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EFFECTS OF SOME PLANT EXTRACTS ON VARIOUS TUMOR CELL LINES

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ABSTRACT. Fresh vegetables were obtained from local producers. The preparation of the extracts is alike for the most of used plants, because we have the same target: to characterize them with HLPC and GC techniques, and treat our cell lines cultivating them in these extracts’ solutions. We prefer extracts with low content of cell-damaging components, which could arise from used preparation technology. We have worked with many dilutions of the indicated vegetables and fruits we were looking for the dose response effect. We tried at first with the low concentrations of the extracts then we decide to change them with more concentrated, because we couldn’t demonstrate any change looking the growth and the proliferation of the treated cells. The aim of our investigations is to identify with bioanalytical and biochemical tools the qualitative and quantitative composition of our extracts, and using the DNA microarray technique we would like to show the effect of the vegetal extracts on transcription of the whole genome. The last years we created the possibility of real time imaging microscopy, we can directly follow the changes in life processes of cells induced by vegetal extracts, especially those of cell cycle regulation. Where we want to get? We want direct results of chemopreventive effects of bioactive substances from vegetal extracts, and use these results in functional foods developing.
Keywords: leafy vegetable, human nutrition, quality, chemical

INTRODUCTION

It is well-known, that many simple or complex bioactive extracts from plants, induce apoptosis in tumoral cell lines (stomach, colon, lung, epidermis (Ramos et al., 2007). These chemopreventive properties of fruits and vegetables arise from their high content of phytochemicals such as polyphenols, anthocyanins, carotenoids. If we can demonstrate differences looking some important cell processes of treated and not-treated cells, the next step will be to identify the molecular mechanism that lead to this different behavior.

MATERIALS AND METHODS

Used vegetables and fruits: Sour cherry *(Prunus cerasus)*, Black currant *(Ribes nigrum)*, Red beet *(Beta vulgaris)*, Pumpkin from Nagydobos *(Cucurbita maxima)*, Broccoli *(Brassica oleracea italica)*.

Fresh vegetables were obtained from local producers. The preparation of the extracts is alike for the most of used plants, because we have the same target: to characterize them with HLPC and GC techniques, and treat our cell lines cultivating them in these extracts’ solutions. We prefer extracts with low content of cell-damaging components, which could arise from used preparation technology. We have worked with many dilutions of the indicated vegetables and fruits we were looking for the dose response effect. We tried at first with the low concentrations of the extracts then we decide to change them with more concentrated, because we couldn’t demonstrate any change looking the growth and the proliferation of the treated cells. After lots of trials we chose and used consistently the following dilutions of our extracts: 5%; 2.5%; 1.25%; 0.62%; 0.31%; 0.15%; 0.07%. We prepared the dilutions using as solvent the culture medium from the cell lines, and we added 1%DMSO to each dilution to enhance the permeability of cell membrane to extracts. Our model system was established using human HT-29 colon cancer cells (ATCC, HTB 38), grown in McCoy’s 5A medium. These cells have a short cell cycle (approx. 20 hours), and are very stable between less favorable conditions too. The 10% dilutions of the extracts were too acids (pH=3), because of that, we decided to throw up them in our experiences. We filtered the initial, 5% dilution through a 0.45 μm filter, in this way we sterilized them and clear away the small particles which could be source of wrong absorbance values. From pumpkin we prepared two kind of extracts in two different way (with enzyme and Tween).

In our experiences usually we used the HT-29 cells, T-47D cells and ZR-75-1 cells, and few times the NCM460 cell line. Our experience shows that the extracts from fruits don’t inhibit the growth of NCM 460 cells, their proliferation is stimulated rather than inhibited. In our new experiences we use the NCM460 cells as vehicle together with colon carcinoma cells. The proliferation of Caco-2 cells was inappropriate we suspected to be infected with *Mycoplasma*. From this reason we used these cells only later in our experiences, when we tested the effect of pumpkin extract.

