Research Article

Anti-metastasis After Bee Venom and Melittin by Upregulation of BRMS1 and DRG1 Genes, With Downregulation of WNT7B in Breast Cancer Cells

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Breast cancer is one of the common cancers in women around the world, and metastasis potential of cancer is the main shortcoming for a high rate of survival. Apitherapy as an alternative medicine is promised to deal with cancer. Bee venom and its major component, melittin, are known to be effective for cytotoxicity in cancer cells. In this study, the expression profiles of three anti-metastatic genes including *BRMS1*, *DRG1* and *KAI1/CD82* were revealed for the first time after bee venom and melittin treatment, and two pro-metastatic genes including *EGFR* and *WNT7B* in metastatic breast cancer cells (MDA-MB-231) were also examined while comparing to normal breast epithelial cells (MCF10A). Selective cytotoxicity of bee venom and melittin were higher compared to cisplatin. Melittin at 0.5 µg/ml was effective at 24h for anti-metastatic function whereas 4 µg/ml was significant in treatments with bee venom or cisplatin. Melittin induced overexpression of *BRMS1* and *DRG1*, however bee venom induced *DRG1* and *KAI1/CD82* expression in breast cancer cells. *WNT7B* was downregulated in bee venom-treated breast cancer cells. These results suggest that both bee venom and melittin may act via upregulation of some anti-metastatic genes (*BRMS1*, *DRG1* and *KAI1/CD82)*, and down-regulation of a pro-metastatic gene, *WNT7B*.

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Introduction

Cancer is one the most chaotic diseases affecting many more people in the world year by year. Breast cancer commonly occurs in women and the survival rate is low [1]. Conventional therapies include

surgery and chemotherapeutics. These sometimes are supported with immunotherapy strategies. However, alternative medicine may be applicable in addition to complementary therapies. Apitherapy is an alternative medicine that uses beehive products including propolis, royal jelly, honey, pollen and venom for a range of diseases [2][3]. Bee venom has been known to have potentials against cancer [4][5][6], inflammation [7], and pain [8]. Melittin peptide, the major compound in the bee venom, is considered to have an anti-cancer function [4][9][10]. One of the main handicaps of cancer is its metastable characteristics, and this is the top challenge that needs to be focused in new developments of cancer therapies. Therefore cancer cells can be more strictly controlled.

There are a vast number of studies suggesting the use of bee venom and its major component, melittin, for effective therapy of cancer to deal with uncontrolled cell proliferation and metastable potential. These indicate the necessity for revealing detailed molecular effects of bee venom or melittin on different cancer cells to conclude specific targets or pathways for each (sub) type of cancer. To the best of knowledge there is no study revealing the expression levels of three anti-metastatic genes, *BRMS1* (breast cancer metastasis suppressor-1)[11], *DRG1* (Differentiation-related gene-1), and *KAI1/CD82* genes in breast cancer after bee venom and melittin. Two specific upregulated markers in MDA-MB-231 cells, the expressions of *WNT7B* and *EGFR*, were also investigated after bee venom, melittin and cisplatin in comparison with MCF10A.

Results and Discussion

First of all, the cytotoxic effects of bee venom, melittin and cisplatin on MDA-MB-231 and MCF10A cells were determined as mentioned in the MTT method. The viability of cells decreased in a dose-dependent manner **(Figure 1)**. IC50 values for cisplatin was calculated as 12 µg/ml for both cells. IC50 values for bee venom were 36 µg/ml and 8 µg/ml in MCF10A and MDA-MB-231 cells, respectively. IC50 values for melittin were 20 µg/ml and 8.45 µg/ml in MCF10A and MDA-MB-231 cells, respectively **(Figure 1)**. These suggest that selective indices (SI) for bee venom (4.5) and melittin (2.6) were more than cisplatin (1) **(Table 1)**. The greater the value of SI the more selectivity for cancer, therefore high SI values indicate selective potential of drug candidates.

