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The role of disaccharide complexes in triggering programmed cell death in cancer cells MCF-7 and HeLa

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Summary. Previous *in vitro* studies have confirmed that exposure of human endothelial cells to high glucose concentration induces significant cell death via apoptosis. The present studies therefore explore the options for treating cancer with cellobiose complex and natural compounds containing disaccharides. Our results show that the cellobiose complex containing cellobiose with vitamin C, sulphur and selenium isolated from a mixture of two cold plant extracts *Capsicum chinense* and *Allium sativum* induces cell death in HeLa cancer cells by activating caspase-3 and loss of mitochondrial integrity, but not in MCF-7 cancer cells or the immortalized cell line HaCaT. When we treated HeLa cancer cells with the laboratory-produced cellobiose alone, we found that a 10,000 x higher concentration was needed (100 mg/mL - 1,000 mg/mL), suggesting the important synergistic role of the other mentioned molecules in the complex, which enable the molecule of cellobiose to be more effective at lower dosages. This study therefore for the first time suggests that the complex formed of cellobiose, selenium, sulphur and vitamin C has an apoptotic and necrotic effect, which could be due to the modulation of a mitochondria-mediated intrinsic apoptotic pathway.

Key words: apoptosis, cancer, cellobiose complex, cytotoxicity, necrosis

«Il ruolo dei complessi disaccaridici nell'attivazione della morte cellulare programmata in cellule tumorali MCF-7 e HeLa»

Riassunto. Studi in vitro hanno confermato che l'esposizione di cellule endoteliali umane ad un'alta concentrazione di glucosio induce ad una significativa morte cellulare tramite apoptosi. I presenti studi esplorano le opzioni di trattamento del cancro con composti a base di complessi di cellobiosio e composti naturali contenenti disaccaridi. I nostri risultati dimostrano che il complesso di cellobiosio contenente vitamina C, zolfo e selenio isolato dall'estratto vegetale *Capsicum chinense* e *Allium sativum* induce morte cellulare nelle cellule tumorali HeLa, tramite attivazione della caspase-3 e la perdita dell'integrità mitocondriale, ma non nelle cellule tumorali MCF-7 o nella linea HaCaT. Trattando le cellule tumorali HeLa con la sola molecola di cellobiosio sintetica prodotta in laboratorio, abbiamo dimostrato che era necessaria una concentrazione 10.000 volte maggiore del composto (100 mg/mL - 1.000 MG/mL) per ottenere gli stessi risultati. Ciò suggerisce l'importanza del ruolo sinergico di altre molecole menzionate nel complesso, che consentono alla frazione di cellobiosio di essere più efficace a bassi dosaggi. Il presente studio dimostra inoltre, per la prima volta che il complesso formato da cellobiosio, selenio, zolfo e vitamina C ha effetto apoptotico e necrotico che potrebbe essere dovuto alla modulazione di un percorso biomolecolare intrinseco mediato dai mitocondri.

Parole chiave: apoptosi, cancro, complessi di cellobiosio, citotossicità, necrosi

Abbreviations used:
Ac – DEVD - AFC: acetyl – Asp – Glu – Val – Asp – 7 – amino – 4 -trifluoromethylcoumarin; C: solution with the cellobiose synthesized in the laboratory; Ctrl: cells grown only in DMEM medium without being treated with the plant extract, fraction of cellobiose complex (cellobiose, vitamin C, sulphur and selenium) or cellobiose itself; DMEM: Dulbecco's modified Eagle's medium; DTT: dithiothreitol; FBS: fetal bovine serum; HaCaT: immortal human keratinocyte cell line; HeLa: Henrietta Lacks adenorcarcinoma cells; MCF-7: breast cancer cell line standing for Michigan Cancer Foundation - 7; MTS: 3 - (4,5 –dimethylthiazol – 2 - yl) – 5 - (3 - carboxymethoxyphenyl) – 2 - (4 -sulfophenyl) - 2H - tetrazolium; KDMEM: Knock out Dulbecco's modified Eagle's medium; PBS: phosphate buffered saline; R: phycoerythrin conjugate; RIPA: radioimmunoprecipitation assay buffer; 7 - AAD: 7 - Amino-actinomycine; V - PE annexin: annexin V; S: sulphur; Se: selenium; VC: vitamin C; z – VAD - fmk: N – benzyloxycarbonyl – Val - Ala Asp(Ome) fluoromethylketone

1. Introduction

Its high prevalence, mortality and unsatisfactory treatment options make cancer a major health issue of our time, responsible for more than 10% of deaths worldwide and more than 25% in some countries (1, 2). Lung cancer remains the commonest cancer worldwide, accounting for 1.2 million new cases per year, followed closely by breast cancer and colorectal cancer with around 1 million new cases (1).

The high incidence of this disease, its life-threatening nature, and often unsatisfactory management has motivated academic researchers and those from the biotechnology and pharmaceutical industries to focus on the causes and potential treatments of cancer on a scale unparalleled in almost any other disease area (1). At present there are almost 500 products in clinical trials of which 100 are in phase III, breast cancer and non-small-cell lung cancer receiving most attention (1, 2).

In the organism homeostasis is dependent on equilibrium between cell proliferation through cell division and cell loss through apoptosis (programmed cell death, PCD). Defects in apoptosis signaling may hinder this balance, thereby enhancing tumor development and metastasis to distant sites like the seminal vesicle, urinary bladder, rectum, bone, lymph nodes via the blood and brain, and in the more aggressive forms resulting in death (2). Chemotherapeutic agents exert their effect by killing cells that are rapidly dividing (1, 2). The agents are therefore not tumor-cell specific and show their toxic effect by killing normal cells that are dividing, for example, hair follicle cells and gastrointestinal mucosa. In recent years, our understanding of the molecular pathways controlling growth of both normal and tumor cells has improved significantly (1). By exploiting the differences between normal and malignant cells, we can target pathways and receptors unique to cancer cells, thus avoiding the indiscriminate universal killing of dividing cells as practiced by conventional cytotoxics. We are therefore entering the era of biologically targeted therapies (1), where monosaccharides and disaccharides would seem to play an interesting part in cancer treatment (3). Previous *in vitro* studies have confirmed that exposure of human endothelial cells to high glucose induces significant cell death via apoptosis (3). There is evidence that apoptosis is particularly prominent in models of hyperglycemia injury, though the mechanism and clinical complications still remain unclear (3).

The present study therefore looks into the options for treating cancer by a cellobiose complex containing cellobiose with vitamin C, sulphur and selenium isolated from a mixture of two cold plant extracts of *Alium sativum* and *Capsicum chinense*, plants that are rich in disaccharides and other cytotoxic molecules (4, 5). The study aims to elucidate the pivotal apoptotic and necrotic role of disaccharide molecule cellobiose together with sulphur, selenium and vitamin C, which are abundant in the plants mentioned (6, 7). It also sheds some light on the mechanism triggered by these molecules in various different types of cancer cells such as human adenocarcinoma cells HeLa and human breast cancer cells MCF-7.

