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CENTRE D'ÉTUDE DE L'ÉNERGIE NUCLÉAIRE

Research Report : Study of the solubility of Duolife® and preliminary tests on its effect on human cancer cells.

May 15, 2006

Research performed within the Laboratory of Radiobiology and Microbiology with the contribution of :

Sarah Baatout, MSc, PhD
Jasmine Buset, lab technician
Mieke Neefs, lab technician
Hanane Derradji, Ir, PhD student

CONTENT

SUMMARY

Over the past 15 years, some potential anticarcinogenic and anti-inflammatory effects of antioxidants have been defined. Antioxidants are known to act as powerful free-radical scavengers. Free radicals are able to induce DNA strand breaks and oxidative modifications of DNA bases and are produced naturally in the cell following a stress or respiration. The present preliminary study was undertaken in order to explore whether the product Duolife containing antioxidants could enhance apoptosis (or programmed cell death) in cancerous cells. Duolife was first solubilised in water (for the extraction of water soluble components of the product) or in methanol (for the extraction of lipid soluble components of the product). Duolife was tested on two human cancerous cell lines: HELA (derived from cervix carcinoma) and IM-9 (derived from multiple myeloma) at various concentrations ranging from 0 to 500 µg/mL. The parameters investigated were cell proliferation, morphological changes and apoptosis.

HELA and IM-9 cells exposed to a water or a methanol extract of Duolife for 24 or 48 hours resulted in a significant enhancement of apoptosis correlated with a decrease of proliferation. All these effects were dose-dependent. In both cell lines, methanol extracts of Duolife induced more apoptosis than water extracts for the same concentrations. Taking into account the various parameters, IM-9 cells appeared as more sensitive to Duolife than the HELA cells. In conclusion, these preliminary results show that depending on the concentration and, to a lesser extent on the cell line, water and methanol extracts of Duolife can induce apoptosis in HELA and IM-9 cancerous cell lines.

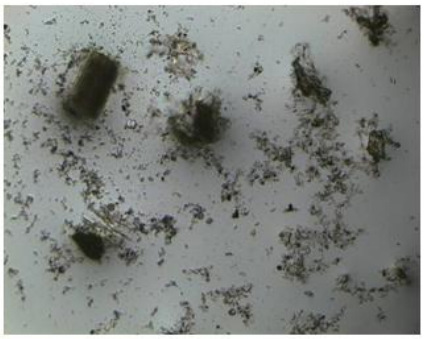
I – OPTIMISATION TESTS CONCERNING THE PREPARATION OF WATER AND METHANOL EXTRACTS OF VITENZA DUOLIFE.

Yellow and green tablets of Duolife were first grainded separately with a pestle and a mortar as represented in the figure below.

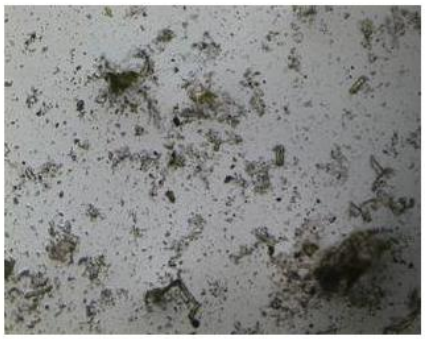
Solubility tests of Duolife in water (for water extraction) and in DMSO and in methanol (for lipid extraction) were performed. Various times of sonication (1, 2.5 and 5 minutes) were tested and a sonication of 5 minutes was needed to ensure that a maximum of Duolife was solubilised. The pictures hereunder illustrate the results of solubilisation of the suspension of Duolife in water, methanol and DMSO, respectively. In function of the time of sonication, the size of the particles decreased in the three conditions. Methanol was chosen for lipid extraction since it presented a better solubility thereafter in the culture medium.

Duolife : Water extracts after 1, 2.5 and 5 minutes of sonication

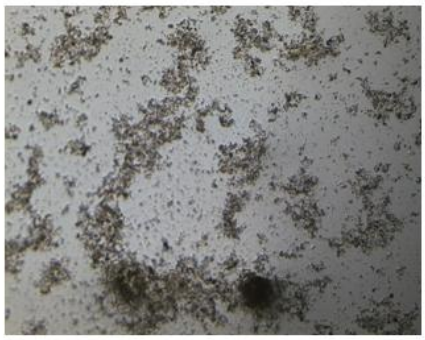
Sonication time : 1 minute



Sonication time : 2.5 minutes

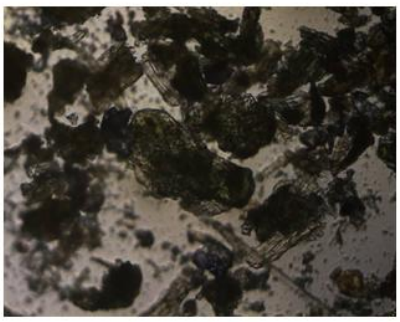


Sonication time : 5 minutes

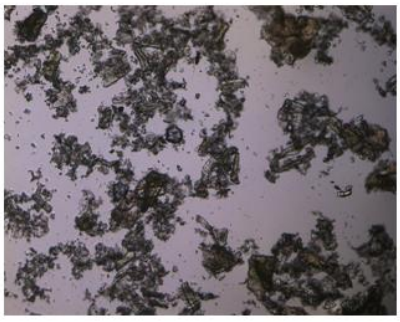


Duolife : Methanol extracts after 1, 2.5 and 5 minutes of sonication

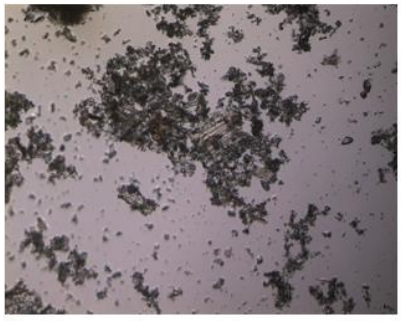
Sonication time : 1 minute



Sonication time : 2.5 minutes

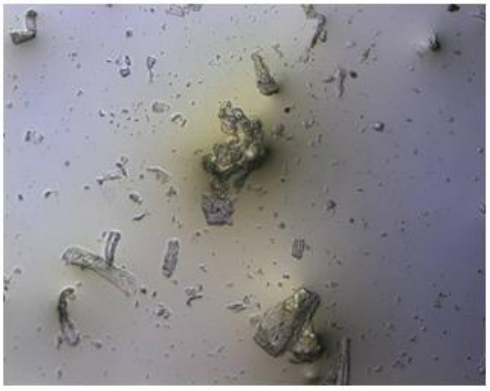


Sonication time : 5 minutes

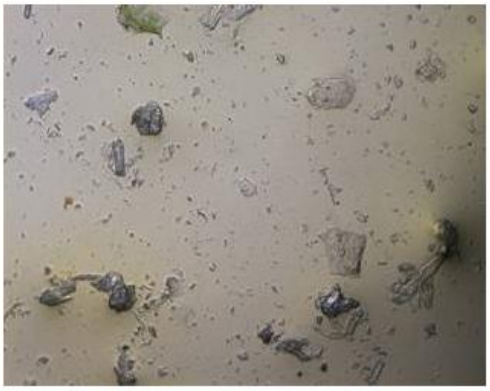


Duolife : DMSO extracts after 1, 2.5 and 5 minutes of sonication

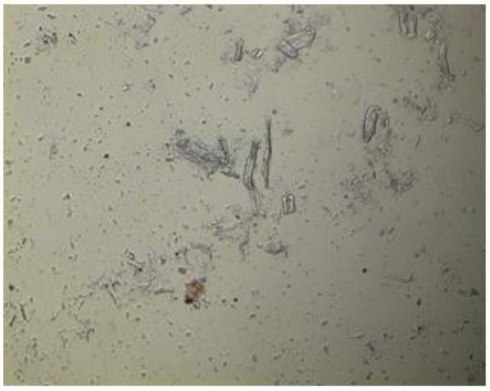
Sonication time : 1 minute



Sonication time : 2.5 minutes



Sonication time : 5 minutes



II – EFFECTS OF VITENZA DUOLIFE ON CULTURES OF HELA AND IM-9 CELL LINES.

II.1- HELA and IM-9 cell lines

The HELA cell line.

The HeLa cell (also Hela or hela cell) is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer in 1951. HeLa are considered "immortal": they do not die of old age and can divide an unlimited number of times as long as basic cell survival conditions are met (i.e. being maintained and sustained in a suitable environment). There are many strains of HeLa cells as they continue to evolve by being grown in cell cultures, but all HeLa cells are derived from the same tumour cells removed from Lacks. It has been estimated that the total mass of HeLa cells today far exceeds that of the rest of Henrietta Lacks' body.

The IM-9 cell line.

The IM-9 cell line is derived from B-lymphoblastoid cells from a bone marrow removed from a female patient with multiple myeloma in 1967. The cells synthesise IgG at a rate of 2.4ug/1,000,000 cells/day and have receptors for insulin, calcitonin and human growth hormone. Cells express bcl-2 mRNA. They also exhibit surface markers and receptor sites characterised of B lymphocytes. IM-9 cultures are single or clumped cells in suspension.

II.2- Culture conditions

HELA (cervix carcinoma) and IM-9 (multiple myeloma) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in DMEM in 175 cm^3 flasks from Falcon (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C in a humidified incubator containing 5% CO_2 in air. The DMEM medium was supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were sub-cultured at 1:3 ratio for routine maintenance and experiments were performed twice a week.

Exponentially growing HELA cells were plated overnight into 24-well culture plates (10^5 cells/cm²) containing supplemented DMEM medium to allow for cell attachment. Exponentially growing IM-9 cells, which do not require cell attachment, were plated at 10^5 cells/cm²/ml just before Duolife extracts.

Fresh medium (5 ml/well) containing different concentrations of Duolife (from 0 to 500 µg/mL) was added and cells were further cultured for 1 or 2 days. HELA cells were harvested by trypsinisation (trypsin 0.25% and EDTA 0.11 % from Life Technologies) and washed with PBS. Adherent and non-adherent HELA cells were then pooled. IM-9 are growing in suspension and were then harvested with PBS.

II.3- Cell counting

The numbers of viable cells were evaluated at the beginning of exposure to Duolife as well as after 1 or 2 days using flow cytometry and beads.

Flow cytometry equipment and sample preparation.

Counting were performed on the flow cytometer EPICS XL MCL (Coulter-Immunotech Diagnostics) equipped with single air-cooled argon laser with an excitation line at 488 nm. System II software was used for data acquisition and data evaluation in list mode.

Flow Count Fluorospheres.

Flow Count fluorospheres (Coulter-Immunotech) consist of polystyrene spheres with a diameter of 6 µm. They are resuspended in an aqueous medium containing a surfactant and 1% formaldehyde. Each fluorosphere contains fluorescent dyes with a broad emission spectrum of 510-700 nm when excited at 488 nm by an argon laser. The assayed concentration of Flow Count fluorospheres is enclosed to each lot at delivery. Fluorospheres were warmed up to room temperature without any exposure to strong light and properly vortexed. Fluorospheres were used without any preparation or dilution step. The pipette was calibrated before use and pipetting was done in single steps with an accurate and precise

technique without repeater or reverse-pipetting strategies. Excessive resuspension of fluorospheres by high-speed vortexing was avoided to minimize the formation of air bubbles and foam. Flow Count fluorospheres were added in a volume (20µL) to each cell sample (1000 µL) with the use of the same pipetting procedure, then vortexed again for 5s to 50% of tube height immediately before flow cytometric analysis. The flow rate was always set to "medium" to ensure constant processing.

Flow Cytometric Analysis.

The flow cytometer settings were established for logarithmic amplification of light scatter and fluorescence channels. The cells were separated on the basis of their size (forward-angle light scatter) and their internal structure (side-angle light scatter). The threshold was defined in the forward scatter to exclude cell debris. A gate was set to include all cells. The principle of the Flow Count fluorospheres is based on the precise mixing of these microparticles, of which both concentration and volume are known, with any one already prepared sample of identical volume to determine the concentration of the cell population of interest by establishing a ratio of cells to fluorospheres. The concentration of the target events is calculated using the following formula:

$$\frac{\text{Cells}}{\mu\text{L}} = \frac{\text{Total number of target cells}}{\text{Total number of fluorospheres}} \times \frac{\text{Fluorospheres}}{\mu\text{L}}$$

The correct assayed concentration of fluorospheres has been applied as a calibration factor in the software program, and the EPICS XL flow cytometer calculated the absolute count for the specimen automatically. Results were reported on histogram printouts.

