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Comparison of the in vitro anticancer effect of habanero pepper extract containing capsaicin with that of pure capsaicin in selected dog neoplastic cell lines

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Abstract: The aim of this article was to assess the anticancer effectiveness of habanero pepper extract containing capsaicin on two dog cancer lines, DAN (fibroblasts isolated from osteosarcoma) and D-17 (epithelium cells of osteosarcoma obtained from pulmonary metastatic tumors), under in vitro conditions. The experiment was carried out in a suspension of D-17 and DAN line cells with a density of 12 × 105 cells/mL in a culture fluid with 10% of FBS. After 24 h of incubation (37 °C, 5% CO2), the fluid above the culture was removed, and diluted habanero pepper extract was added. The concentrations of capsaicin in the extract were 10, 20, 50, 100, 150, and 200 µM (in the culture fluid with 10% of FBS). The cells were incubated for 24–96 h (37 °C, 5% CO2). After the incubation period, cytotoxicity assessment of the habanero pepper extract and cell proliferation impairment by the habanero pepper extract in a suspension of cell lines, in MTT test, as well as the cytometric examination of viability of cancer cells exposed to the habanero pepper extract were performed. The results indicate that the extract shows better anticarcinogenic activity in vitro compared with pure capsaicin. Confirmation of the anticancer effectiveness of the extract on the cells is a starting point for wide clinical observations and a good indication for further research.

Key words: Capsaicin, habanero pepper, neoplasma, in vitro study

1. Introduction

Cancer pharmacotherapy is a dynamically developing branch of both human and veterinary medicine. Currently, the directions chosen in oncological treatment aim to make cancer a curable disease, changing it from a lethal disease into a chronic disease with a long time frame. Therefore, it is not surprising that new substances are tested in order to achieve these oncological goals, and these should combine high effectiveness with low manufacturing cost. On this aspect, much recent attention has been given to capsaicin. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is an organic compound belonging to the alkaloids, obtained from plants from the genus *Capsicum*, with the chemical formula $C_{18}H_{27}NO_3$ and molecular mass of 305.41 g/mol. Relatively large amounts are present in fruits of various pepper variants, and the compound itself is responsible for the spicy taste of this plant. Capsaicin bonds with transient receptor potential vanilloid subtype 1 (TRPV1) (1), which is present in large amounts in the hypothalamus, at the endings of the sensory neurons, in the dorsal root ganglia, and in the trigeminal nerve. It is also present in the kidneys, liver, urinary bladder, and pancreas (2). Capsaicin, after bonding with the TRPV1 receptor, causes the cation channel to open, as a result of which cations can flow into the cell and depolarize it. The resulting action potential is passed to the spinal cord and is responsible for the feeling of warmth and pain. Capsaicin may dilate the skin vessels and increase heat exchange, which results in the development of hypothermia, although this alkaloid may also increase metabolism, stimulating heat generation in the body and increasing internal body temperature (3).

It has been proved that capsaicin modulates the metabolism of carcinogenic and mutagenic compounds, which made this alkaloid an object of study for oncologists as a substance that could potentially be used in cancer prevention and therapy (4). In human medicine, it has

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been shown that capsaicin is effective in cancer therapies, including breast, pancreatic, bladder, and prostate cancers (5–7). In veterinary medicine, current research on habanero pepper extract and capsaicin itself has been limited mainly to the safety of its application in laboratory animals (8–11).

The aim of this study was to assess the anticancer effectiveness of habanero pepper extract containing capsaicin on two dog cancer lines, DAN (fibroblasts isolated from osteosarcoma) and D-17 (epithelium cells of osteosarcoma obtained from pulmonary metastatic tumors), under in vitro conditions, and to compare the anticancer effectiveness of habanero pepper extract with that of pure capsaicin.

2. Materials and methods

The test substance was ground and dried ecologically grown habanero fruit from the Mexican states of Jukatan and Quintana Roo. The capsaicinoids content in the substance measured at a specific HPLC was 7.64 mg/g dry matter (capsaicin and dihydrocapsaicin). After grinding, the test substance was suspended in peanut oil.

