

## IN VITRO IMPACT OF SOME NATURAL COMPOUNDS ON HT-29 COLORECTAL ADENOCARCINOMA CELLS

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### Abstract

Relatively recent research has robustly proved the role of phytochemicals present in vegetable and whole grain diets, fruit, spices and various herbal teas in approach of cancer related risk. In recent times, this has been further supplemented with research regarding the pharmacological effects of herbal medicinal products from the chemo-preventive perspective. The aim of our study was to evaluate the potential effects that the association of silymarin and capsaicin could induce in HT-29 colorectal adenocarcinoma cells. For this, we treated HT-29 colorectal adenocarcinoma cells with silymarin, capsaicin and with a combination of both compounds to investigate the cell viability, cell morphology as well as the DNA fragmentation as a result of apoptosis. We found that both silymarin and capsaicin alone significantly decreased cell viability, determined morphological changes and induced apoptosis. However, when used in combination, they mutually cancelled out their toxic effects on HT-29 cells.

### Rezumat

Cercetările relativ recente au demonstrat rolul substanțelor fitochimice prezente în dietele cu legume, cereale integrale și fructe în abordarea riscului de cancer. În ultimul timp, acest lucru a fost documentat prin cercetarea efectelor farmacologice ale produselor din plante din perspectiva chemopreventivă. Scopul acestui studiu a fost evaluarea potențialelor efecte ale utilizării unei combinații de silimarina și capsaicină asupra unor celule tumorale de cancer de colon aflate în cultură. După tratamentul celulelor *in vitro* cu silimarina, capsaicină și combinația lor, am investigat viabilitatea și morfologia celulelor precum și gradul de fragmentare a ADN-ului ca urmare a apoptozei. Rezultatele noastre arată că silimarina și capsaicina administrate singure induc modificări majore ale viabilității și morfologiei celulare, precum și un grad ridicat de apoptoză în cultură. Totuși, administrați împreună, cei doi compuși par să își atenueze reciproc efectele toxice, deoarece modificările observate nu au fost la fel de accentuate ca în cazul tratamentelor individuale.

**Keywords:** silymarin, capsaicin, colon cancer, apoptosis, HT-29 colorectal adenocarcinoma cells

### Introduction

In the search for comprehensive means to fight cancer over the years, relatively recent research has robustly proved the role of phytochemicals present

in vegetable and whole grain diets, fruit, spices and various herbal teas in approach of cancer related risk. In more recent times, this has been further supplemented with research regarding the pharmacological effects of

herbal medicinal products from the chemopreventive perspective. In fact, either as such or combined with conventional chemotherapeutic agents, herbal products and change in food intake are proved successful for cancer prevention, spread or treatment [1, 2].

One such natural agent, whose benefits are well-established in its long history of use, is silymarin. This compound has been recognised for its contribution in liver protection from toxic effects and treatment of hepatic conditions from hepatitis to cirrhosis. Silymarin is extracted from milk thistle seeds (*Silybum marianum* (L.) Gaertn.) (*Asterceae* Family). This extract contains the so-called silymarin complex of flavonolignans (characteristically silybin but also flavonoid taxifolin, silychristin, silydianin and isosilybin) and some amounts of flavonoids (making up to 65 - 80%), accompanied by fatty acids and polyphenolic compounds accounting for the remaining 20 - 35% [3]. Silymarin and/or silibinin have been proved to interfere with regulators of the cell cycle and apoptosis-involved proteins, thus controlling cell survival-apoptosis imbalances. The mechanism is supplemental to silymarin capacity to modulate specific proteins, rendering its anti-inflammatory and anti-metastatic properties [4].

Spices have been studied from the perspective of cancer prevention and therapy as well. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), for instance, which is a homovanillic acid derivative, is the main spice in chili peppers, so commonly used in many diets. Given its long age dietary use, its medicinal properties have also become apparent and lately it has increasingly been used as analgesics [5, 6], antioxidant [7], anti-inflammatory [8] and anti-obesity [9] agent. In this context, capsaicin's cancer related action has been also extensively examined [10], but results of epidemiological studies and evidence from basic research have been conflicting, which renders capsaicin effects on carcinogenesis rather controversial. In spite of laboratory reports of capsaicin properties as a cancer prevention and therapeutic agent [11-14], also substantiated by results of *in vivo* research on rodent models, giving evidence of its anti-tumorigenic effects [15-19], capsaicin potential has also been shown as a carcinogen or co-carcinogen [20, 21].