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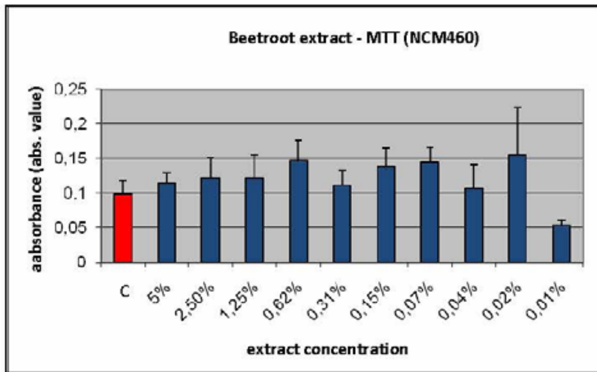

Fig. 1 The effects of beetroot extract on viability and proliferation of NCM460 normal colon mucosa cells. *Note: After 48 h incubation there are no significant changes looking the proliferation of NCM460 cells incubated with beetroot extract correlating with the control cells*

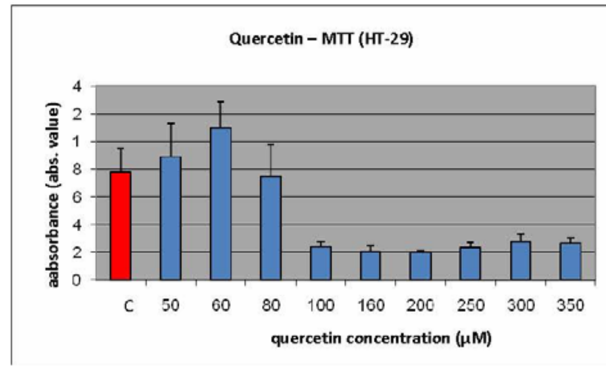

Fig. 2 The biphasic modulation of HT-29 colorectal adenocarcinoma cell proliferation by quercetin. *Note: The proliferation of HT-29 cells is biphasic modulated by quercetin, corresponding to datas communicated in international publications: the solutions of 50-60 μM stimulate the proliferation, the quercetin of 80-350 μM concentration is inhibiting the cell proliferation*

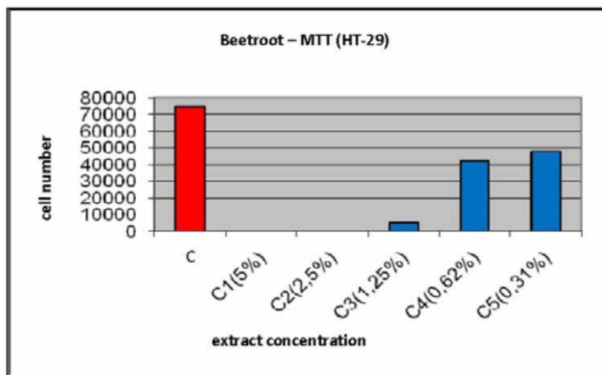

Fig. 3 The effect of beetroot extract on HT-29 cells proliferation. *Note: The proliferation of cells treated with 5% and 2,5% extract is zero; the cells are beginning to proliferate when the concentration of the extract became more diluted, but in all used concentrations (1,25- 0,31%) stay below of control. The counting of cells was performed after 48 h incubation*

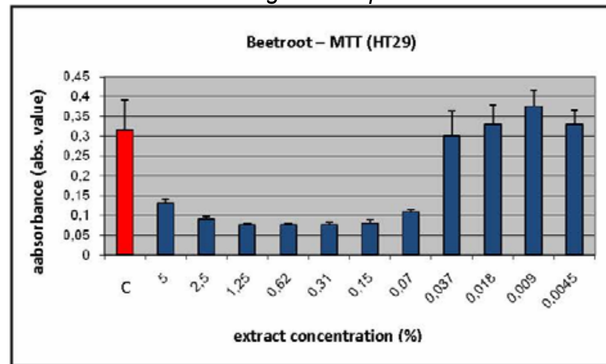

Fig. 4 The effect of beetroot extract on HT-29 colorectal adenocarcinoma cell viability and proliferation. *Note: The beetroot extract was the most efficient regarding the HT-29 cells:beside the inhibiting effect of high concentrations, in relatively low concentrations (0,62% - 0,07%) inhibits significantly the cell proliferation. Between 0,037%-0,0045 % the extract has an accentuated stimulating effect on cell proliferation (biphasic effect)*