The anticancer effectiveness of habanero pepper extract was examined using the MTT colorimetric test to evaluate cell metabolic activity (12) (assessment of cytotoxicity and the influence of the extract on the proliferation of cancer cells), while the assessment of cell viability in the cultures was performed with a cytometric test.

2.1. Cytotoxicity assessment of habanero pepper extract – MTT test

The cytotoxicity assessment of habanero pepper extract was carried out in a suspension of D-17 and DAN cells with a density of 12×10^5 cells/mL in the culture fluid with 10% of FBS (POCH, Poland). The suspension was poured onto a 96-well plate (100 µL/well) (Iwaki, Japan) and then incubated for 24 h (37 °C, 5% CO_2) in order for the cells to attach to the bottom of the plate. After the incubation period, the fluid above the culture was removed, and diluted habanero pepper extract was added. The concentrations of capsaicin in the extract were 10, 20, 50, 100, 150, and 200 µM (in the culture fluid, with 1% of FBS). Capsaicin analytical standard (Sigma, UK) was used as the positive control, while the negative control was the culture fluid itself. The cells were incubated (37 °C, 5% CO_2) for 24–48 hours, after which 15 µL of the MTT solution (5 mg/mL with PBS with Ca^{2+} and Mg^{2+} ions; POCH, Poland) was introduced into each well on the plate. After a 3-h incubation period at 37 °C, 100 µL of 10% SDS in 0.01 N HCl with pH 7.4 was added to the plate wells in order to achieve cell lysis and dissolve the formazan crystals. The plate was placed in the incubator again for 24 h, and then a spectrophotometric reading was performed of suspension absorbency at a wavelength of λ = 570 nm.

2.2. Examination of cancer cell proliferation using the MTT test

The assessment of cancer cell proliferation impairment by habanero pepper extract was carried out in a suspension of D-17 and DAN line cells with a density of 4×10^5 cells/mL in the culture fluid with 10% of FBS (POCH, Poland). The suspension was poured onto a 96-well plate (100 µL/well) and then incubated for 24 h (37 °C, 5% CO_2) in order for the cells to attach to the bottom of the plate. After the incubation period, the fluid above the culture was removed, and diluted habanero pepper extract was added. The concentrations of capsaicin in the extract were 10, 20, 50, 100, 150, and 200 µM (in the culture fluid, with 10% of FBS). Capsaicin analytical standard (Sigma, UK) was used as the positive control. The cells were incubated for 96 h (37 °C, 5% CO_2), after which 15 µL of the MTT solution (5 mg/mL with PBS with Ca^{2+} and Mg^{2+} ions) (POCH, Poland) was introduced into each well on the plate. After a 3-h incubation period at 37 °C, 100 µL of 10% SDS solution in 0.01 N HCl with pH 7.4 was introduced to the plate wells in order to achieve cell lysis and dissolve the formazan crystals. The plate was placed in the incubator again for 24 h, and then a spectrophotometric reading was performed of suspension absorbency at a wavelength of λ = 570 nm.

2.3. Examination of cancer cell viability using flow cytometry (PI dye)

The examination of the viability of cancer cells exposed to habanero pepper extract was carried out in a suspension of D-17 and DAN cells with a density of 12×10^5 cells/mL, in a culture fluid with 10% of FBS. The suspension was poured onto a 24-well plate (1 mL/well) and then incubated for 24 h (37 °C, 5% CO_2) in order for the cells to attach to the bottom of the plate. After the incubation period, the fluid above the culture was removed, and the diluted habanero pepper extract was added. The concentrations of capsaicin in the extract were 10, 20, 50, 100, 150, and 200 µM (in the culture fluid, with 1% of FBS). Capsaicin analytical standard (Sigma, UK) was used as the positive control. After 24 h of incubation, the fluid was collected from the culture, and the cells were flushed with PBS with Ca^{2+} and Mg^{2+} ions, after which a solution of Trypsin-EDTA (POCH, Poland) was added to the culture to unstick the cells from the plate bottom. The cells were then moved to cytometric test tubes, centrifuged, and suspended with 1 mL of PBS (POCH, Poland). A total of 5 µL of PI was added to each test tube (final concentration 1 µg/mL) and the test tubes were incubated for 5 min. The samples were then ready for analysis in a flow cytometer (BD FACSVerse, Becton Dickinson, UK). Data gathering and analysis were carried out using BD FACSuite Software.

