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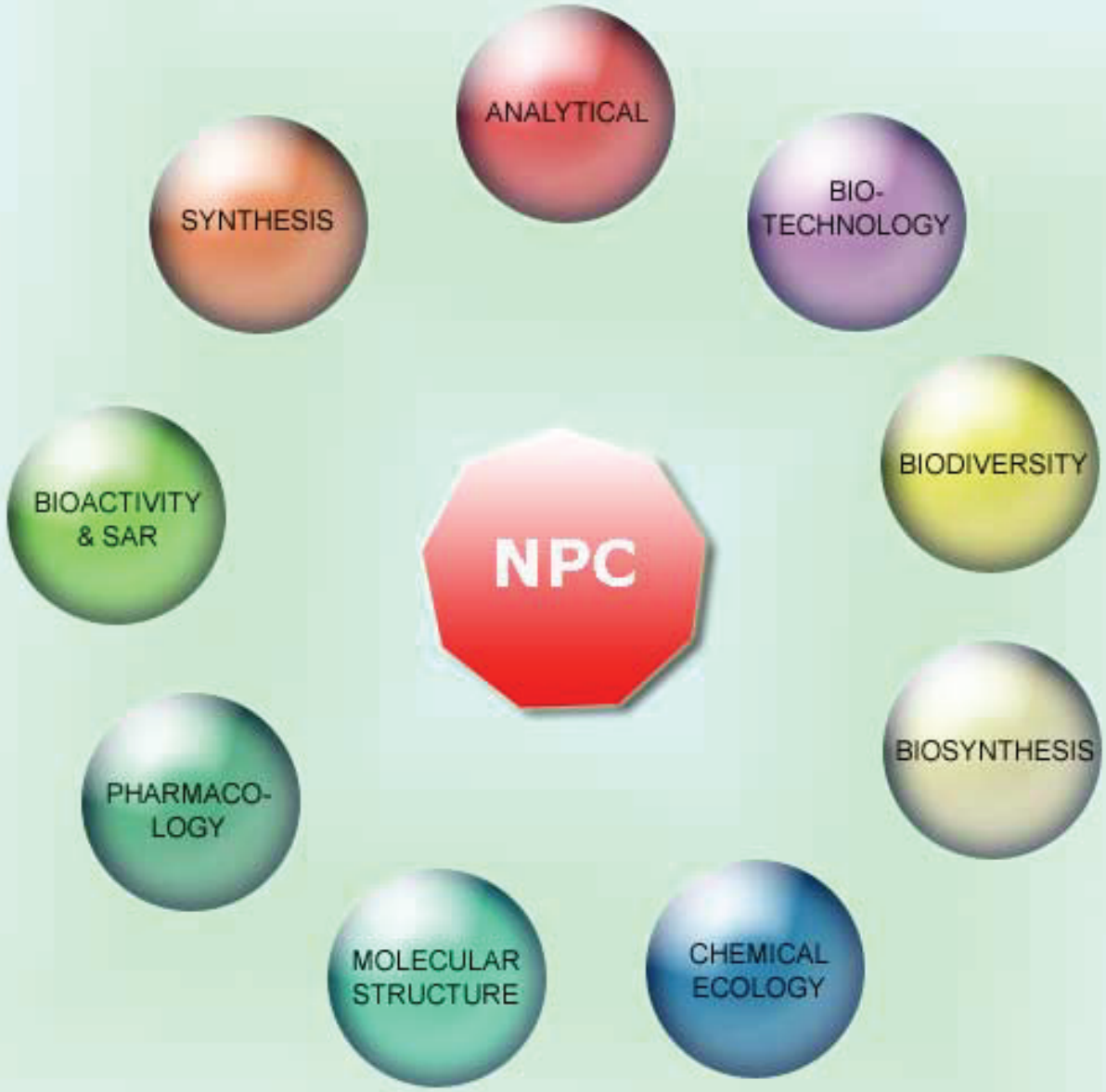

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Cytotoxic Effect of Aeruginosin-865, Resveratrol and Capsaicin on Mouse Fibroblasts and Cells Derived from Fallow Deer