2. Materials and methods

2.1 Materials

In our research we used the following materials: MTS from Promega (USA), Bradford reagent from Bio - Rad (Germany), z – VAD - fmk, Ac – DEVD - AFC, DTT from Bachem (Switzerland), FBS from Gibco (USA), antibiotics penicillium, streptomycin

and glutamax from Gibco (USA); annexin V-PE and 7-AAD for flow cytometry were purchased from BD Biosciences (USA). Fluorescent organelle-specific probe Mitotracker CMXRos came from Molecular Probes (Eugene, OR, USA) and cellobiose, vitamin C, sulphur and selenium were from Calbiochem Germany. All other reagents (RIPA buffer, caspase buffer, KDMEM, Tryple Select, PBS) were prepared at the institute according to standard procedures (8, 9).

2.2 Cell lines

The cell lines used in the experiments were human andenocarcinoma cells HeLa (ATCC), immortal human keratinocyte cell line HaCaT (ATCC) and breast cancer cells MCF-7 (ATCC). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% bovine serum (FBS), 1% penicilium/streptomycin and 1% glutamax to obtain 90% confluency. The cells used in our experiments were grown in DMEM, free of any kind of supplement of animal origin.

2.3 Preparation of plant extract

Capsicum chinense and *Allium neapolitanum* were grown in a greenhouse. Once the plants grown in the greenhouse blossomed and produced first fruits, the whole plant was collected and dried. Our calculation was as follows: 100 g of *Alium sativum* contained 1 g cellobiose, 31.2 mg vitamin C, 14.2 μg of selenium and 10 μg of sulphur, while 100 g of *Capsicum chinense* contained 76.4 mg of vitamin C, 8.8 μg of selenium and 36.6 g of cellobiose (9-11). The dry plants were ground into a powder. The powder was suspended in DMEM to which penicillium and streptomycin were added, together with glutamax (1% v/v each of antibiotic and glutamax in the final mixture). The mixture was left at room temperature for 3 days and shaken several times in between. After 3 days the extract was filtered and by these means became ready for experimental use.

2.4 Cell culture

All cells were grown in 10 cm plates (N=3) to obtain 90% confluency and afterwards washed with 1 x PBS and treated with Tryple Select 5-10 minutes (depending on the cell line). In the next step the cells were transferred into 96-well (N=1 for 96-well plate, each concentration in a 96 well-plate was represented by N=3 repeats) and 6-well plates (N=1 for 6-well plate, each concentration in a 96 well-plate was represented by N=3 repeats) at a ratio of 10,000 cells/well (HaCaT, HeLa and MCF-7) and grown overnight. The next day the cells were treated with the plant extract, and later with the solution of cellobiose in the range of 0.001 mg/mL–1000 mg/mL (raising concentrations by a factor of 10; N= for each concentration) and incubated for 48 hours (depending on the cell line and the onset of apoptosis). We used two control cells. The first were grown only in DMEM (N=3) and the other ones in DMEM with penicillium/streptomicinum in a 1% v/v (N=3). All experiments were repeated 3 times. Control cells were grown in DMEM alone.

2.5 Determination of cell death

Cells were cultured at 1×10^6 in 6-well plates (N=3) overnight before treatment with the plant extract at a final concentration (N=3 for each concentration) depending on the cell line. For comparative control experiments the cells were incubated overnight in DMEM alone. After incubation with the plant extract, specific for each cell line, the cells were observed by light microscopy (Olympus IX71, Japan, magnification 40 and 60). To prepare the extracts, cells were collected in three parallels, pelleted by centrifugation at 1000 rpm for 5 minutes and washed twice with 1 × PBS. Whole-cell extracts were prepared in RIPA buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1% (v/v) SDS, 1% (v/v) Nonidet P-40 0.5% v/v deoxycholic acid, 1mM EDTA) in three parallels. Following 10 minutes incubation on ice, the insoluble material was removed by centrifugation at 14,000 rpm for 10 min. Cytosolic extracts were prepared as previously described (12). Total protein concentration was determined using the Bradford assay.

The first apoptosis impression was measured by addition of 20 μL of MTS to 100 μL samples in the 96-well plate (N=3 for each concentration). After 45 minutes' incubation we measured the absorbance at 490 nm with a 96-well plate reader (TECAN). For further analysis we chose concentrations of plant extracts

that induced 20-50% cell death and in the next step we measured caspase activity in these samples (N=3). We gathered 40 μg of protein cells, untreated and treated with plant extract in the presence or absence of inhibitors z – VAD - fmk, to determine caspase activity and measuring the proteolytic cleavage of the fluorogenic substrate Ac – DEVD - AFC (Bachem) (12).

Apoptosis was quantified by flow cytometry measurements of phosphatidylserine exposure and 7-AAD incorporation, and by measurement of the DEVD - ase activity of the caspases. Briefly, 100 μL aliquots of cells were labeled with annexin V - PE and 7 - AAD according to the manufacturer's instructions. The cells were then subjected to flow cytometry analysis using a FACScalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software.

To determine by which apoptotic pathway (intrinsic, extrinsic) the plant extract triggers apoptosis we used Mitotracker Red CMXRos to assess and monitor the integrity of mitochondria. Mitotracker CMXRos was added to the cells at a final concentration of 20 nM. Following 30 minutes incubation at 37°C the cells were washed in 1 x PBS and again re - suspended in 1 x PBS for the measurement by flow cytometer. Here we measured the red fluorescence of 5,000 or 10,000 cells (depending on the cell line chosen) per sample, corresponding to the mitochondria using the FL3 channel.

2.6 Analysis and statistics

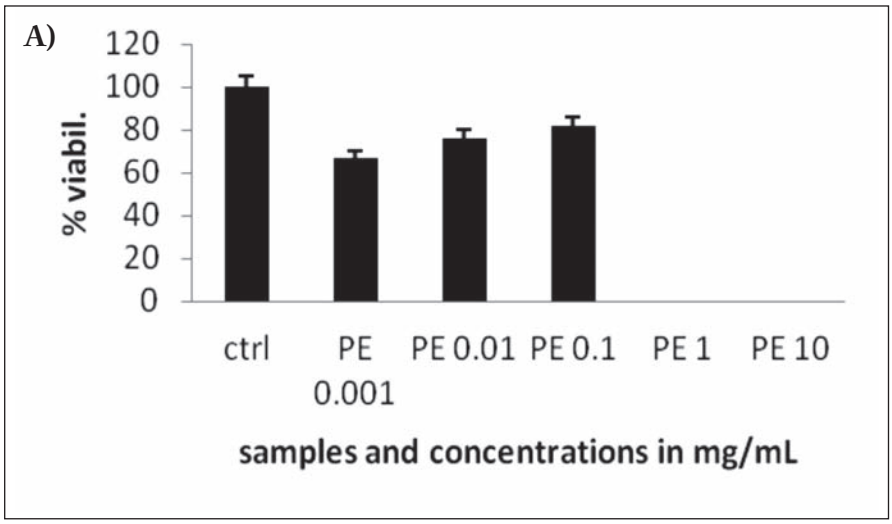
The plant extract concentrations were prepared according to the mass of pulverized plant to be diluted in DMEM in order to reach the desired final concentration. Apoptotic and necrotic cells were first detected by Olympus IX71 light emission microscope (magnification 1,000 x) before being further analyzed by MTS. Samples of treated cells were prepared in 3 parallels and each experiment was repeated 3 times. After quantification of apoptotic and necrotic cells by the MTS test and flow cytometry analysis, the average percentage of all parallel samples was calculated. For quantification of damaged mitochondria by MitoTracker RedCMXRos we calculated the average percentage of damage in all 3 parallels of selected cells (HeLa and MCF-7). The percentage of apoptotic and necrotic cells were reported as mean ± standard error. Data were analyzed using a one-way analysis of variance (ANOVA). Differences between experimental and control groups and among different exposure groups were regarded as statistically significant at the level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

