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SYNERGISTIC EFFECTS INDUCED BY COMBINED TREATMENTS OF AQUEOUS EXTRACT OF PROPOLIS AND VENOM

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Abstract

Background and aims. *Breast cancer is a heterogeneous disease and the leading cause of cancer mortality worldwide. Triple negative breast cancer (TNBC) is considered to be one of the most aggressive breast neoplasia due to failure of chemotherapy response. Thus, there is an urgent need of finding alternative therapies for TNBC. This study was designed to evaluate the synergistic effect induced by propolis and bee venom on luminal (MCF-7) and TNBC (Hs578T) cell lines.*

Methods. *In order to evaluate the synergistic effect of aqueous extract of propolis and bee venom, we treated in combination two breast cancer cell lines: MCF-7(luminal subtype) and Hs578T (TNBC subtype).*

Results. *Our results indicate that both cell lines exhibited similar sensitivity to the aqueous extract of propolis at a dilution of 0.072-0.09 mg/ml. The results concerning IC50 for bee venom on MCF-7 cells was 1 mg/ml, 20 times higher than 0.05 mg/ml in Hs578T cells. By combining the aqueous extract of propolis with bee venom, we obtained synergistic effects at a higher concentration, which was 5 and 2 times stronger than the two treatments alone.*

Conclusion. *Overall, the results from our study indicated that the combination of aqueous extract of propolis and bee venom treatments induced synergistic antiproliferative effects in a concentration-dependent manner in breast cancer cells. Thus we can hypothesize that the combination of honeybee propolis and venom might be involved in signaling pathways that could overcome cells resistance to therapy.*

Keywords: propolis, venom, breast cancer, apoptosis

Background and aims

Breast cancer continues to represent the most frequently diagnosed cancer in women in almost all regions of the world. With more than 1.7 million new cases diagnosed in 2012, breast cancer represented 25% of all new cancers cases in women [1]. Unfortunately, breast cancer also represents the leading cause of cancer mortality, more than a half a million deaths were reported for 2012 worldwide.

Breast cancer represents a heterogeneous disease, with several molecular portraits. Gene expression profiling revealed four main molecular subtypes with different clinical implications [2,3,4,5]. Two subtypes, named luminal A and B are characterized by positive estrogen receptor (ER, PR) status, the HER2 enriched group is characterized by the amplification of the HER2 gene and triple negative breast cancer (TNBC) subtype is characterized by negative expression of HER2, ER and PR [6]. According to this classification, three therapeutic approaches including hormonal therapy for luminal tumors, HER2-targeted therapy for HER2 positive subtype and chemotherapy for TNBC are currently applied in clinical practice. A 15-year retrospective survival analysis has showed that TNBC patients have the worse five years overall survival [7]. TNBC represents up to 20% of all breast cancers, and it is considered to be one of the most aggressive breast neoplasia due to the failure of the chemotherapy response [8]. The real problem related to TNBC it is given by the lack of treatment strategies. If drugs such as trastuzumab, lapatinib, and pertuzumab are available for HERs positive patients and aromatase inhibitors (anastrozole, exemestane, letrozole), estrogen receptor modulators (tamoxifen, raloxifene, toremifene) and downregulators of estrogen receptor (fulvestrant) are available for estrogen receptor positive breast cancer patients, there is no specific therapy developed for TNBC patients as yet [9]. Therefore, there is a great challenge to develop new approaches based on both conventional and unconventional compounds, to improve the treatment for TNBC. Unconventional therapies based on propolis and bee venom become a subject of debate regarding their application for cancer treatment.

The antitumoral properties of honeybee propolis including cell cycle arrest, activation of apoptosis [10] induction of mitochondrial stress [11], inhibition of tumor cell growth and proliferation was recently demonstrated [12,13,14,15]. The chemical composition of propolis includes resins, waxes, volatile oils, pollen, sugars, fatty, aliphatic and aromatic acids, vitamins, terpenes, alcohols, esters and impurities [16,17]. Moreover, propolis was found to be rich in phenolic compounds, mainly in flavonoids and derivatives of cinnamic acid [18].

Bee venom, another beekeeping compound, has an antitumoral activity both *in vivo* and *in vitro* models by suppressing tumor growth and proliferation [19,20]. It represents a complex mixture of proteins, peptides and low molecular weight components such as phospholipase A2 (PLA2), melittin, apamin, peptides, histamine and dopamine [21].