2.4. Statistical analysis

The Mann–Whitney rank test was used to demonstrate the differences between cytotoxicity and the differences between levels of apoptosis induction of habanero pepper extract and pure capsaicin in DAN and D-17 cells. Changes were considered statistically significant at $P < 0.05$. Statistica 10.0 PL software was used for the calculations.

3. Results

In the MTT test, it was shown that the cytotoxicity of the habanero pepper extract and its effect on the inhibition of cancer cell proliferation were higher than the cytotoxicity and the capacity to inhibit cancer cell proliferation of pure capsaicin.

In 24- and 48-h cultures of DAN cells, habanero pepper extract in the concentration range of 10–150 µM induced statistically stronger cytotoxicity than pure capsaicin (from P = 0.011668 to P = 0.019244), while in the 96-h culture this was seen in the concentration range of 50–200 µM (from P = 0.011160 to P = 0.011926) (Figures 1a–1c).

In 24- and 48-h cultures of D-17 cells, habanero pepper extract induced statistically stronger cytotoxicity than pure capsaicin in the concentration range of 10–150 µM (from P = 0.010910 to P = 0.012186), while in 96-h culture this was seen in the concentration range of 50–200 µM (from P = 0.010910 to P = 0.011926) (Figures 2a–2c).

The cytotoxicity of the habanero pepper extract at higher concentrations (100–200 µM) did not change at all and was around 80% (only 20% of the living cells remained in the culture). In the 96-h system, the highest cytotoxicity in cell cultures was demonstrated at extract concentrations of 150–200 µM (Figures 1c and 2c). This effect was evident regardless of the used cell line (D-17 or DAN).

The results of the tests carried out on cell cultures were confirmed cytometrically. The introduction of the pepper extract containing capsaicin into the DAN and D-17 cell cultures induced stronger apoptosis of cancer cells in the culture than pure capsaicin (standard).

A statistically stronger induction of apoptosis by habanero pepper extract compared with pure capsaicin (from P = 0.011668 to P = 0.011926) in both the DAN and D-17 cell lines was observed for concentrations from 50 to 200 µM.

In the DAN cell cultures incubated with pure capsaicin at concentrations of 50, 100, 150, and 200 µM, the percentage of apoptotic cells was 18%, 25%, 30%, and 36%, respectively. When the capsaicin-containing extract at concentrations of 50, 100, 150, and 200 µM was introduced to the culture, the percentage of apoptotic cells was higher at 59%, 67%, 85%, and 91%, respectively (Figures 3a and 3b). A similar relationship was observed in the D-17 cell line culture. Incubation of cells with pure capsaicin at concentrations of 50, 100, 150, and 200 µM resulted in apoptosis of 7%, 25%, 28%, and 43% of cells, respectively, while capsaicin-containing extracts at concentrations of 50, 100, 150, and 200 µM induced apoptosis in 10%, 72%, 87%, and 92% of cells, respectively (Figures 3c and 3d).

4. Discussion

Capsaicin is used to prevent the development of cancer but it also shows a beneficial impact and, to a certain degree, effectiveness in terms of already existing cancer diseases. In this aspect, its effect is related to the induction of cancer cell apoptosis by the inhibition of the last stage of the cell breathing process taking place in the mitochondria. The apoptosis of cancer cells is a consequence of the inhibitory