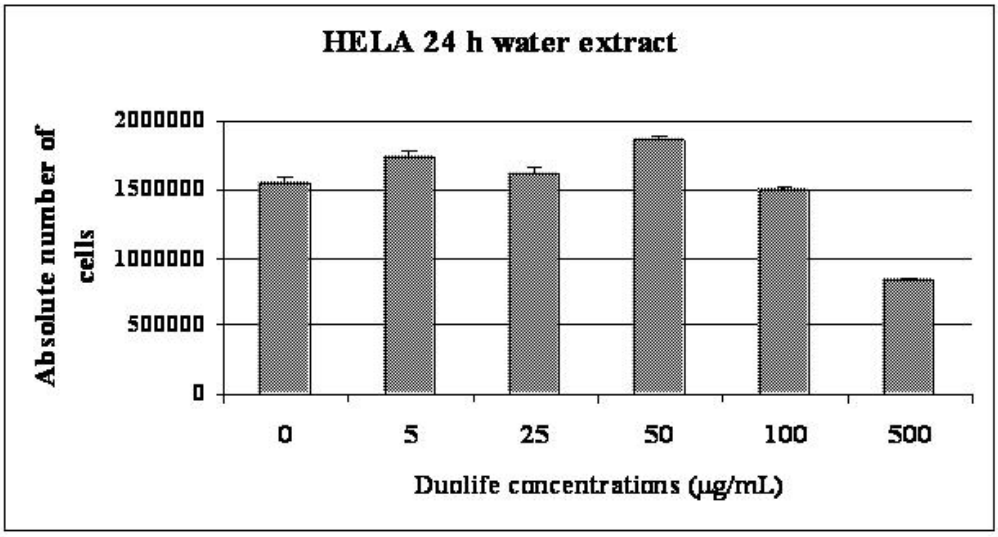
Results.

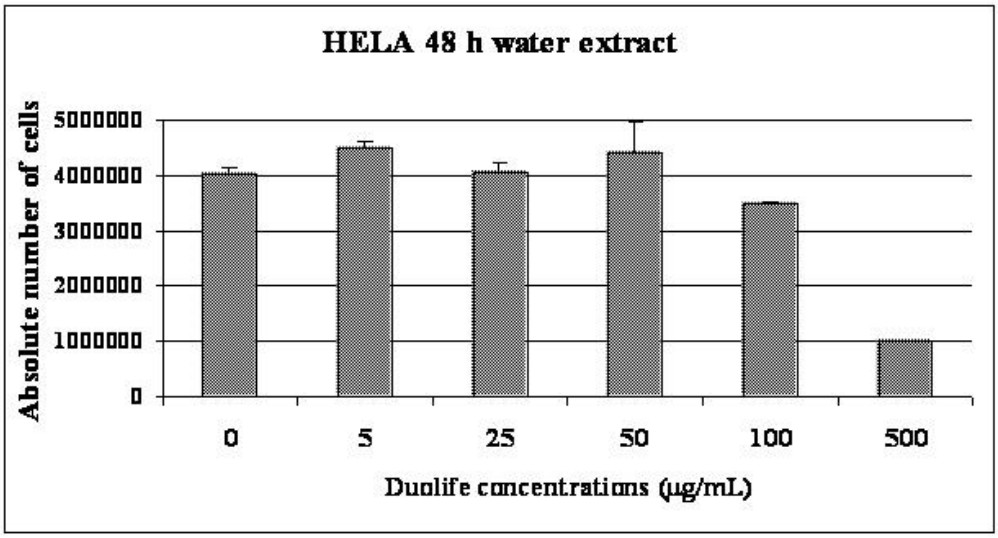
Hereunder are shown the results of the effect of **water** extracts of Duolife in **HELA** cells after 24 (top) and 48 (bottom) hours of culture. HELA cells were treated with Duolife at various concentrations: 0 (control), 5, 25, 50, 100 and 500 µg/mL. The number of viable cells was

counted. The means for four independent experiments ± SD are shown. A significant reduction in the number of cells is observed after 24 hours at a concentration of 500 µg/mL Duolife. This effect is even emphasized at 48 hours with a significant reduction of the number of cells from 100 µg/mL onwards.

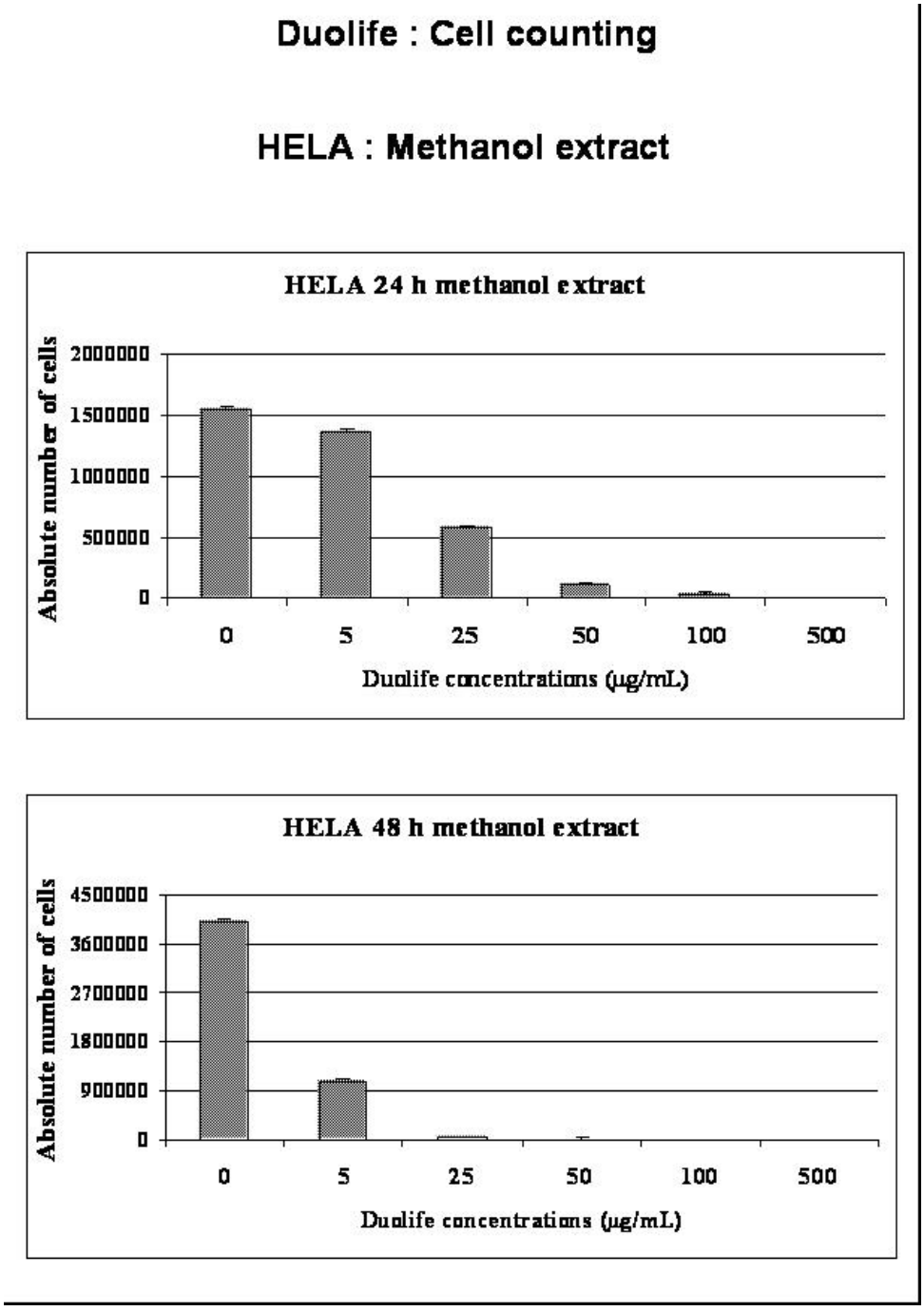
Duolife : Cell counting

HELA : Water extract

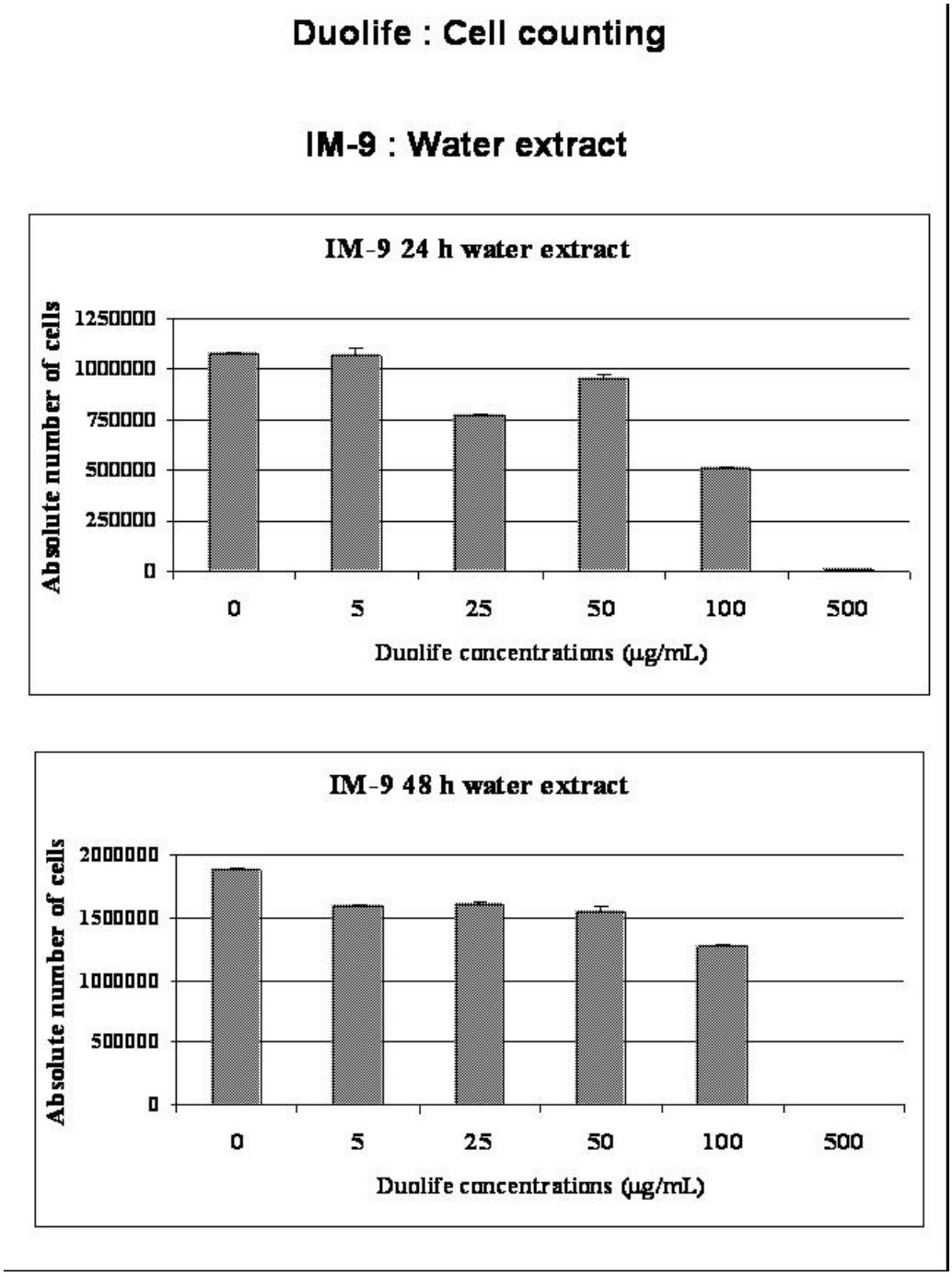




Hereunder are shown the results of the effect of **methanol** extracts of Duolife in **HELA** cells after 24 (top) and 48 (bottom) hours of culture. HELA cells were treated with methanol extracts of Duolife at various concentrations: 0 (control), 5, 25, 50, 100 and 500 µg/mL. The number of viable cells was counted. The means for four independent experiments ± SD are shown. In comparison with the effect of the water extracts of Duolife, a significant reduction in the number of cells was already observed after 24 hours from a concentration of 5 µg/mL methanol extracts of Duolife onwards. This effect is even emphasized at 48 hours.