Objectives

The aim of this study was to evaluate the synergistic effect induced by propolis and bee venom on luminal and TNBC cell lines.

Materials and methods

Cell culturing

Two breast cancer cell lines including Hs578T and MCF-7 (ECACC) were cultured in DMEM and MEM medium respectively, supplemented with 10% Fetal Bovine Serum, 1% Glutamine and 1% Penicillin-Streptomycin at 37° in 5% CO_2 incubator. A quantity of 0.01 mg/ml insulin was added to Hs578T cells media while 1% nonessential amino acids (NEEA) was added to MCF-7 media. All cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Treatment

30% aqueous extract of Propolis (S.C. Phenalex S.R.L., Oradea, Romania) and bee venom (S.C. Apilife RO S.R.L., Sibiu) were obtained as gifts from Bisboaca Simona Elena and Dostetean Cornelia respectively. Honeybee venom was obtained through electrostimulation method. The propolis treatment was applied as follows: the aqueous solution was initialy centrifuged at 5000xg for 10 minutes to remove the sediment, after that the supernatant was passed through a 2 μm filter to remove any residual sediment or contaminants. The bee venom was suspended in RNAse/DNAse free water to a stock solution of 10 mg/ml. The propolis and bee venom were further diluted with RNAse/DNAse free water to serial concentrations of 0.1:100-30:50 ratios.

In order to investigate a synergistic effect induced by propolis and bee venom, we proposed a combination therapy according to each treatment half maximal inhibitory concentration (IC_{50}), for the two cell lines, and performed subsequent serial dilutions in a similar manner.

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Toxicity evaluation

MTT assay was used to measure the anticancer activity of the two treatments. $2x10^4$ cells were pre-plated in 96 well plates in 200 µl culturing media one day before treatment. Before treatment, 200 µl of fresh media containing the above described increasing concentrations of either propolis, bee venom or their combination was added to the wells. After an incubation period of 24 hours, the medium was removed, and 100 µl MTT was added to each well for one hour. The formed formazan salts were eluted in 150 µl DMSO and the absorbance was read in a Tecan Sunrise plate reader at 490 nm.

Results

The half maximal inhibitory concentrations (IC_{50}) were established by plotting the cells absorbance against the concentrations of each treatment and identify the concentrations at which 50% of the cells were dead. According to the dose-response relationship curves, we identified that the two cell lines have similar sensitivity to the aqueous extract of propolis. A dilution of 0.072-0.09 mg/ml was necessary to kill half of the cells (Figure 1).

On the other hand, when the cells were treated with bee venom, they presented different sensitivities. According to the toxicity data (Figure 2), MCF-7 cell was less responsive to bee venom treatment when compared to Hs578T cells. The IC_{50} established for MCF-7 (1 mg/ml) cells was 20 times higher than the one observed for Hs578T cells (0.05 mg/ml).

Once we identified the working concentrations for each treatment and cell line, we investigated whether the combination of the two treatments could induce synergistic effects in the two cell lines and improve cells sensibility. For that, we combined the two treatments in the corresponding IC_{50} concentration for each cell line and performed serial dilutions. Therefore, one treatment contained 0.072 mg/ml aqueous extract of propolis and 0.05 mg/ml bee venom (Figure 3) and a second treatment including 0.09 mg/ml aqueous extract of propolis and 1 mg/ml bee venom were established (Figure 4).

Our data showed that the cells did not present increased sensitivity to the first tested concentration (0.072 mg/ml aqueous extract of propolis and 0.05 mg/ml bee venom), only the undiluted treatment has reduced cell population by 50% (Figure 3). For the second concentration (0.09 mg/ml aqueous extract of propolis and 1 mg/ml bee venom) of combinatory treatment we observed an increased effect in both MCF-7 and Hs578T cells by 5 and 2 times respectively (Figure 4).