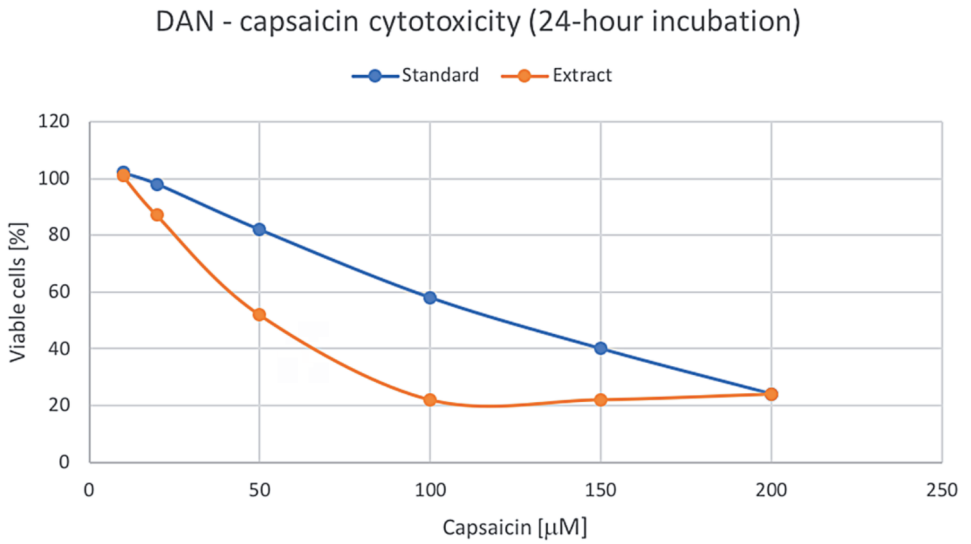


Figure 1a. The results of the cytotoxicity assessment: habanero pepper extract vs. pure capsaicin in a DAN cell culture, after a 24-h incubation period with the examined extracts.

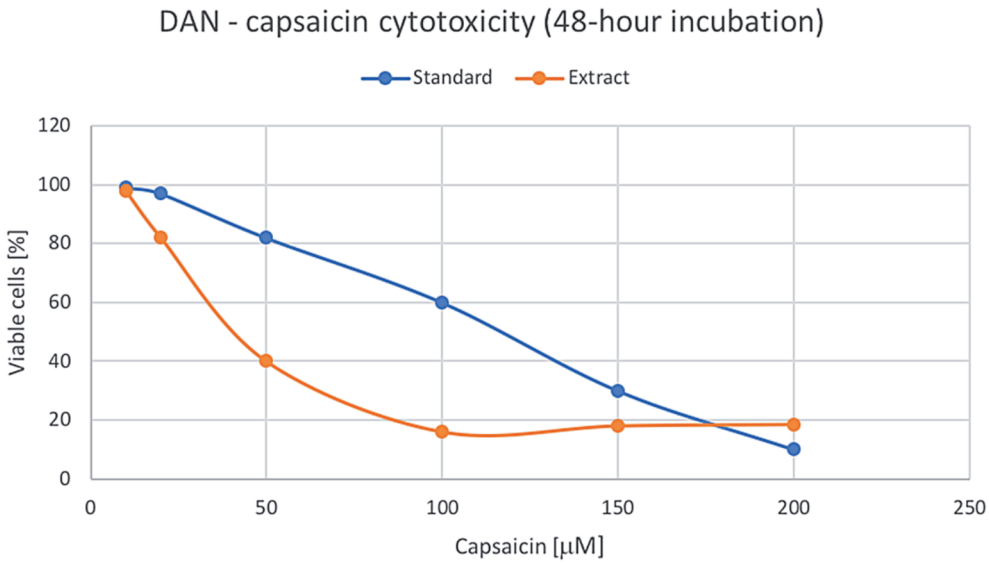


Figure 1b. The results of the cytotoxicity assessment: habanero pepper extract vs. pure capsaicin in a DAN cell culture, after a 48-h incubation period with the examined extracts.

