The use of bioactive compounds from propolis and venom as therapeutic options in human breast cancers

SUMMARY OF PhD. THESIS

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INTRODUCTION

Cancer is a somatic cell genetic disease which is manifested by an accumulation of cells as a result of clonal expansion. Internationally, breast cancer presents the highest incidence globally (FERLAY, 2013) and it is the leading cause of death in Europe and second in USA (ALTHUIS, 2005).

Conventional therapy for breast cancer involves surgery, therapeutic schemes based on chemotherapy, hormonal, immunological and/or radiation, but each of these therapies has its own limitations in achieving a complete recovery. The use of non-conventional therapies based on natural compounds with antitumor and immune-stimulating effects can increase the antitumor effect of conventional therapy.

Developing new therapies based on both traditional and non-traditional compounds to improve the treatment of triple negative breast cancer represents a considerable challenge. Currently, non-traditional therapies based on propolisand bee venom compoundsrepresent a subject of debate for their applicability in the treatment of cancer. Recent studies have described the antitumoral properties of propolis in cell cycle arrest, activation of apoptosis (SAWICKI, 2012), induction of mitochondrial stress (BENGUEDOUAR, 2008), inhibition of growth and tumor proliferation (BIRT, 2001; CHEN, 2004 SZLISZKA, 2009), and bee venom by inhibiting tumor growth and proliferation (LIU, 2002 ORSOLIC, 2003c). Targeting multiple signaling pathways through natural compounds has opened new approaches to anti-tumor therapies suggesting new opportunities for non-conventional treatments (SARKAR, 2009).

Apitherapy uses honey, propolis, wax, royal jelly and bee venom to treat and prevent various diseases of the body. Apitherapy uses natural bee products, without any processing, by utilizing apitherapy standardized extracts or drugs. Apitherapy and its applications represents a natural alternative treatment and the interest in this approach is increasingly widespread worldwide.

Keywords: breast cancer, bee venom, propolis, apoptosis, antitumor activity
THESIS STRUCTURE

The paper entitled "The use of bioactive compounds from propolis and venom as therapeutic options in human breast cancers" contains 122 pages and is written according to the rules in two parts.

The first part contains 18 pages, structured into one chapter and summarizes the current general knowledge related to breast cancer, the characteristics of tumor cells, proliferation and survival of tumor cells, the extrinsic and intrinsic pathway of apoptosis induction, supervision of the immune system, the immune response: molecular mediators of the immune response, conventional therapies in breast cancer, alternative therapies with natural compounds: apitherapy, propolis: therapeutic properties, antitumor activity, bee venom: the therapeutic properties, antitumor activity.

The second part, extended to 85 pages, contains the personal research conducted during 2012-2015, focused on two research directions. The first one focuses on characterizing the role of the immune surveillance in breast cancer and the second on identifying the strategies for targeting cell proliferation and survival and stimulating the immune response in breast tumor cells. Each chapter is divided into subchapters describing the aim and objectives, materials and methods used, results obtained with talks on their novelty compared to other studies and partial conclusions of the present study. The research results are illustrated in 39 figures and summarized in 18 tables. The paper concludes with a bibliography (183 titles).

RESEARCH RESULTS

The third chapter entitled "Identification of gene expression particularities in whole blood collected from patients with breast cancer and their correlation with clinicopathological data is structured on three main objectives: correlation of clinicopathological data with Her2 expression in primary tumors of the patients, identifying specific Her2 expression profiles in primary tumors of the patients with breast cancer, identifying the molecular mechanisms associated with response to standard therapy.

For this study, were selected 30 patients diagnosed with breast cancer at the Oncology Institute "Prof. Dr. Ion Chiricuță" from Cluj-Napoca (IOCN) between 2010-2012. The Ethics Committee from the Oncology Institute "Prof. Dr. Ion Chiricuță" from Cluj-Napoca approved this study, and all patients included in the study gave their written consent.

