

IN VITRO EVALUATION OF ANTIPROLIFERATIVE AND CYTOTOXIC PROPERTIES OF PTEROSTILBENE AGAINST HUMAN COLON CANCER CELLS

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Abstract: Colon cancer has been remaining the second leading cause of cancer mortality in Poland in the last years. Epidemiological, preclinical and clinical studies reveal that dietary phytochemicals may exert chemopreventive and therapeutic effect against colorectal cancer. There is a growing interest in identifying new biologically active agents from dietary sources in this respect. Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a naturally occurring stilbene, that has been found to have antioxidative, anti-inflammatory and antiproliferative properties. Compared to other stilbenes, pterostilbene has greater bioavailability, and so, a greater potential for clinical applications. Recent studies showed that pterostilbene exhibits the hallmark characteristics of an anticancer agent. The aim of this study was to analyze antiproliferative and cytotoxic effects of pterostilbene on human colon cancer Caco-2 cells. They were cultured using standard techniques and exposed to increasing doses of pterostilbene (5–100 μM) for 48 and 72 h. Cell proliferation was determined by sulforhodamine B assay. The growth of treated cells was expressed as a percentage of that of untreated control cells. Pterostilbene decreased proliferation rate of Caco-2 cells in a dose- and time-dependent manner. Its concentrations = 25 μM did not affect cell growth after 48 h treatment period. Significant growth inhibition was observed in cultures incubated with higher concentrations of pterostilbene (40–100 μM). Pterostilbene at all concentrations used (5–100 μM) caused significant inhibition of cell proliferation when the experimental time period was elongated to 72 h. The maximum growth reduction was observed at 100 μM pterostilbene. The cytotoxicity of pterostilbene was evaluated in 48 h cultures based on lactate dehydrogenase (LDH) leakage into the culture medium and showed dose-related pattern. The findings of this study showed significant dose-dependent antiproliferative and cytotoxic effects of pterostilbene against human colon cancer cells *in vitro*.

Keywords: pterostilbene, colon cancer, proliferation, Caco-2 cell line

Colon cancer remains the second leading cause of cancer mortality in Poland in the last years (1). Epidemiological, preclinical and clinical studies reveal that dietary phytochemicals may exert chemopreventive and therapeutic effect against colorectal cancer (2, 3). There is a growing interest in identifying new biologically active agents from dietary sources in this respect.

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a naturally occurring stilbene, found in fruits such as grapes and berries (4). It is a structural analogue of another highly studied stilbene, resveratrol (5). The structural difference between these compounds is that pterostilbene contains one hydroxy group and two methoxy groups while resveratrol has three hydroxy groups. Due to the presence of methoxy groups pterostilbene exhibits

an increased oral absorption, better metabolic stability, greater lipophilicity and higher potential for cellular uptake. Pterostilbene administered orally, shows 95% bioavailability while resveratrol has only 20% bioavailability (4, 6). Compared to other stilbenes, pterostilbene has greater bioavailability, and so, a greater potential for clinical applications (7). Pterostilbene has been found to possess several pharmacological activities such as antioxidative, anti-inflammatory and antiproliferative (5, 8). Recent studies showed that pterostilbene exhibits the hallmark characteristics of a valuable anticancer agent (9).

The aim of this study was to evaluate antiproliferative and cytotoxic effects of pterostilbene on human colon cancer Caco-2 cells *in vitro*.

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EXPERIMENTAL

Cell line and culture conditions

The Caco-2 human colon adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC). Cells were grown routinely in medium containing the following composition: 90% modified Eagle's medium (MEM, Sigma Aldrich), 10% fetal bovine serum (FBS, PAA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma Aldrich), and 10 mM HEPES (Sigma Aldrich). The cell cultures were cultivated as a monolayer at 37°C in a humidified atmosphere containing 5% CO₂.

Cell growth determination

The effect of pterostilbene on Caco-2 cell proliferation was analyzed by *In Vitro* Toxicology Assay Kit, Sulforhodamine B (SRB) based (Sigma Aldrich). The SRB is a dye staining cellular proteins and thus the amount of the incorporated dye is an indirect measure of total biomass and consequently, cell number and proliferation (10). Pterostilbene was purchased from Sigma Aldrich. Stock solution of pterostilbene was prepared in dimethyl sulfoxide

(DMSO) and further diluted in sterile culture medium to desired concentrations immediately before use. The final DMSO concentration in the working solutions was 0.01%.

To study the cell proliferation, colonocytes were seeded at an initial density of 1×10^3 cells/well in 200 µL MEM medium complemented with the components given above and allowed to attach and grow. After 24 h, the medium was aspirated and cells were exposed to the freshly prepared medium containing pterostilbene (5; 10; 25; 40; 50; 60; 75 and 100 µM) for 48 or 72 h. Subsequently, cells were washed with PBS and fixed in 10% trichloroacetic acid (4°C, 1 h), followed by 5 washes with deionized water. Cells were stained by the addition of 0.4% SRB in 1% acetic acid at room temperature for 30 min. Afterwards, plates were washed with 1% acetic acid and air-dried. After the liberation of the incorporated SRB by the addition of 10 mM Tris-HCl, absorbance was measured at 570 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies). The growth of treated cells was expressed as a percentage of untreated control cells.