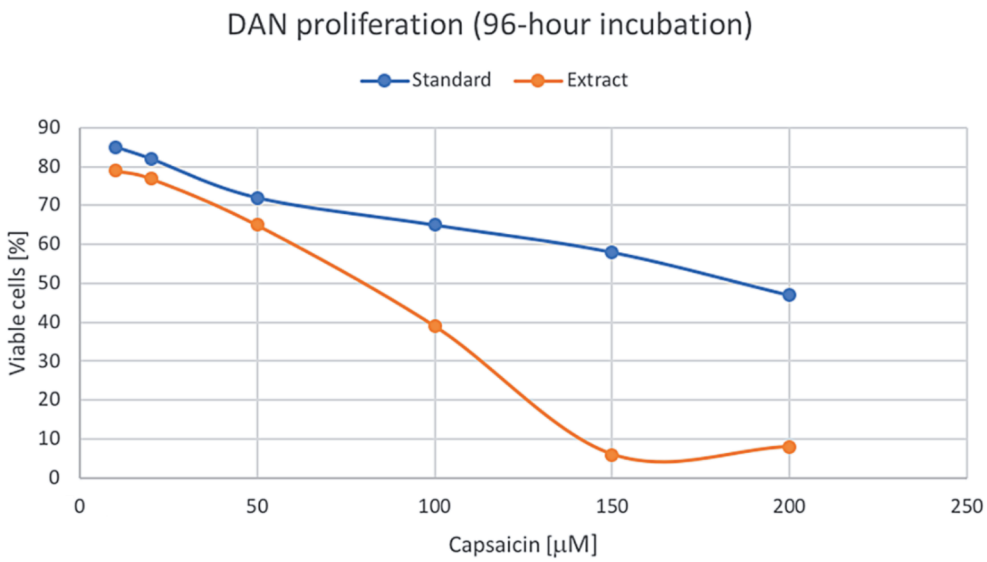


Figure 1c. The impact of habanero pepper extract vs. pure capsaicin on DAN cell line proliferation, after a 96-h cell incubation period with the examined extracts.

effect of capsaicin on the electron transport from NADH to ubiquinone, or direct binding of capsaicin with co-enzyme Q, which causes change in the electron flow direction and the creation of excessive amounts of reactive oxygen forms. This results in the dispersion of the transmembrane potential in the mitochondria (13), which is crucial for the functioning of these organelles. Apoptosis starts from the transmembrane potential breakdown, while the forms of active oxygen may cause damage to the structure and impairment of the mitochondrial function and, in consequence, the death of the cancer cell.

The apoptosis of cancer cells caused by capsaicin is related to the ability of this alkaloid to stimulate the transcription of, for instance, the p53 gene. Such a mechanism of operation, accompanied by DNA fragmentation in cancer cells, was confirmed under in vitro conditions in the case of stomach cancer cells (14).

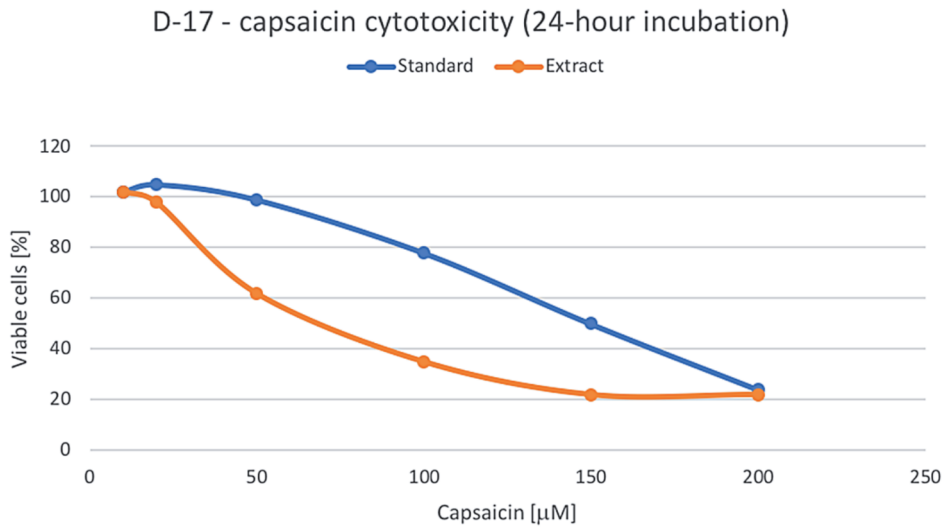


Figure 2a. The results of the cytotoxicity assessment: habanero pepper extract vs. pure capsaicin in a D-17 cell culture, after a 24-h incubation period with the examined extracts.