Regarding the mechanisms responsible for capsaicin anticancer action, several suggestions made so far have concerned its effects on apoptosis as key deterrent of cancer cell development and progression, given the strong association between malignancy and deficit of apoptotic signalling by disruption of apoptotic pathways and boost of anti-apoptotic pathways contributing to cancer cell resistance to apoptosis [22]. Consequently, the aim of our study was to evaluate the potential effects that the association of silymarin and capsaicin could induce in HT-29 colorectal adeno-

carcinoma cells. In this view, we treated the HT-29 cells with silymarin, capsaicin and with a combination of both compounds and we investigated the cell viability, cell morphology as well as the DNA fragmentation as a result of apoptosis.

## Materials and Methods

### *Cell culture model and drugs treatment*

HT-29 human colon adenocarcinoma cells (ATCC<sup>®</sup> HTB-38<sup>™</sup>) (ATCC – American Type Culture Collection) were maintained as a monolayer at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin and subcultured weekly.

For all experiments, cells were collected by enzymatic treatment with trypsin/EDTA and seeded in 25 cm<sup>2</sup> culture vessels at a final density of 2 x 10<sup>3</sup> cells/cm<sup>2</sup>. After 24 h of culturing, the culture medium was removed and capsaicin (200 µM) and silymarin (25 µg/mL) treatments were added alone or in combination. For experimental controls, the culture medium was replaced with fresh complete culture medium.

### *Live/Dead Fluorescence Microscopy assay*

The cytotoxic effects of the treatments were investigated by Live/Dead (Invitrogen) fluorescence assay. In this view, after 24 h and 48 h of treatment, the culture medium was removed and the monolayers were washed with phosphate buffer saline (PBS) and then stained for 15 minutes at room temperature and darkness with a solution containing calcein-AM and ethidium bromide, freshly prepared according to the manufacturer's protocol. The stained cell cultures were analysed by fluorescence microscopy using an Olympus IX73 inverted microscope and images were captured with CellSens Imaging Software.

### *HT-29 morphology evaluation*

The morphological changes triggered by the exposure of the cells to treatments were evaluated by the fluorescent labelling of the actin filaments. In this view, after 24 h and 48 h of treatment the cellular monolayers were fixed with 4% paraformaldehyde for 10 minutes and cells membranes were permeabilized with 2% bovine serum albumin/0.1% Triton X-100. Next, the samples were incubated with 1 h at 37°C with Alexa Fluor 488 phalloidin (ThermoFisher Sci.) for labelling the actin filaments. After cell nuclei were stained with DAPI for 20 minutes, the samples were inspected in fluorescence microscopy using an Olympus IX73 inverted microscope. Image capturing was performed using CellSens software.

### *Evaluation of DNA fragmentation of apoptotic cells by flow cytometry*

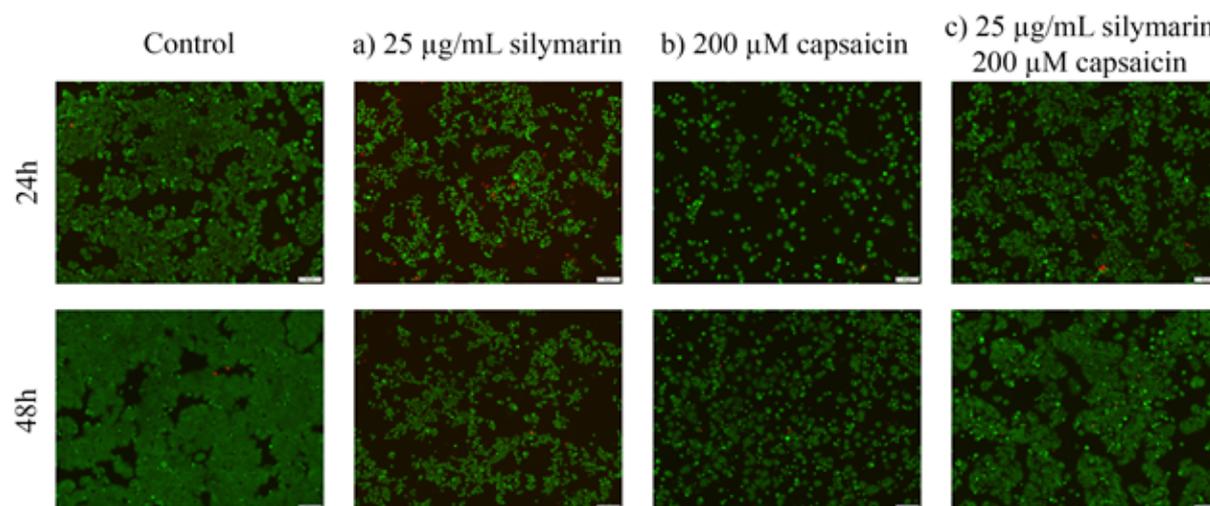
The pro - apoptotic potential of the treatments was evaluated by labelling the DNA strand breaks using