Histopathological diagnosis and subtyping of the tumors was evaluated according to AJCC criteria (American Joint Committee on Cancer). The status for estrogen, progesterone and Her2 receptor was determined
by immunohistochemistry (IHC). In this study were included only patients with negative receptors for estrogen and progesterone. 18 patients included in this study had triple negative subtype (ER-, PR-, HER2-) and 12 patients had Her2 +non-luminal subtype (ER-, PR-, and HER2 +). The blood sample harvesting from 30 patients included in this study has been previously undertaken before administering any therapies, in vacutainers with EDTA anticoagulant. After removing the plasma and red blood cells, nucleated cells were processed for isolation of total RNA. Total RNA extraction was performed using the classical method with TriReagent (Invitrogen), chloroform and alcohol, based on a previously validated protocol. Subsequently, the obtained RNA was purified on silica gel columns, using the RNeasy Mini Kit (Qiagen). The concentration of extracted RNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and the integrity evaluation was performed using 2100 Bioanalyzer (Agilent).

For the synthesis of complementary DNA (cDNA) was used an amount of 300 ng of RNA and reagents from RT2 First Strand Kit according to the manufacturer's protocol (Qiagen). After the synthesis of cDNA, the obtained samples were diluted to 1/10 and amplified on PCR array plates with 96 wells using the Human Breast Cancer PAH-131Z (Qiagen) kit containing a set of 84 genes involved in breast cancer. The settings for the amplification program were established according to manufacturer's specifications and performed using LightCycler 480 II apparatus (Roche, Romania). For fluorescent detection it was used SYBR green, and the inflection point specific to the number of amplification cycles (Ct) were determined by automatic analysis method called second derivative method.

Correlations between clinical data were analyzed using Fisher's exact test or chi square test. PCR array results were analyzed for calculating the fold-change value by the ΔΔCt method for Ct values with a cut-off at 35 cycles. Genes of interest were considered with a 1.5 <fold change> 1.5 and an adjusted p value <0.05 obtained by Benjamin Hochberg method. Patients were grouped according to their Her2 expression in the primary tumor at diagnosis. Differentially expressed genes were analyzed for functional interactions with Ingenuity Pathway Analysis software (Ingenuity Pathway Analysis IPA). By using the information stored in Ingenuity Database (Ingenuity Knowledge Base IKB), the genes were grouped into molecular networks, canonical pathways and biological functions. The importance of the association between genes/networks and biological functions was assessed by using Fisher's exact test (p <0.05).

According to the immunohistochemical analysis, Her2 receptor was expressed in primary tumors of 18 patients, whereas the rest of the patients were found negative for protein expression. Twenty-eight patients were diagnosed with invasive ductal carcinoma, and two patients had invasive lobular carcinoma. Approximately 75% of the patients were
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diagnosed with stage III breast tumors. Most of the patients presented positive lymph nodes without secondary distant metastases. Statistically significant values were identified between lymph nodes status and HER2 expression (p < 0.03), fewer HER2-positive patients had lymph nodes mobility than Her2 negative patients.

According to the transcriptional analysis it was identified a group of 15 genes differentially expressed between the group of patients with HER2- and patients with HER2+. For patients with Her2- all the genes were underexpressed except Cyclin A1 which was overexpressed. These genes encode for cell cycle regulators: CCNA1, JUN, MKI67, RASSF1A, SFN; molecules involved in cell adhesion: CDH1, CTNNB1, HER2, transcription factors: CTNNB1, GATA3, HIC1, JUN, NOTCH1; or signal transducers: GLI1.

Based on the analysis from gene expression, cell cycle (p=9.21E-15-3.99E-04) is the most important function regulated by these genes, followed by cell motility (p = 4.13-13-6.01E-04) and cell growth (p = 2.40, 11-5,69-04). These genes have been included in the molecular signaling of the canonical pathways involved in cancer, in particular the signaling mechanisms in the reproductive system (p = 1.68, 13-4.22E-04), as well as signaling mechanisms in development and functionality of the circulatory system (p = 1.18-05-5.17E-04).

