

***In-Vitro* assessment of anticancer potential of Lapatinib and Bee venom against breast (MCF-7) and Prostate (PC-3) cancer cell lines: *In-Vitro* study**

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Abstract: Breast and prostate cancer are heterogeneous diseases and the leading cause of death worldwide. They are considered to be one of the most aggressive cancers due to failure of chemotherapy response. Thus, there is an urgent need of finding alternative therapies for breast and prostate cancer. In the present study, the anti-cancer properties and cytotoxicity of Lapatinib and bee venom on breast MCF-7 and prostate PC-3 cancer cell lines were evaluated using MTT assay and it was noticed that the cytotoxicity was concentration dependent recording IC₅₀ of 60 µg and 81 µg and 288 µM and 345 µM for MCF-7 and PC-3 respectively. Also, synergetic activity of both BV and Lap to the other recording a significant decreased IC₅₀ values in the order of 1.04 µg and 0.59 µg for BV and 178 µM and 234 µM for Lapatinib post MCF-7 ad PC-3 respectively. The cell cycle arrest profile and specific cellular apoptosis markers were monitored (MMP1, CDK2, HER2, and P53) in bee venom and Lapatinib IC₅₀ values pretreated MCF-7 and PC-3 cells using real time PCR and flow cytometric analysis. By analyzing the cell cycle arrest and related gene expression pattern, it was noticed that the main phase of cell cycle arrest was found to be G0/G1 in MCF-7 cell line. An S-phase arrest was also observed in bee venom pretreated breast MCF-7 cell line to a greater extent than that observed in cells only treated with Lapatinab. Up regulation of pro-apoptotic and down regulation of anti-apoptotic genes in bee venom pretreated cells were significantly enhanced as compared to cells treated with Lapatinab. In this study, we suggest that Lapatinib via its synergistic action with bee venom might be used as an enhancer of the anticancer properties of Lapatinib.

Key words: Bee venom, Lapatinab, apoptosis, MCF-7 and PC-3 cell lines, Cell cycle, cytotoxicity, anticancer activity, MMP1, CDK7, HER2, P53.

Introduction

Breast is the most common cancer among women while prostate is the most common among men. The American Cancer society estimated that for

breast cancer 255,180 new breast cancer cases and 41,070 breast cancer deaths were expected to occur in the United States in 2017 (Siegel *et al.*, 2016). Furthermore, according to the National Cancer Institute in 2016, approximately 12 percent of men will be diagnosed with prostate cancer. Moreover, several studies have reported that breast cancer in young women have more aggressive features, such as larger tumor size, poor differentiation, positive lymph nodes, high proliferation rates, higher prevalence of Human Epidermal Growth Factor Receptor 2 (HER2/neu) overexpression, and much more tumors of the basal-like histologic subtype (Walker *et al.*, 1996 ; Pollán., 2010). On the other hand, Many studies conducted that men with prostate cancer may experience common problems such as BPH (benign prostatic hyperplasia), acute and chronic bacterial prostatitis and chronic prostatitis (Rycaj and Tang., 2015).

However, the most common types of cancer treatment include Chemotherapy, surgery, and radiation- therapy. There are two types of chemotherapy: The first type is cell cycle specific which affects cells only when they are dividing, on the other hand the second type is cell cycle non-specific which affects cells when they are at rest. According to Puliye *et al.*, (2015), chemotherapy drugs mainly work through damaging the DNA or the RNA of the cells. However, these types of cancer treatment contain many side effects that have a psychological, physical, emotionally, and economic impact on the patient's life. Cancer patients typically experience "Break-Through" nausea, vomiting, hair loss, weight loss or gain, feeling constantly tired, mouth ulcers, chemo brain (problems with concentration or memory), hearing loss, fertility issues, and heart and lung problems as a result of the active pharmacology imposed by Intravenous Chemotherapy (Siegel *et al.*, 2012).

Nonetheless, bee venom (BV) (api-toxin) is known to be one of the strongest natural substrates that are very useful in cancer treatments. Also it has been widely used in the treatment of some immune-related diseases, as well as in recent times in treatment of tumors. Several cancer cells, including

renal, lung, liver, prostate, bladder, and mammary cancer cells as well as leukemia cells, can be targets of bee venom peptides such as melittin and phospholipase A2. The cell cytotoxic effects through the activation of PLA2 by melittin have been suggested to be the critical mechanism for the anti-cancer activity of BV. The induction of apoptotic cell death through several cancer cell death mechanisms, including the activation of caspase and matrix metalloproteinases, is important for the melittin-induced anti-cancer effects. The conjugation of cell lytic peptide (melittin) with hormone receptors and gene therapy carrying melittin can be useful as a novel targeted therapy for some types of cancer, such as prostate and breast cancer. This review summarizes the current knowledge regarding potential of bee venom and its compounds such as melittin to induce cytotoxic, antitumor, immunomodulatory, and apoptotic effects in different tumor cells in vivo or in vitro (Oršolić., 2011).