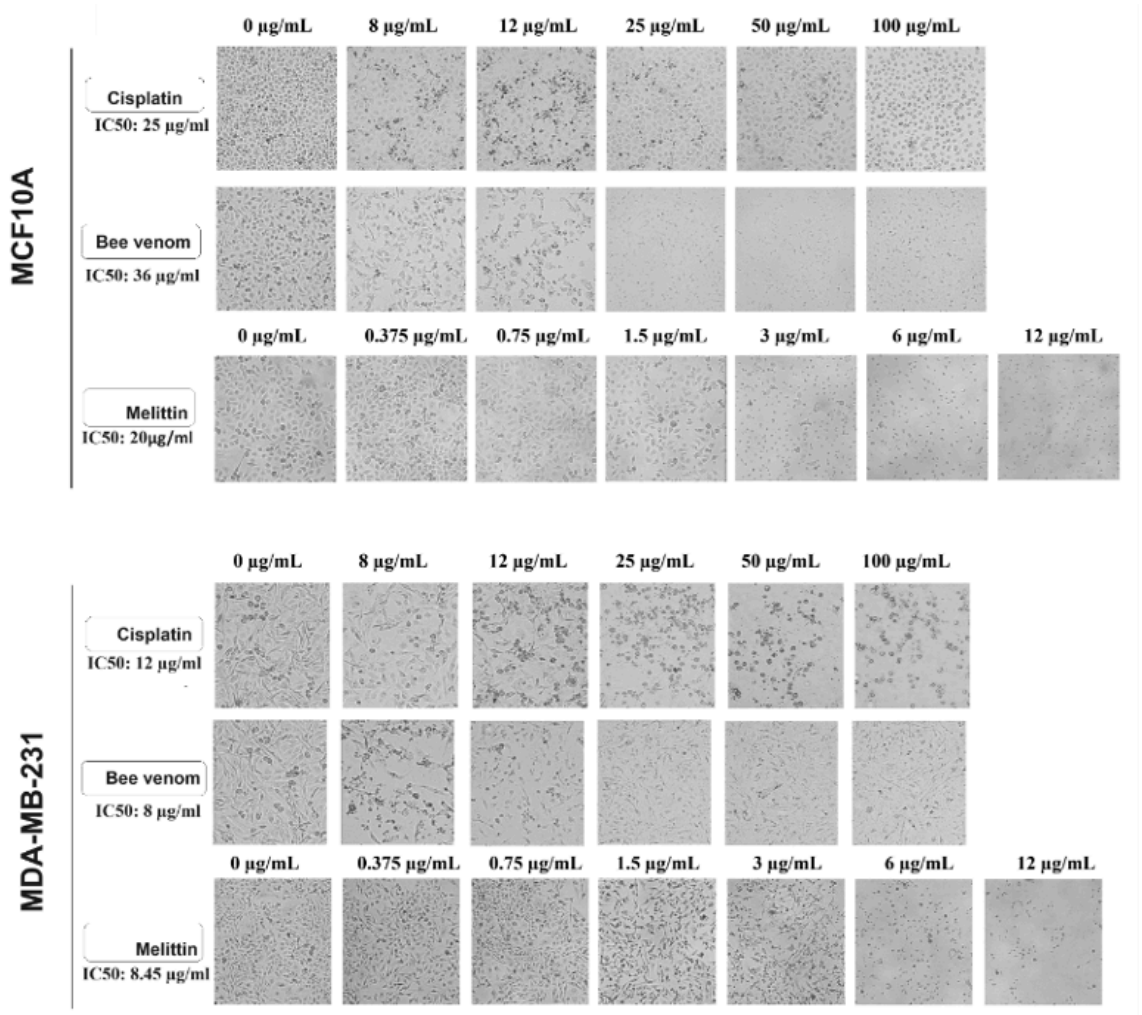

Figure 3. Cytotoxicity profiles of MCF10A and MDA-MB-231 cells after cisplatin, bee venom and melittin. Cisplatin and bee venom treated at 8 µg/ml, 12 µg/ml, 25 µg/ml, 50 µg/ml or 100 µg/ml for 24h, and melittin treated at 0.375 µg/ml, 0.75 µg/ml, 1.5 µg/ml, 3 µg/ml, 6 µg/ml or 12 µg/ml for 24h. IC50 values are given for each agent in the cells.