the APO – BrdU TUNEL Assay Kit (ThermoFisher Scientific). Briefly, after 24 and 48 h of treatment the culture medium was collected together with the cell monolayers detached from the culture vessels by enzymatic treatment with trypsin and centrifuged at 1500 rpm for 5 minutes. The obtained cell pellets were resuspended in PBS and fixed on ice for 15 minutes with 1% paraformaldehyde solution. After centrifugation, the cells membranes were permeabilized with 70% ethanol and left 1-day prior labelling at  $-20^{\circ}\text{C}$  in the ethanol solution. After ethanol removal and washing steps, samples were stained with DNA – labelling solution freshly prepared according to the manufacture's recommendations and incubated for 4 h at  $37^{\circ}\text{C}$ . Next, the samples were stained with Alexa Fluor 488 labelled anti – BrdU antibody staining solution freshly prepared after the manufacture's protocol at room temperature in darkness. The samples were analysed using a CytoFLEX flow cytometer (Beckman Coulter) and the data was acquired and analysed using the CytExpert software.

## Results and Discussion

### *HT-29 cell viability after the treatment with silymarin and/or capsaicin*

The potential cytotoxic effects of the treatment with  $25\ \mu\text{g/mL}$  silymarin,  $200\ \mu\text{M}$  capsaicin and their combination on HT-29 colon cancer cells was investigated in terms of cell viability decrease by the concomitant fluorescent labelling of both living and dead cells. The monolayers were stained after 24 h and 48 h of treatment and analysed under a fluorescence inverted microscope. The images are presented in Figure 1 and reveal that silymarin and capsaicin treatments alone decrease HT-29 cell viability as compared with the untreated sample. Interestingly, the combined treatment with both silymarin and capsaicin did not trigger a decrease in cell viability in a higher proportion than the compounds alone. However, after the exposure to the combined treatment, HT-29 cells were not able to aggregate as they do in pure culture medium.



**Figure 1.**

Fluorescence microscopy images of untreated HT-29 colon cancer cells (control) and of HT-29 colon cancer cells treated for 24 h and 48 h with: (a)  $25\ \mu\text{g/mL}$  silymarin, (b)  $200\ \mu\text{M}$  capsaicin and (c)  $25\ \mu\text{g/mL}$  silymarin and  $200\ \mu\text{M}$  capsaicin (green fluorescence - living cells; red fluorescence - dead cells)

### *HT-29 cell morphology after the treatment with silymarin and/or capsaicin*

The potential morphological changes that might occur as a result of the treatment's cytotoxicity were investigated after the fluorescent staining of the cytoskeleton's actin filaments. The investigations were performed after 24 h and 48 h of treatment with  $25\ \mu\text{g/mL}$  silymarin,  $200\ \mu\text{M}$  capsaicin and their combination on HT-29 colon cancer cells. As shown in Figure 2, both silymarin and capsaicin treatments caused major alteration of the actin filaments organization and protein expression. Actin filaments are well and homogeneous represented inside the untreated cells, while in the silymarin and capsaicin

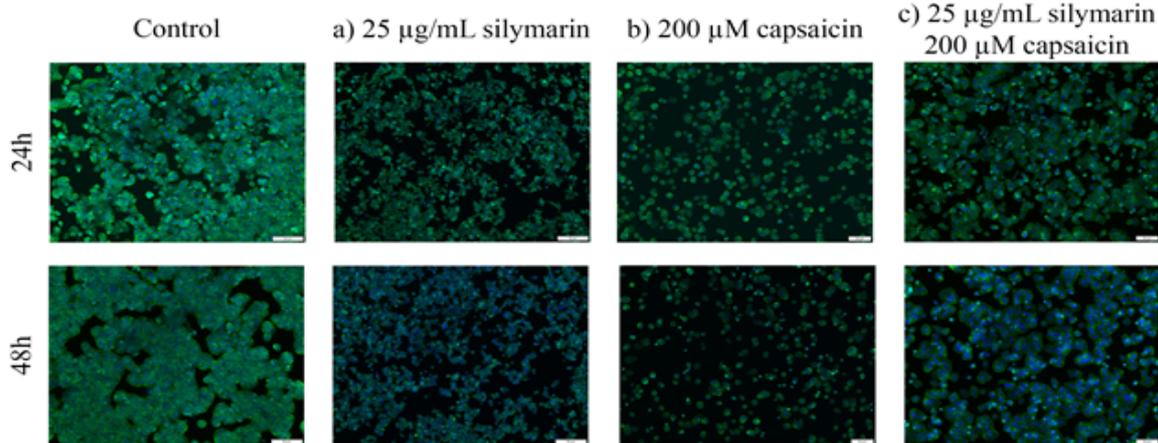
treated cells the expression of actin was identified only at the periphery, probably in the area of the adhesion contacts with the substrate. Furthermore, the combination treatment of silymarin and capsaicin induced a decrease of actin protein expression, but not as much as each compound alone.