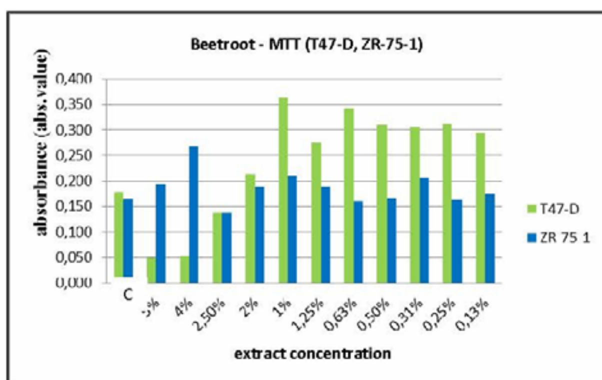

Fig. 5 The effect of beetroot extract on T47-D and ZR-75-1 mammary adenocarcinoma cells viability and proliferation *Note: The two different cell line's reaction to beetroot extract was specific. This extract in higher concentrations (5%-2,5%) inhibited the T47-F cell line proliferation, whereas in lower concentrations stimulated it. The proliferation of ZR-75-1 mammary adenocarcinoma cell line wasn't inhibited in any concentration by beetroot extract on the contrary it stimulated the cell proliferation.*

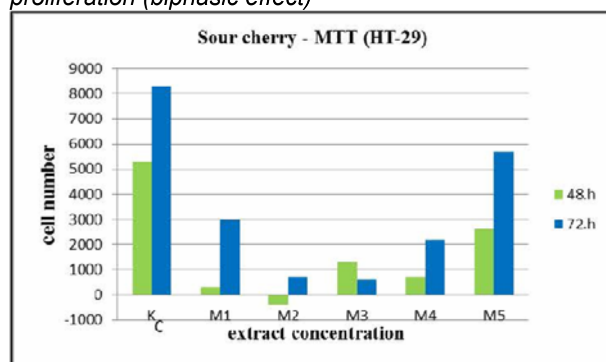

Fig. 6 The proliferation of HT-29 cells incubated 48-72 h with sour cherry extract. *Note: after 48 h incubation in sour cherry extract+ medium, the number of colorectal tumoral cells is low, comparing with control cell number. After 72 h incubation between the same conditions, the proliferation is more visible but remains significantly below the control in every dilution. Parallel with attenuation of the concentration (M5) the proliferation is stronger*

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The cells were cultivated in their appropriate culture medium, at 37°C under a humidified atmosphere containing 5% CO2. We needed a 70-80 % cell confluence for our trials. If it was necessary we changed the medium every second day, to eliminate the toxic substances from cellular metabolism and enrich the medium with nutrients for cellular processes. The culture medium contained 10%FBS, and antibiotic-antimycotic supplement (1ml/100 ml medium)to avoid the infection with bacteria and fungus. The duration of different cells' cell cycle is various: Ht-29 cells need 20-24 hours for replication, the mammary carcinoma cells 30-80 hours. When the cells haven't the usual aspect, and their replication became slow,without the sign of bacterial or fungal infection, we tested them for *Mycoplasma* with PCR Mycoplasma test (Venor GeM/Minerva Biolabs).

RESULTS AND DISCUSSIONS

We investigated how the different vegetal extracts influence the normal and tumor cell lines' viability and proliferation. It is important to relieve that we do not know very much about the action mechanism of vegetal extracts at cellular level, that is why our experiments could be considered like novelty. To realize our targets, we developed the model system based on human cell lines, introduced the past year. Based on our previous experiences we supposed, that the isolated vegetal extracts, inhibit the growth of human tumor cells and reduce their viability. We have done our experiences using the following human cell lines:

Used human cell lines	Used culture media
HT-29 (ATCC –HTB-38): colon adenocarcinoma	Mc Coy's 5A
Caco-2 (ATCC- HTB-37): colon adenocarcinoma	EMEM
NCM460 (INCELL): normal colon mucosa	M3F Base
T-47D (ATCC-HTB133): mammary gland adenocarcinoma	RPMI1640
ZR-75-1 (ATCC-CRL-1500™): mammary gland adenocarcinoma	RPMI1640

The most important criteria of the malignant transformation are the unrestricted and unregulated proliferation of the cells. The capacity of cells to apoptosis is changed, their survival is raised. That's why we cultured our human tumor cells in medium containing vegetal extracts with potentially antitumor effect, and we studied the changes that occur in their proliferation, viability, apoptosis, comparing with untreated cells (vehicle).

For determination of proliferation, cells were seeded at a density of 5x103 per well onto 24 well cell culture plates, and allowed to adhere for 24 or 48 h incubated at 37°C under a humidified atmosphere containing 5% CO2. We could follow the adherence with microscope: until they are in suspension, the cells are round, when they adhere, took their characteristic, mostly polygonal form. After they have adhered we counted them (day 0) to decide how many of the introduced cells survived. Thereafter the medium was replaced by fresh culture medium containing (excepting the control cells) the plant extracts in different concentrations and cells were allowed to grow for another 48 and 72 h at 37°C under a humidified atmosphere containing 5% CO_2. After 48 and 72 h we counted the cells stained with Trypan Blue 0.1% (Sigma) for each concentration of the tested vegetal extract (N=3), and we calculated the differences in cells number between day 0 and culture of 48 h and 72 h in %: (B2-A2)/ABS (A2)*100. In this way we could conclude which extract in which concentration was the most efficient in cell proliferation's inhibition. We represented our results on diagrams (columns) (fig. 3 and 5)

Cell viability and the proliferation, the cytotoxicity of the extracts were determined also by assaying the mitochondrial activity of treated cells after 48 h incubation, with MTT assay. We elaborated the method based on data from the international literature and experiences using the quercetin (Hester van der Woude et al.: Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans; Cancer Letters 200(2003) 41-47): for the colon carcinoma cell line HT-29 at relatively high concentrations a significant decrease in cell proliferation was observed, providing a basis for claims ont he anti-carcinogenic activity of quercetin. At lower concentrations, a subtle but significant stimulation of cell proliferation was observed. Briefly, yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was added to each well of treated cells and to the control cells too. The MTT is reduced to purple formazan in living cells. A solubilization solution, an acidified ethanol, is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at 570 nm by a spectrophotometer. Because these reductions take place only when mithocondrial reductase enzymes are active, the intensity of formazan (absorbtion values) solution is used as a measure of viable cells. We calculated our results repeating the tests 3x8 times (significance was calculated with SPSS, Mann-Whitney U test)

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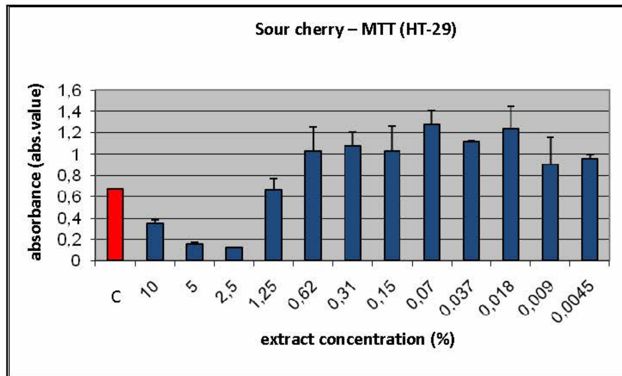

Fig. 7 The biphasic modulation of HT-29 cell proliferation by sour cherry extract. *Note: The sour cherry extract inhibits the HT-29 cell proliferation only in three concentrations, the highest ones (10%, 5%, 2.5%). The other members of the serial dilution significantly stimulate the cell proliferation*