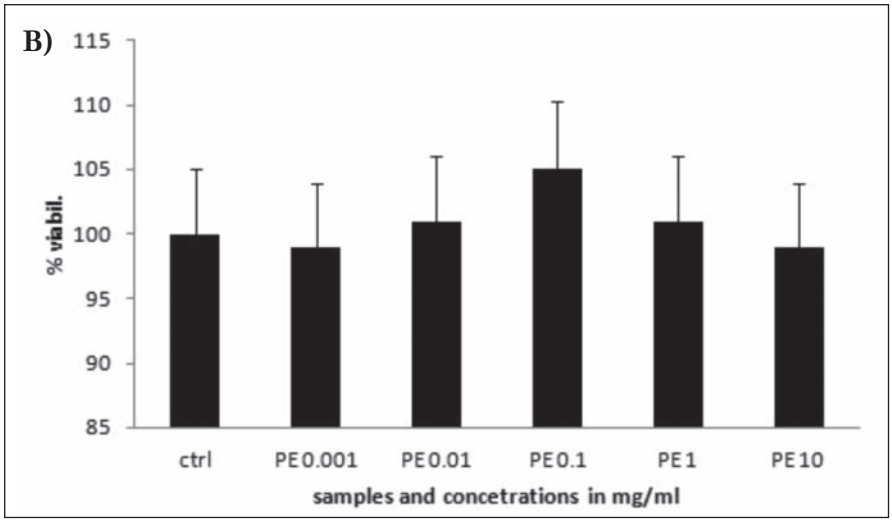
3. Results

3.1 Experiments with plant extract containing cellobiose

The aim of the present study was to investigate whether the plant extracts *Capsicum chinense* and *Allium sativum* can trigger programmed cell death and necrosis, and to show that there are active molecules in plant extract responsible for PCD. We were also interested in finding out the differences in response to plant extract by various types of cancer cells. Thus cancer cells HeLa, HaCaT and MCF-7 were treated with the plant extract containing cellobiose complex, one isolated fraction being treated with cellobiose complex (cellobiose, sulphur, selenium and vitamin C as determined in previous studies) and in next experiment with pure cellobiose. Cell death was assayed by MTS test and FACS flow cytometry determination of viability. To assess the molecular basis of PCD, measurement of DEVD - ase activity and of mitochondria integrity was performed.

In order to determine whether plant extract and cellobiose induced growth inhibition due to PCD, we evaluated the apoptotic indices at the indicated times of treatment (i.e. 48 h) by microscopic analysis. The extent of apoptosis was quantified by the MTS test and flow cytometric analysis where HeLa cancer cell lines were labeled with annexin - V and 7AAD. In an MCF-7 cancer cell line and an HaCaT immortalized cell line the plant extract showed only a slight decrease in viability, which was not statistically significant and therefore these two cell lines were not studied in further experiments. In Hela there was a different outcome. The plant extract with the concentration of 1 μg/mL induced 33% apoptotic cells, 0.1 mg/mL 23% while plant extract with the concentration of 1 mg/mL induced 18% apoptotic cells. It was seen that as the concentration range grew, the number of apoptotic cells decreased, because the rate of necrosis was higher





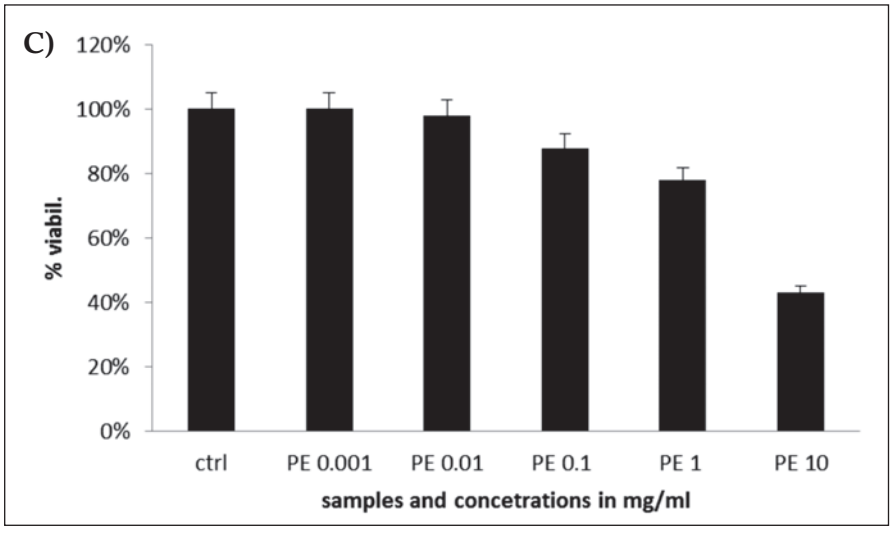


Figure 1. Viability of HeLa, HaCaT and MCF-7 cancer cells determined by MTS test after treatment with plant extracts of *Alium sativum* and *Capsicum chinense* together (marked as PE) containing cellobiose. While in MCF-7 the viability was retained, there was a 33% decrease in viability in HeLa cancer cells with the concentration 1 μg/mL. In HaCaT the viability was retained the same as in MCF-7. In higher concentrations there was a higher rate of necrosis, where cells burst (HeLa and HaCaT cell line).

A) HeLa viability after treating cells with concentrations of plant extract (marked as PE) from 0.001 mg/mL to 10 mg/mL measured by MTS test. Error bars denote mean ±SEM.

B) MCF-7 viability after treating cells with concentrations of plant extract (marked as PE) from 0.001 mg/mL to 10 mg/mL measured by MTS test. Error bars denote mean ±SEM.

C) HaCaT viability after treating cells with concentrations of plant extract (marked as PE) from 0.001 mg/mL to 10 mg/mL measured by MTS test. Error bars denote mean ±SEM.

