reduced MTT in the absence of living cells (Peng *et al.*, 2005; Weyermann *et al.*, 2005; Bruggisser *et al.*, 2002); (!!) Tested compounds could interfere with mitochondrial dehydrogenase activity and therefore lead to underestimation (inhibition of mitochondrial dehydrogenases) such as the cases of chloroquine, β-amyloid, and interferons (Ulukaya *et al.*, 2008; Weyermann *et al.*, 2005) or overestimation (activation of the MTT-reducing dehydrogenases) as shown for genistein in cell cultures (Liu *et al.*, 1997). As PI has been described manifold in literature to penetrate injured membranes and cell walls, the dye is often applied to distinguish living from dead microorganisms. The PI assay is based on the detection of the loss of cell membrane capability to exclude this dye. Early apoptotic cells exclude PI. Only necrotic and late apoptotic cells stain with PI. Thus, early apoptotic cells, although for all practical reasons they are dead (certainly reproductively dead), are recognized as live cells by this assay. In the absence of DNA replication the cells grow in size, including the increase in mitochondrial mass and activity. Such cells are are moribound - at certain degree of growth unbalance they are irreversibly commited to die. By the MTT assay, however, not only such cells are detectable as live, but with time one sees their increased capacity to reduce MTT. It may appear, therefore that cells in cultures proliferate (this can be seen for up to three days), whereas in fact they are reproductively dead. These findings constitute an important issue when researches carry out their experiments to evaluate the cytotoxic effect of natural and synthetic compounds for pharmaceutical and biological uses.

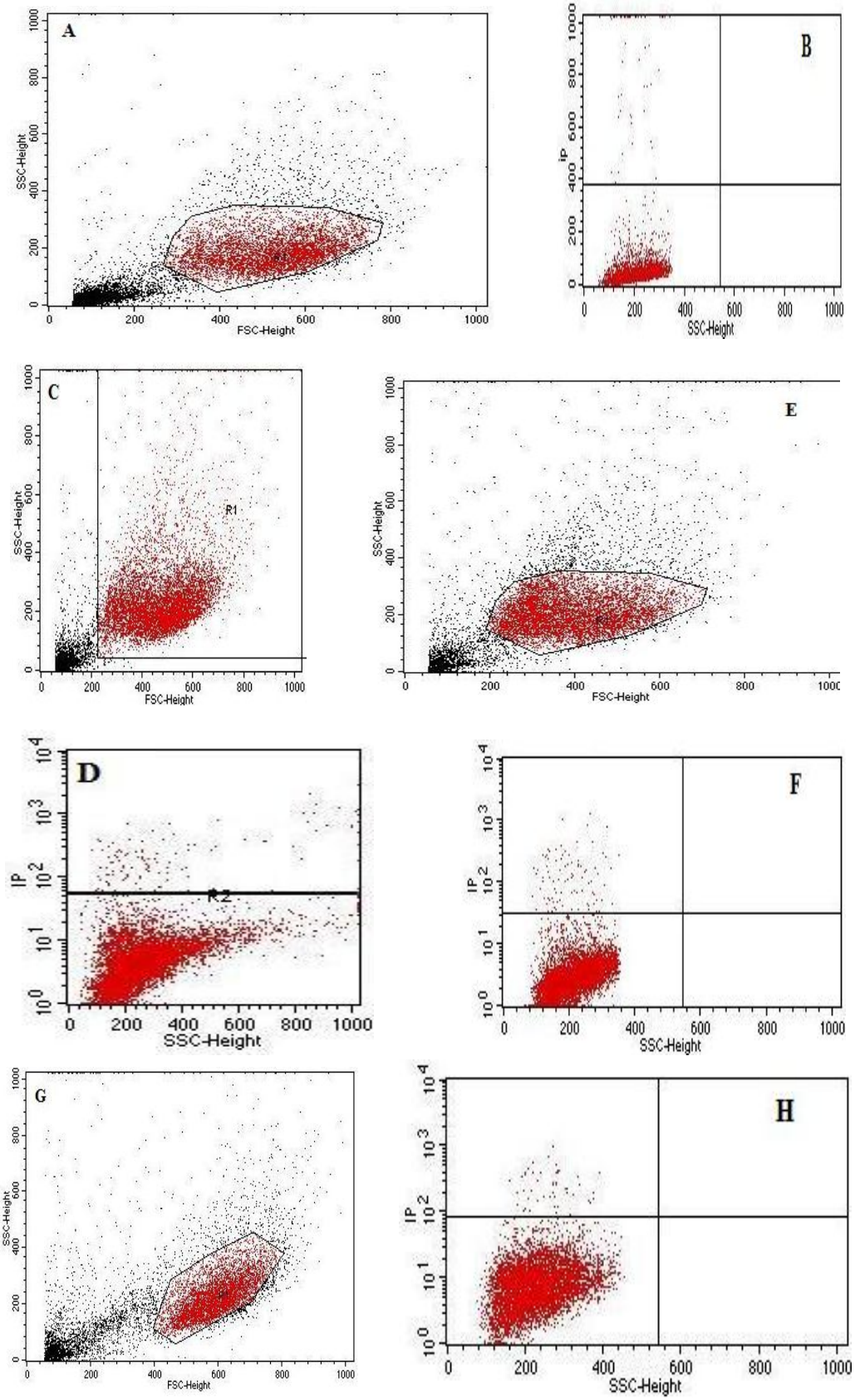

Fig. 3: assessment of cell viability by flow cytometry using PI dye.

CONCLUSION

To better evaluate the cytotoxic effect of natural compounds *in vitro*, several experiments need to be undertaken to overcome the problem of the underestimation or the overestimation of the cell death. In addition, for pharmaceutical and biological use of these natural products, there is a great need to carry out *in vivo* experiments on laboratory animal to better evaluate their cytotoxicity in interaction with cell metabolites followed by histological analysis. We need to mention here that there is no ideal analysis which can give perfect results, however the in vivo and in vitro results can make an acceptable approach to evaluate the cytotoxic effect of a natural compound.

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