### *Flow cytometry investigation of HT-29 cells apoptosis after silymarin and/or capsaicin treatment*

The pro-apoptotic effect of the treatments was investigated by flow cytometry in terms of DNA fragmentation degree based on BrdU-FITC labelling of the DNA strand breaks. The intensity of the green fluorescence recorded by the flow cytometer is directly correlated with the amount of the BrdU

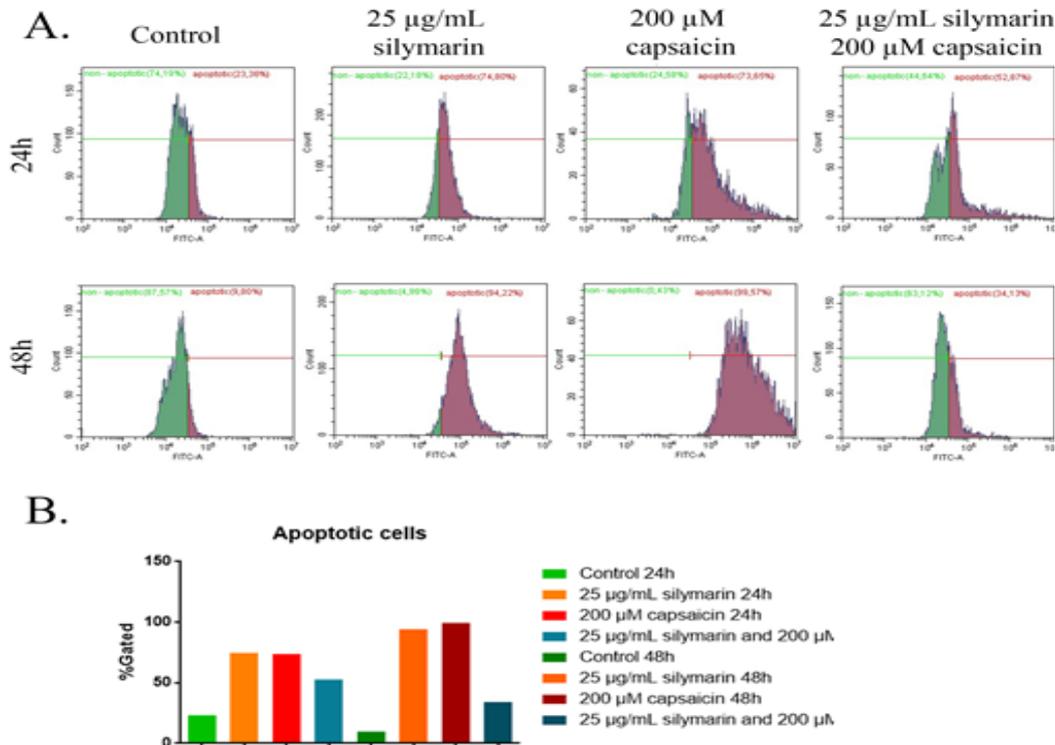
which has been bound to the DNA fragments. Consequently, as the DNA is more fragmented (as a result of apoptosis), the more BrdU will incorporate and the intensity of the green fluorescence will be higher. Our flow cytometry results confirm the fluorescence microscopy images and offer additional quantitative data regarding the amount of apoptotic cells in the samples. As shown in Figure 3, in the untreated sample the amount of non-apoptotic cells

was significantly higher than the apoptotic cells, both at 24 h and 48 h. Furthermore, both silymarin and capsaicin treatments dramatically increased the amount of apoptotic cells in the samples. Notably, capsaicin seems to exert higher pro-apoptotic effects on HT-29 cancer cells than silymarin. However, when combining the compounds, the treatment induces a significant increase of the apoptotic cells, but not as much as each compound alone.