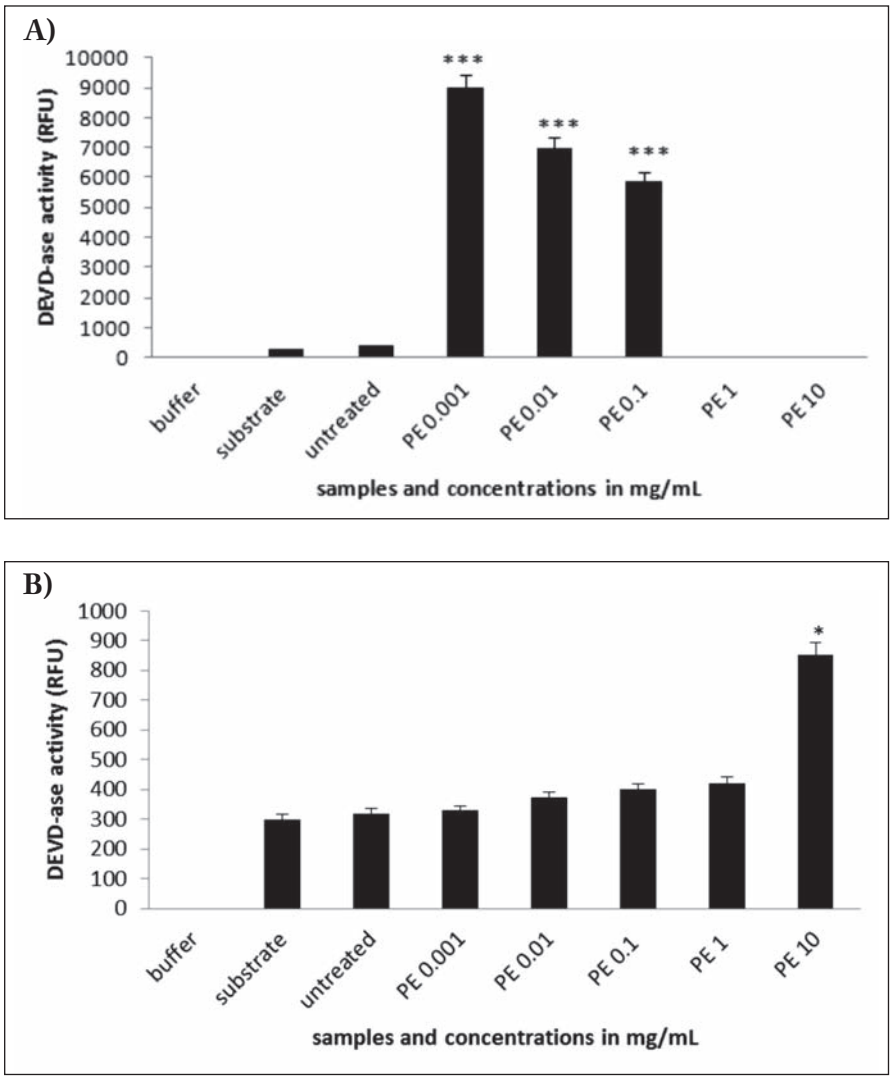
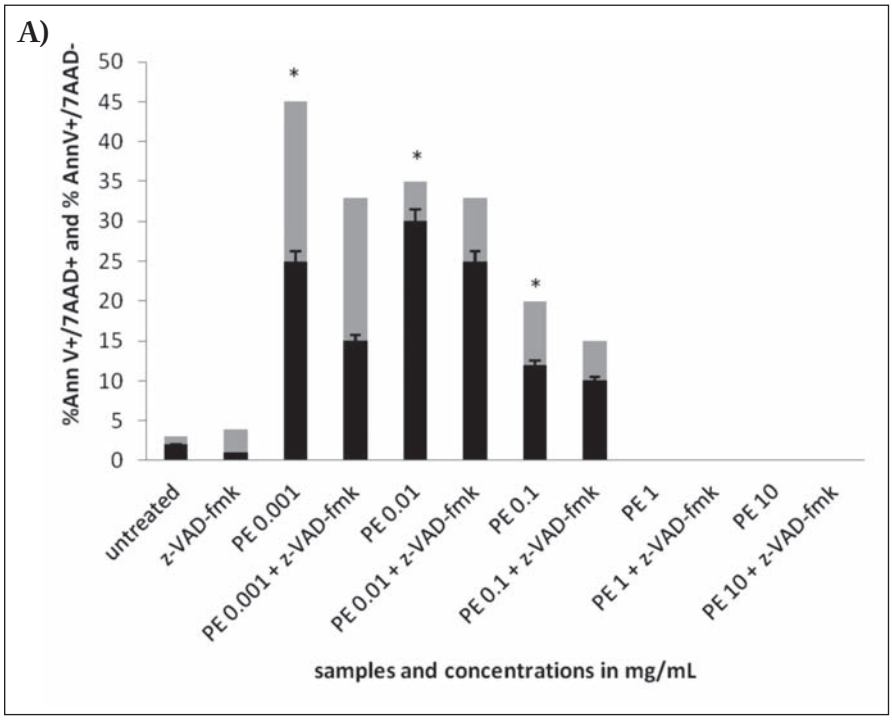


Figure 2. DEVD - ase activity in HaCaT and HeLa cancer cell lines after treatment with the plant extract (marked as PE) containing cellobiose. In HaCaT there was no activation, while in HeLa the greatest activation was with the concentraton of 1 μg/mL.
Error bars denote mean ± SEM.
*p < 0.05; ** p < 0.01; *** p < 0.001.
A) HeLa caspase activity after treating cells with plant extract (marked as PE) in the concentration range from 0.001 mg/mL to 10 mg/mL measured in arbitrary units (RFU).
B) HaCaT caspase activity after treating cells with plant extract (marked as PE) in the concentration range from 0.001 mg/mL to 10 mg/mL measured in arbitrary units (RFU).

and many cells burst in the process of treatment (Figure 1). In MCF-7 there was very low response to the treatment with plant extract and the number of apoptotic cells varied from 5-10%, which was comparable to control cells (untreated cells). The results of the DEVD - ase activation (Figure 2) were in correlation with the viability tests, showing that the caspase - 3 was most highly activated only in HeLa with the concentration 1 μg/mL, where activation reached up to 9,000 RFU (± 0.15; p < 0.001). While treating the cells with the pan- caspase inhibitor z –VAD - fmk we saw that the blockade was not achieved in HeLa cancer cells (Figure 3), suggesting the apoptotic pathway triggered with the concentration of plant extract of 0.001 mg/mL and 0.01 mg/mL was triggered through different means and that the majority of cells died via necrosis (p < 0.05), which was also seen while studying the integrity of mitochondria, where the mitochondria were not damaged or only damaged to a low percentage (up to 4%) which was comparable to control cells (Figure 4).

3.2 Experiments with pure cellobiose solution

Finally we repeated the experiments with the molecule cellobiose alone (a molecule synthesized in the laboratory). We found that only concentrations from 10 mg/mL to 1000 mg/mL (N=3 parallels and repeats) induced cell death in HeLa, which was con-