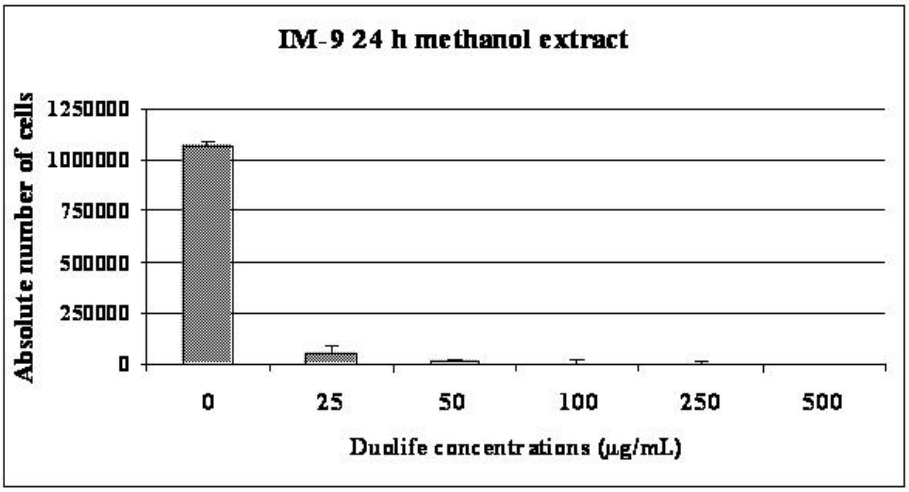
Hereunder are shown the results of the effect of **water** extracts of Duolife in **IM-9** cells after 24 (top) and 48 (bottom) hours of culture. IM-9 cells were treated with Duolife at various concentrations: 0 (control), 5, 25, 50, 100 and 500 µg/mL. The number of viable cells was counted. The means for four independent experiments ± SD are shown. A significant reduction in the number of cells is observed after 24 hours at a concentration of 25 µg/mL Duolife. This effect is even emphasized at 48 hours with a significant reduction of the number of cells from 5 µg/mL onwards.

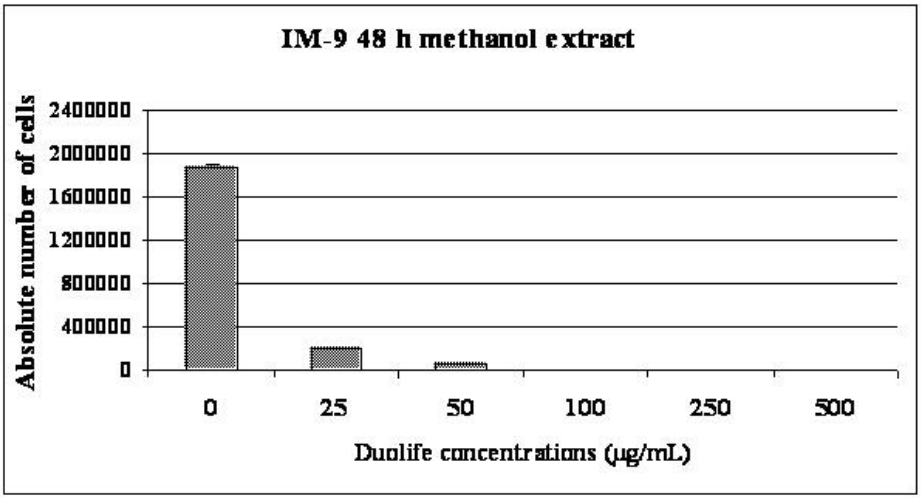


Hereunder are shown the results of the effect of **methanol** extracts of Duolife in **IM-9** cells after 24 (top) and 48 (bottom) hours of culture. IM-9 cells were treated with methanol extracts of Duolife at various concentrations: 0 (control), 5, 25, 50, 100 and 500 µg/mL. The number of viable cells was counted. The means for four independent experiments ± SD are shown. In comparison with the effect of the water extracts of Duolife, a significant reduction in the number of cells was already observed after 24 hours from a concentration of 5 µg/mL methanol extracts of Duolife onwards. This effect is even emphasized at 48 hours.

Duolife : Cell counting

IM-9 : Methanol extract





II.4- Cell morphology

Apoptosis.

In biology, apoptosis (from the Greek words *apo* = from and *ptosis* = falling) is one of the main types of programmed cell death (PCD). As such, it is a process of deliberate life relinquishment by an unwanted cell in a multicellular organism. In contrast to necrosis, which is a form of cell death that results from acute cellular injury, apoptosis is carried out in an ordered process that generally confers advantages during an organism's life cycle. For example, the differentiation of human fingers in a developing embryo requires the cells between the fingers to initiate apoptosis so that the fingers can separate. The way the apoptotic process is executed facilitates the safe disposal of cell corpses and fragments.

Since the beginning of the 1990s, research on apoptosis has grown spectacularly. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. *Too much* apoptosis causes cell-loss disorders, whereas *too little* results in uncontrolled cell proliferation, namely cancerous tumors.

Not all forms of PCD share the characteristic shapes (the morphology) and sequences of apoptosis, but all types of PCD are highly-regulated processes

Morphology : apoptotic process.

A cell undergoing apoptosis shows a characteristic morphology that can be seen under a microscope:

1. The cell becomes round (circular). This occurs because the protein structures that conform the cytoskeleton are digested by specialized peptidases (called *caspases*) that have been activated inside the cell.
2. Chromatin (DNA and its packaging proteins in the cell nucleus) undergoes initial degradation and condensation (see the article by Madeleine Kihlmark *et al.*).
3. Chromatin undergoes further condensation into compact patches against the nuclear envelope. At this stage, the double membrane that surrounds the nucleus still appears complete; however, as observed by Kihlmark and colleagues, specialized caspases have

already advanced in the degradation of nuclear pore proteins and have begun to degrade the lamin that underlies the nuclear envelope. It must be noted, also, that, while the previous stage of initial chromatin condensation has been observed in nonapoptotic forms of programmed cell death, this advanced stage (called pyknosis) is considered a hallmark of apoptosis.

4. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented (a process referred to as karyorrhexis). The nucleus breaks into several discrete *chromatin bodies* or *nucleosomal units* due to the degradation of DNA.

5. Plasma membrane blebbings

6. The cell is phagocytosed, *or*

7. The cell breaks apart into several vesicles called *apoptotic bodies*, which are then phagocytosed.

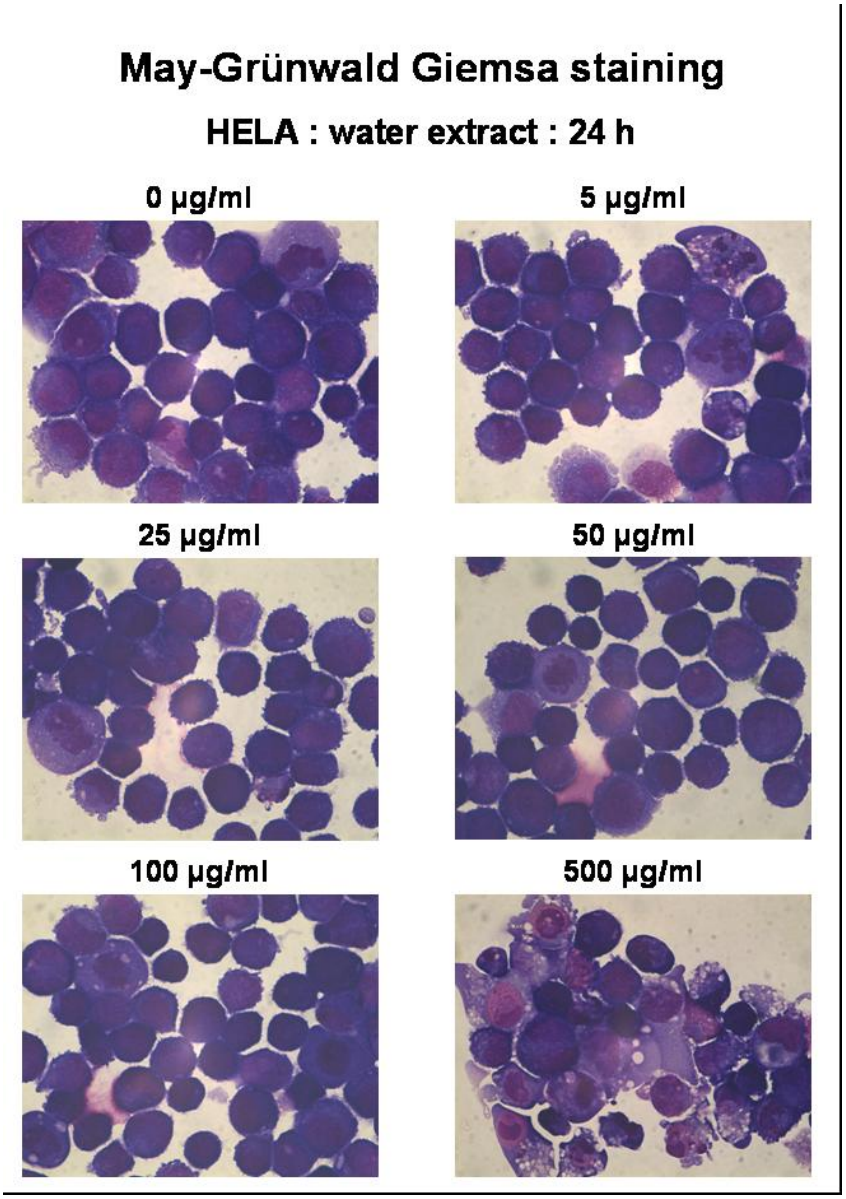
Staining

May-Grünwald Giemsa stain is a classical blood film stain for peripheral blood smears and bone marrow specimens. Erythrocytes stain pink, platelets show a light pale pink, lymphocyte cytoplasm stains sky blue, monocyte cytoplasm stains pale blue, and leukocyte nuclear chromatin stains magenta. May-Grünwald Giemsa stain is a mixture of methylene blue and eosin. It is prepared from commercially available powder.

For morphological observations, cells were first centrifuged on cytospin slides. Then, they were fixed with methanol absolute (5 min), stained with May-Grünwald (5 min in a mixing solution of 50% May-Grünwald and 50% buffer pH 6.2) and then with Giemsa (5 min in 16.6% of Giemsa and 83.4% of buffer pH 6.7). Buffer pH 6.2 consists of 1.9% (v/v) of Na_2HPO_4 (6 x 10^{-2} M) and 8.1% of KH_2PO_4 (6 x 10^{-2} M). Buffer pH 6.7 consists of 3.6 % Na_2HPO_4 (6 x 10^{-2} M) and 6.4% KH_2PO_4 (6 x 10^{-2} M). Cells were then rinsed in buffer pH 6.7 for 15 sec and in distilled water for 30 sec and dried. Cells were mounted with a glass coverslip with DePeX (EMS, Fort Washington, PA, USA) and analysed under a Zeiss microscope (Axioskop) and pictures were taken at 400x magnification.

Results

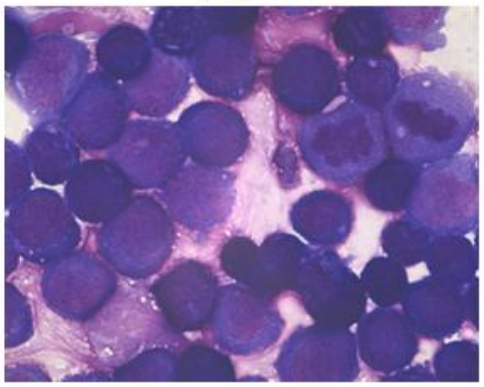
Hereunder is shown the morphology of **HELA** cells 24 or 48 hours after incubation with **water** extracts of Duolife at concentrations from 0 to 500 µg/ml. Cells were stained with May-Grünwald Giemsa. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells contained a single nucleus rounded in shape. Phenotypically, apoptosis is characterised by cell shrinkage, chromatin compaction, nuclear blebbing and collapse of the nucleus into small intact fragments (apoptotic bodies). In control conditions, apoptotic cells were only sporadically visible in the cell line. In comparison with control conditions, the treatment with high concentrations of water extracts of Duolife induced an increasing number of apoptotic cells with the presence of a high number of cell debris in the cell line.