Wound healing experiments were performed after revealing the cytotoxic profiles of the cells after relevant treatments. The concentrations below IC50 values were used for wound healing. **Figure 2** shows the wound healing response of MDA-MB-231 cells against bee venom, melittin and cisplatin. Anti-wound healing profiles were defined after bee venom at 4 µg/ml, cisplatin at 4 µg/ml and melittin 0.5 µg/ml **(Figures 2 and 3).** Statistical comparisons suggest that incubation up to 24h induced more anti-healing than extended incubations **(Figure 3)**. The most significant doses for anti-healing were defined by the UNIANOVA test **(Supplementary 1)**. In MCF1A cells, wounds were still

unhealed after bee venom and cisplatin at 4 µg/ml for 24 h, but melittin at 1 µg/ml for 24h incubation was effective for anti-healing **(Figure 4 and 5)**. Detailed statistical comparisons by UNIANOVA are given in **Supplementary 2**.

	Bee Venom	**Melittin**	**Cisplatin**
Selective index for breast cancer	4.5	2.36	1

Table 1. Selective index (SI) values for each agent.

RNAs were isolated at the conditions that resulted in significant anti-wound healing compared to untreated control cells **(Table 2, Supplementary 1-2).** QPCR were performed after cDNA synthesis using RNA isolates. *KAI1/CD82* and *DRG1* expressions increased after bee venom but cisplatin only induced *KAI1/CD82* upregulation in MDA-MB-231 cells **(Figure 8B, C)**. *KAI1/CD82* has been shown to function in cancer prevention in particular angiogenesis, therefore considered as an anti-metastatic player [12][13].

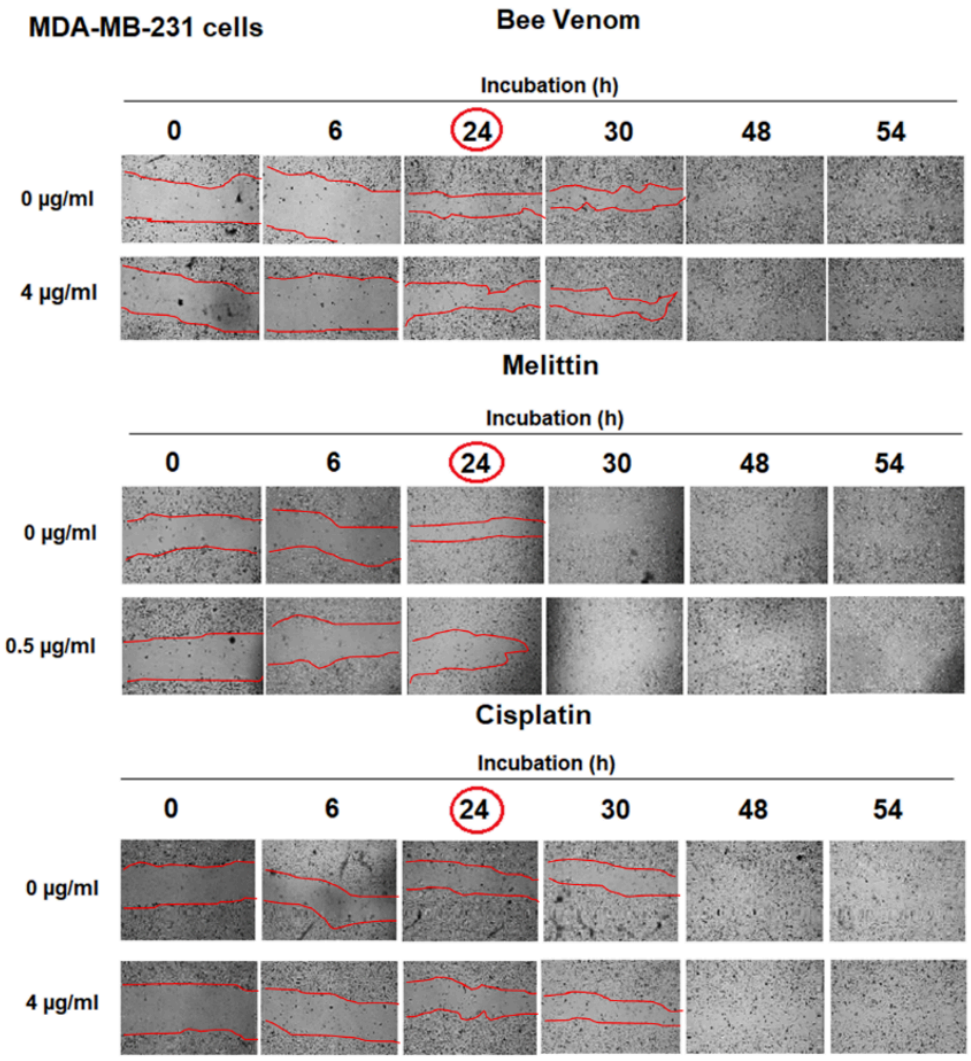

Figure 2. Representative wound healing profiles in MDA-MB-231 cells after bee venom, melittin or cisplatin treatments.