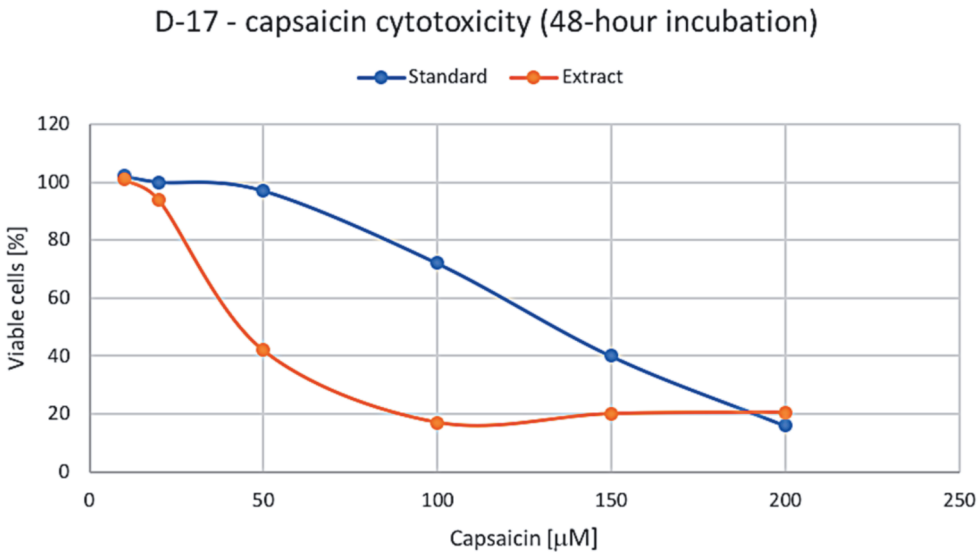


Figure 2b. The results of the cytotoxicity assessment: habanero pepper extract vs. pure capsaicin in a D-17 cell culture, after a 48-h incubation period with the examined extracts.

In the course of in vitro examinations carried out on T24 cancer cells of the bladder in mice, it was proved that, depending on the dose, capsaicin may lead to the depolarization of the mitochondrial membrane, the result of which was the death of a cell (not apoptosis). It is suggested that this alkaloid may be used in urinary bladder cancer treatment (15).

The case is similar to that of prostate cancer, for which this alkaloid shows a strong antiproliferation effect. Capsaicin induces apoptosis both in cancer cells with the androgen receptor (AR) and without it. At the same time, it increases the concentration of the p53, Bax, and p21 proteins, which participate in the control of the cell cycle. The substance decreases the expression of prostate-specific antigen (PSA), which is the well-known human serine protease from the family of kallikreins, produced in the prostate and present in the blood in increased concentrations during prostatitis, benign prostatic hyperplasia, or prostate cancer, for example (16).

The observations above confirm the results of this research, where it was shown that the habanero pepper extract containing capsaicin both manifests cytotoxicity