Furthermore, Lapatinib (INN) is used in the form of Lapatinib ditosylate (USAN) is an orally active drug for breast cancer and other solid tumors. It is a dual tyrosine kinase inhibitor which interrupts the HER2/neu (human epidermal growth factor receptor 2) and EGFR (epidermal growth factor receptor) pathways (Medina & Goodin., 2008). It is used in combination therapy for HER2-positive breast cancer. It is given with chemotherapy tablet called capecitabine, or in combination with hormonal therapy. It is used for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpress HER2. Over expression of HER2/neu can be responsible for certain types of high-risk breast cancer induction in women. Like Sorafenib, Lapatinib is a protein kinase inhibitor shown to decrease tumor-causing breast cancer stem cells by binding to the ATP-binding pocket of the EGFR/HER2 protein kinase domain, preventing self-phosphorylation and subsequent activation of the signal mechanism. It was recorded that bee venom, melittin and propolis showed clear anticancer potentials as they up/down regulate of P53, BAX pro-apoptotic genes and Bcl2 anti-apoptotic gene, respectively (Aydiner., 2016).

However, these side effects of chemotherapy resulting from the drug effects itself in addition to the drug administration in large amounts lead the scientists to think about a solutions in order to reduce these adverse effects. One of these solutions is the emerging or combining Lapatinab that aimed to overcome the problems stated above by using natural compounds such as bee venom, which may affect multiple targets with reduced side effects and which are effective against several cancer types

such as breast cancer. However, natural compounds from various sources including plants, animals, and microorganisms offer a great opportunity for discovery of novel therapeutic candidates for the treatment of breast cancer. Accordingly, the aim of the present work is to *In-vitro* evaluate the anticancer potential of Lapatinib as anticancer drug against breast (MCF-7) and Prostate (PC-3) cancer cell lines, in addition to the evaluation of the enhancement potential of bee venom to the anticancer property of test drug and related gene profile.

Materials and methods

Breast Cancer of Human (MCF-7) cell line, Prostate Cancer of Human (PC-3) cell line, Bee venom, and Lapatinib were kindly supplied from cell culture unit and Helwan Animal House (VACSERA-Egypt), respectively.

Preparation of bee venom and Lapatinab

Bee venom was dissolved in RPMI 1640 medium as 2 mg / ml, and sterile filtrated using syringe filter (Millipore –USA). Also, Lapatinib was dissolved in (DMSO) and prepared as 10 mM/ ml

Cytotoxicity (MTT assay)

Human Breast (MCF-7) and prostate (PC-3) cancer cells were propagated in 75 cm² cell culture flasks using RPMI-1640 medium (GIBCO-USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO-USA) and incubated in 5% (v/v) CO₂ incubator at a temperature of 37°C. Confluent cells were detached using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylenediaminetetraacetic acid (GIBCO-USA) for 5 min. Cells were plated at a concentration of 2 x 10⁵ cells/ml in 96-well cell culture plates and incubated at a temperature of 37°C for 24 hours to achieve confluence state. The medium was decanted and fresh medium containing various concentrations of bee venom and Lapatinib was added for cytotoxicity determination using colorimetric MTT reduction assay. Dead cells were washed out using phosphate-buffer saline (PBS), and 50 µl of MTT stock solution (0.5 mg/ml) were added to each well. After 4 h incubation period, the supernatants were discarded and the formazan precipitates were solubilized by addition of 50 µl per well of dimethyl sulfoxide (DMSO). Plates were incubated in the dark for 30 min at a temperature of 37°C, and absorbance was determined at a wavelength of 570 nm using microplate reader (ELX -800, Biotek-USA). The cell viability percentage was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD of treated wells} \times 100}{\text{OD of control wells}}$$

The synergetic potentials of bee venom and Lapatinib was examined by evaluating the cytotoxic potential of bee venom and Lapatinib in MCF-7 and PC-3 cells pretreated with the safe concentration of each of them for 24 h. Morphological alterations of cells were analyzed using an inverted microscope (Nikon-Japan). The cell viability (%) was blotted against the tested bee venom and Lapatinib concentrations.

Statistical analysis

Statistical significance between treated and untreated cells was determined using one way ANOVA. Differences at P values less than 0.05 were considered significant. The IC₅₀ values of test bee venom and Lapatinib were determined using Masterplex-2010 software program.