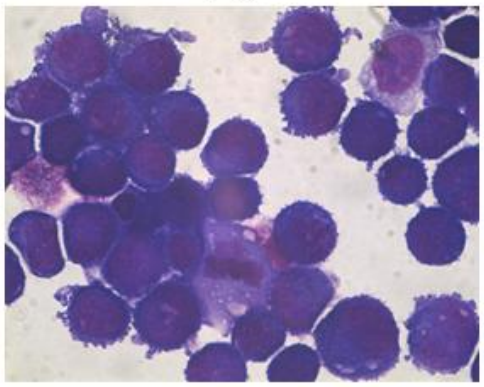
May-Grünwald Giemsa staining

HELA : water extract : 48 h

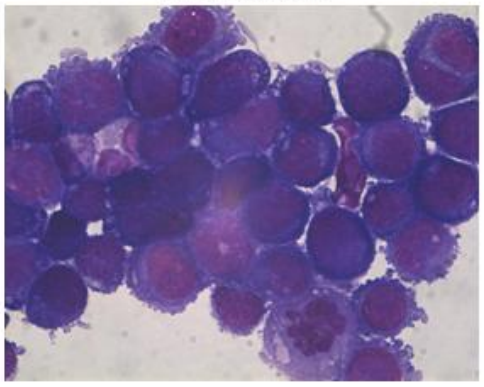
0 µg/ml



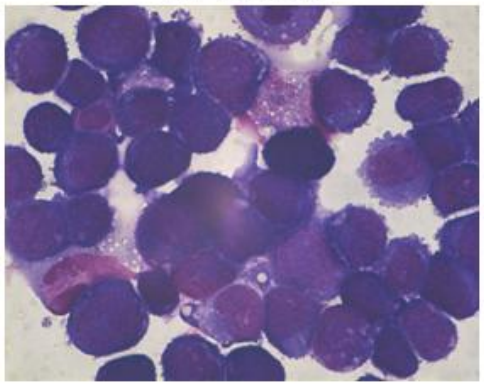
5 µg/ml



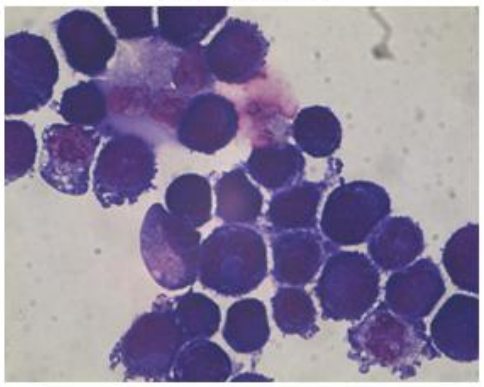
25 µg/ml



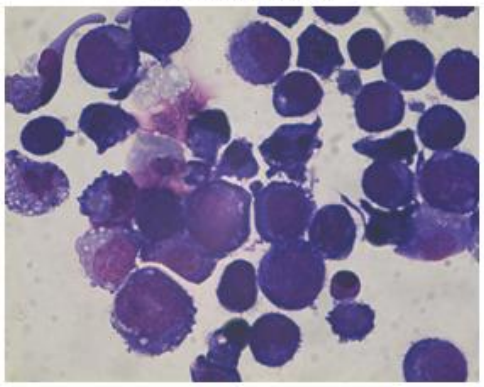
50 µg/ml



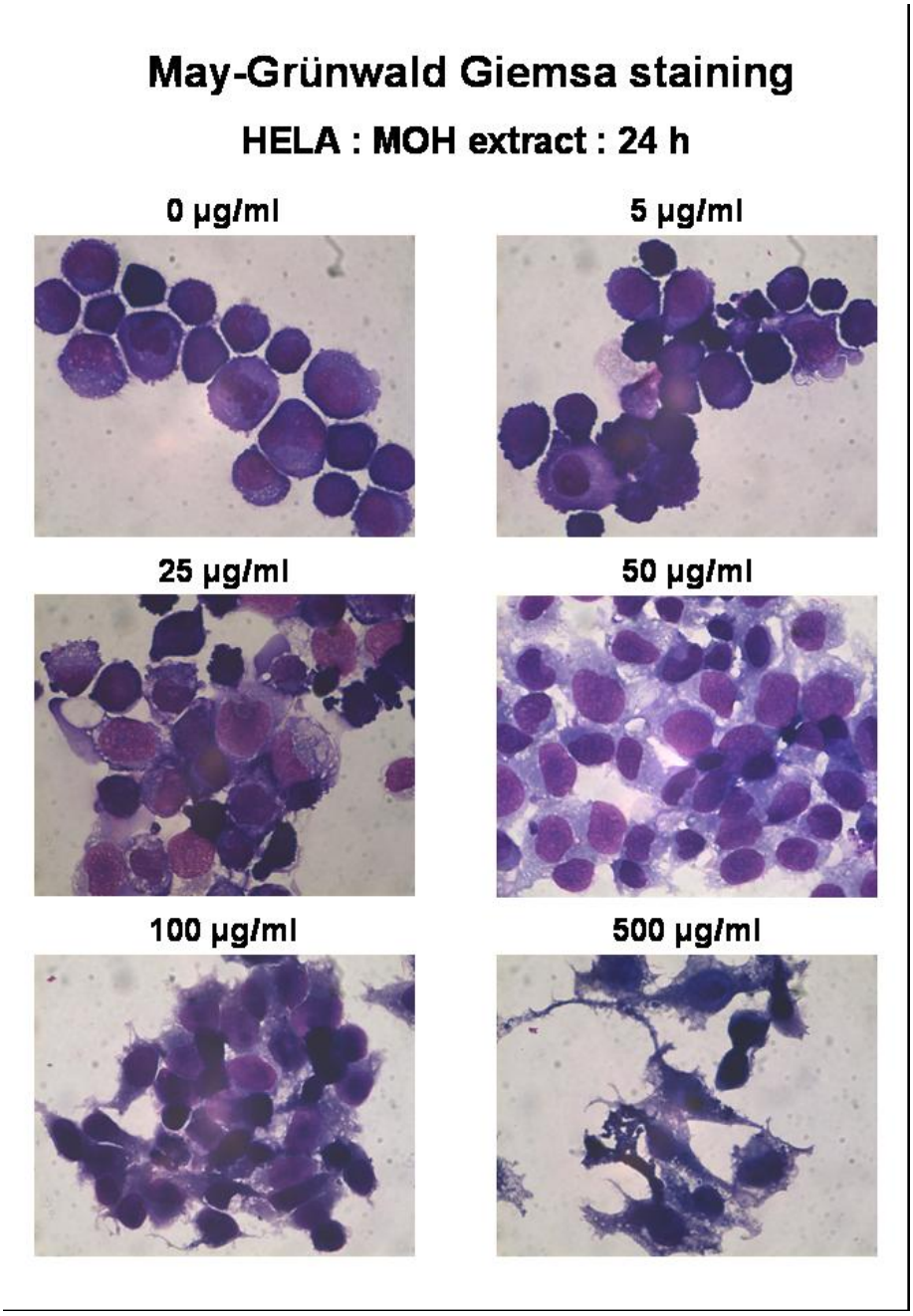
100 µg/ml



500 µg/ml



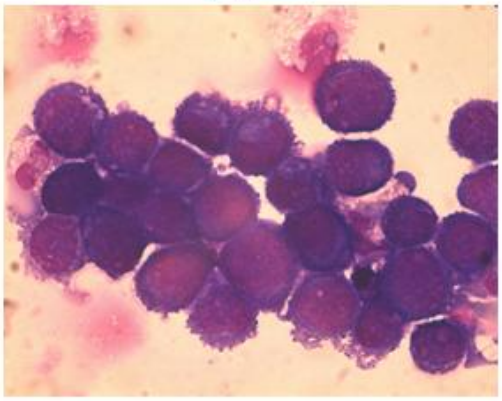
Hereunder is shown the morphology of **HELA** cells 24 or 48 hours after incubation with **methanol** extracts of Duolife at concentrations from 0 to 500 µg/ml. Cells were stained with May-Grünwald Giemsa. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells contained a single nucleus rounded in shape. In control conditions, apoptotic cells were only sporadically visible in the cell line. In comparison with control conditions, the treatment with concentrations of methanol extracts of Duolife higher than 50 ug/mL induced an increasing number of apoptotic cells with the presence of a high number of cell debris in the cell line.