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Natural substances offer interesting bioactivity patterns including antiproliferative, antioxidant or cytotoxic effects. However, the safety profile of many of them has not been extensively determined. In this study, the cytotoxic effect of Aeruginosin-865, resveratrol and capsaicin at different concentrations was tested on normal mouse cells (NIH/3T3) and tumour fibroblasts (WEHI-13VAR) as well as on liver- and kidney-derived cells from fallow deer. A lactate dehydrogenase cytotoxicity assay kit was used to measure cell death in response to treatment with the test substances. It was found that NIH/3T3 cells tolerated Aeruginosin-865 (10–200 μM) and resveratrol (5–100 μM) treatment without any cytotoxic effect, while capsaicin exerted a cytotoxic effect only at the highest tested concentration (200 μM). Mouse fibrosarcoma cells were more sensitive to the cytotoxic effect of all three compounds where Aeruginosin-865 (100–200 μM) and resveratrol (50–100 μM) showed high-dose cytotoxicity and capsaicin showed low- and high-dose cytotoxicity (25 μM and 200 μM). The three tested compounds at the highest concentrations were found to be cytotoxic to both liver- and kidney-derived cells from fallow deer. Overall, the results indicate that the cytotoxic effects of the three tested natural substances on cells derived from fallow deer and mouse tumour fibroblasts differ significantly from those exerted on normal fibroblasts. The results demonstrate the potential of these natural compounds as therapeutic agents and pave the way for future *in vivo* toxicological investigations.

Keywords: Aeruginosin-865, Resveratrol, Capsaicin, Fibroblast, Fallow deer, Cell culture, Lactate dehydrogenase, Cytotoxicity.

Natural substances offer interesting biological properties such as antiproliferative, antioxidant or cytotoxic activities. However, their potential utilization as therapeutic agents requires determination of their safety profiles. In this research article, we focus on the effects of Aeruginosin-865, resveratrol and capsaicin. The aeruginosin family represents more than 500 aeruginosin variants that have been isolated from several cyanobacteria and marine sponges thus far [1, 2]. Cyanobacteria produce numerous secondary metabolites, which have many different functions, and several of them are cytotoxic [3]. On the other hand, a recently described class of linear peptides called aeruginosins exhibit varying degrees of inhibitory activity against serine proteases [2]. Nearly all of the aeruginosins are composed of four subunits: an N-terminal hydroxy or acidic group, a large hydrophobic amino acid, a 2- carboxyperhydroindole core and a C-terminal guanidine-containing group [4]. The most studied aeruginosin variants have been isolated from *Nodularia* or *Microcystis* strains [5, 6]. Aeruginosin-865, a tetrapeptide isolated for the first time from the terrestrial cyanobacterium *Nostoc,* has been shown to have anti-inflammatory effects mediated by inhibition of the NF- κB signalling pathway [7], which subsequently lead to inhibition of transcription of genes playing a role in cell survival or inflammation progression. However, the exact mechanism underlying the anti-inflammatory effect of Aeruginosin-865 has not yet been fully elucidated.

Capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide), an alkaloid found in the fruit of the Capsicum plant family, is the main molecule responsible for the typical pungency of these plants. Therefore, it represents a natural defence mechanism against herbivores and fungi. The effect of capsaicin on animals and humans has been studied for more than a century, yielding promising results mainly in pain relief, inflammation, obesity and even cancer treatment or cancer prevention [8]. The anticancer property of capsaicin was tested on more than 80 different cell lines, predominantly of human origin. Most of the published studies agree that capsaicin shows an inhibitory effect on cancer cells, whereas "normal" cells tolerate capsaicin treatment without any effect on their viability and growth. The mechanism by which capsaicin provides an anticancer effect is still not fully elucidated, and there are probably additional modes of action. In fact, many authors have addressed cell-cycle arrest, inhibition of cell growth and proliferation or apoptosis induction [9, 10] as the possible mechanisms underlying the anticancer effect. Capsaicin has also been shown to have an anti-inflammatory effect similar to that of aeruginosine-865 [11].

Resveratrol is a polyphenol compound synthesized by various plant species such as grapevine, cranberries, broccoli or garlic, and the mechanisms by which it can prevent, arrest or delay tumour development have been elucidated [12,13]. As in the case of capsaicin, the mechanism of resveratrol action is still not satisfactorily explained. However, the disruption of mitochondrial transmembrane potential, increase in production of oxygen radicals or increase in the intracellular calcium concentration [13-15] may play essential roles in the benefits exhibited by these two compounds.