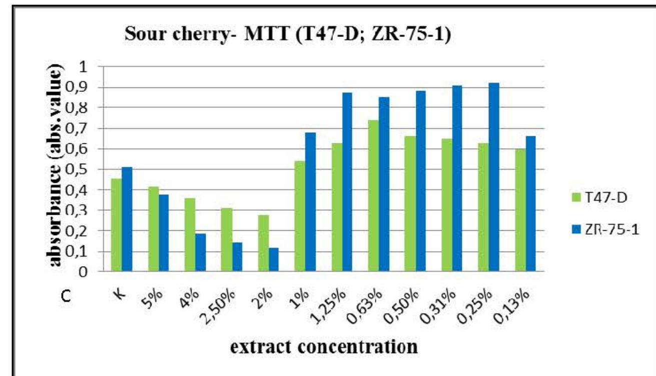

Fig. 8 The biphasic modulation of T47-D and ZR-75-1 cells proliferation by sour cherry. *Note: From all of used extracts in our experiments, the sour cherry has the narrowest effect zone, the viability and proliferation of mammary carcinoma cells is inhibited significantly only by 5%-2% dilutions. The other concentrations (1% - 0.13%) stimulate the proliferation*

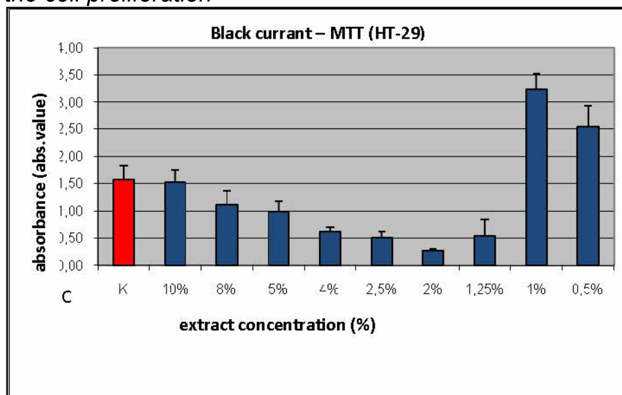

Fig. 9 The biphasic modulation of HT-29 cell proliferation by black currant extract. *Note: The black currant extract in concentrations 8%-1.25% significantly inhibits the proliferation of HT-29 colorectal adenocarcinoma cells. The proliferation is stimulated by more diluted extracts (1%, 0.5%): it would be interesting what is happening in cells incubated in 1.25% and 1% dilutions, because they show a different aspect, even the two concentrations are very close each other*

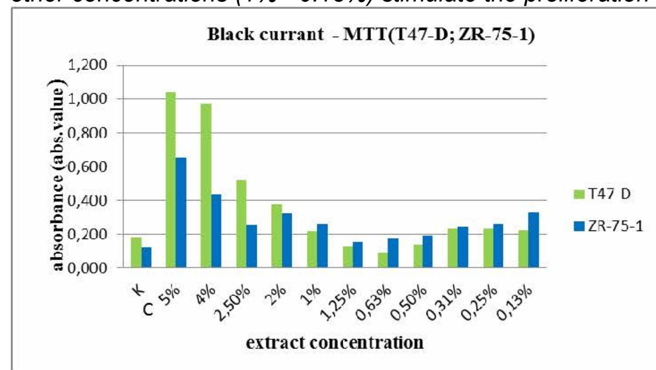

Fig. 10 The effect of black currant extract on T47-D and ZR-75-1 mammary adenocarcinoma cells viability and proliferation. *Note: The biphasic modulation of T47-D cell proliferation exists but it is „reverse": the most concentrated solutions stimulate the proliferation some of the diluted ones (1.25%, 0.63%, 0.50%) inhibit it. We can conclude the same, treating with black currant extract the ZR-75-1 cells. May be there was a cell manipulating or/and a detection problem: the control cells' number is unusual reduced. May be we loosed some cells when we changed the medium after 24 h, our posterior experience show that these cells need more than 24 h to adhere*