doi.org/10.32388/0Y7O4S

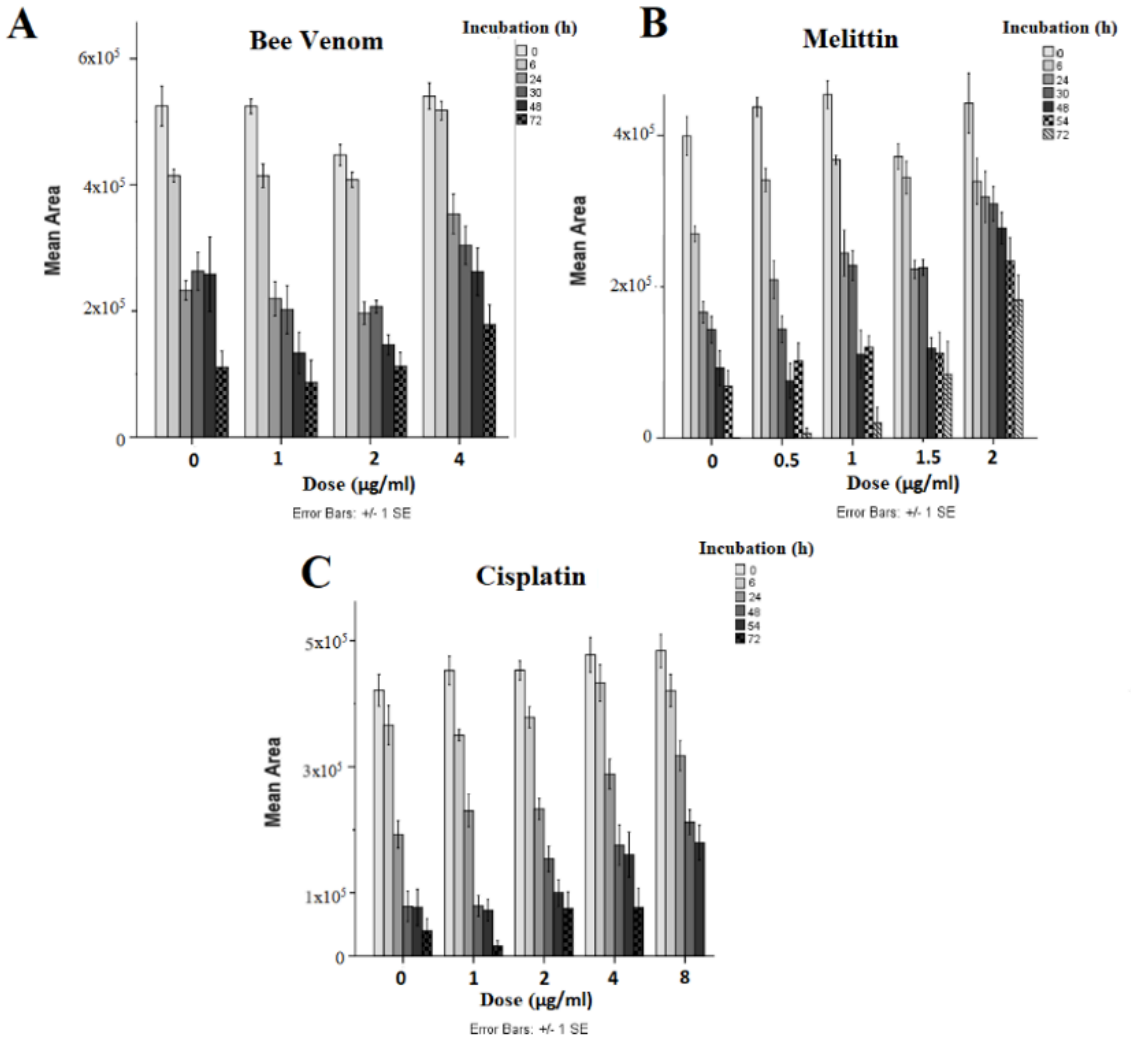

Figure 3. Wound healing area in MDA-MB-231 cells after bee venom (A), melittin (B) or cisplatin (C) treatments.