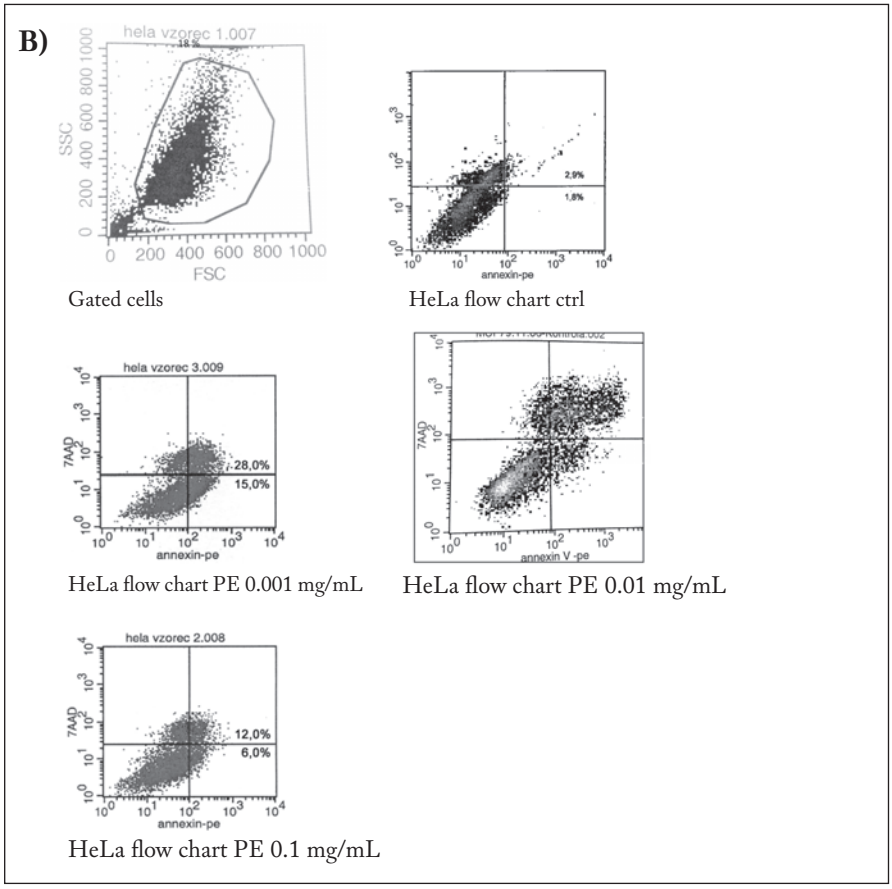


Figure 3. A) Flow cytometry analysis and number of apoptotic cells (grey bars) and necrotic cells (black bars) in HeLa cancer cell line after treatment with plant extract (marked PE) containing cellobiose. Error bars denote mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. B) Flow cytometry analysis and number of apoptotic (lower right quadrant) and necrotic cells (upper right quadrant) in HeLa cancer cell line after treatment with plant extract (marked PE) containing cellobiose. Error bars denote mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

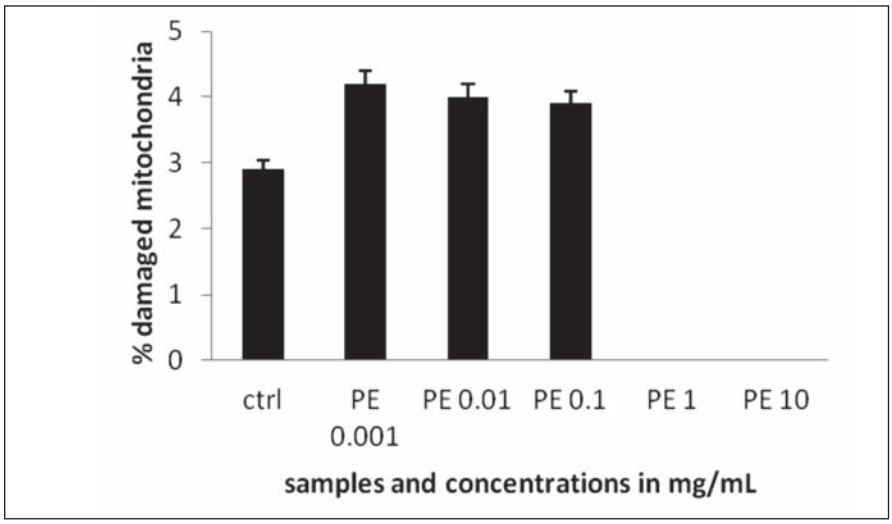
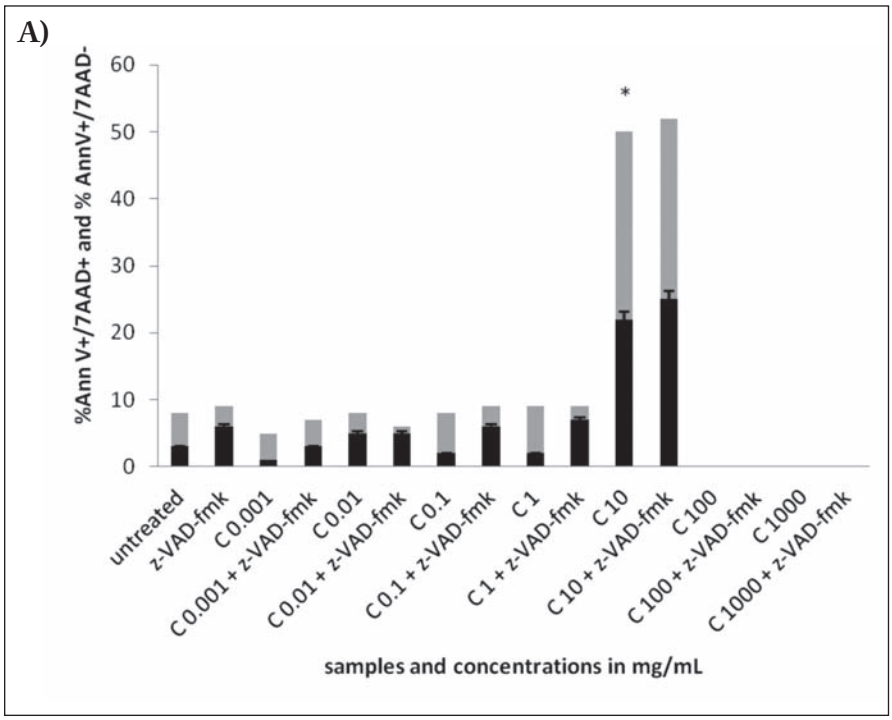


Figure 4. Analysis of the integrity of mitochondria in HeLa cancer cells after the treatment with plant extract (marked as PE) containing cellobiose. Final concentrations of plant extract are in the concentration range from 0.001 mg/mL to 10 mg/mL. Error bars denote mean ± SEM.

firmed by flow cytometry (Figure 5), where we discovered that the concentration of 10 mg/mL (N=3 parallels and repeats) induced cell death in 50% (± 0.15; $p < 0.05$) of cells, while 1000 mg/mL of cellobiose (N=3 parallels and repeats) induced necrosis ending in cell debris. Pre-treatment of cells with z – VAD - fmk did not rescue the cells from PCD in any of the above-mentioned concentrations (Figure 5). DEVD - ase activity was confirmed in concentrations from 0.001 mg/mL up to 10 mg/mL ($p < 0.01$), but in concentrations above 10 mg/mL caspase activity was not detected at all, because necrosis led to cell debris (Figure 6).

3.3 Experiments with cellobiose complex (cellobiose, vitamin C, sulphur and selenium)

As we had shown in our previous studies (13) that cellobiose itself is not the main molecule triggering apoptosis and necrosis, we further investigated which molecules might also be present in the active fraction with cellobiose, making it effective in triggering apoptosis and necrosis within as little as 3 hours and at very low concentrations (13). We discovered there are many other molecules abundant in the plant family *Solanaceae*, which are crucial for the synergistic effect (9-11). Hence we repeated the experiments with cellobiose together with vitamin C, sulphur and selenium according to NMR results in our previous studies (13). The combination of these molecules is responsible for triggering apoptosis and necrosis in the human liver cancer cell line HepG2 (13).We have shown that even low concentrations from 0.001 mg/mL to 0.1 mg/mL (N=3 parallels and repeats) induced cell death in HeLa, which was confirmed by flow cytometry (Figure 7), where we discovered that the concentration of 0.1 mg/mL (N=3 parallels and repeats) induced cell death in 54% (± 0.15; $p < 0.05$) of cells, while 1 mg/mL of cellobiose with vitamin C, sulphur and selenium (N=3 parallels and repeats) induced necrosis ending in cell debris. However we noticed that individual solutions of cellobiose, vitamin C, sulphur and selenium did not induce the same rate of cell death as was achieved when combining the molecules mentioned all together (Figure 7). Pre-treatment of cells with z – VAD - fmk did not rescue the cells from PCD in any of the above-mentioned concentrations (Figure 7). DEVD - ase activity was confirmed in concentrations from 0.001 mg/mL to 0.1 mg/mL ($p < 0.01$), but at higher concentrations it was not detected at all, showing that cells were completely destroyed (Figure 7 and 8). When we studied the integrity of mitochondria, we discovered the highest damage was achieved with the concentration of 0.1 mg/mL of cellobiose together with vitamin C, sulphur and selenium ($p < 0.05$). Higher concentrations of cellobiose, with vitamin C, sulphur and selenium induced necrosis leading to cell debris, which was not achieved in individual solutions of specified molecules (Figure 9).