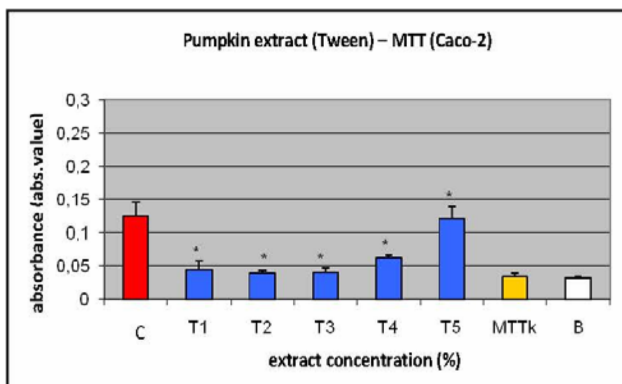

Fig. 11 The effect of pumpkin extract (Tween) on Caco-2 colorectal adenocarcinoma cell viability and proliferation. *Note: The pumpkin extract (Tween) significantly inhibits the Caco-2 cell viability and proliferation*

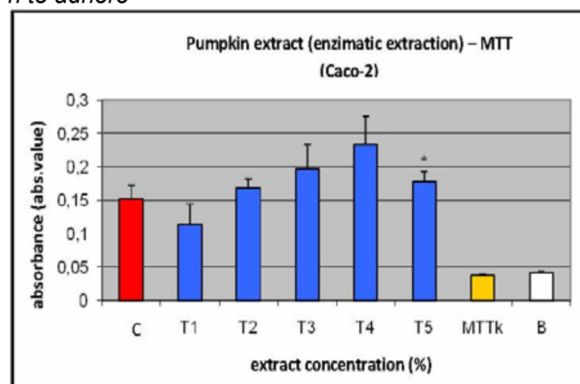

Fig. 12 The effect of pumpkin extract (enzymatic extraction) on Caco-2 cell viability and proliferation. *Note: The most concentrated enzimatic pumpkin extract (5%) shows inhibitory effect on Caco-2 cell proliferation. The rest of serial dilutions (2,5%-0,31 %) stimulate cell proliferation*

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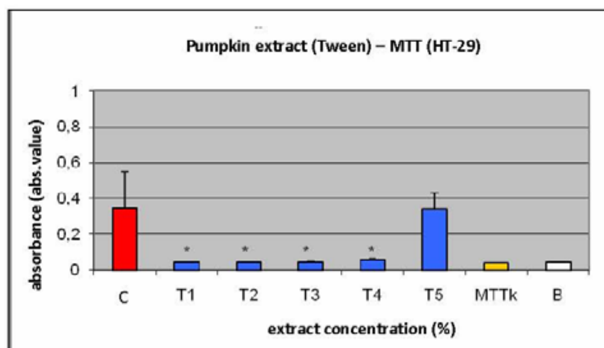

Fig. 13 The effect of pumpkin extract (Tween) on HT-29 colorectal adenocarcinoma cell viability and proliferation. *Note: The colorectal tumor HT-29 cells, cultivated with pumpkin extract (Tween), are totally inhibited in their proliferation with the exception of most diluted solution (0.31%). Treating the cells with this concentration of our extract, the MTT test show a cell proliferation equal to the control cells*

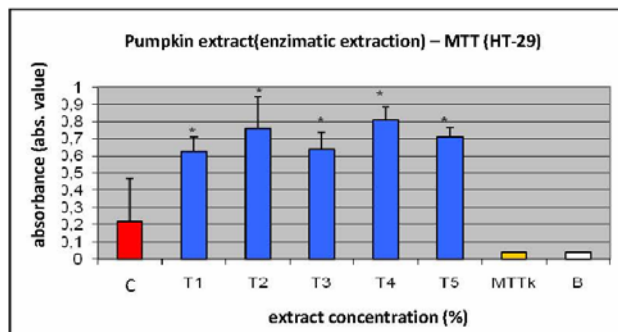

Fig. 14 The effect of pumpkin extract (enzymatic extraction) on HT-29 colorectal adenocarcinoma cell viability and proliferation. *The pumpkin extract prepared with enzimatic extraction produce a significant stimulating effect on proliferation of HT-29 cells in every used concentration of the serial dilution. There is no biphasic m odulation of the cell proliferation by pumpkin extract*