In the present study, the cytotoxic effects of Aeruginosin-865, resveratrol and capsaicin were investigated on two mouse cell lines: normal fibroblasts and tumour-transformed fibroblasts, which are a suitable animal model of carcinogenesis. Due to the increasing incidence of cancer in humans and animals, the results obtained could contribute to the use of these natural substances as potential

therapeutics in both human and veterinary medicine. Moreover, the effects of these compounds were tested on liver- and kidney-derived cells from fallow deer. We focused primarily on the effect of aeruginosin produced by cyanobacteria, because their intensive growth in freshwater supplies and terrestrial soil can lead to water and plant contamination, so grazers such as fallow deer are at the greatest risk [16]. Therefore, examination of the compounds produced by cyanobacteria is at the forefront of recent research. This study compares the cytotoxic effects obtained using a standard experimental model and a wildlife cell model.

We hypothesized that cells of different origins would show a variable response to the adverse effects of Aeruginosin-865, resveratrol and capsaicin, i.e. substances with potential medical applications. We tested this by using a range of concentrations and assaying cytotoxicity through lactate dehydrogenase activity (LDH).

The cytotoxicity of Aeruginosin-865, capsaicin and resveratrol to mouse fibroblasts is shown in Figure 1. 3T3 mouse fibroblast cells tolerated Aeruginosin-865 and resveratrol treatment without any cytotoxic effects, while capsaicin showed cytotoxicity only at the highest concentration ($p < 0.05$). Mouse fibrosarcoma cells were more sensitive to the cytotoxic effects of all three compounds. Aeruginosin-865 and resveratrol showed high-dose cytotoxicity. Statistical significance ($p < 0.05$) was only observed in the case of Aeruginosin-865, while the effect of resveratrol was nonsignificant ($p > 0.05$). Cancer cells were more responsive to the cytotoxic effect of capsaicin when compared with Aeruginosin-865 or resveratrol treatment. We detected low- and high-dose cytotoxicity on fibrosarcoma cells when treated with capsaicin with the level of significance $p < 0.05$.

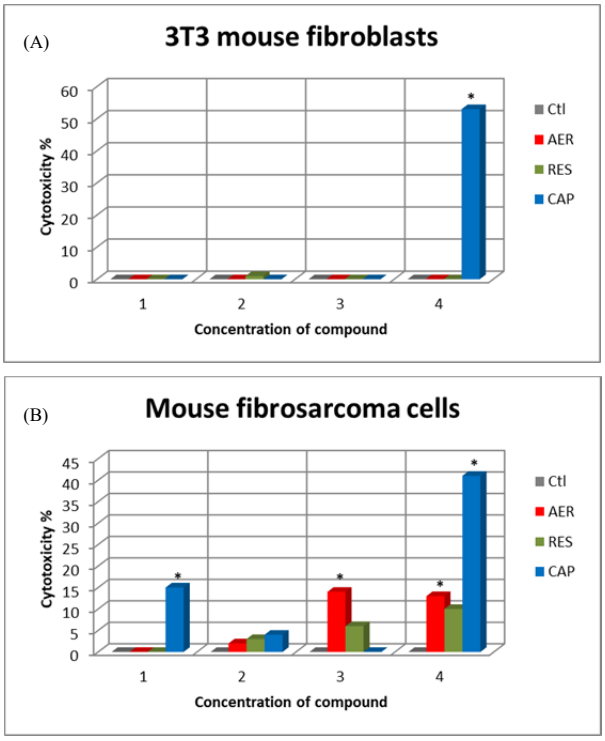

Figure 1: Cytotoxicity of Aeruginosin-865, resveratrol and capsaicin on 3T3 mouse fibroblasts (A) and mouse fibrosarcoma cells (B). Cells were treated with the test compounds at different concentrations for 24 hours. Group 1 – Aeruginosin-865 10 □M, resveratrol 5 □M, capsaicin 25 □M. Group 2 – Aeruginosin-865 50 □M, resveratrol 10 □M, capsaicin 50 □M. Group 3 – Aeruginosin-865 100 □M, resveratrol 50 □M, capsaicin 100 □M. Group 4 – Aeruginosin-865 200 □M, resveratrol 100 □M, capsaicin 200 □M. Controls represent untreated cells incubated with 0.1%–2% DMSO. * = $p < 0.05$ when compared with the control group.