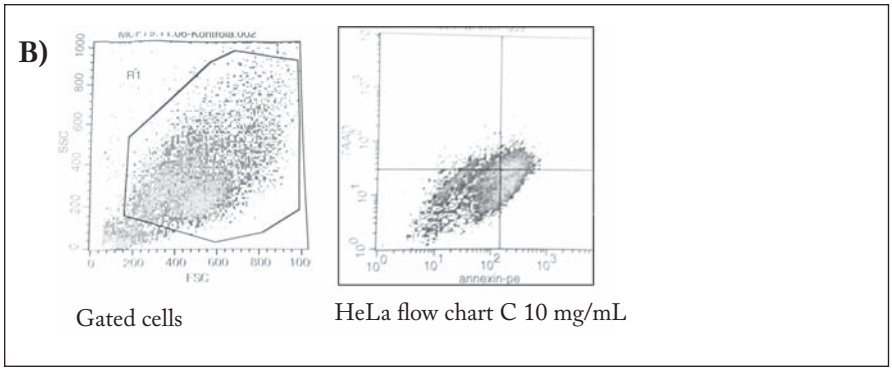


Figure 5. A) Flow cytometry analysis and number of apoptotic cells (grey bars) and necrotic cells (black bars) in HeLa cancer cell line after treatment with cellobiose (marked as C) in the concentration range from 0.001 mg/mL to 1000 mg/mL together with possible blockade of DEVD - ase activity by z – VAD - fmk. Error bars denote mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
B) Flow cytometry analysis and number of apoptotic (lower right quadrant) and necrotic cells (upper right quadrant) in an HeLa cancer cell line after treatment with a solution of cellobiose in the concentration 10 mg/mL (marked C). Only results, which were statistically significant are shown. Error bars denote mean ± SEM.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Discussion

The search for promising agents that may reduce the incidence and burden of cancer has become increasingly important in recent years. Immense interest has been generated in saccharides especially as concerns d-allose, monosaccharides and disaccharides in view of their role in attenuating the risk of developing cancer, as demonstrated by Naha *et al.* (2). Previous studies have shown that sugars are in some cases pro-apoptotic in a variety of cancer cells (3, 14-16). We are one of the groups focusing on the effect of mixing two cold plant extracts from the plant family *Solanaceae* in comparison to cellobiose synthesized in the laboratory and later on in comparison to a solution of cellobiose with vitamin C, sulphur and selenium.

To see whether cellobiose induces PCD, we next evaluated apoptosis in two cancer cell lines HeLa and MCF-7. Agents that can modulate apoptosis may be able to affect a steady-state cell population, which may be useful in cancer therapy (2, 3). By using HeLa and MCF-7 cell lines we showed the effect of the mix-

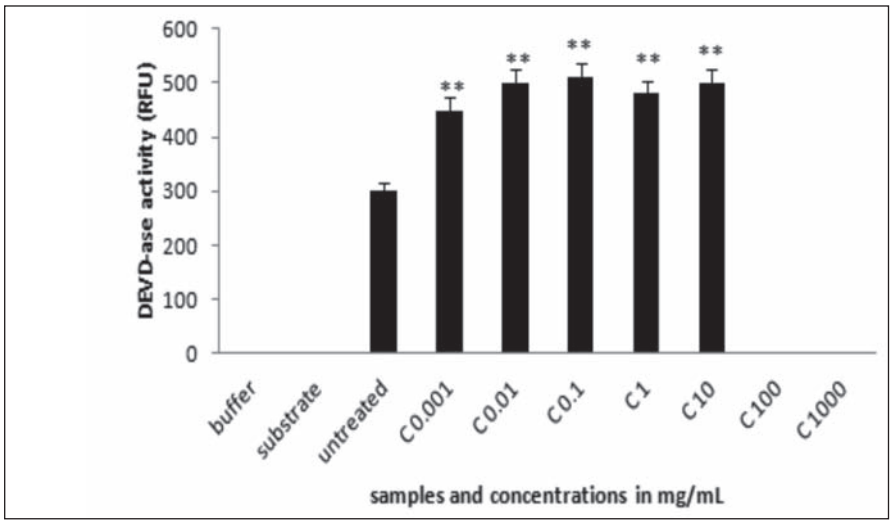


Figure 6. The activation of caspase - 3 (DEVD-ase activity) in HeLa cancer cells after treatment with solutions of cellobiose (marked as C) in the concentration range from 0.001 mg/mL to 1000 mg/mL. Error bars denote mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

ture of two cold plant extracts from *Alium sativum* and *Capsicum chinense* on the onset of apoptosis and necrosis. We demonstrated there was a different response to plant extract in MCF-7 and HeLa, where a MCF-7 cancer cell line was resistant to treatment with the plant extract mixture in contrast to HeLa, where 33% of apoptotic cells were detected even with a concentration of 1 μg/mL, which correlates with the results of other research groups (17, 18). This by no means correlated with treatment with cellobiose alone, where much higher concentrations of 10 mg/mL or more induced PCD and necrosis in 48 hours in contrast to the already mentioned low dose of the plant extract containing cellobiose. This fact suggested there are other molecules in the plant extract, which cause apoptosis and necrosis of HeLa cancer cells. According to our data there is a difference between different cancer cell lines as well as immortalized cell lines, which respond differently to the same plant extract and to the combination of individual molecules present in these plant extracts. This was clearly seen in experiments for the identification of molecular pathway of apoptosis and necrosis as also shown by Lamy *et al.* (19). While in MCF-7 cancer cell line there was no response as well as not in HaCaT cell line in HeLa there was a significant response at the lowest concentration of 10 μg/mL of plant extract. Furthermore there was no significant damage to mitochondria in HeLa cancer cells, suggesting the plant extract and cellobiose itself triggers PCD mainly through necrosis. This fact is correlates with the already observed cytotoxicity of a high concentration of saccharides, especially glucose in the case of cultured vascular endothelial cells (16). One of the studies also confirmed that increased cell death in HUVEC by exposure to high glucose was caused by apoptosis (2, 3), which was also indicated in our case, where apoptosis (27%) and necrosis (16%) was determined in HeLa, when the cells were treated with 10 μg/mL of the plant extract. When comparing the experiments with plant extract to those with the pure molecule of cellobiose (13), we discovered we needed 10,000 fold higher concentration of cellobiose to induce comparable results – something that Yang and Naha have also shown in their studies with glucose, suggesting that high dosages of sugars induce programmed cell death (2, 3). What was common in all concentrations was the fact that z – VAD - fmk did not rescue the cells from PCD. Noting that the best effect with cellobiose was in HeLa cancer cells, we discovered that caspase - 3 was only weakly activated in the concentration range 0.001 mg/mL – 10 mg/mL, which led us to conclude that cellobiose itself in lower concentrations does not trigger PCD partly because cellobiose is degraded to glucose (2, 3) and because programmed cell death is not triggered only by one molecular pathway as Lamy has likewise shown (19). If the concentration was 100 mg/mL or higher, cells burst and we could not detect either them or caspase - 3 activity (13).