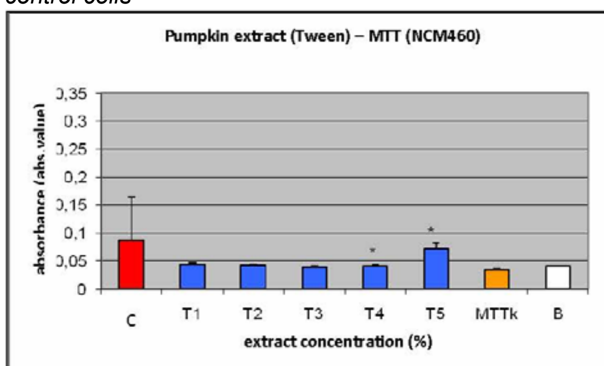

Fig. 9 The effect of pumpkin extract (Tween) on NCM460 normal colon mucosa cell viability and proliferation. *Note: The pumpkin extract (Tween) inhibits the proliferation of NCM460 cells. This is interesting because in our earlier experience (Fig.1) we treated the same cell line with beetroot extract, and we could see that the beetroot extract stimulate the normal colon mucosa cell proliferation. We shall investigate in our further experiments the molecular basis of this effect*

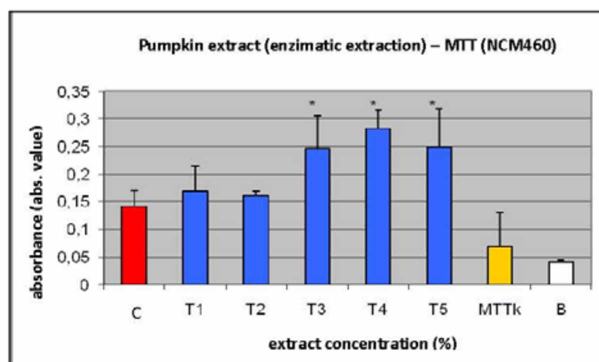

Fig. 10 The effect of pumpkin extract (enzymatic extraction) on NCM460 normal colon mucosa cell viability and proliferation. *Note: The pumpkin extract prepared by enzymatic extraction stimulates the proliferation of NCM460 cells as we can see from MTT test's results. The same cell line comports in a different way treated with the two pumpkin extracts, most probably because of the different composition (quantitative and qualitative) of the extracts, determined by the different way of extraction.*

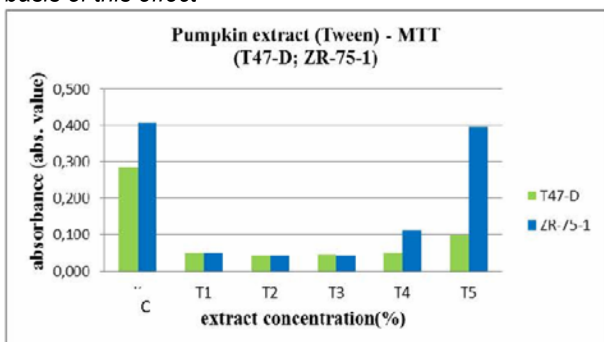

Fig. 16 The effect of pumpkin extract (Tween) on viability and proliferation of T47-D and ZR-75-1 mammary adenocarcinoma cells. *Note: The pumpkin extract (Tween) inhibits the mammary adenocarcinoma T47-D cell proliferation in each concentration of serial dilution. Excepting the most diluted solution (0.62%),which give an absorbance equal to the control, the same effect we can see on ZR-75-1 mammary adenocarcinoma cell line.*