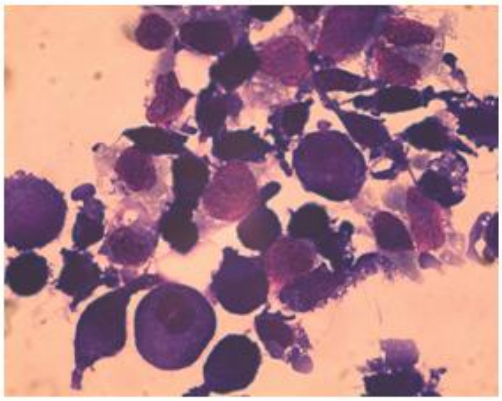
May-Grünwald Giemsa staining

HELA : MOH extract : 48 h

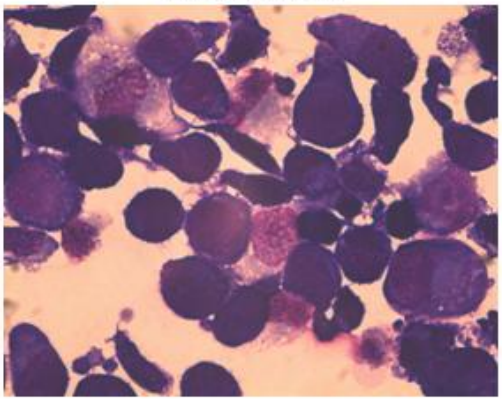
0 µg/ml



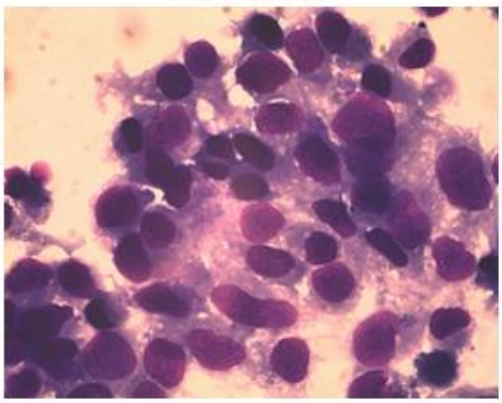
5 µg/ml



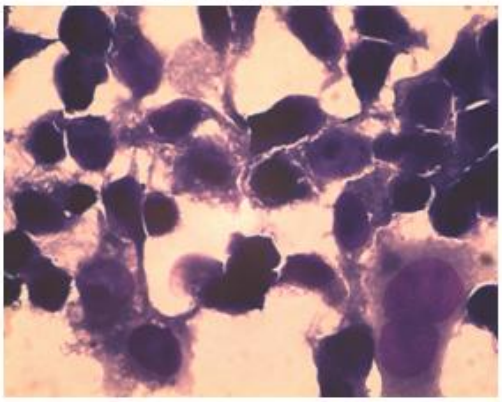
25 µg/ml



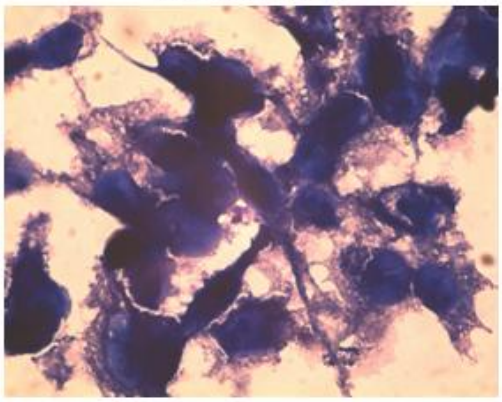
50 µg/ml



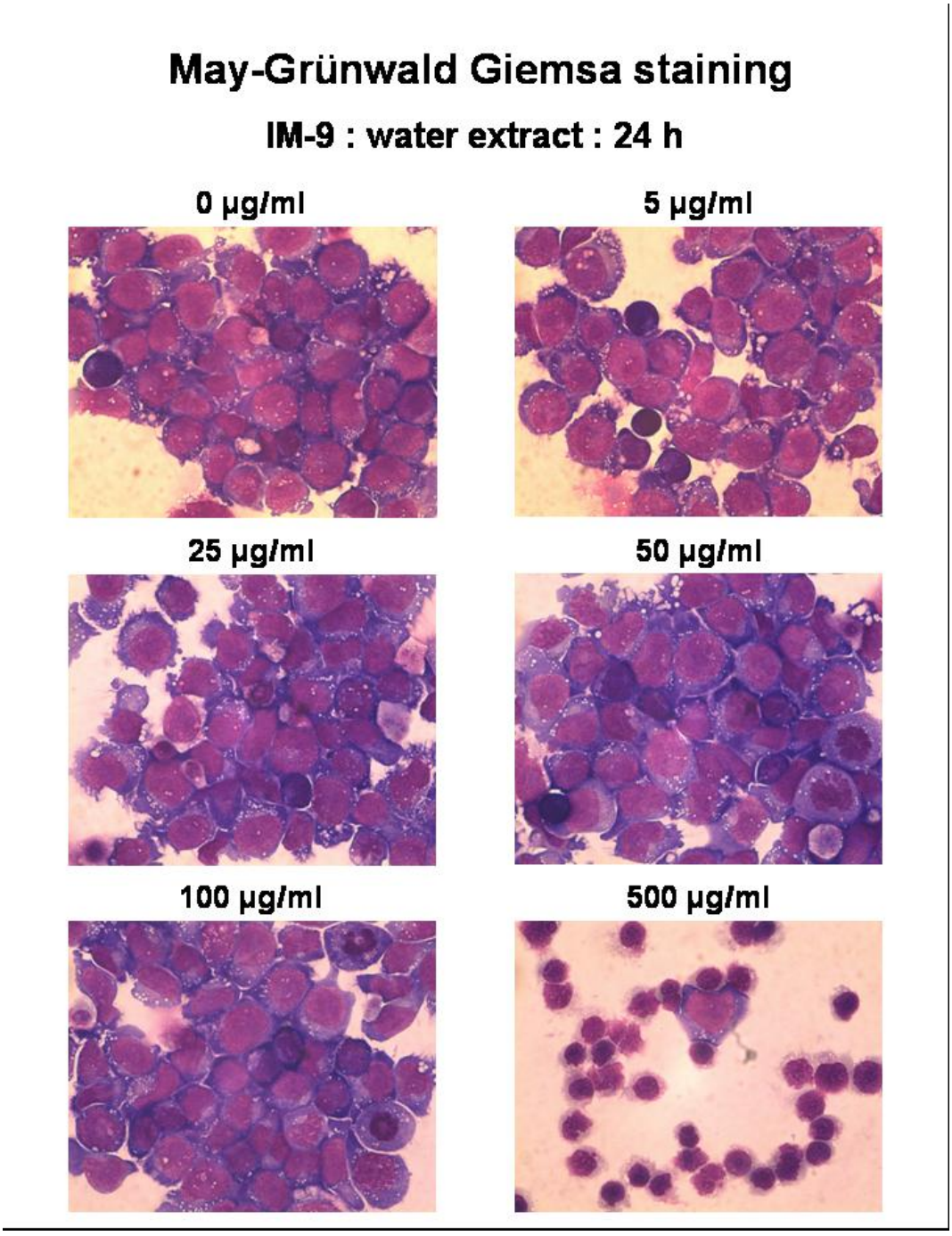
100 µg/ml



500 µg/ml



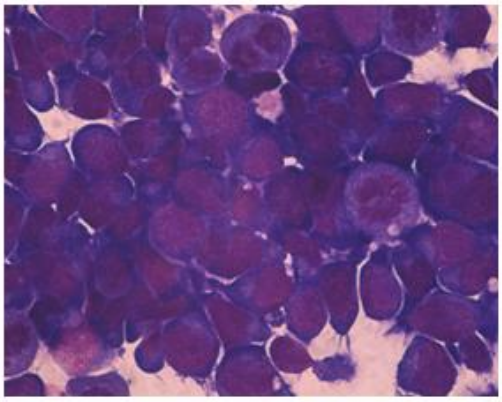
Hereunder is shown the morphology of **IM-9** cells 24 or 48 hours after incubation with **water** extracts of Duolife at concentrations from 0 to 500 µg/ml. In control cultures (0 µg/mL), cells contained a single nucleus rounded in shape and apoptotic cells were only sporadically visible in the cell line. In comparison with control conditions, the treatment with a concentration of 500 µg/mL water extracts of Duolife induced an increasing number of apoptotic cells with the presence of a high number of cell debris in the cell line.



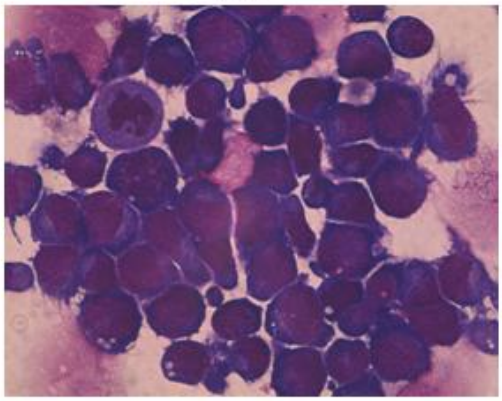
May-Grünwald Giemsa staining

IM-9 : water extract : 48 h

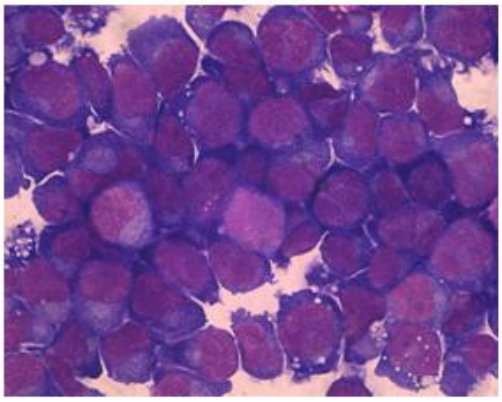
0 µg/ml



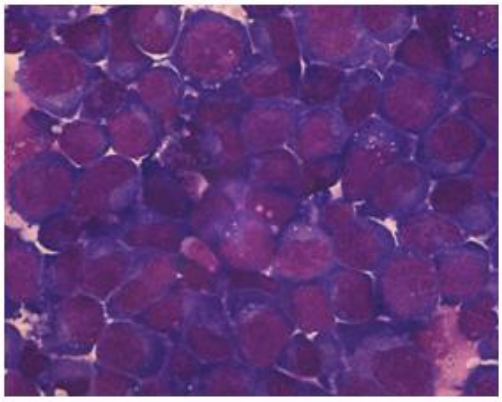
5 µg/ml



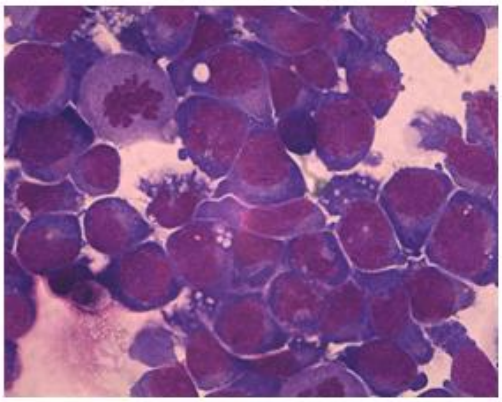
25 µg/ml



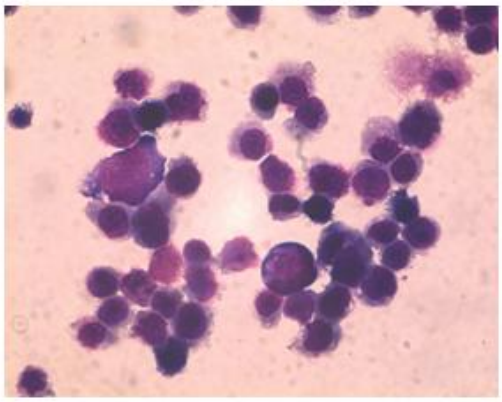
50 µg/ml



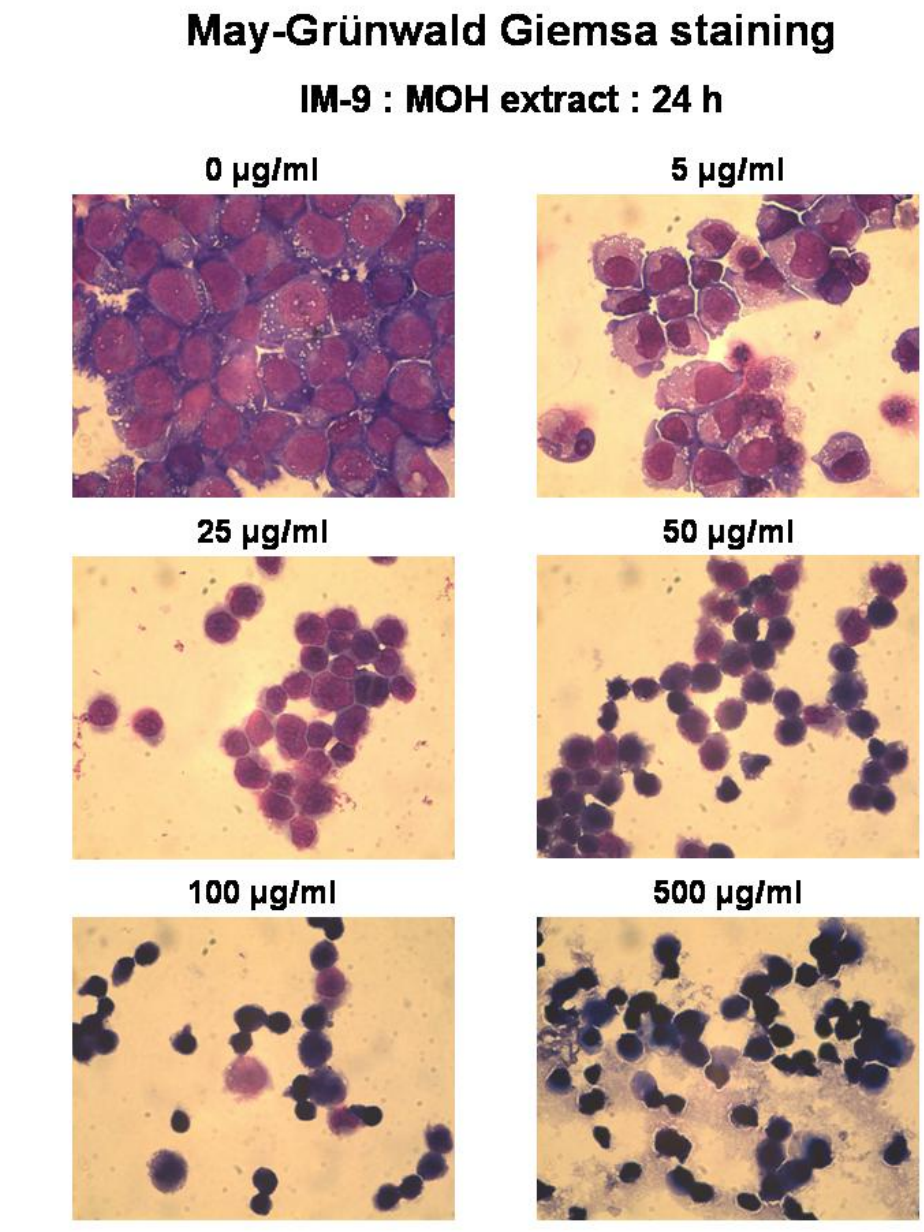
100 µg/ml



500 µg/ml



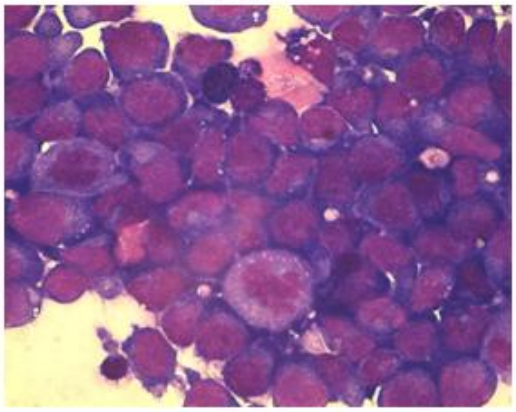
Hereunder is shown the morphology of **IM-9** cells 24 or 48 hours after incubation with **methanol** extracts of Duolife at concentrations from 0 to 500 µg/ml. Cells were stained with May-Grünwald Giemsa. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells contained a single nucleus rounded in shape. In control conditions, apoptotic cells were only sporadically visible in the cell line. In comparison with control conditions, the treatment with concentrations of methanol extracts of Duolife higher than 5 ug/mL induced an increasing number of apoptotic cells with the presence of a high number of cell debris in the cell line.