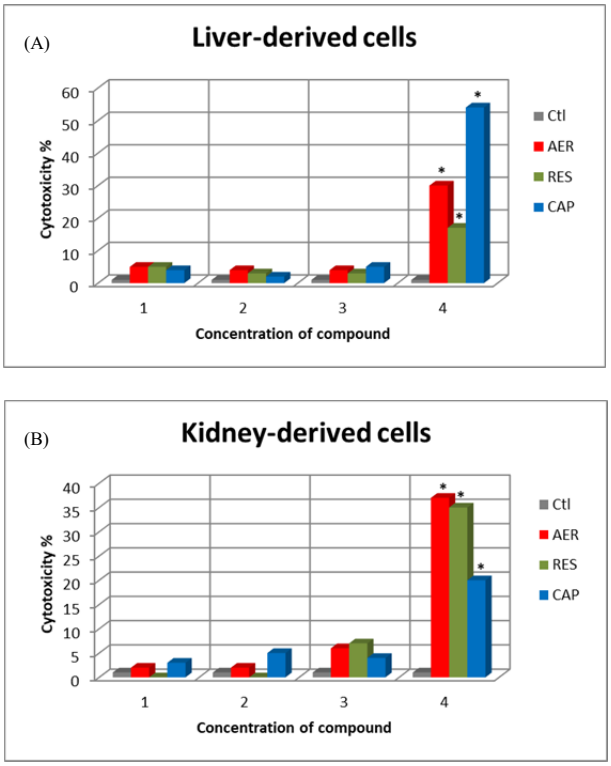

Figure 2: Cytotoxicity of Aeruginosin-865, resveratrol and capsaicin for fallow deer liver-derived cells (A) and kidney-derived cells (B). Cells were treated with test compounds at different concentrations for 24 hours. Group 1 – Aeruginosin-865 10 □M, resveratrol 5 □M, capsaicin 25 □M. Group 2 – Aeruginosin-865 50 □M, resveratrol 10 □M, capsaicin 50 □M. Group 3 – Aeruginosin-865 100 □M, resveratrol 50 □M, capsaicin 100 □M. Group 4 – Aeruginosin-865 200 □M, resveratrol 100 □M, capsaicin 200 □M. Controls represent untreated cells incubated with 0.1%–2% DMSO. * = $p < 0.05$ when compared with the control group.

The cytotoxicity of Aeruginosin-865, capsaicin and resveratrol to liver- and kidney-derived cells from the fallow deer is shown in Figure 2. The three compounds exerted a similar profile of cytotoxic effects in both tested cell lines. Aeruginosin-865, capsaicin and resveratrol did not show any significant effect at low concentrations but were found to exhibit a statistically significant cytotoxic effect ($p < 0.05$) on both liver- and kidney-derived cells. Liver-derived cells were twice as sensitive to capsaicin as kidney-derived cells. A similar effect was observed for resveratrol, but in this case kidney-derived cells were twice as sensitive as liver-derived cells. The cytotoxic effects of Aeruginosin-865 in liver- and kidney-derived cells were almost identical.

This work utilised an *in vitro* animal model of carcinogenesis to evaluate the cytotoxic effects of three different natural substances and simultaneously compared an *in vitro* mouse experimental model and a wildlife model represented by cells derived from fallow deer. According to Kapuścik *et al*. [7] Aeruginosin-865 did not show any cytotoxic effect in human lung microvascular endothelial cells (HLMVECs). A similar outcome was also confirmed by Faltermann *et al*. [3] for Aeruginosin-828A from *Planktothrix* strains in Huh7 human hepatoma cells. Furthermore, acute toxicity of Aeruginosin-828A to the crustacean *Thamnocephalus platyurus* has been documented [17, 18], but this effect has not been confirmed in zebrafish embryos or zebrafish liver organ cultures [3]. The current study presents for the first time the effect of Aeruginosin-865 on NIH/3T3 mouse normal fibroblasts and WEHI-13VAR mouse tumour fibroblasts. Aeruginosin-865 showed no cytotoxic effect on normal mouse fibroblasts, even at the highest concentration used in the test, in agreement with other studies [3, 7]. Concurrently, tumour-transformed mouse fibroblasts were sensitive to