Chapter 4, entitled “Evaluation of the molecular mechanisms of action from alternative treatments with propolis, venom or their combination in triple negative breast cancer treatment” is divided into four major objectives: obtaining and purification of the propolis and bee venom aqueous solutions, evaluation of the effects from propolis and bee venom treatment on breast cancer in vitro models, identifying the molecular mechanisms modulated by these treatments, investigating the cellular and molecular mechanisms of synergic action of the two treatments.

The obtained results from determination of total polyphenols, total flavonoids and measuring the antioxidant capacity of aqueous and alcoholic propolis extract reveal that the highest value for the extraction yield was obtained for 96% alcohol propolis extract and the greatest amount of biologically active compounds (polyphenols and total flavonoids) and antioxidant activity (DPPH and FRAP) was detected in 20% alcoholic propolis extract in 70% ethyl alcohol.

According to high liquid performance chromatography analysis (HPLC) from the aqueous propolis extract, three main phenolic acids were identified: caffeic acid, p-coumaric acid and ferulic acid, while in bee venom were identified melittine (62.32%), phospholipase A2 (21.84%) and apamin (4.36%). The main free amino acids in bee venom detected through liquid chromatography technique coupled with mass spectrometer detector (LC-MS) were proline, cysteine, alanine, arginine, while in 30% aqueous propolis extract were found proline, asparagine, glutamic acid and valine. The results obtained by using atomic absorption spectrometry for minerals
determination revealed that bee venom contains rich amounts of Mg (28297.57 µg/kg), Na (4243.08 µg/kg), Ni (4162.61 µg/kg), Ca (3636.06 µg/kg), while in 30% aqueous propolis extract were predominantly identified Mg (21436.13 µg/kg), Mn (5744.11 µg/kg) (5249.00 µg/kg) and Ca (4173.93 µg/kg).

In order to conduct the *in vitro* study, we selected two representative breast cancer cell lines: Hs578T (triple negative subtype) and MCF-7 (luminal subtype) (ECACC), which were grown in DMEM culture medium, respectively MEM, supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin at 37°C in a 5% CO2 incubator. The MTT assay was used to evaluate the antitumor activity of the two treatments. The value of half maximal inhibitory concentrations (IC_{50}) were determined by representing the absorbance graph concentrations of each treatment applied to cell lines and by identifying concentrations at which 50% of cells are viable. According to the graphic curves obtained from the dose-response relationship, it was found that in order to induce cell death in half of the cell population it is required a concentration of 1 mg/ml in the case of MCF-7 cell line and 0.05 mg/ml for Hs578T cell line.

After identifying the working concentrations for each treatment and cell line, it has been investigated the possibility of inducing a synergistic effect by combining the two treatments on these two cell lines. Based on this goal, we combined these two treatments according to the IC_{50} dose established for each cell line and serial dilutions were performed. Therefore, the first combined treatment was established at a concentration of 0.072 mg/ml aqueous extract of propolis respectively 0.05 mg/ml bee venom, and for the second combined treatment we used a concentration of 0.09 mg/ml aqueous extract of propolis and 1 mg/ml bee venom. When the second concentration for the combined treatment was tested (0.09 mg/ml aqueous extract of propolis and 1 mg/ml bee venom), it was observed an increased effect of 5 respectively 2 times in both cell lines MCF-7 and Hs578T.