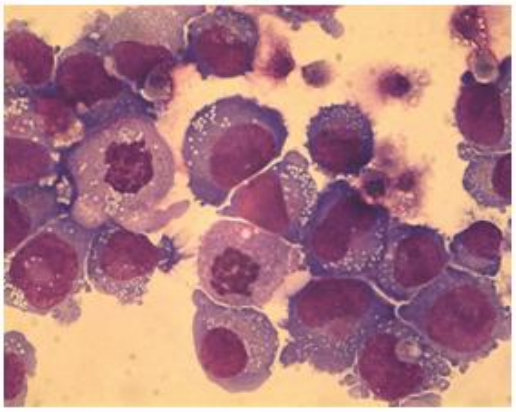
May-Grünwald Giemsa staining

IM-9 : MOH extract : 48 h

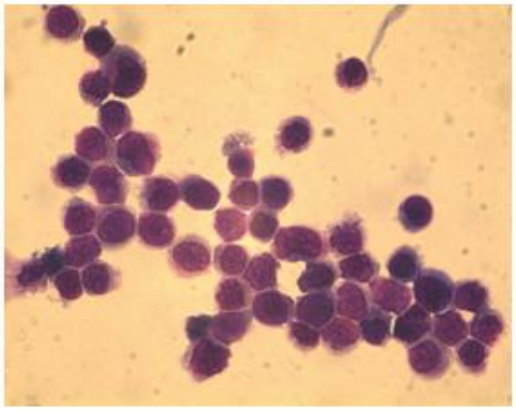
0 µg/ml



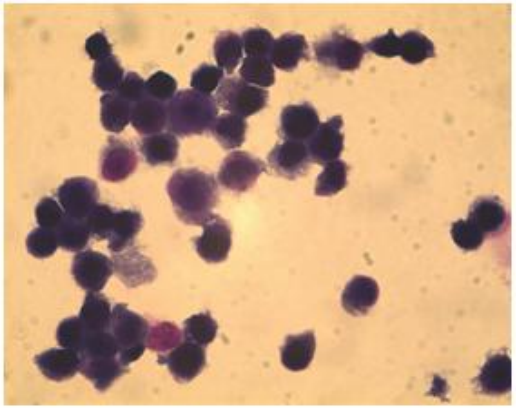
5 µg/ml



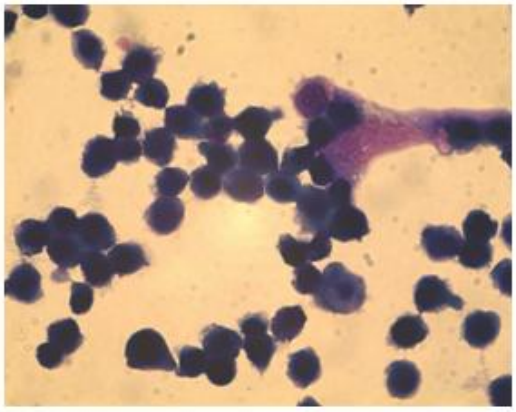
25 µg/ml



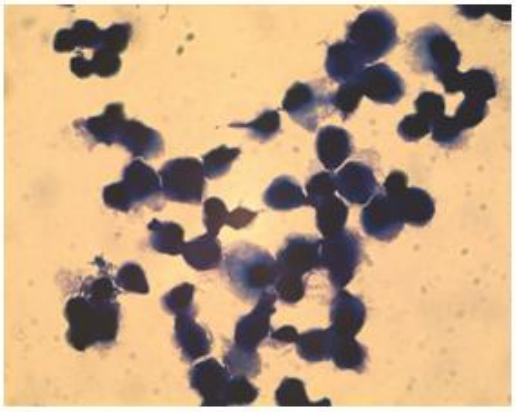
50 µg/ml



100 µg/ml



500 µg/ml



II.5- Double staining AO/PI

Chromatin condensation associated to apoptosis and membrane permeability loss associated to late apoptosis and necrosis can be detected using fluorescent dyes that bind DNA.
One such dye, acridine orange (AO), is a vital dye that stains both viable and dead cells. Acridine Orange (3,6-dimethylaminoacridine) is a nucleic acid selective metachromatic stain useful for cell cycle determination. It is a DNA and RNA binding compound due to its intercalation abilities.
Propidium iodide (PI) stains cells which have lost membrane permeability and therefore stain late apoptotic cells undergoing secondary necrosis. Propidium iodide (or PI) is a fluorescent biomolecule that can be used to stain DNA. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. It can be used to differentiate necrotic, apoptotic and normal cells.

Acridine orange stained cells appear green under a wide band fluorescein (FITC) filter (520-700nm), PI stained cells appear red under a wide band FITC filter, PI fluorescence can be visualised with AO fluorescence simultaneously.

Fluorescence microscopy

A mixture of acridine orange and ethidium bromide (both purchased from Sigma and dissolved at 2 µg/ml of PBS) is added to the culture medium of unfixed cells, and the cells are viewed immediately under a wide band fluorescein filter and photographed immediately.

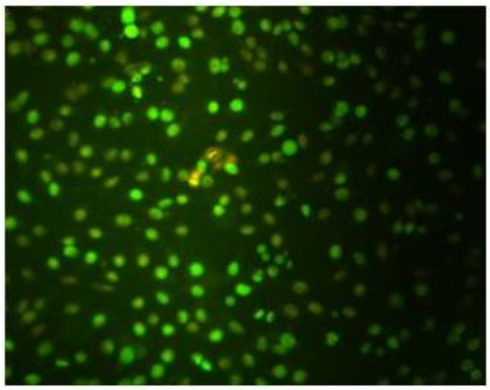
Results

Hereunder are shown **HELA** cells 24 or 48 hours after incubation with **water** extracts of Duolife at concentrations from 0 to 500 µg/ml, the cells being stained with acridine orange/propidium iodide. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells are healthy and therefore all appear green. The treatment with concentrations of water extracts of Duolife at 500 µg/mL induced a slight increase in the number of apoptotic cells with the appearance of some red cells. The effect of Duolife was even increased after 48 hours.