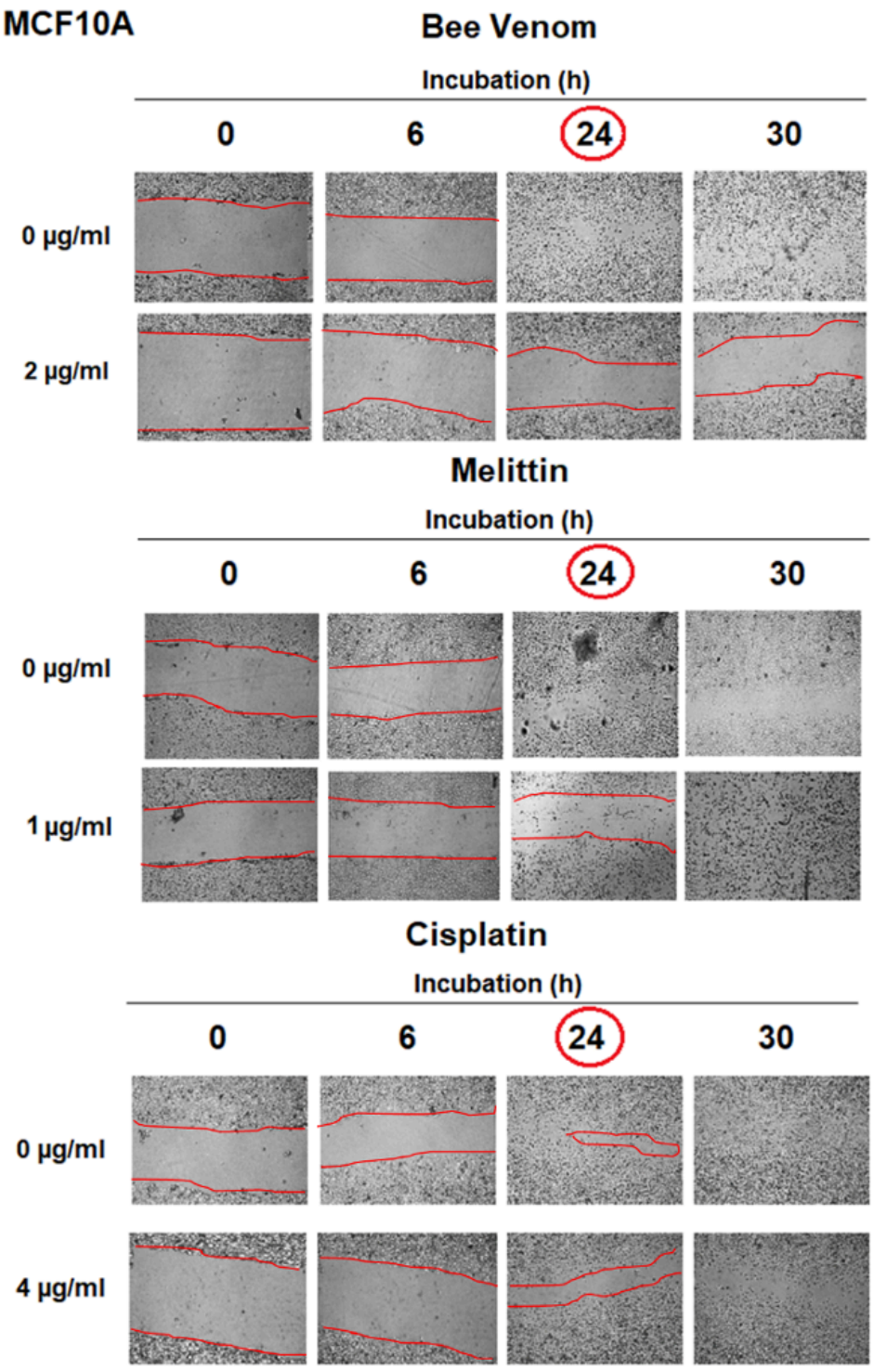

Figure 4. Representative wound healing profiles in MCF10A cells after bee venom, melittin or cisplatin treatments.