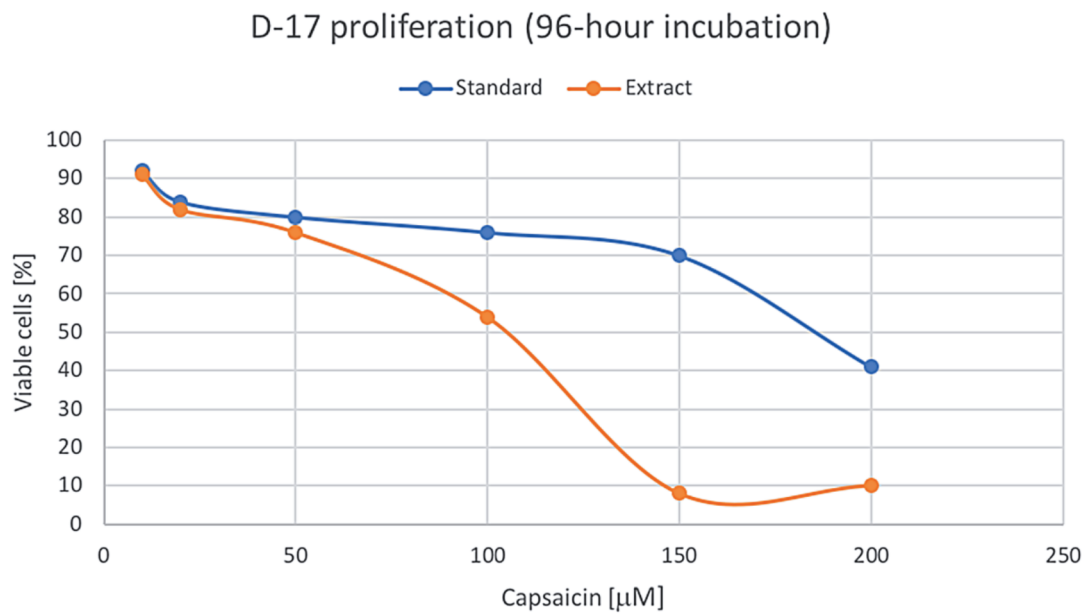


Figure 2c. The impact of habanero pepper extract vs. pure capsaicin on D-17 cell line proliferation, after a 96-h cell incubation period with the examined extracts.

for cancer cells and significantly inhibits their proliferation. To confirm this activity, the MTT assay was used in this research. The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. A yellow tetrazole is reduced to purple formazan in living cells (12). A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measurement at a certain wavelength by a spectrophotometer.

The statistically stronger anticancer effect of habanero pepper extract than pure capsaicin may be due to the fact that, apart from capsaicin itself, it also contains other capsinoids such as dihydrocapsaicin, norcapsaicin, or nornorcapsaicin, which may exacerbate the antitumor effect of pure capsaicin (17,18).

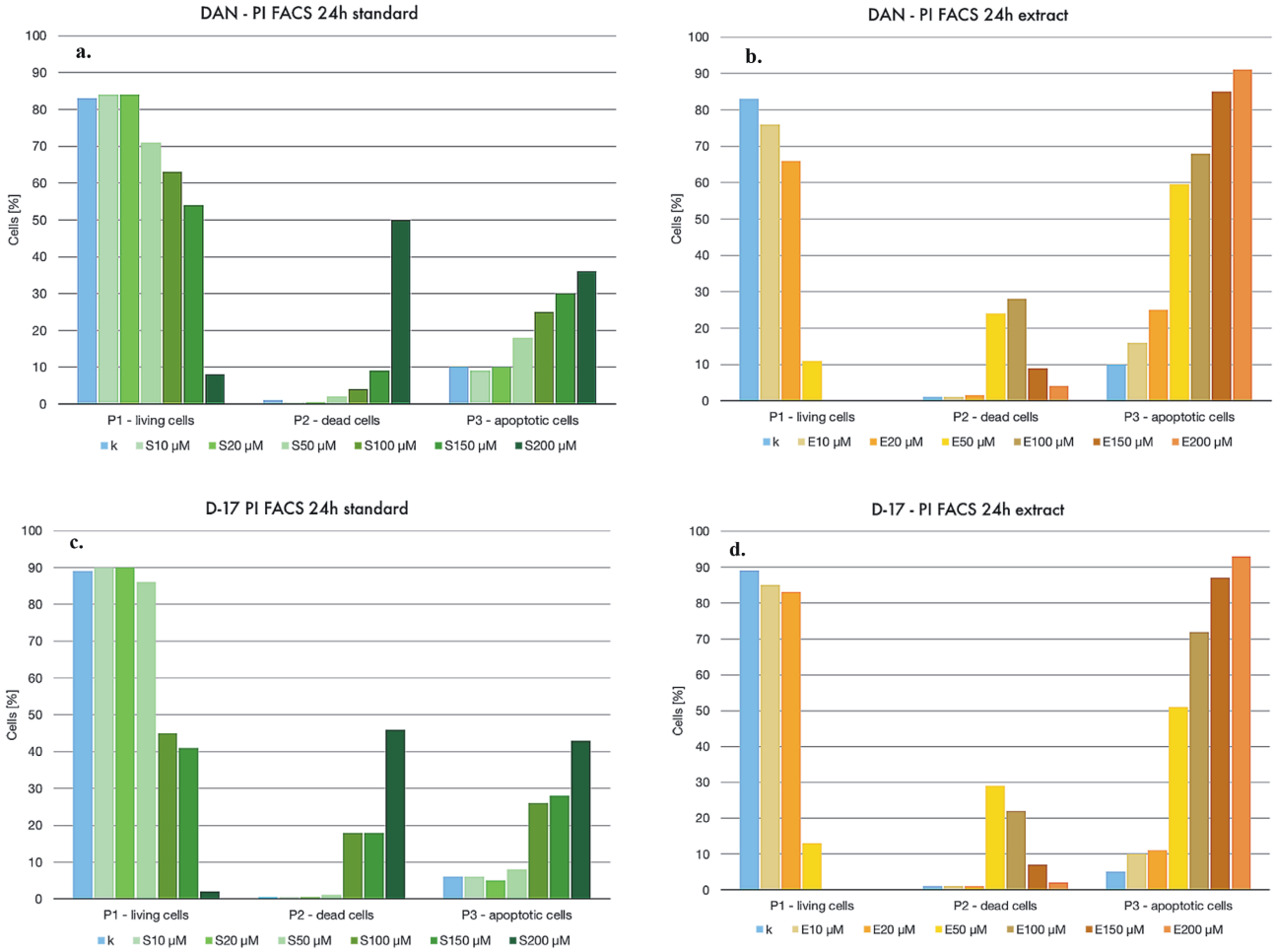
Another aspect of the anticancer effect of capsinoids is related to blocking angiogenesis. Angiogenesis is a key stage in tumor development and metastasis. Proliferation of cancer cells increases their need for oxygen supply. After some time, the existing vascular system becomes insufficient to provide a sufficient amount of oxygen for the cancer cells. Therefore, the formation of additional blood vessels becomes necessary. The study by Min et al. (19) confirmed without doubt that capsaicin blocks angiogenesis both under in vitro and in vivo conditions. The alkaloid works as an inhibitor of the angiogenic paths induced by vascular endothelial growth factor (VEGF). VEGF is the main regulator of angiogenesis, not only in cancer cells but also in healthy cells. Therefore, it is suggested that capsaicin may be used for angiogenesis-dependent diseases.