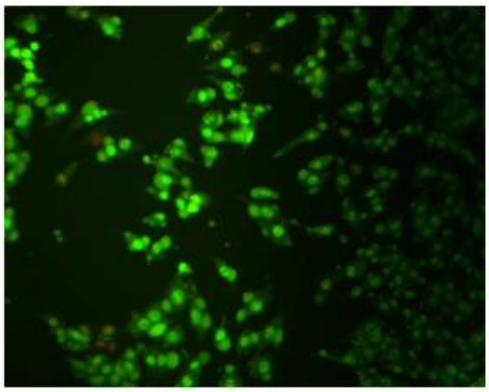
Duolife : Double staining AO/PI

HELA : 24 hours Water extract

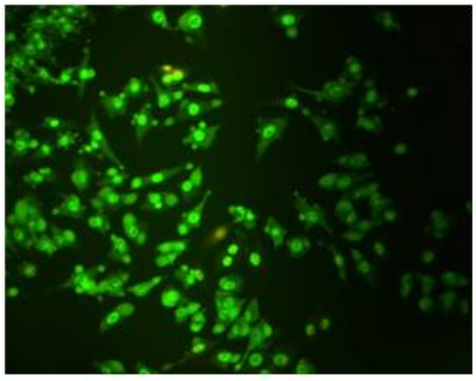
0 µg/mL



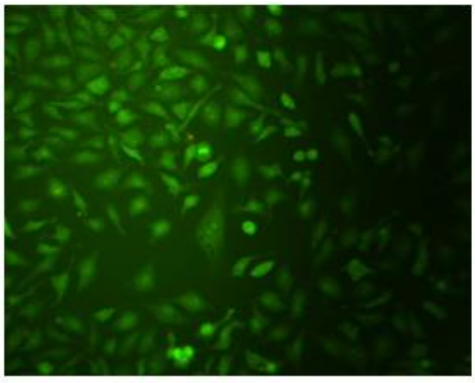
5 µg/mL



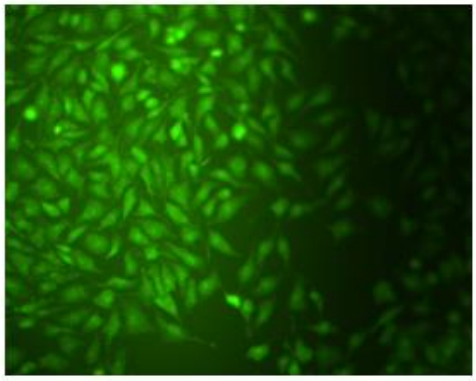
25 µg/mL



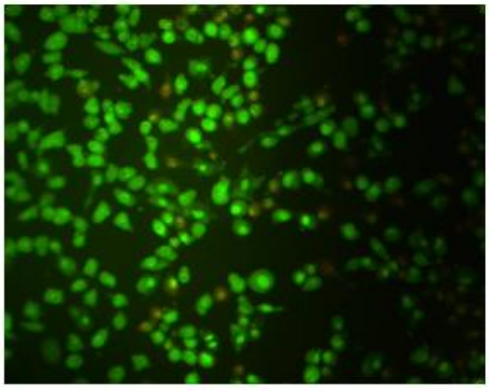
50 µg/mL



100 µg/mL



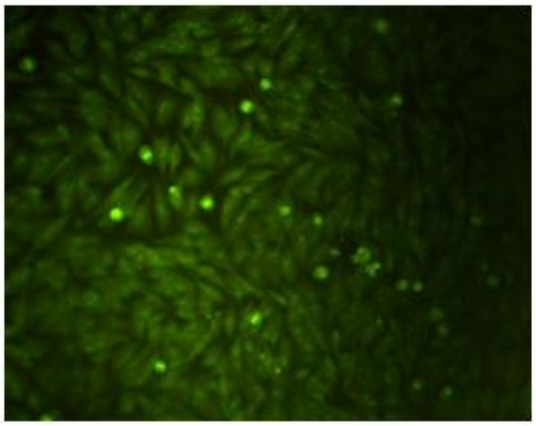
500 µg/mL



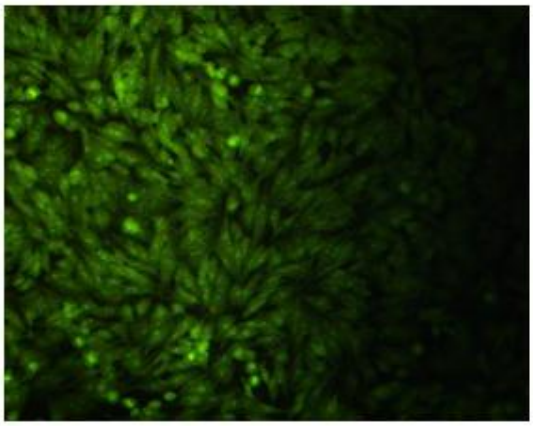
Duolife : Double staining AO/PI

HELA : 48 hours Water extract

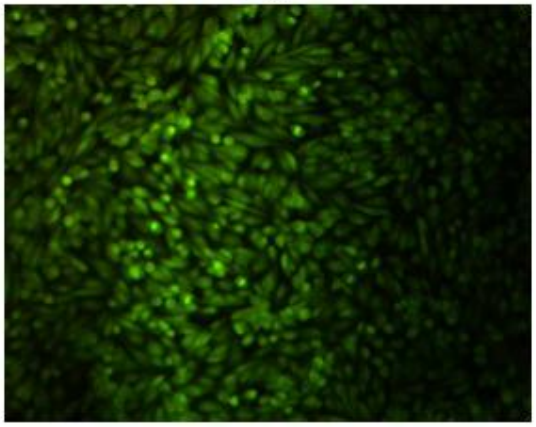
0 µg/mL



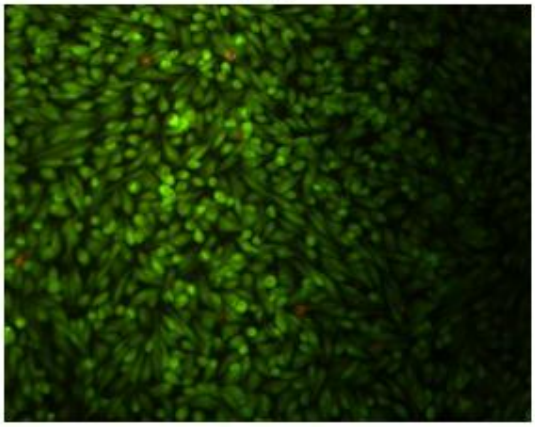
5 µg/mL



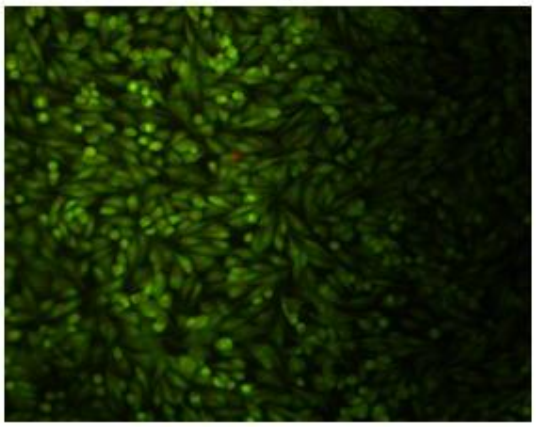
25 µg/mL



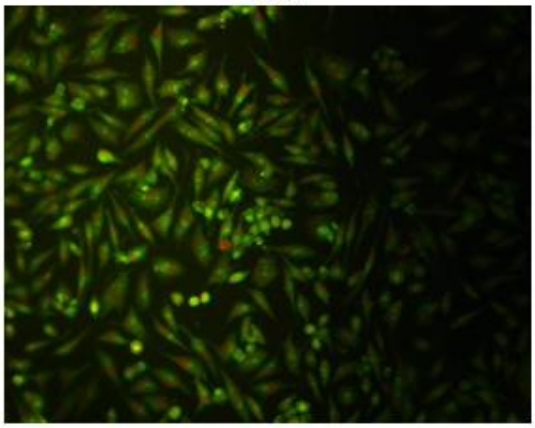
50 µg/mL



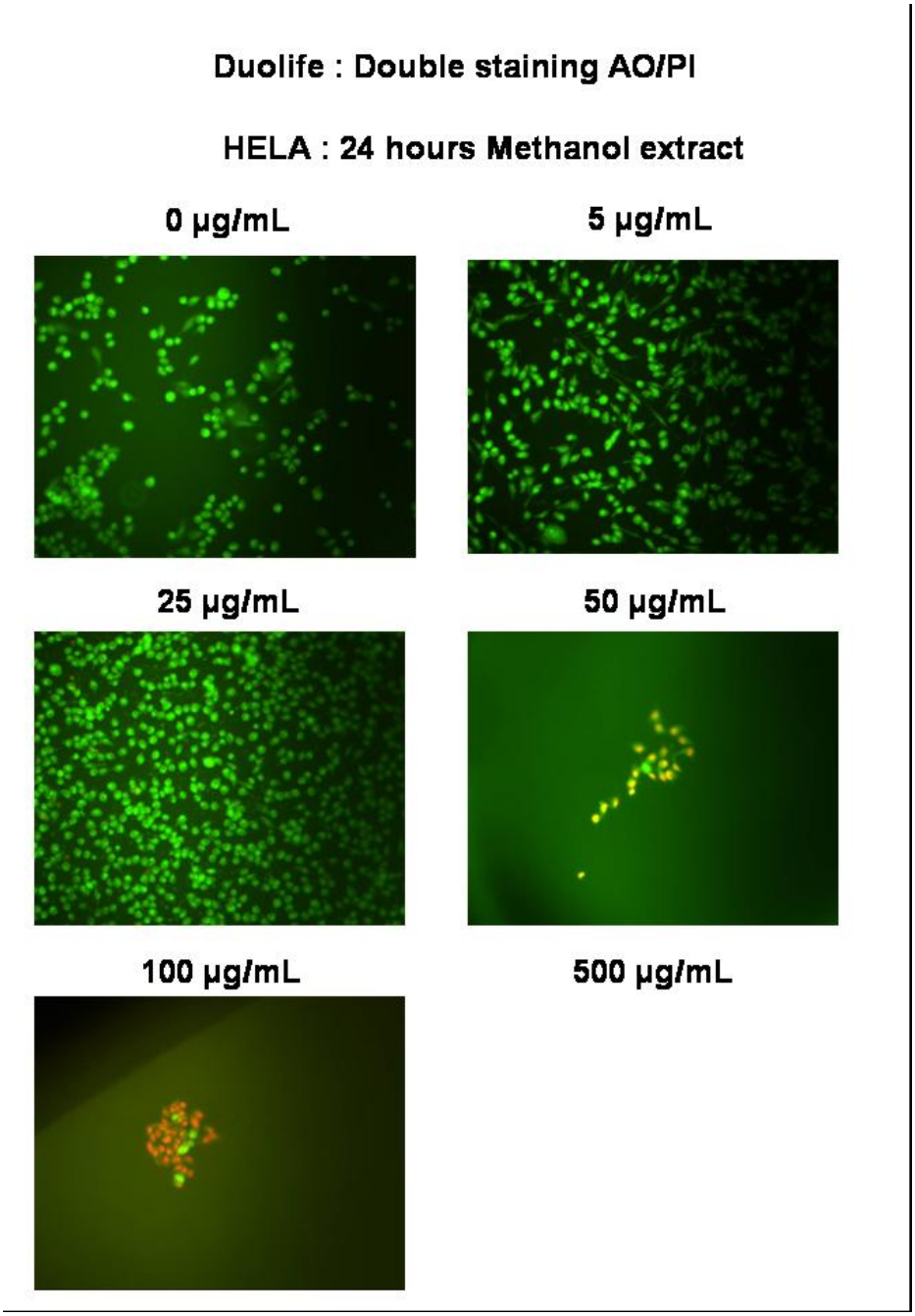
100 µg/mL



500 µg/mL



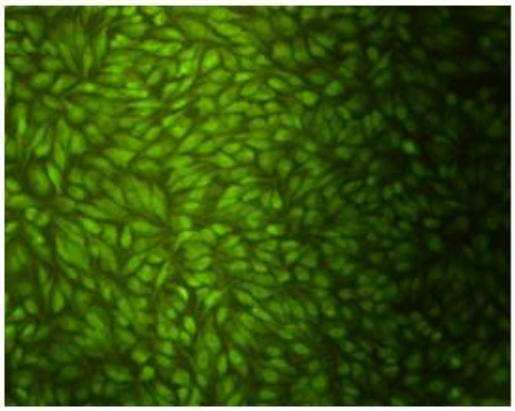
Hereunder are shown **HELA** cells 24 or 48 hours after incubation with **methanol** extracts of Duolife at concentrations from 0 to 500 µg/ml, the cells being stained with acridine orange/propidium iodide. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells are healthy and therefore all appear green. The treatment with concentrations of methanol extracts of Duolife at 5 µg/mL induced a slight increase in the number of apoptotic cells with the appearance of some red cells. The effect of Duolife was even increased after 48 hours.



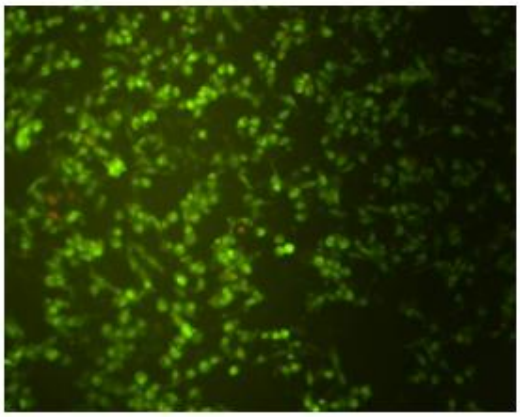
Duolife : Double staining AO/PI

HELA : 48 hours Methanol extract

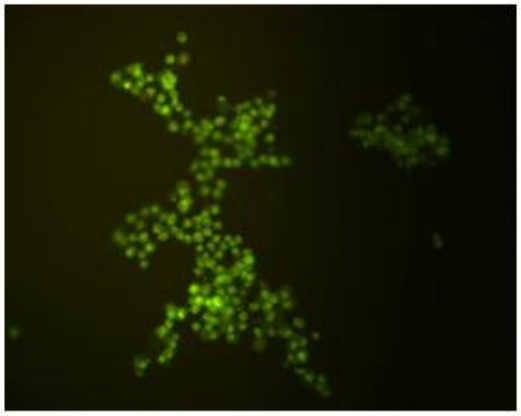
0 µg/mL



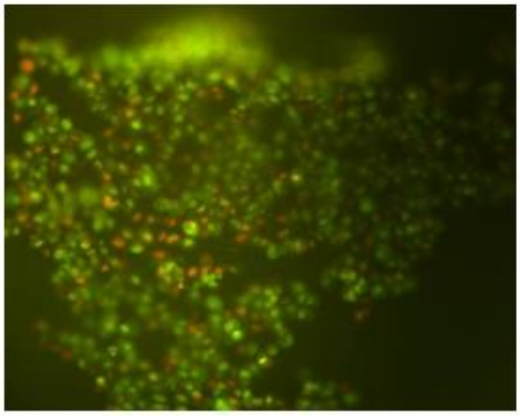
5 µg/mL



25 µg/mL

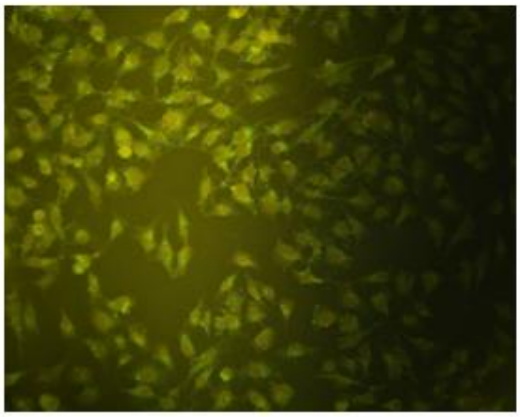


50 µg/mL

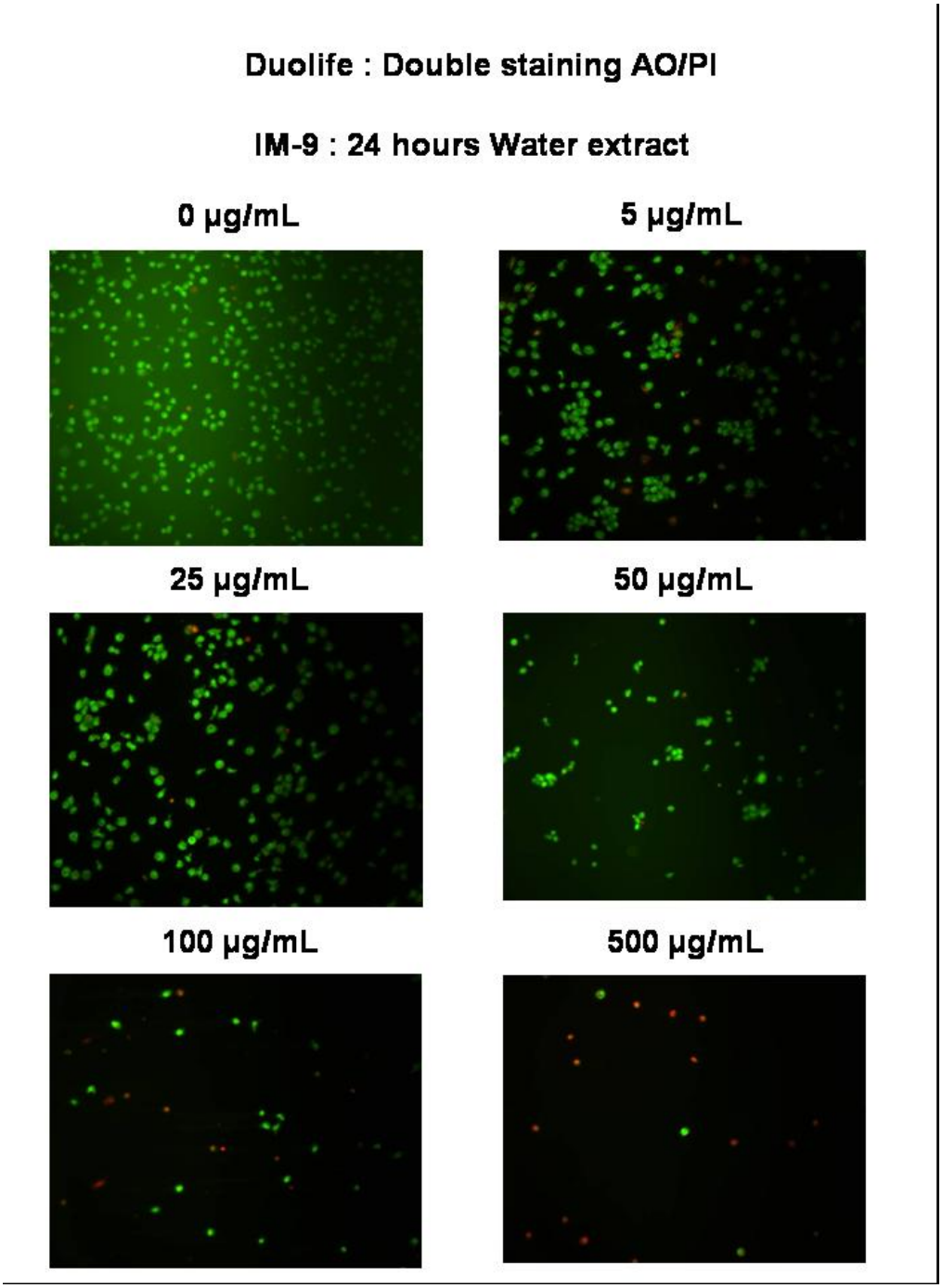


100 µg/mL

500 µg/mL



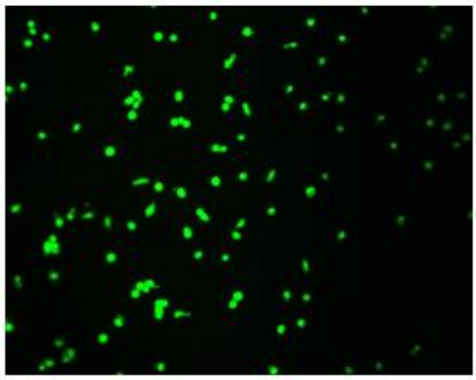
Hereunder are shown **IM-9** cells 24 or 48 hours after incubation with **water** extracts of Duolife at concentrations from 0 to 500 µg/ml, the cells being stained with acridine orange/propidium iodide. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells are healthy and therefore all appear green. The treatment with concentrations of water extracts of Duolife from 50 µg/mL induced a slight increase in the number of apoptotic cells with the appearance of some red cells. The effect of Duolife was even increased after 48 hours.