Cell cycle analysis

MCF-7 and PC-3 cells pre-cultured in 25 cm² cell culture flasks were treated with an IC₅₀ of test venom dissolved in RPMI-1640 medium, for 24h. For cell cycle analyses, the cells were harvested and fixed gently with 70% (v/v) ethanol in PBS, maintained at a temperature of 4°C overnight and then re-suspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase and 0.1% (v/v) Triton X-100 in a dark room. After 30 min at 37°C, the cells were analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle and sub-G1 group were determined and analyzed, as described previously.

mRNA Expression levels of cell apoptosis-related genes

Total RNA was extracted from control and treated MCF-7 and PC-3 cells using the GeneJET RNA Purification kit (Fermentus-UK) according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed spectrophotometrically at 260/280 nm ratio and by gel electrophoresis, respectively. First-strand cDNA was synthesized with 1 µg of total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) in accordance with the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80°C until use for determination of the expression levels of MMP1, CDK7, HER2, and P53 genes using real-time PCR. Quantitative real-time PCR was performed on a Rotor-Gene Q cyler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse

primers for each gene. The nucleic acid sequences of the primers were as follows :human MMP-1 (F 5'-ctggccacaactgccaaatg-3' , R 5'-ctgtccctgaacagcccagctactta-3') & CDK7 CDK2 (Cyclin-dependent kinase 2) (F 5'-GCTAGCAGACTTTGGACTAGCCAG -3' , R 5'-AGCTCGGTACCACAGGGTCA -3') & P53 (F 5'-CCCCTCCTGGCCCCTGTCATCTTC -3' , R 5'-GCAGCGCCTCACAACCTCCGTCAT-3'.) as well as anti-apoptotic gene HER2 (F 5'-ATCTGCCTGACATCCACG-3' , R 5'-GCAATCTGCATACACCAGTTC-3') compared to β -actin as a housekeeping gene (F 5'-TTCCTGGGCATGGAGTC-3' , R 5'-CAGGTCTTTGCGGATGTC-3'). Real-time PCR mixture consisted of 12.5 µL 2x SYBR Green PCR Master Mix, 1 µL of each primer (10 pmol/µL), 2 µL cDNA, and 8.5 µL Rnase-free water in a total volume of 25 µL. Amplification conditions and cycle counts were a temperature of 95° C for 15 min for the initial activation, followed by 40 cycles of denaturation at 94° C for 15 s, annealing at 60° C for 30 s, and elongation at 72° C for 30 s. Melting curves were performed after real-time PCR to demonstrate the specific amplification of single products of interest. A standard curve assay was performed to determine the amplification efficiency of the primers used. Relative fold changes in the expression of target genes (MMP1, CDK7, HER2, and P53) were accomplished using the comparative 2- $\Delta\Delta$ Ct method [22] with the β -actin gene as an internal control to normalize the level of target gene expression. $\Delta\Delta$ CT is the difference between the mean Δ CT (treatment group) and mean Δ CT (control group), where Δ CT is the difference between the mean CT gene of interest and the mean CT internal control gene in each sample. Logarithmic transformation was performed on fold change values before being statistically analyzed, using the fold change values of three replicates for each gene measured.

Statistical analysis

All experiments were carried out in three independent tests. Data were expressed as the mean \pm standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). The results were considered statistically significant at probability < 0.05.

Results

Cytotoxicity

The cytotoxic effect of test bee venom was assessed by recording the various morphological changes of cells. Induced cell toxicity was accompanied with morphological changes in the culture fields showing a polygonal shape with

distinct boundaries and homogenous cellular contents. On the other hand, a number of morphological abnormalities of cells were observed 24 h post treatment with the bee venom. At the lowest concentrations, cells lost their characteristic appearance, became rounded and detached out of the culture surface, while other cells retained their normal morphological appearance. Increasing bee venom concentrations resulted in increased cellular irregularities and larger areas devoid of cells. At the highest concentrations, cells showed obvious deterioration and deformation with severe shrinkage and condensation of their cellular

contents. The viability % of treated cells was concentration-dependent and the IC₅₀ values of Bee venom was 60 and 81 µg/ml and 288 µM and 345 µM for mMCF7 and PC-3 post treatment with bee venom and Lapatinib respectively and the MCF-7 and PC-3 cells pretreatment with Lapatinib maximized the cytotoxic effect of bee venom significantly (P<0.05) recording 1.04 and 0.59 µg for MCF7 and PC-3 and the IC₅₀ of Lapatinib was decreased significantly post cell pre-treatment with BV safe concentration recording 178 µM and 243 µM for MCF-7 and PC-3 respectively. [Fig. 1-2]

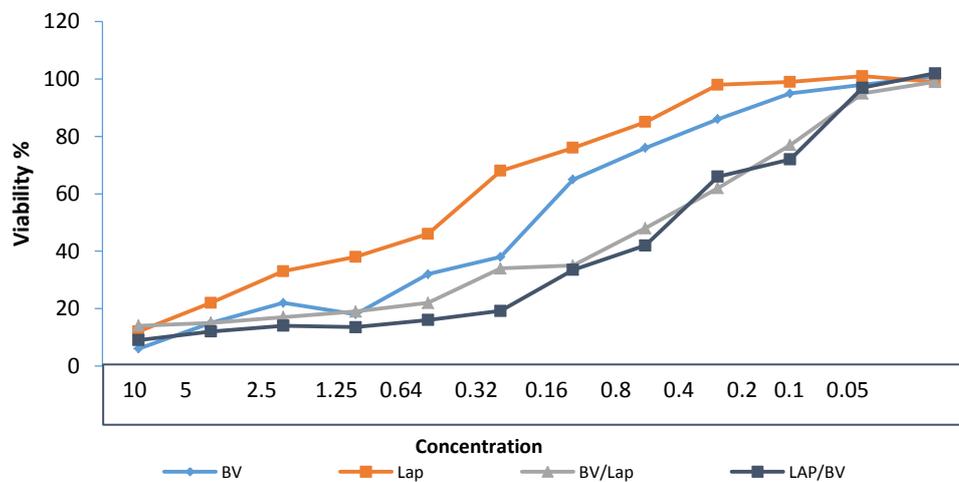


Fig.1: Evaluation of cell viability relative to Concentration.