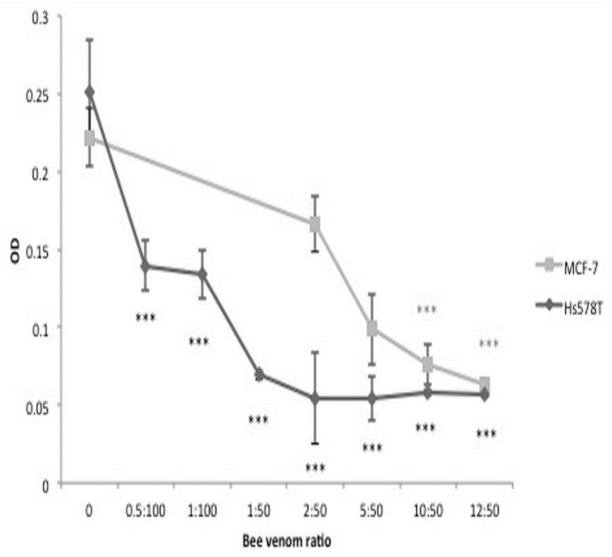

Figure 1. Influence of the aqueous extract of propolis on cell proliferation of the two cell lines.

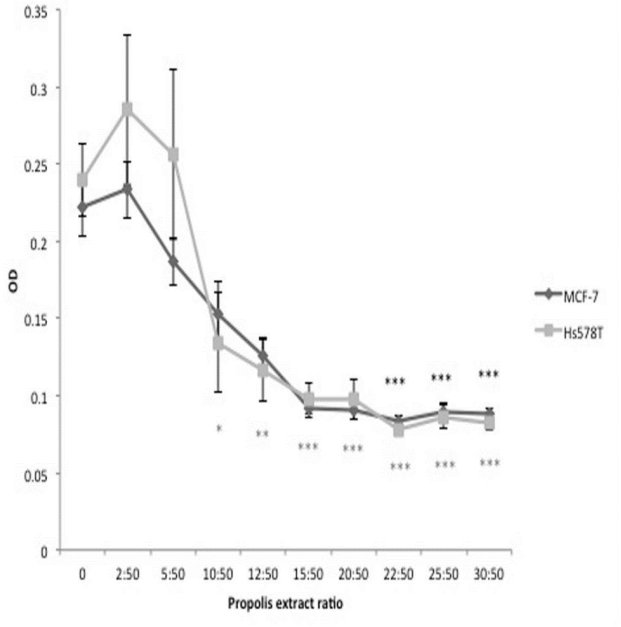

Figure 2. Influence of bee venom on cell proliferation of the two cell lines.

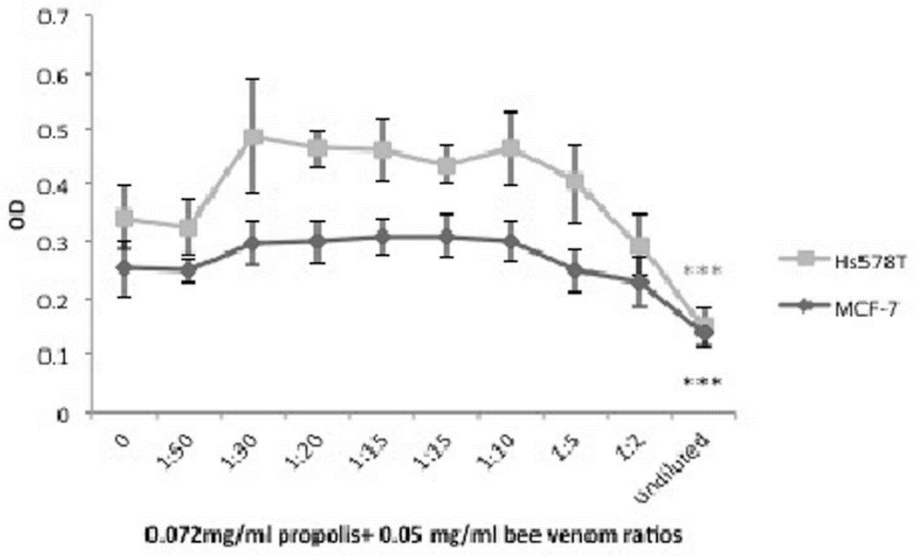

Figure 3. Synergistic effects of combined treatments of aqueous extract of propolis and bee venom on cell proliferation of the two cell lines.