Because the patients with triple negative breast cancer subtype have the weakest response to current therapies, the molecular studies from this work focused on Hs578T triple negative breast cancer cell line, in order to use combined treatments of propolis and bee venom extracts as targeted therapy for this breast cancer subtype. For the molecular biology analysis through the qRT PCR technique, a number of 2.5x10^5 Hs578T cells were plated in 12 well plates one day before treatment. On the day of treatment, the cell culture medium was replaced with fresh medium containing 0.072 mg/ml aqueous extract of propolis, 0.05 mg/ml bee venom, or 0.072 mg/ml aqueous extract of propolis plus 0.05 mg/ml bee venom. The cells were incubated for 24 hours with the established treatment and then the cells were lysed in Trizol and processed for total RNA extraction using the classical method with phenol-chloroform. According to the quantitative
RNA evaluation using NanoDrop ND-1000 spectrophotometer, the concentration of extracted RNAs was between 171.37 - 441.29 ng/µl. According to the qualitative RNA analysis using Agilent 2100 Bioanalyzer, the RIN (RNA Integrity Number) value ranged between 8.9 - 9.6. For complementary DNA (cDNA) synthesis we used an amount of 500 ng of total RNA from each sample and Transcriptor First Strand cDNA Synthesis Kit, following the manufacturer's protocol (Roche Technologies). The obtained complementary DNAs were diluted 1:10 and amplified for each gene of interest using LightCycler TaqMan Master kit in LightCycler 480 device (Roche Technologies). The Ct values were calculated by the Absolute Quantification Second Derivative max method, after that the levels of expression for each gene were normalized to 18S housekeeping gene and calculated using 2^(-ΔΔCt) method.

In order to elucidate the molecular mechanisms of action in the case of propolis aqueous extract and bee venom, we investigated the gene expression changes induced by these two individual treatments and the combined treatment of these two extracts using a panel of 22 genes involved in apoptosis and immune response. The investigation of the apoptotic process was chosen based on specialized literature data in which these therapies act primarily by inducing cell death. In order to identify a treatment that stimulates the compromised immune response of triple negative breast cancer patients identified in the previous section study, we investigated whether the treatment with propolis and/or bee venom regulates the transcription of signaling genes from the immune response. Therefore, we identified and tested a panel of 7 genes that have been described to regulate apoptosis main signaling pathways, 8 genes involved in signaling of the immune response, and 7 genes that regulate both the process of apoptosis and the immune response.

The results show that the treatment with aqueous extract of propolis induces overexpression of FAS, ATM, SPP1, IL8, VEGF, TGFB2, CSF2, IL4 and TGFB1 genes and underexpression of PCNA, PTTG1, p53 and EGF genes. Treatment with bee venom stimulates gene transcription for DRAM1, TGFB2, CSF2 and MYC and represses gene transcription for SPP1. The combined treatment of these two extracts has a synergistic effect of stimulation on CSF2 and MYC genes and a inhibitory effect for EGF, p53, PTTG1 and PCNA genes. Also, the combined treatment of aqueous extract of propolis and bee venom exerts antagonistic effects on SPP1 gene, causing the loss of any effect. In general, the regulated genes after the treatment with aqueous propolis extract are also maintained regulated in the case of combined treatment. Moreover, DRAM1 and BCL2 genes which are activated due to treatment with venom lose their effect when adding propolis, these data suggesting that treatment with propolis is decisive in the transcriptional regulation of these genes. Also, if a gene is regulated by
both treatments similarly, the synergistic treatment does not lead to an increase in signaling, as observed in the case of ATM, IL8, IL4, TGFB1 genes.

By contrast, in the case of FAS and VEGFA genes, which are strongly activated in propolis treatment, this effect disappears at synergistic treatment, suggesting that these genes are mainly regulated by the treatment with venom. A similar effect is observed when PTGS2 and MYC genes, which aren’t expressed after treatment with propolis, become active after treatment with venom or synergic. These data suggest that these two treatments act on specific signaling pathways and their synergistic effect is controlled selectively by one or other treatment.

Genes and the fold change values corresponding to each treatment were loaded into Ingenuity Pathway Analysis (IPA) for a prediction analysis of the transcriptional regulation of these genes and their signaling network organization, also for the prediction of the biological effects modulated by these genes. The analyzed genes were organized in three main signaling networks, the score assigned to each network being dependent on the number of genes identified in each network.