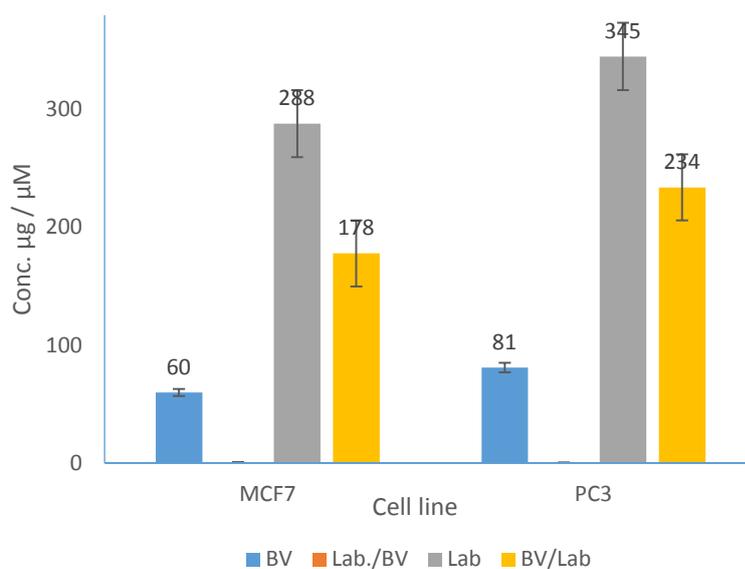


Fig.2: Evaluation of IC50 of sole bee venom and Lapatinib and their synergetic activity Cell cycle analysis

Toxicity of bee venom and Lapatinib was accompanied by cell cycle arrest in both MCF-7 and PC-3 cell lines. Cell cycle analyses have demonstrated that there was an accumulation of arrested cells in the G2/M phase in both cell lines either after cellular treatment with bee venom alone or in the case of Lapatinib pretreated cells. In addition, S phase arrest was found to be significantly higher in MCF-7 cells with 24.51%

and 17.04% in the case of bee venom and Lapatinib pretreated cells as compared to untreated cells (24.37 %). PC-3 cells exhibited an increase in the percentage of apoptotic cells as indicated by G0-G1 phase recording 56.66% and 57.66%, in addition to elevated G0-G1 phase that was observed in MCF-7 cells in the order of 55.33% and 53.56 post bee venom treatment and Lapatinib pretreatment, respectively, compared to control cells [Fig.4&5].

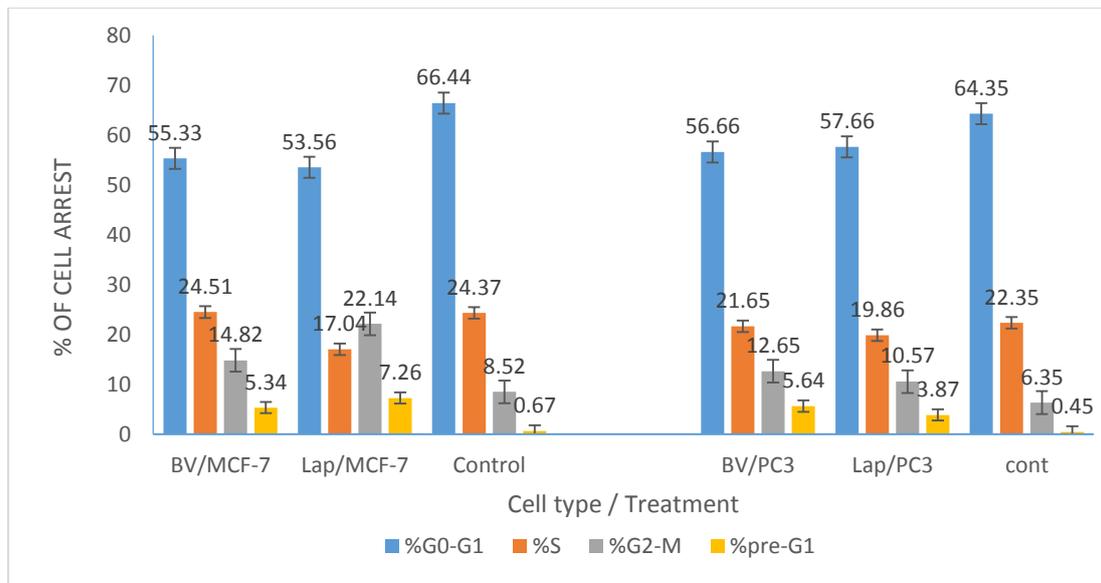
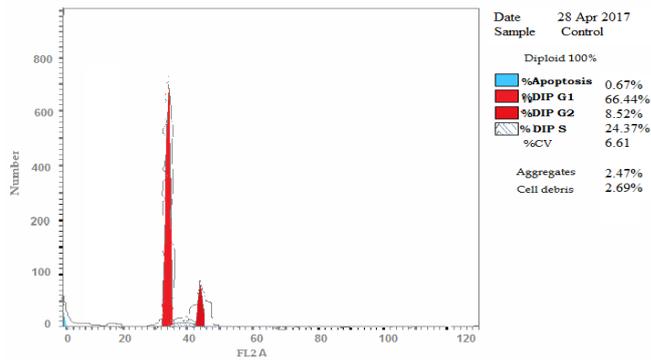
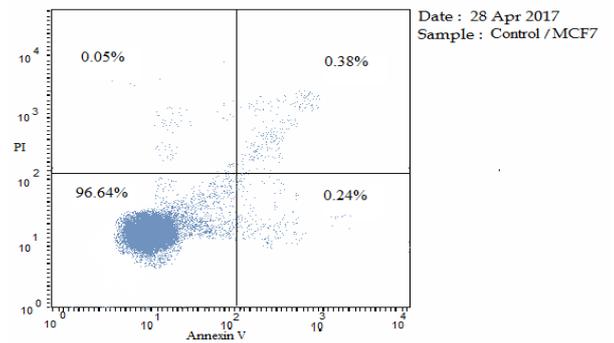