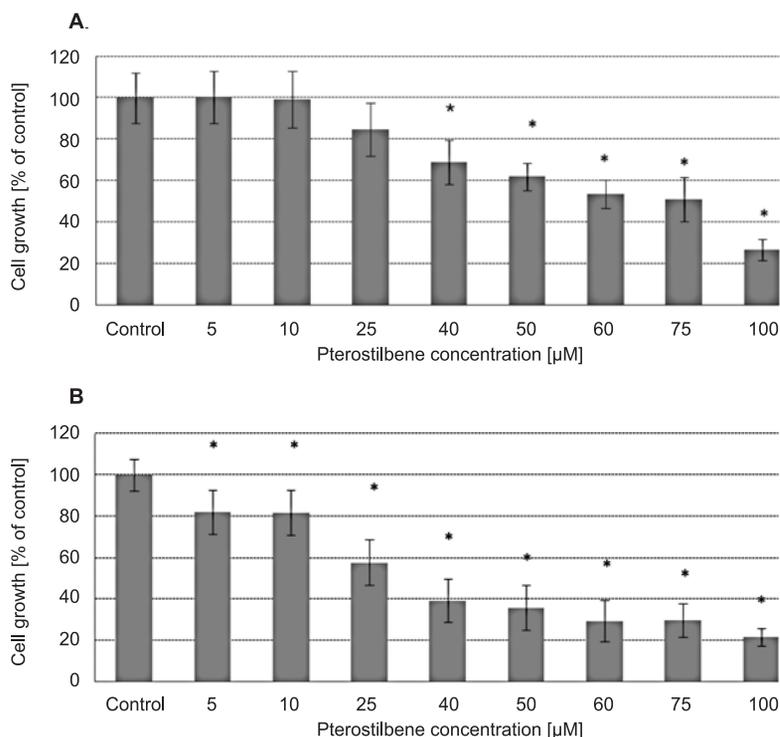


Figure 1. Growth inhibitory effect of various concentrations of pterostilbene on Caco-2 cells after 48 (A) and 72 (B) hours treatment. The results are expressed as percentage of untreated control (the means \pm SD; * $p < 0.05$ vs. control).

LDH cytotoxicity assay

Cytotoxicity induced by pterostilbene was assessed by lactate dehydrogenase (LDH) leakage into the culture medium by a commercially available *In Vitro* Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma Aldrich). Increased LDH activity in the supernatants of cell cultures is a result of a damage to the cellular membrane and correlates with the percentage of dead cells. For the cytotoxicity test, Caco-2 cells were seeded at a density of 1×10^4 cells per well in 200 μL culture medium in a 96-well plate and cultured for 24 h. Afterwards, media were replaced with the fresh ones containing various concentrations of pterostilbene (5–100 μM). Following a 48 h period, LDH activity was measured in both culture media and cell lysates according to manufacturer's instruction. Colorimetric results were read on a multiplate reader MRX Revelation (Dynex Technologies) at 492 nm and 690 nm (reference wavelength). The results were shown as percentage of total LDH released into the medium. The LDH release (%) was calculated using the following equation:

$$\text{LDH release (\%)} = \frac{\text{Extracellular LDH}}{\text{Total LDH}} \times 100\%.$$

Statistical analysis

Statistical analysis was performed with the use of Statistica PL ver. 9.0 Software (StatSoft). The examined parameters were first evaluated for normal distribution (Shapiro-Wilk test). One-way analysis of variance (ANOVA) with NIR's *post-hoc* test was used to evaluate significances between examined groups. Comparison of two data sets was performed by t-test. Results were expressed as the

means \pm standard deviation (SD). Differences were considered statistically significant when the probability value p was lower than 0.05.

RESULTS

In the present study, Caco-2 cells were used as the colon cancer cell model *in vitro*. The effect of pterostilbene on their growth was evaluated using the SRB assay. Cells were incubated in the presence of different concentrations of pterostilbene (5; 10, 25; 40; 50; 60; 75 and 100 μM) for 48 and 72 h (Fig. 1). The effect of this compound on Caco-2 cell proliferation after 48 h is shown in Figure 1A. Its concentrations = 25 mM did not affect cell growth and higher concentrations (40–100 mM) significantly decreased cellular density. Pterostilbene at all concentrations used (5–100 μM) caused significant inhibition of cell proliferation when the experimental time period was elongated to 72 h (Fig. 1B) and the maximum growth reduction (78%) was observed at 100 mM. The effect of DMSO on the growth of Caco-2 cell line was also evaluated by culturing cells in medium containing 0.01% DMSO but no influence of this compound was found (data not shown). Taken together, this data demonstrated that pterostilbene decreased proliferation rate of Caco-2 cells in a time- and dose-dependent manner.