In human medicine, capsaicin has been used in the treatment of various types of cancer. Its ability to block the VEGF-induced path was used, for example, in the therapy of lung cancer. Apart from reducing angiogenesis, the alkaloid contributed also to oxidative damage of the DNA of the cancer cells in the lungs and inhibited their proliferation (20,21).

The alkaloid also induced apoptosis of cancer cells in acute lymphoblastic (lymphoid) leukemia (22) and was efficient in the therapy of cancers of the pancreas (23), colon and rectum (24), and urinary bladder (25).

In veterinary medicine, the anticancer efficiency of capsaicin was studied mostly on laboratory animals serving as models for human medicine. It was proved that the alkaloid is efficient in the therapy of prostate adenocarcinoma in mice (26) and prevents the development of benzo(a)pyrene-induced lung cancer in mice (27).

A recent study by Adaszek et al. (28) proved the anticancer efficacy of a capsaicin-containing habanero pepper extract in dogs. The study was conducted on



P1 – Living cell population, P2 – dead cell population, P3 – apoptotic cell population.

Figure 3. a–d) Results of the cytometric examination presenting the impact of habanero pepper extract including various capsaicin concentrations (10–200 µM) vs. standard capsaicin on cancer cells from the D-17 and DAN lines.

a group of 50 dogs diagnosed with tumors and 20 dogs forming a control group. All animals were administered a diet supplement based on a habanero pepper extract containing capsaicin. Observations were conducted for a period of 6 months, during which time the general condition of the animals administered the extract was monitored and hematological as well as biochemical examinations were conducted at 2-week intervals in order to assess the tolerance of the animals to the extract. In the test group, the tumor sizes were measured at monthly intervals.. As a result of habanero pepper extract administration, the tumor size decreased by 5%–50% in 15 dogs and the tumor size remained unchanged in 29 dogs, whereas tumor size increased by 10%–30% in 5 dogs despite the administration of the extract. The extract was well tolerated by the animals.

This research involved the use of two cell lines (D-17 and DAN), which represent two of the most often diagnosed cancers in dogs. In the available literature, there are no reports on the effectiveness of the habanero pepper extract containing capsaicin on dog cancer cells. The confirmation of the anticancer effectiveness of the extract on the cells is a starting point for wide clinical observations and a good indication for further research.

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