Aeruginosin-865, mainly at the highest concentrations. These findings identify Aeruginosin-865 as a potential therapeutic agent for use in the prevention of cancer or as part of a combined therapy with radiation and chemotherapeutics. However, additional research is needed to better understand the exact mechanism of action and biological behaviour of Aeruginosin-865 in cancer cells. As the majority of aeruginosin variants are produced by aquatic cyanobacteria, it is understandable that environmental toxicity has been studied in sensitive freshwater organisms such as the crustacean *Thamnocephalus platyurus* [17, 18] or zebrafish [3]. Aeruginosin-865 is a novel class of aeruginosin variant that was discovered in a strain of soil cyanobacterium (*Nostoc* sp.) [7]. The lack of cell toxicity and anti-inflammatory properties of Aeruginosin-865 [7] encouraged the investigation of this compound in fallow deer-derived cells. Considering that the liver and kidney represent metabolically active organs and the pharmocodynamics of Aeruginosin-865 is not fully understood, we performed experiments on liver- and kidney-derived cells. Cytotoxicity of Aeruginosin-865 was found only at the highest used concentration (200 □M) in both cell types. On the other hand, toxicity of Aeruginosin 828A was reported for the crustacean *Thamnocephalus platyurus*, with lethal doses starting at 22.4 and 34.5 □M, respectively [17, 18]. Based on the cytotoxicity observed in liver- and kidney-derived cells, it may be hypothesized that possible structural modifications in aeruginosin variants might act as a factor contributing to the cytotoxic effect. Furthermore, freshwater organisms might be more sensitive to such compounds than terrestrial species.

Bley *et al*. [14] summarized the results of studies describing apoptotic or growth inhibitory effects of capsaicin which were selective for cancerous cells and left normal or noncancerous cells unharmed. Another study revealed variable sensitivity of different cell lines including human dermal fibroblasts (HDF) and mouse embryonic fibroblasts (NIH/3T3) and some cancerous cell lines. Capsaicin had no effect on the viability of dermal fibroblasts, whereas mouse embryonic fibroblasts were sensitive to capsaicin treatment, with the first cytotoxic effect observed at a dose of 50 μM and an IC_{50} around 200 μM. Among the cancerous cell lines, human breast carcinoma cells (MCF7) were the most sensitive, already displaying a cytotoxic effect at the lowest concentration (5 μM) [19]. Ghosh and Basu [20] compared the pro-apoptotic effects of capsaicin on fibrosarcoma cells (Meth A, CMS5) and mouse embryonic fibroblasts (MEFS). The authors reported that capsaicin treatment induced apoptosis only in fibrosarcoma cells with increasing reactive oxygen species (ROS) production. In the present study, normal fibroblasts showed a low sensitivity to capsaicin, but cancerous cells were sensitive to capsaicin even at a low concentration. This discontinuity between low- and high-concentration cytotoxicity of capsaicin may suggest that mouse fibrosarcoma cells respond to capsaicin exposure using a different mechanism when compared with normal fibroblasts. Isolated rat hepatocytes treated with capsaicin showed a cytotoxic effect only at a high concentration (LD_{50} 400 μM), whereas hepatoma cells (Hep G2) were eight times more sensitive (IC_{50} 50 μM) [21]. Similar results using a normal hepatic cell line (L-02) and a human hepatoma cancer cell line (SMMC-7721) were documented [22]. Normal hepatocytes showed a very slight decrease in viability (approximately 90%) at 300 μM of capsaicin whereas the viability of cancerous cells was markedly decreased (approximately 20%). The impact of capsaicin treatment on kidney cells has not been well studied. Cochereau *et al*. [23] described the cytotoxic effect of capsaicin in monkey kidney cells (Vero cells) when capsaicin at a concentration of 68 μM reduced the cell number by half compared with the control. In the current study, kidney-derived cells from fallow deer were, in contrast, more resistant to the cytotoxic effect of a high concentration of capsaicin when compared with liver-derived cells. Further studies are needed to detect a possible toxic effect of capsaicin in different cells from different species and to clarify the mechanisms of its action.