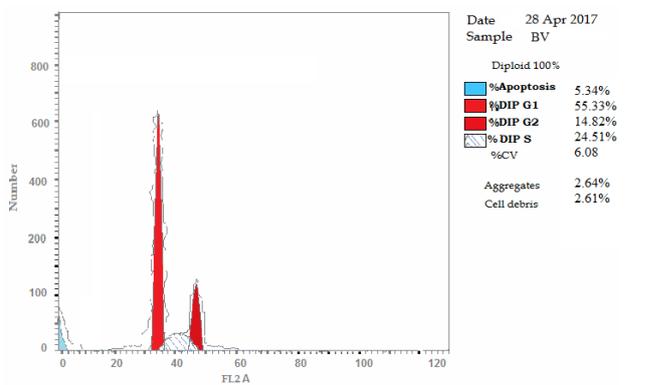
Figure 3: the arrest % at each of the cell cycle phases of MCF-7 and PC-3 cell lines after treatment with bee venom and lapatinib in comparison to control.



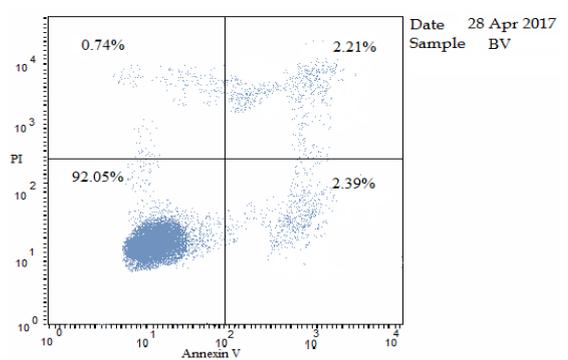
A (1): Control sample MCF-7



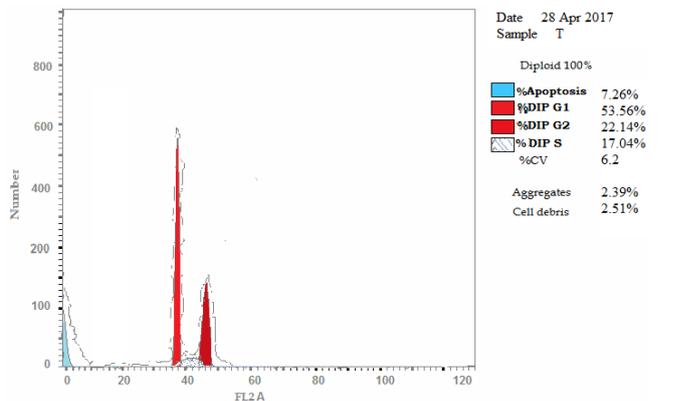
A (2): Control sample MCF-7



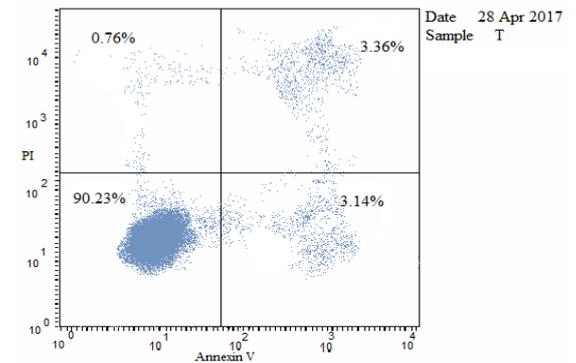
B (1): MCF-7 with Bee venom



B (2) MCF-7 with Bee venom

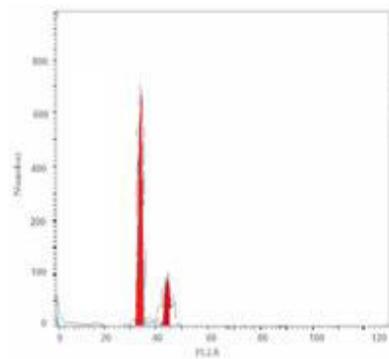


C (1): MCF-7 with Lapatinib

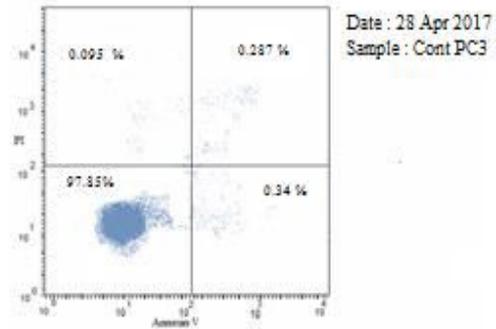


C (2): MCF-7 with Lapatinib

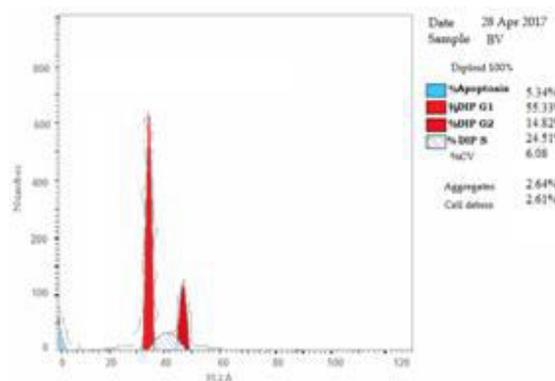