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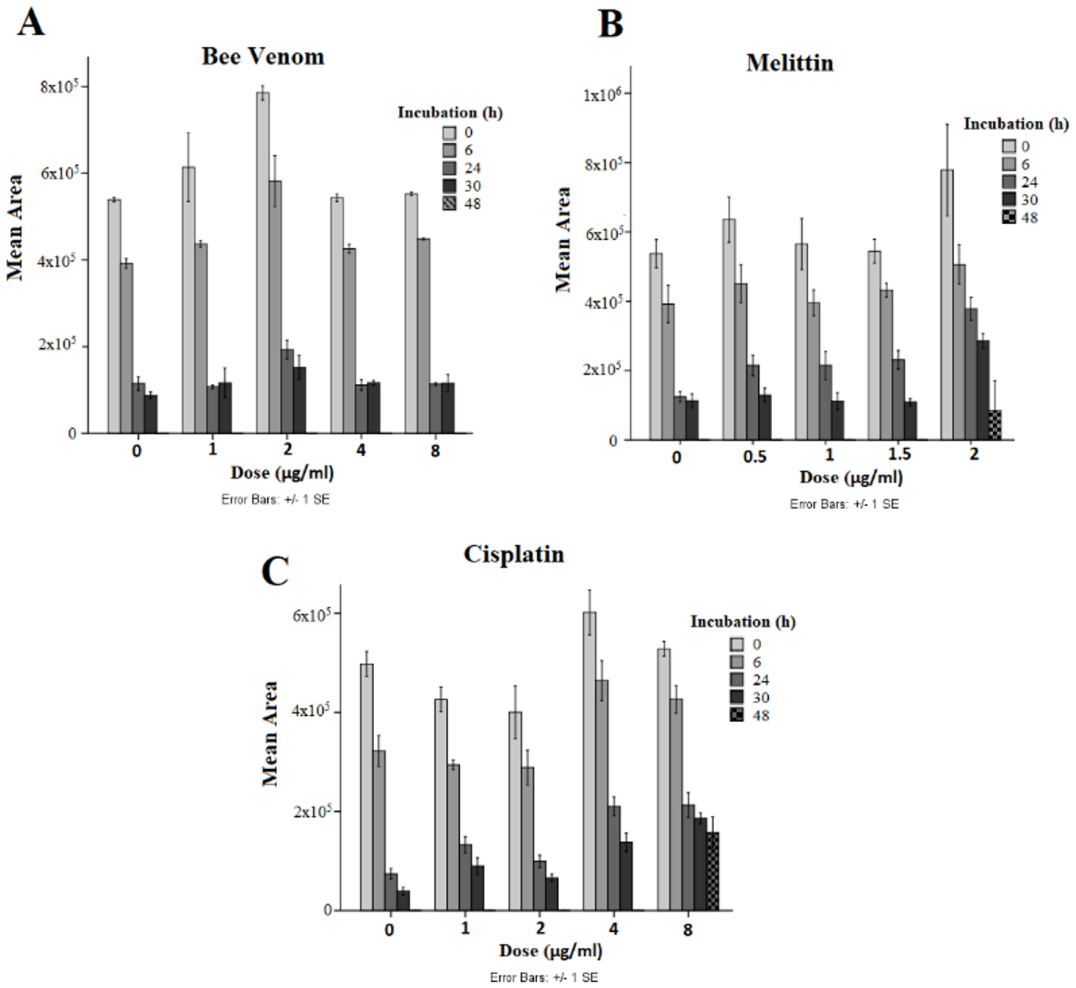

Figure 5. Wound healing area in MCF10A cells after bee venom (A), melittin (B) or cisplatin (C) treatments.

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Cell	The conditions for RNA isolations (with isolations from untreated control cells)		
	Bee Venom	**Melittin**	**Cisplatin**
MDA-MB-231	4 µg/ml 24 hours	0.5 µg/ml 24 hours	4 µg/ml 24 hours
MCF10A	2 µg/ml 24 hours	1 µg/ml 24 hours	4 µg/ml 24 hours

Table 2. The significant conditions for anti-metastatic profiles after treatments that used for RNA isolations.