Resveratrol was shown to inhibit growth and proliferation in many cancer cell lines with limited cytotoxicity toward normal cells [13, 15]. Other related compounds, oxyresveratrol or stilbene-based resveratrol analogues, are also considered to have antiproliferative or anticancer properties [24, 25]. Resveratrol decreased cell viability and induced apoptosis in HT1080 fibrosarcoma cells [26-28]. The effect on the cell viability was dose-dependent, showing a 50% decrease in the viable cell count at a dose of 50 μM [26, 28]. In our study we confirmed the dose-dependent inhibitory effect of resveratrol on mouse fibrosarcoma cells, but without any significant result at any concentration tested. In normal mouse fibroblasts resveratrol did not induce any cytotoxic effects. In liver- and kidney-derived cells from fallow deer, the highest concentration of resveratrol significantly increased cytotoxicity, leading to reduction of cell viability.

Our findings indicate that Aeruginosin-865, resveratrol and capsaicin differ significantly in their cytotoxic effect on cells derived from fallow deer; moreover, the dissimilar mechanism of action is observed in mouse fibrosarcoma cells in comparison with normal mouse fibroblasts. Further studies are necessary to clarify and better understand the effect of these natural substances on normal and/or tumour cells in different animal species.

Experimental

Cell cultures: The commercially available mouse normal cell line NIH/3T3 (in the collection of the Department of Physiology) and the tumour fibroblast cell line WEHI-13VAR (ATCC® CRL-2148 ™) were tested. Cell lines were cultured in MEM Alpha Medium (Thermo Fisher Scientific, Waltham, MA, USA) for normal cells and RPMI-1640 medium (Sigma-Aldrich, St. Louis, Missouri, USA) for tumour cells. Liver- and kidney-derived cells from fallow deer were obtained as previously described [29] and cultured in DMEM/F12 medium (Biosera, Boussens, France). Each medium was supplemented with 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA) and 1% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were cultured in 96-well plates in the appropriate complete medium and placed in a 5% CO_2 incubator at 37°C.

Natural substances: Aeruginosin-865 was provided by the Laboratory of Algal Biotechnology, Institute of Microbiology, Czech Academy of Sciences in Trebon, where it was obtained according to a previously described isolation procedure [7]. Resveratrol (554325) and capsaicin (M2028) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). These substances were applied in parallel to all types of cells at different concentrations and incubated for 24 hours. DMSO (D4540, Sigma-Aldrich, St. Louis, Missouri, USA) was used to dissolve the natural substances in the concentration range of 0.1%–2% to prepare a stock solution. Exposure solutions were prepared immediately prior to the experiment. We used Aeruginosin-865 in 10 □M, 50 □M, 100 □M and 200 □M concentrations, resveratrol in 5 □M, 10 □M, 50 □M and 100 □M concentrations and capsaicin in 25 □M, 50 □M, 100 □M and 200 □M concentrations.

Treatment schedule for cytotoxicity and LDH test: To evaluate the cytotoxicity, we used LDH cytotoxicity assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer´s instructions. At first, an optimal cell concentration was tested in a

preliminary experiment. Then the cells were seeded in 96-well plate at a density of 2 x 10^4 cells/well in the appropriate medium and incubated overnight. On the second day, test solutions of Aeruginosin-865, resveratrol and capsaicin at different concentrations were applied, and the cells were further cultured for 24 hours at 37°C in a 5% CO_2 atmosphere. The next day, the reaction mixture was added to each sample. The reaction was performed for 30 min at 37°C protected from light and then stopped by the addition of Stop Solution. The absorbance was read at 490 nm and 680 nm on a SynergyHT (BioTek, USA) instrument.

LDH released from the cytosol of damaged cells induces tetrazolium conversion to a red formazan of intensity proportional to the amount of LDH released. A positive control (maximum LDH activity) was established by treatment with Lysis Buffer (10X) provided by the kit, and a negative control (spontaneous LDH activity) was established by treatment with sterile distilled water. To exclude a potential cytotoxic effect of the solvent, we performed a solvent control reaction with 0.1%–2 % DMSO on untreated cells.

Statistics: The statistical significance of the difference between each concentration *vs* the control treated sample was evaluated using MedCalc statistical software. We applied T-test for statistical analysis. Differences with a $p < 0.05$ were considered statistically significant.

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