Figure 4: Cell cycle analysis of MCF-7 (B 1,2) and (C 1,2) cell line after treatment with lapatinib and bee venom in comparison to control A (1,2) samples, detected by flow cytometry.



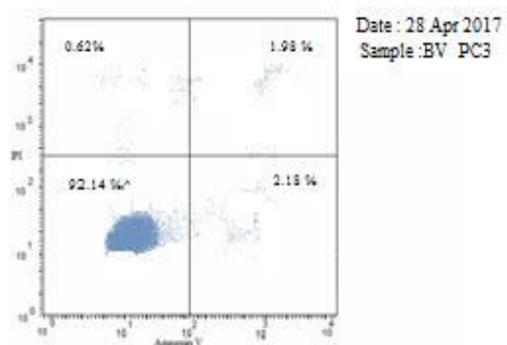
A (1): Control sample PC-3



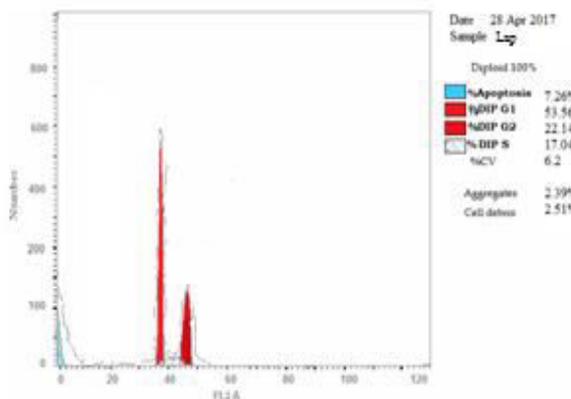
A (2): Control sample PC-3



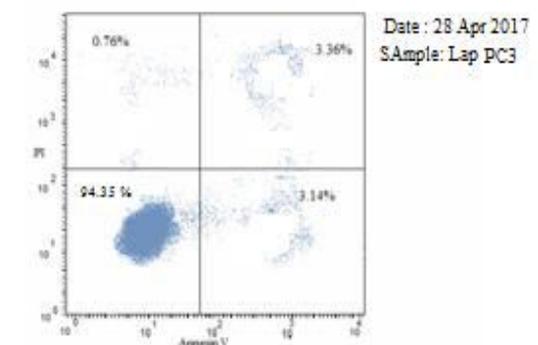
B (1): PC-3 with Bee venom



B (2): PC-3 with Bee venom



C (1): PC-3 with Lapatinib



C (2): PC-3 with Lapatinib

Figure 4: Cell cycle analysis of PC-3 (B 1,2) and (C 1,2) cell line after treatment with lapatinib and bee venom in comparison to control A (1,2) samples, detected by flow cytometry.

mRNA expression levels of apoptosis-related genes.