BRMS1 was downregulated after bee venom whereas melittin treatment induced its upregulation **(Figure 6A)**. *EGFR* expression did not change after any of the agents **(Figure 6E)**, but *WNT7B* was downregulated after bee venom **(Figure 6D)**. In contrast, *EGFR* expression increased after both bee venom and melittin in MCF10A cells **(Figure 6J)**. *WNT7B* and *BRMS1* expressions were down after both cisplatin and melittin compared to untreated counterparts **(Figure 6F and I)**. Cisplatin also induced a decrease in *DRG1* expression which was upregulated after melittin **(Figure 6H)**. *CD82* and *BRMS1* were overexpressed after melittin in MCF10A cells **(Figure 6F and G)**. DRG1 protein regulates the functioning of CD82 by inhibiting the ATF3 transcription factor, which causes the inhibition of the *CD82* gene [14] suggesting that DRG1 is the positive regulator of CD82. CD82 and BRMS1 are the negative regulator of EGFR and WNT7B is a negative regulator of CD82 [14]. Dysregulation of *DRG1* was found to be related to breast cancer progression in patients [15], however its downregulation after knocking-out resulted in metastasis in MCF7 breast cancer cell line, but no significant effect on MDA-MB-231 cells [15]. In our study we found DRG1 was upregulated after bee venom and melittin.

Honeybee venom has been known to be effective on cancer cell death and considered as a promising medicine for cancer therapy [16]. This may function as a cytotoxic agent or an anti-metastatic agent in a range of cancers, such lung cancer [17]. Its anti-metastatic function on lung metastasis of

osteosarcoma was shown to be regulated by the action of melittin, the major peptide component of bee venom, that participated Wnt/β-catenin signaling [18] or Wnt/BMP associated pathway in gastric cancer preventing the transition of cells from epithelial stage to mesenchymal stage [19]. Melittin has been also found to inhibit cell migration in bladder cancer [20] and in liver cancer by the inhibition of the Rac-1 pathway [21]. Melittin may affect epigenetic modifications as it functions by the regulation of DNA demethylation on a non-coding RNA gene, *ADAMTS9-AS2*, in hepatocellular carcinoma [22]. Melittin itself induced cancer cell proliferation and invasion in melanoma by inhibiting PI3K/AKT/mTOR [9][23] and MAPK pathways as well as bee venom induced [23].