The effect of pterostilbene on Caco-2 cell membrane integrity was examined by determining the percentage of LDH released into the culture medium following 48 h treatment. As seen in Fig. 2, pterostilbene at concentrations up to 40 μM did not cause significant increase in the LDH release and at higher concentrations (= 50 μM) statistical-

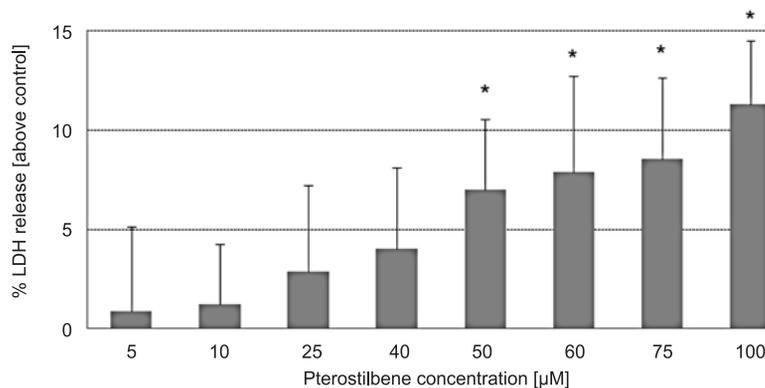


Figure 2. Cytotoxicity of pterostilbene evaluated by lactate dehydrogenase (LDH) release from Caco-2 cells after 48 h of incubation. Data are expressed as the % LDH release above control, * $p < 0.05$ vs. control.

ly significant increase in LDH release as compared with control cells (7–11.3% LDH released above control). The highest accumulation of LDH in media was observed after treatment of Caco-2 cells with 100 μ M pterostilbene. DMSO at concentration of 0.01% did not affect LDH release in this model (data not shown). The study indicated that pterostilbene exerted cytotoxic activity on colon cancer cells, but this effect depended on its concentration

DISCUSSION

Colorectal cancer is one of the most common tumors in the industrialized countries. Diet strongly influences the risk of this cancer, and changes in food habits might reduce up to 70% of colon cancer incidence rate (11). In addition, a number of studies point to an increased risk of colorectal cancer with low consumption of vegetables and fruits (12). Recently, pterostilbene found in fruits such as grapes, berries, has been a subject of intense investigation as cancer chemopreventive agent. The latest clinical trial showed that pterostilbene is well-tolerated and safe for use in humans (13).

Several studies in animal models have demonstrated that dietary administration of pterostilbene inhibited colon tumor development. Diet containing pterostilbene has been shown to significantly reduce colon tumor multiplicity when treatment of rats with pterostilbene was commenced after injection of colon-specific carcinogen – azoxymethane (AOM) (14). Supplementation of a diet with pterostilbene inhibited formation of AOM-induced colonic aberrant crypt foci and adenomas also in mice (15). Furthermore, colon tumors from pterostilbene-fed animals showed reduced expression of a cell proliferation marker, proliferating cell nuclear antigen (PCNA) (14).

In cancer biology, cell proliferation is an important and commonly studied parameter reflecting cellular vitality. Dysregulation of cell proliferation is a hallmark of cancer cells and assessment of cellular proliferation is a key component in discovery and development of anticancer drugs (16). As observed in this study, pterostilbene demonstrated time- and dose-dependent growth inhibitory effect on human colon cancer cells Caco-2. Significant decrease of cell proliferation was observed in cultures incubated with the concentrations of pterostilbene = 40 mM for 48 h treatment period. The prolongation of cells exposure to stilbenoid up to 72 h resulted in their considerable growth inhibition compared to untreated control cells at all concentrations

used (5–100 mM). These data concur with the results published by Paul et al. (17), who observed reduced cell proliferation of colon cancer cells HT-29 after 24, 48 and 72 h incubation with pterostilbene in a dose-dependent manner and the most pronounced growth inhibition after 72 h treatment. In addition, pterostilbene revealed greater inhibitory potency toward HT-29 cells proliferation than resveratrol.

Furthermore, in the current study, the cytotoxic effect of pterostilbene was evaluated by measuring the LDH leakage out of cells. This method is commonly used as an indicator of cell membrane integrity and serves to assess cytotoxicity of chemical compounds (18). Percentage of LDH released to the medium was measured to find out whether the growth inhibitory effect of pterostilbene on Caco-2 cells was due to its cytotoxicity. Pterostilbene at concentrations up to 40 μ M did not cause increase in the percentage of LDH release after 48 h treatment. Higher concentrations of this stilbene (= 50 μ M) statistically significantly enhanced the leakage of enzyme resulting from a loss of membrane integrity. Pterostilbene was reported to reduce cell viability in colon cancer cells HT-29, HCT116 and Caco-2 and its concentration required to achieve 50% inhibition of cell viability after 48 h incubation period was about 15 μ M in HT-29, 12 μ M in HCT116 and 65 μ M in Caco-2 cells (19). These results differ from the findings by Harun and Ghazali (20), who did not observe toxic effects of pterostilbene at concentrations up to 100 μ M against HT-29 cells. This might be, however, due to the shorter incubation period (24 h) with pterostilbene.

In conclusion, the present study show antiproliferative and cytotoxic properties of pterostilbene against colon cancer cells *in vitro*. Hence, it can be suggested that pterostilbene, an active constituent of berries, holds great promise in the field of chemoprevention of colon cancer.

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