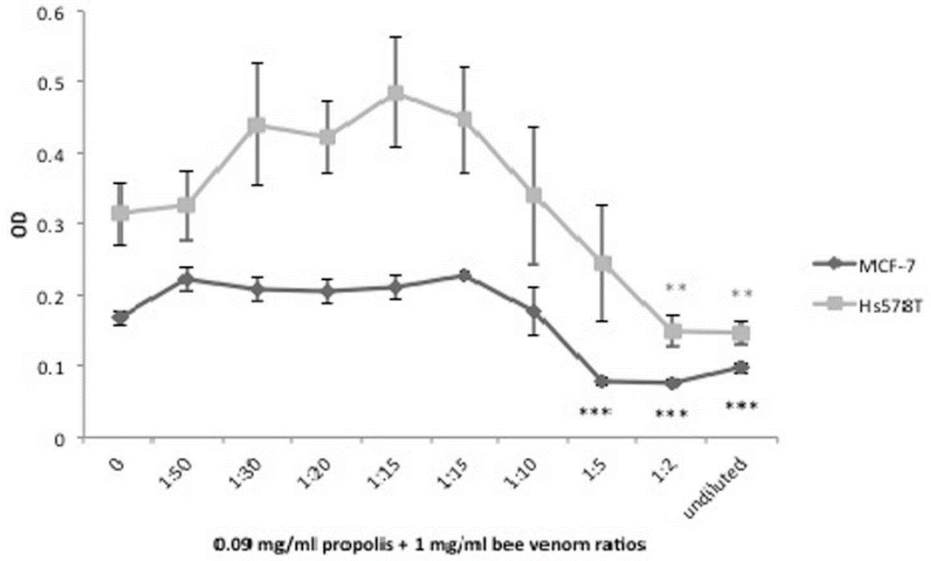

Figure 4. Synergistic effects of combined treatments of aqueous extract of propolis and bee venom on cell proliferation of the two cell lines.

Discussion

The healthy eukaryotic systems are characterized by a complex transduction of cell signals, and the proper function of these cells is limited by their ability to maintain a tight control over the signaling pathways. Cancer cells present multiple alterations in the communication networks, which are often regulated by positive and negative feedback loops called compensatory mechanisms. This is the main reason why specific inhibitors that target only one pathway, most often, failed in cancer treatment. Therefore, it is important to find strategies that target multiple cellular signaling pathways to eradicate cancer cells.

In recent years, natural compounds have received much attention in anticancer treatment, both *in vitro* and *in vivo* studies have demonstrated that these natural products act through multiple mechanisms and have inhibitory effects on various human and animal cancers [22,23,24,25]. Targeting multiple signaling pathways by natural products has opened new approaches to cancer therapies, and it appears to hold great promise [26].

The anticancer activity of both propolis and bee venom [10,27] is mainly mediated by apoptosis induction [21,20,23,28] or proliferation suppression [29,30]. Propolis has been reported to regulate apoptosis in a type

and concentration-dependent manner, irrespective of the cellular type investigated. Therefore, the fact that both cell lines, MCF-7 and Hs578T present similar sensitivity to propolis extract was not surprising. On the other hand, MCF-7 seems to be 20 times less sensitive to bee venom than Hs578T cells. However, the influence of bee venom on different cell subtypes has not been specifically reported so far. Furthermore, studies have shown that bee venom treatment cannot differentiate between normal and tumor cells [31,32]. MCF-7 cells are positive for progesterone expression, they belong to the luminal A breast cancer subtype while Hs578T cells are triple negative. Whether the bee venom could differentiate based on receptor expression of breast cancer subtypes remain to be investigated, but this could be an explanation for the observed differences in sensitivity.

The combination treatment of water extract of propolis with bee venom, induced synergistic effects based on the bee venom concentration. At lower bee venom concentrations, no synergy was observed, while at higher concentrations, the synergic effect was 5 and 2 times more pronounced than the two treatments alone. The combination of these two treatments has not been reported previously, thus we hypothesize that at lower concentrations of bee venom, there is a saturation of the mechanism of action. Bee venom inhibits tumor growth by activating apoptosis, necrosis and lysis of tumor cells, so the precise mechanisms are not yet fully elucidated [33].

However, at higher concentrations additional signaling could be activated to overcome cells reduced sensitivity.

Conclusions

The combination of propolis and bee venom treatments induces synergistic antiproliferative effects in a concentration-dependent manner in breast cancer cells. Our data point toward an activation of several additional signaling mechanisms that could overcome cells resistance to therapy, and this hypothesis remains to be investigated in future molecular studies.

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