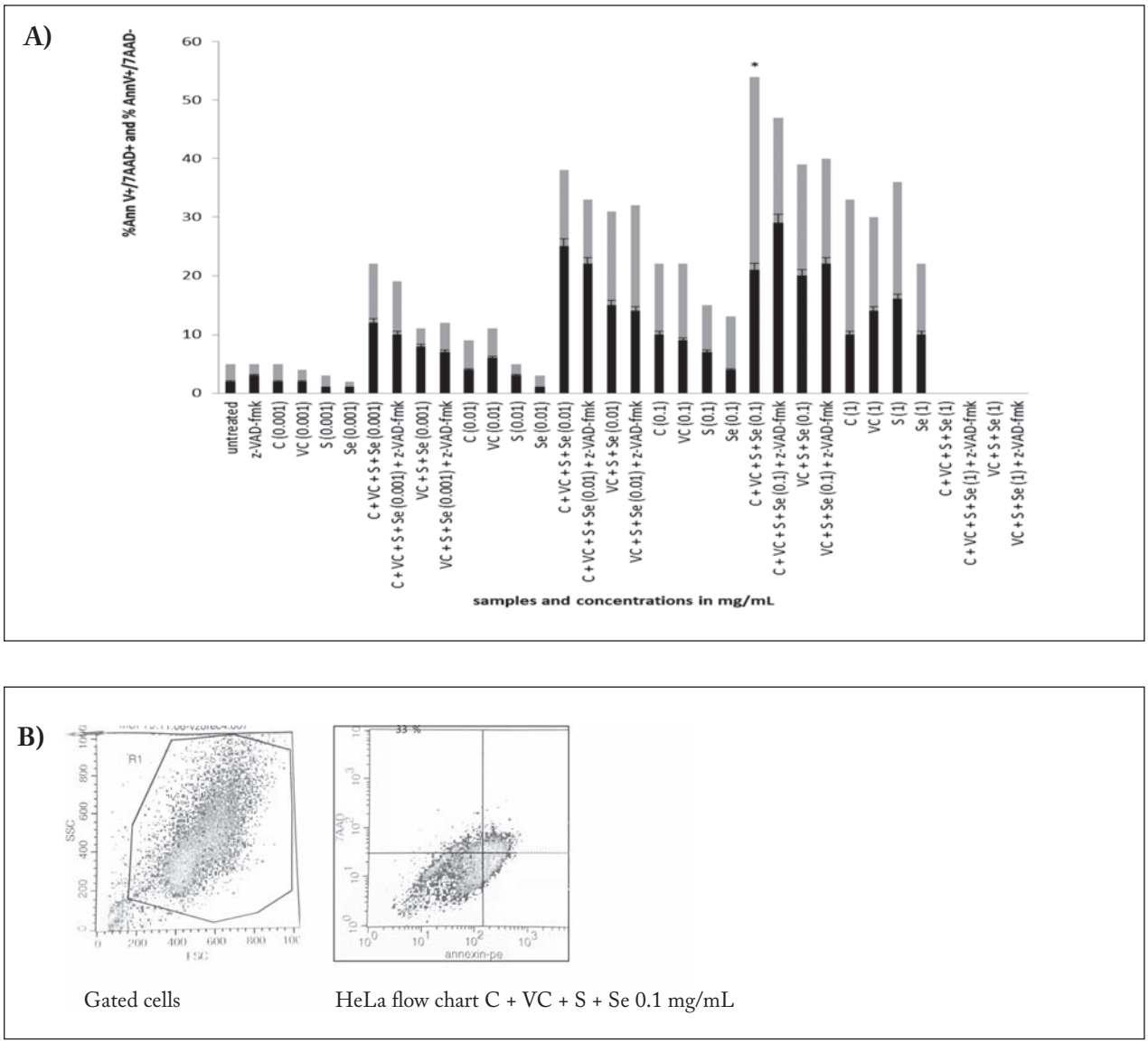


Figure 7. A) Flow cytometry analysis and number of apoptotic cells (grey bars) and necrotic cells (black bars) in an HeLa cancer cell line after treatment with cellobiose (marked as C), vitamin C (marked as VC), sulphur (marked as S), selenium (marked as Se) and cellobiose complex (marked as C + VC + S + Se) together with the possible blockade of DEVD - ase activity by z – VAD - fmk. Error bars denote mean ± SEM. *p < 0.05; ** p < 0.01; *** p < 0.001.
B) Flow cytometry analysis and number of apoptotic (lower right quadrant) and necrotic cells (upper right quadrant) in HeLa cancer cell line after treatment with a solution of cellobiose, vitamin C, sulphur and selenium (marked as C + VC + S + Se) in the concentration 0.1 mg/mL. Only results which were statistically significant are shown. Error bars denote mean ± SEM. *p < 0.05; ** p < 0.01; *** p < 0.001.

Our further research therefore confirmed that cellobiose itself is not the main molecule triggering apoptosis and necrosis in HeLa. In light of our previous research on the cancer cell line HepG2 (13), we repeated our experiments using cellobiose complex which was cellobiose with vitamin C, sulphur and selenium. This combination proved to be one that triggered apoptosis in the HeLa cancer cell line, but not in MCF-7. We observed loss of viability when HeLa cells were treated with cellobiose complex for 48 hours, where lower