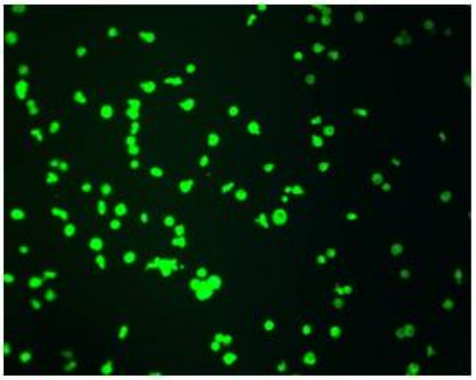
Duolife : Double staining AO/PI

IM-9 : 48 hours Water extract

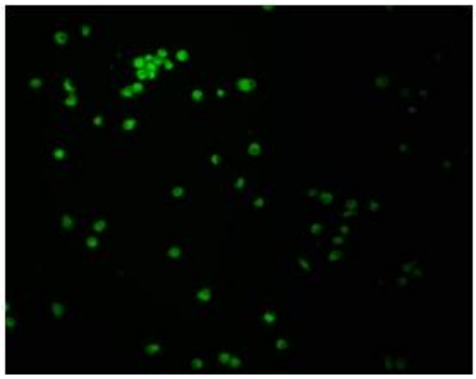
0 µg/mL



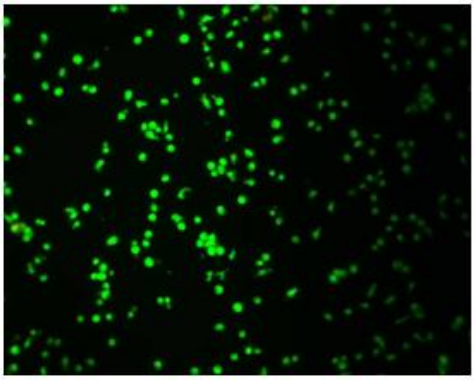
5 µg/mL



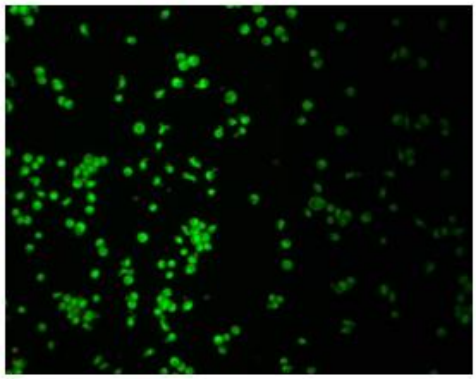
25 µg/mL



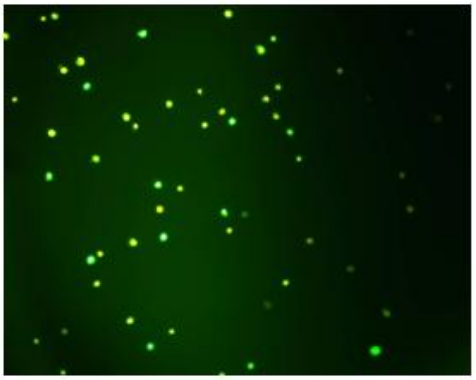
50 µg/mL



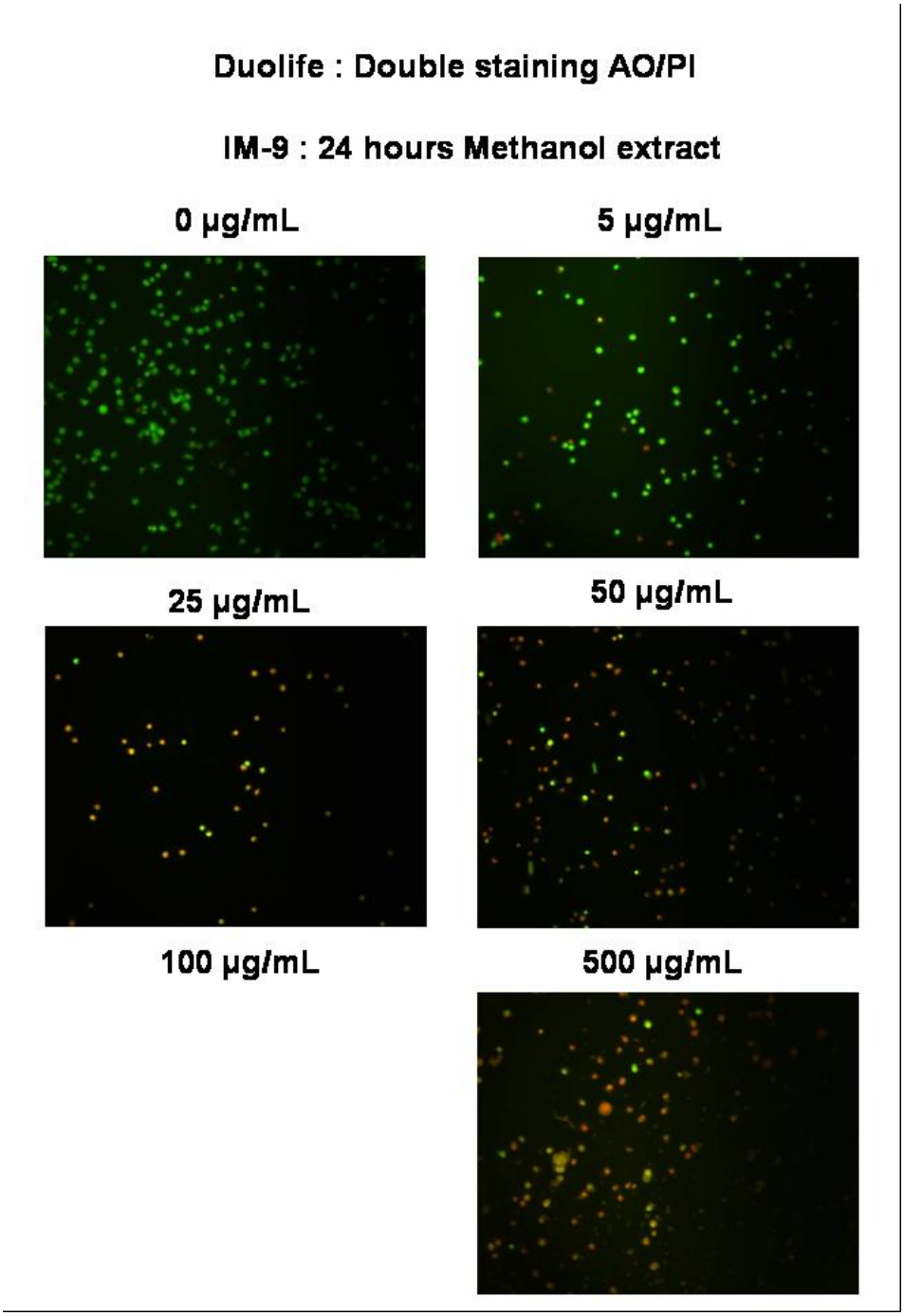
100 µg/mL



500 µg/mL



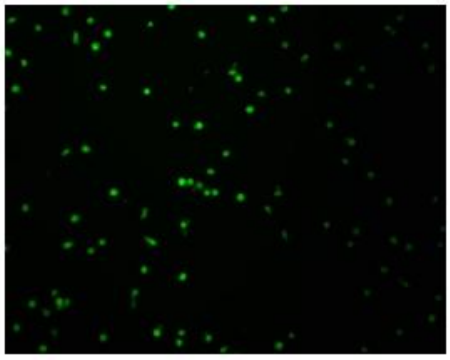
Hereunder are shown **IM-9** cells 24 or 48 hours after incubation with **methanol** extracts of Duolife at concentrations from 0 to 500 µg/ml, the cells being stained with acridine orange/propidium iodide. Representative slides for independent experiments are shown. In control cultures (0 µg/mL), cells are healthy and therefore all appear green. The treatment with concentrations of methanol extracts of Duolife at 5 µg/mL induced a slight increase in the number of apoptotic cells with the appearance of some red cells. The effect of Duolife was even increased after 48 hours.



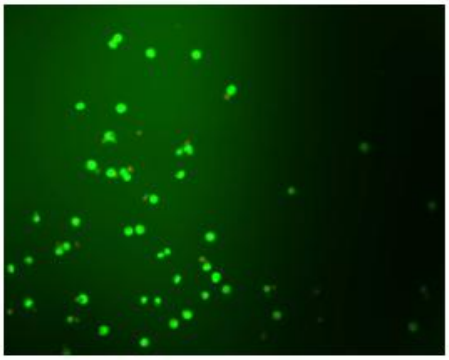
Duolife : Double staining AO/PI

IM-9 : 48 hours Methanol extract

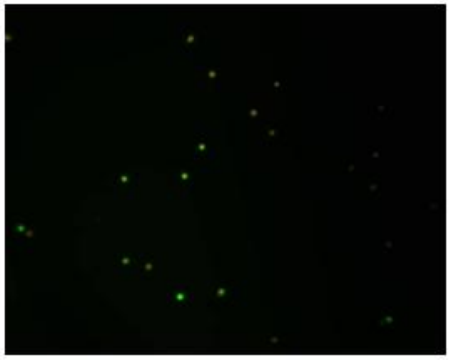
0 µg/mL



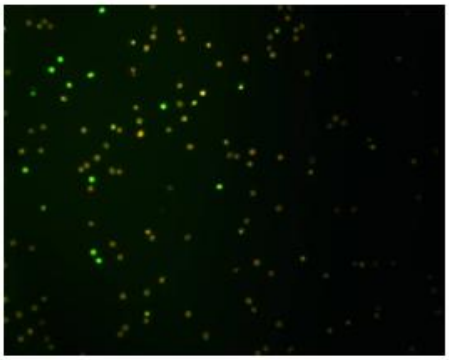
5 µg/mL



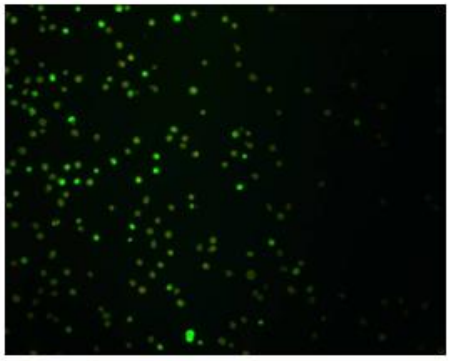
25 µg/mL



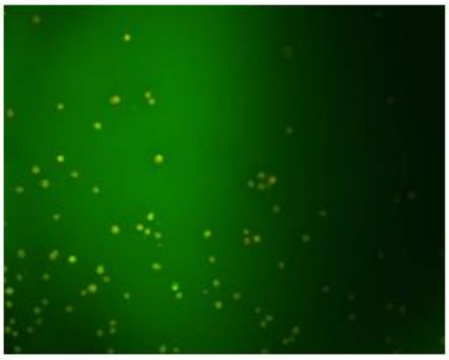
50 µg/mL



100 µg/mL



500 µg/mL



III- DICUSSION AND CONCLUSION

The concept that cancer can be prevented or certain diet-derived substances can postpone its onset is currently eliciting considerable interest. The preliminary results of this study show that Duolife (methanol and to a lesser extent water extracts) could induce apoptosis in two human cancer cell lines in a dose dependent manner. However, further research is needed to study the intrinsic toxicity of Duolife on normal cells.

Over the years, cancer therapy had witnesses many exciting developments, but cure of cancer has still remained as complex as the disease itself, since the mechanisms of tumor killing are still not fully realised. Identification of new substances leading to tumor cell death as well as increasing radiation sensitivity of the cells may be of immense help to selectively induce apoptosis in cancer cells. Knowledge acquired from this kind of study on the effect of Duolife may, therefore, lead us one step forward towards that goal.

IV- REFERENCES

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