Data reported that pretreatment of MCF-7 and PC-3 cancer cells with Lapatinab potentiates the apoptotic activity of bee venom. The level of

expression of pro-apoptotic genes (MMP1, HER2, CDK2, and P53) was clearly up-regulated, while the expression level of anti-apoptotic genes was down-regulated ($P < 0.05$) indicating its potential efficacy in directing cancer cells towards programmed death [Fig.5-6].

GENE EXPRESSION RT-PCR

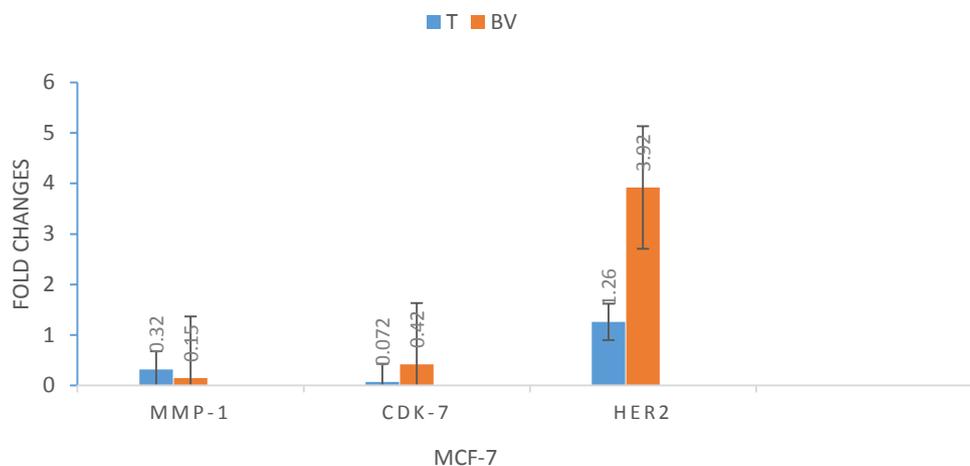


Figure 5: Gene expression level of MMP1, CDK2, and HER2 in Breast (MCF-7) cancer cells after the treatment with bee venom and lapatinib, using real time PCR.

GENE EXPRESSION RT-PCR

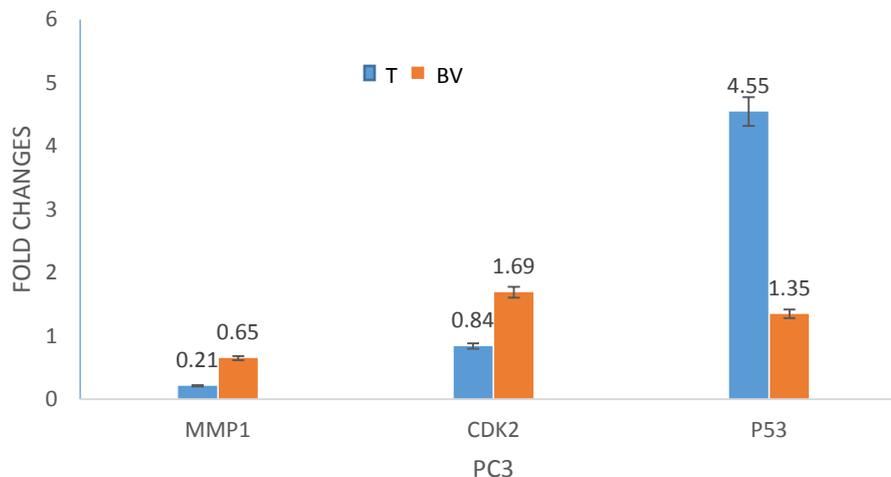


Figure 6: Gene expression level of MMP1, CDK2, and P53 in Prostate (PC-3) cancer cells after the treatment with bee venom and lapatinib, using real time PCR.

Discussion

Combined chemotherapy is known to be one of the most effective treatments for numerous types of cancer, especially when it is combined with a natural compounds that are believed to have multiple specific targets with minimal acceptable side-effects. Moreover, it decreases the likelihood of resistant cancer cells to develop. In the same way combination of anticancer drug with proteinacious products or fractions enhance the anticancer activity as conducted by (Ayman *et al.*,

2017) recording that pre-treatment of PC-3 cells with BCG / DDP derived protein enhanced the toxicity and anticancer potential of cerastes cerastes venom of about 150 fold and in turn proapoptotic genes.

In addition, the cell cycle profiles obtained during this research stating that bee venom has an anti-proliferative and cytotoxic effect on prostate cancer cells are found to be similar to the published work of Park *et al.*, (2010) in which bee venom was tested on several human prostate cancer cell lines

including LNCaP, DU145, and PC-3. The results obtained suggested that bee venom inhibited the tumor growth and the activity of NF- κ B accompanied with apoptotic cell death. Therefore, bee venom mediated the induction of apoptotic cell death as well as increased the expression levels of P53 (pro-apoptotic) and decreased levels of MMP-1 and CDK7 (anti-apoptotic).