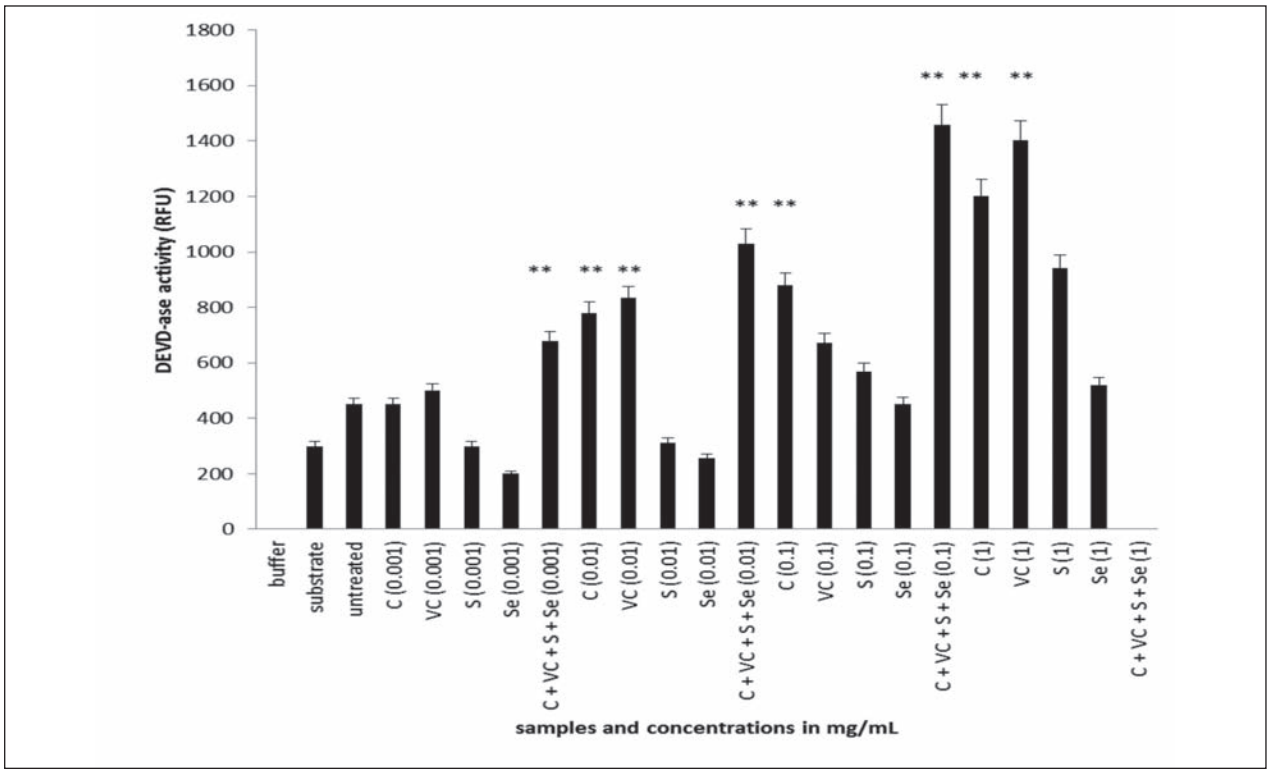


Figure 8. The activation of caspase-3 in HeLa cancer cells after treatment with solutions of cellobiose (marked as C), vitamin C (marked as VC), sulphur (marked as S), selenium (marked as Se) and cellobiose complex (marked as C + VC + S + Se) in the concentration range from 0.001 mg/mL to 1 mg/mL. Error bars denote mean ± SEM. *p < 0.05; ** p < 0.01; *** p < 0.001.

concentrations of 0.001 mg/mL to 0.1 mg/mL induced from 22 to 54% of apoptotic and necrotic cells. The findings were correlated with caspase-3 activation, where higher concentrations of 1 mg/mL induced bursting of cells, making enzyme activity no longer detectable.

These results suggested that a cellobiose such as disaccharide alone is not enough to trigger different pathways leading to cell death, but a rich composition of the mixture of plant extract, which was later shown to be the cellobiose complex of cellobiose, sulphur, selenium and vitamin C, is the crucial main step activating apoptosis and necrosis as well.

5. Conclusion

Taken together, our results show that the mixture of two cold plant extracts of *Capsicum chinense* and *Allium sativum*, which naturally contain cellobiose vitamin C, sulphur and selenium, induce cell death in HeLa cancer cells, but not in MCF-7 cancer cells. However, when treating HeLa cancer cells with the pure molecule of cellobiose, it was demonstrated that 10,000 fold higher concentrations were needed. Later we discovered other molecules (sulphur, selenium and vitamin C) present in plant extracts, which produce a synergistic effect with cellobiose, are more effective in triggering cell death of HeLa cancer cells even in lower dosages such as 1 μg/mL. When studying the molecular basis of cell death, we clearly demonstrated that cell death activation took place not only via caspase - 3 activation, but there was also another non-caspase dependent apoptotic pathway present due to the fact that z – VAD - fmk did not rescue the cells from apoptosis and necrosis, a fact which correlated with the findings of other research groups.

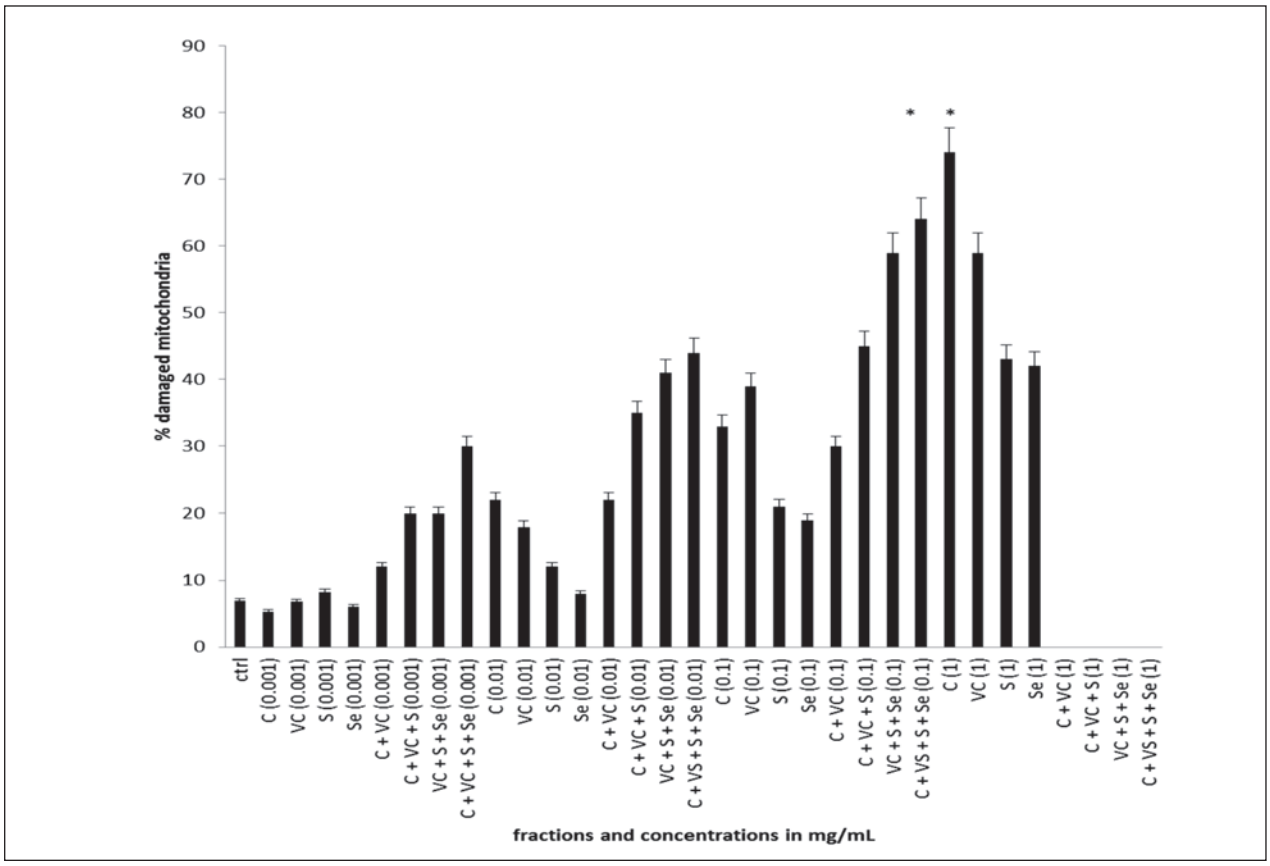


Figure 9. Analysis of the integrity of mitochondria in HeLa cancer cells after treatment with cellobiose (marked as C), vitamin C (marked as VC), sulphur (marked as S), selenium (marked as Se) and cellobiose complex (marked as C + VC + S + Se) in the concentration range from 0.001 mg/mL to 1 mg/mL. Error bars denote mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

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