The treatment with aqueous propolis extract induced underexpression of 4 genes and overexpression of 10. The p53 and PTTG1 genes are direct-acting factors on the process of transcription, their underexpression as a result of the treatment suggests the inhibition of the genes expression regulated by them. PTTG1 regulates negatively the expression of ATM (Chesnokov, 2007) and positively the expression of VEGF (McCabe 2002), the observed data are in concordance with the data provided by the specialized literature, PTTG1 underexpression leads to overexpression of ATM, while data for regulating VEGFA are inconsistent, so although PTTG1 is underexpressed in this experiment, the level of expression for VEGF is increased.

At the same time, the expression level for VEGFA is regulated by p53 gene which inhibits it’s expression (PAL, 2001), our data indicate a dominant regulation of VEGFA through p53 gene. The p53 gene being inhibited due to treatment with propolis allows transcription of VEGFA. Also, p53 regulates negatively the expression of CSF2 (Mori, 1997), TGFB1 (IZZOTTI, 2004), PTTG1 and IL4 and positively the expression of EGF (KOMAROVA, 1998), TGFB2 (BOYKO, 2006), IL8, SPP1, PCNA, ATM and FAS. In turn, the activation of IL4, IL8, CSF2, TGFB1 and TGFB2 genes indicate the release of pro-inflammatory cytokines.

The bee venom treatment induced underexpression of a single gene and overexpression of 9 genes. The transcriptional regulation following this treatment is made apparently by the MYC gene and not by p53 as in the case of propolis. MYC regulates positively the expression of TGFB2 (SHU, 2013) and negatively the BCL2 gene (CORY, 2002) and FAS gene (SHIVAPURKAR, 2002). It was also reported the involvement of MYC in regulating of SPP1 (WANG, 2000) and IL8 (FLORCZYK, 2011), but the
signaling direction has not been described. Also, in the case of bee venom treatment, the activation of the IL4, IL8, CSF2 and TGFB2 genes suggests the induction of pro-inflammatory cytokines.

The combined treatment with propolis and venom induced the underexpression of 4 genes and the overexpression of 8 genes. The combined treatment maintains the molecular characteristics of the individual networks. The transcription is regulated both through p53 and PTG1 such as in the treatment with propolis also through MYC as well as in treatment with venom. Thus, there is noticed a synergistic effect of the molecular regulation of the two treatments.

The prediction analysis involves calculating an activation score that is considered significant when \(-2 \leq \text{activation score} \leq 2\), so when for a given biological process is calculated an activation score \(\geq 2\), this process is considered activated and when the activating score is \(\leq -2\), the biological process is considered to be activated. Based on expression levels of the studied genes is estimated that treatment with propolis decreases apoptosis (activation score of \(-1.928, p = 1.6 \times 10^{-22}\)) and increases tumor cell viability (activation score \(2.658, p = 6.96 \times 10^{-20}\)). These molecular results contradict the biological effects observed at the concentration of propolis used where it was obtained a reduction of 50% in the number of tumor cells at 24 hours of treatment. After analyzing these results we can hypothesize that propolis activates adjacent apoptosis signaling pathways different than the activated through p53, FAS or TNF. One of these signaling pathways could be represented by the preferential regulation through TGFB1, which is the only molecule whose expression level is inversely correlated with the assumed biological effect; increased expression level for TGFB1 is correlated with the induction of apoptosis in tumor cells (Hofmann, 2003).