**Figure 2.**

Fluorescence microscopy images of untreated HT-29 colon cancer cells (control) and of HT-29 colon cancer cells treated for 24 h and 48 h with: (a) 25 µg/mL silymarin, (b) 200 µM capsaicin and (c) 25 µg/mL silymarin and 200 µM capsaicin (green fluorescence - actin filaments stained with Alexa Fluor 488 Phalloidin; blue fluorescence - cells nuclei stained with DAPI)



**Figure 3.**

A. Flow cytometry histograms showing the amount of apoptotic and non-apoptotic cells populations in the 25 µg/mL silymarin, 200 µM capsaicin and 25 µg/mL silymarin and 200 µM capsaicin samples as well as in the untreated control  
 B. Graphical representation of the apoptotic cells variation in the 25 µg/mL silymarin, 200 µM capsaicin and 25 µg/mL silymarin and 200 µM capsaicin samples as well as in the untreated control

Research and experience on cancer approach have revealed the specificity of carcinogenesis and further cancer evolution, i.e. deregulated cell cycle differentiation, proliferation, progression and apoptosis accompanied by higher angiogenic potential, invasion and lastly, metastasis.

Therefore, all potential protective/therapeutic agents studied are assessed for their efficacy against these processes.

In this context, it has been shown that silymarin interferes with the expression of cell cycle and apoptosis regulators, thus controlling cell survival-apoptosis imbalances [4]. Silymarin and silibinin inhibit cell growth by acting on G1 and G2 checkpoints regulators. The mechanism of growth obstruction was found to be dose dependent as low concentrations inhibit extracellular signal-regulated kinases (ERK1/2) and high doses can induce apoptosis by the mitogen-activated protein kinase (MAPK)/c-Jun N-terminal kinase (JNK) pathway [4, 23, 24]. In our study, the treatment with 25 µg/mL silymarin induced in HT-29 colorectal adenocarcinoma cells significant decrease in cell viability and proliferation potential as well as important morphological modifications during 48 h of exposure. More, we found that after 24 h of treatment, 74.8% of the treated cells suffered apoptosis while after 48 h of treatment, 94.22% of the cells were apoptotic.

Capsaicin seems to target a number of proteins involved in the signalling systems and as such, whereas sparing normal cells [10, 12, 18, 25], it may initiate apoptosis in more than 40 distinct cancer cell line types [10], among which the bladder [26], prostatic [27], pancreatic [18], liver [28], colonic [29], skin [30], oesophageal [31], lung [32], leukaemia [25] and endothelial cells [33]. As regards the mechanisms responsible for capsaicin anticancer action, several suggestions made so far have concerned its effects on apoptosis as key deterrent of cancer cell development and progression, given the strong association between malignancy and deficit of apoptotic signalling by disruption of apoptotic pathways and boost of anti-apoptotic pathways contributing to cancer cell resistance to apoptosis [34]. Research has thus revealed that capsaicin activates intrinsic and extrinsic apoptotic pathways mediated by cluster of differentiation 95 (CD95) [11], at the same time withholding expression of the B-cell lymphoma 2 antiapoptotic protein, resulting in loss of potential of the mitochondrial membrane, in activation of caspases -9 and -3, and ensuring increased release of cytochrome c [35].

The effect of capsaicin in sustaining apoptosis is mediated by the transient receptor potential vanilloid 1 (TRPV1) [11, 35-39] and TRPV6 [40, 41], two of the TRPV receptor family determining mitochondrial damage and release of cytochrome c mediated by Ca<sup>2+</sup>. Additionally, research in capsaicin interference with

p53 has revealed its potential to induce phosphorylation of p53 at the Ser-15 residue [25] and increase p53 acetylation by down-regulation of sirtuin 1 [42], determining apoptosis.

In our experiment, we showed that the treatment with 200 µM capsaicin induced significant decrease in HT-29 colorectal adenocarcinoma cells viability and proliferative potential as well as architectural modifications in actin filaments organization. All these observations are accompanied by a highly significant increase of the apoptotic cells in our culture: 73.65% after 24 h and 99.57% after 48 h of exposure to capsaicin treatment.

In spite of laboratory reports of capsaicin properties as a cancer prevention and therapeutic agent [11-14], also substantiated by results of *in vivo* research on rodent models, giving evidence of its anti-tumorigenic effects [15-19], capsaicin potential has also been shown as a carcinogen or co-carcinogen [13, 21]. After exposing HT-29 cells both to silymarin and capsaicin we found that the cells did not suffer such dramatic viability and proliferation potential decrease as after the exposure to individual compounds. More, we found only 52.87% apoptotic cells after 24 h and 34.13% apoptotic cells after 48 h of combined treatment.

## Conclusions

Our results indicate that both silymarin and capsaicin exert pro-apoptotic effects on HT-29 colorectal adenocarcinoma cells, but when used in combination, they mutually cancel out their toxic effects on HT-29 cells.

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