Although, another study conducted by **Jo et al., (2012)** and **Burke et al., (2001)** bee venom was tested for anti-cancer effects and its ability to inhibit the cell growth through the enhancement of death receptor that expressed in the human ovarian cancer cells, SKOV3, and PA-1 post treatment with (1–5 μ g/ml) of bee venom, the expression of death receptor DR3 and DR6 was increased in both cancer cells, nonetheless the expression of DR4 was increased in PA-1 cells only. Furthermore the expression of DR downstream pro-apoptotic proteins that include caspase-3, 8, and Bax was concurrently increased, however, it inhibited the expression of Bcl-2 in SKOV3 and PA-1 cells and the phosphorylation of JAK2 and STAT3. The expression of cleaved caspase-3 has been increased in SKOV3, on the other hand the expression of cleaved caspase-8 has been increased in PA-1 cells. Furthermore, deletion of DR3, DR4, and DR6 with small interfering RNA has significantly reversed bee venom induced cell growth inhibitory effect in addition to down regulation of STAT3 by bee venom in SKOV3 and PA-1 ovarian and breast cancer cells. However, the results showed that bee venom induced apoptotic cell death in ovarian and breast cancer cells through the enhancement of DR3, DR4, and DR6 expression, and also through the inhibition of STAT3 pathway. These results illustrates the importance of bee venom in the induction of apoptotic pathways in breast cancer, and thus making it the perfect candidate for our study.

Moreover, in the present study the cell viability evaluation exhibited that the combination of Lapatinib with bee venom (natural compound) overcomes the drug resistance and inhibits migration of HER2 positive breast cancer cells by interrupting signaling from the epidermal growth factor receptors, EGFR and HER2/neu. Conversely, at the same time it's capable of increasing the probability of Lapatinib-resistant cell selection. These results obey with the work done by **Kaczyńska et al., (2016)** which aims to confirm whether the combinations of Lapatinib with isothiocyanates (natural compound) would apply stronger cytotoxic effect than therapy targeting the receptor only. Upon the investigation of the combination of Lapatinib with any of isothiocyanates significantly decreased cell

viability and inhibited migration of populations consisting of different amounts of drug-sensitive and drug-resistant cells. In case of population entirely composed of Lapatinib-resistant cells the most effective was combination of Lapatinib with natural compound which decreased cell viability and motility, phosphorylation of Akt, S6 and VEGF level more efficiently than each agent alone. In the present study the combination of the bee venom with lapatinib increased the cytotoxic effect on the cancer cells, where the cell viability test showed significant decrease as the dose concentrations increases.

Furthermore, According to a study conducted by **Drigla et al., (2016)** to evaluate the synergistic effect induced by propolis and bee venom on luminal (MCF-7) and TNBC (Hs578T) cell lines. Two breast cancer cell lines: MCF-7(luminal subtype) and Hs578T (TNBC subtype) were treated in a combination. The results indicated that both cell lines exhibited similar sensitivity to the aqueous extract of propolis at a dilution of 0.072–0.09 mg/ml. Also it 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, the synergistic effects were obtained at a higher concentration, which was 5 and 2 times stronger than the two treatments alone. These results can hypothesize that the combination of honeybee propolis and bee venom might be involved in signaling pathways that could overcome cells resistance to therapy. However, these results are matched with the present study despite the use of different cell lines that considered different gene pattern and different combinations.

In the studies conducted by **Drigla et al., (2016)** and **Kaczyńska et al., (2016)**, showed an agreement to our results that suggest that the effect of both bee venom and Lapatinib is concentration, cell type and time dependent. And that both bee venom and Lapatinib have almost the same effects *in vitro*. Furthermore, they showed an elevation in the expression of the pro-apoptotic genes P53 while a down regulation in the anti-apoptotic gene MMP-1 and CDK-7 was noticed in PC-3 cell line. On the other hand, they showed up regulation of MMP-1 and down regulation of CDK-7 and HER2 in MCF-7 cell line as well as a noteworthy cell cycle arrest at G2/M was observed post treatment with bee venom and Lapatinib in both cell lines.

Conclusion:

Novel therapeutic strategies for cancer are one of the most important current concerns. Natural compound mixtures that are believed to have multiple specific targets with minimal side-effects

are now of interest to many researchers. Therefore, the present work could conclude that Both bee venom and Lapatinib are cytotoxic in a concentration and cell type dependent, evaluation of cytotoxicity post pretreatment of MCF-7 cells with bee venom and Lapatinib separately enhanced the cytotoxicity of both than the single use of treatment in a significant way. Also, the use of both bee venom and Lapatinib showed a common cell arrest during the G2-M phase and the little arrest was clear in the S phase in case of cell treatment with Bee venom. In the same time, there was a clear up and down regulation of target genes those responsible for the suppression of cancer metastases and growth namely P53, HER2, MMP-1, and CDK7. Thus, it is recommended to test the Lapatinib on a wide range of cancer cell lines, in addition *in-vivo* application of Lapatinib and the evaluation the synergetic potential of different factions from different types of venoms on the anticancer potential.

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