The analysis of cellular effects induced by bee venom treatment, although they have not resulted in a significant prediction, maintain the orientation of the observed activation in the case propolis treatment: decrease of apoptosis (activation score \(-1.49\)) and increased cell survival (activation score 1.923). Both CSF2 (LOTEM, 1996) and BCL2 (JIANG, 2011) stop the induction of cell death, including mammary cells (RUIZ-RUIZ, 2003). On the other hand, MYC gene was shown to stimulate breast tumor cell apoptosis (BEARSS, 2002; BEARSS, 2000 DEB, 2004), suggesting that in our conditions this process could be adjusted by MYC. In the case of cell survival, all genes whose expression levels were statistically significantly affected after treatment with bee venom indicates an activation of the process of survival, as opposed to actual observed biological effect.

In the case of combined treatment it was observed a molecular synergism of the two treatments, with an activation score of 1.85 for apoptosis, respectively 2.237 for cell survival. The direction of calculated activation of the two processes is preserved, reinforcing the hypothesis that
these processes are regulated by alternative signaling pathways other than those studied.

In order to investigate the molecular effects that treatment with propolis, venom and propolis combined with venom might have on modulating the immune system, the activation score was calculated for two illustrative cases as well as their expansion and proliferation of immune cells.

Following treatment with propolis it was calculated an activation score of 1.689 for the immune cell expansion and 1.159 for their proliferation. Although these scores aren’t significant in terms of prediction, however, these values show that treatment with propolis induces transcription of some genes that signals mainly the modulation of the immune system. Predictive analysis shows that overexpression of genes such as VEGFA (TERME, 2013) and CSF2 can lead to the expansion and proliferation of immune cells (KARMALI, 2011), thus modulating the immune system to fight against tumor growth. On the other hand, p53 regulates in a negative loop activated immune cells through CSF2 (Ishii, 2009), and concomitant underexpression of p53 suggests a mechanism of amplifying the signal.

Following the treatment with bee venom, the proliferation of immune cells does not appear to be significantly affected (activation score 0.737), in contrast with their expansion process (the activation score of 2.4). Although CSF2 (KRMALI, 2011), MYC (CHARLOT-RABIEGA, 2011) and BCL2 (JOSHI, 2007) promote the expansion and proliferation of immune cells, TGFB2 inhibit their proliferation (Beswick, 2011). In the case of the combined treatment of propolis and venom, it was calculated an activation score of 1.977 for the expansion of immune cells and 1.337 for their proliferation, these processes are apparently controlled by p53, MYC and CSF2 genes, the most important regulators of the immune system.
GENERAL CONCLUSIONS

According to the first objective of this thesis, we identified and characterized several genes involved in modulating the response to treatment of patients with triple negative breast cancer. This characterization was made by a non-invasive exploration method, using whole blood as biological material collected from the patients enrolled in this research study. Gene expression data were correlated with clinicopathological features, the identified genes were associated with a decreased response to conventional therapies and a compromised immune system in the case of these patients.

In order to improve the response to treatment for patients with triple negative breast cancer subtype, we performed in vitro studies concerning the anti-tumoral activity of some natural compounds such as propolis and bee venom, on triple negative breast cancer cell cultures. Through these studies we have identified both the individual cellular and molecular mechanisms of signaling in the case of propolis and bee venom and the synergistic mechanisms induced by the combination of both treatments.

Our results have highlighted that the combination of treatment based on propolis and bee venom induce a synergistic antiproliferative effect in a concentration-dependent manner in breast tumor cells.

The analysis of molecular signaling mechanisms shows that propolis acts primarily through p53-dependent signaling pathways and bee venom acts through MYC pathway. By combining these two treatments it was observed a molecular synergism of transcriptional control both by the p53 pathway and by MYC pathway. Moreover, these treatments regulate the transcription of genes involved in signaling the immune response, which may suggest that these treatments may stimulate the immune response in the subtype of triple negative breast cancer cells. These observations are relevant and could be considered for establishing therapeutic schemes for multitarget treatment to reduce the risk of recurrence in patients with triple negative breast cancer, by activating adjacent molecular mechanisms of regulating proliferation and cell survival and stimulating an immune response.
BIBLIOGRAPHY