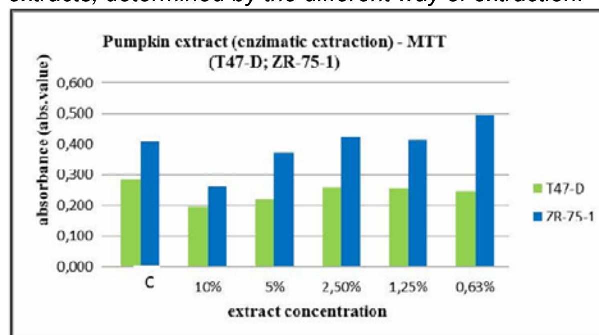

Fig. 17 The effect of pumpkin extract (enzymatic extraction) on viability and proliferation of T47-D and ZR-75. *Note: The pumpkin extract prepared by enzymatic extraction has different effect on the two cell lines even they are both mammary adenocarcinoma cells. The proliferation of T47-D cells' is slightly inhibited by each concentration of the serial dilution. The same extract has biphasic effect on ZR-75-1 cell proliferation: the 10% and 5% dilutions inhibit, the rest stimulate the proliferation.*

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We found that there are considerable differences in the ability of vegetal extracts to inhibit the proliferation of various cancer cells. The antiproliferative activity varies depending on the extract and appears to be dependent ont he cell type employed. Our first investigated extract was the sour cherry: we followed the cherry extract effect on the proliferation and viability of human tumoral cells HT-29, T47-D and ZR-75-1. The sour cherry extract inhibited the proliferation of colon carcinoma cells HT-29 and mammary ductal carcinoma cells T47D and ZR-75-1 in relative high concentrations (10%-2%) The beet root extract was the most effective on HT-29 colorectal adenocarcinoma cells, even in relative low concentrations (0.62%-0.07%). The same biphasic modulation of cell proliferation by beet root extract was shown on T47-D cells. The same extract in NCM460 normal colon mucosa cells and ZR-75-1 mammary ductal carcinoma cells stimulated the proliferation, or the proliferation was equal with values shown by the control. The black currant extract inhibited the proliferation of HT-29 cells in lower concentrations too (1.25%). As we could observe, when the mammary adenocarcinoma cells (T47-D and ZR-75-1) were cultivated in the presence of this extract, they show an „inverse biphasic modulation” of proliferation: in high concentrations the black currant extract stimulated the cell proliferation and viability; in low concentrations it inhibited the same processes. The pumpkin extract prepared using Tween detergent inhibited the proliferation of all tested cell lines. The same extract but prepared in a different way, by enzymatic procedure, shows a different and more complex effect on cell proliferation: stimulated the proliferation when we treated the NCM460 normal colon mucosa cells and the colorectal carcinoma cells Caco-2;on T47-D mammary ductal adenocarcinoma cells the extract doesn’t have the biphasic effect on proliferation, but treating with the enzymatic extract the other mammary cell line, the ZR-75-1, even in only two concentration, but the extract shows the biphasic modulation of proliferation.

CONCLUSIONS

We can conclude that the in vitro effect of vegetal extracts on cell viability and proliferation is a concentration - dependent, biphasic effect: in high concentration these extracts have antiproliferative effects, in low concentrations they stimulate the human tumor cell proliferation. If we would try to represent by a curve this relationship we would obtain a J – shaped curve, characteristic to „dose-response effect” diagrams. (I.J. Calabrese & L.A. Baldwin, Defining hormesis, Human and Experimental Toxicology, 2002, 21, 91-97) Growth-inhibition for almost all extracts was generally (excepting quercetin) not a consequence of cytotoxic effects. These results could rise another problem: how we can use these extracts like dietary supplements in prevention or in the therapy of cancer? We need newer experiments because we must define exactly the molecular effects of bioactive substances from vegetal extracts on human cells. In most cases the identification of such substances that are responsible for these effects is still lacking. There is increasing evidence that the chemopreventive properties of vegetal extracts result from the additive and synergistic effects of several phytochemicals present together in the extracts. The aim of our next investigations is to identify with bioanalytical and biochemical tools the qualitative and quantitative composition of our extracts, and using the DNA microarray technique we would like to show the effect of the vegetal extracts on transcription of the whole genome. The last years we created the possibility of real time imaging microscopy, we can directly follow the changes in life processes of cells induced by vegetal extracts, especially those of cell cycle regulation. Where we want to get? We want direct results of chemopreventive effects of bioactive substances from vegetal extracts, and use these results in functional foods developping.

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