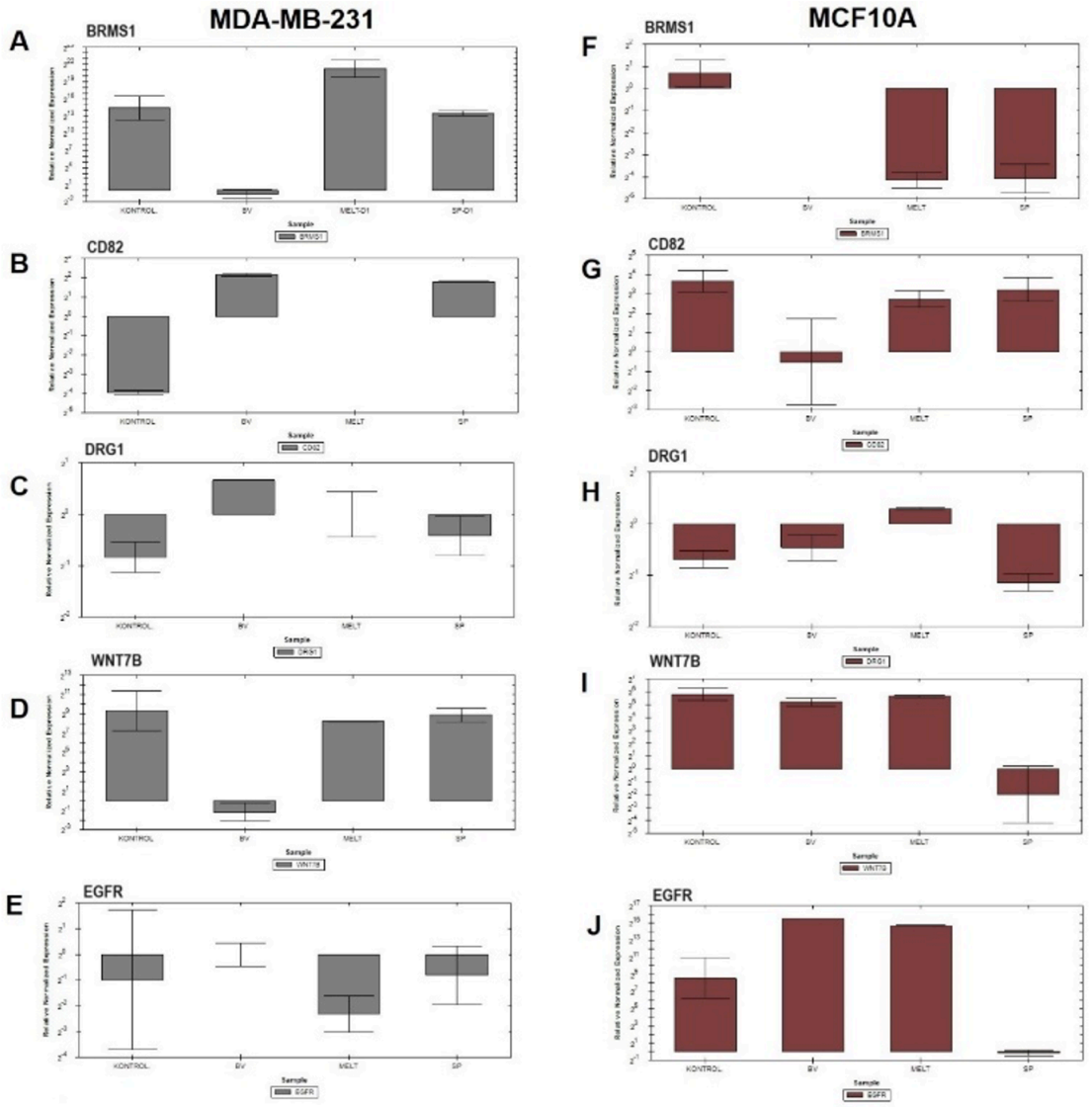

Figure 6. Anti metastatic (*BRMS1*, *DRG1* and *KAI1/CD82*) and pro-metastatic (*WNT7B* and *EGFR*) gene expressions after bee venom, melittin and cisplatin in MDA-MB-231 (A-E) and MCF10A (F-J).

doi.org/10.32388/0Y7O4S

MDA-MB-231 cells					
	Expression changes				
Agent	*BRMS1*	*CD82*	*DRG1*	*EGFR*	*WNT7B*
Bee venom	↓	↑	↑	_	↓
Melittin	↑	n.d.	↑	_	_
Cisplatin	_	↑	_	_	_
MCF10A cells					
	Expression changes				
Agent	*BRMS1*	*CD82*	*DRG1*	*EGFR*	*WNT7B*
Bee venom	n.d	_	_	↑	↓
Melittin	↓	↓	↑	↑	_
Cisplatin	↓	_	↓	↓	↓

Table 3. Summary for changes in gene expression (↑ upregulation, ↓ downregulation compared to untreated cells)

Epidermal growth (EGF) pathway is associated with the progression of a broad range of cancers, and it has been found to be upregulated in breast cancer cells. Bee venom was used to be coupled with nanoparticles including EGFR-targeting peptide to inhibit MEK-ERK pathway mediated by EGFR [24]. Melittin inhibited EGF-mediated cell migration and invasion by blocking PI3K/Akt/mTOR pathway in breast cancer cells [9]. Melittin was also designed to be constructed with a membrane-lytic immunotoxin to suppress EGF function [25]. Therefore, EGF and its receptor (EGFR) are two of the main targets for dealing with aberrant cell proliferation. MDA-MB-231 cells is a cell line that overexpresses EGF and has an activated function of *WNT7B* oncogene (https://www.atcc.org/products/htb-26). In this study, overexpressed two markers specific for this cell line were also investigated along with the anti-metastatic genes. *WNT7B* expression decreased after bee venom in both cells (MDA-MB-231 and MCF10A) suggesting that bee venom is the most effective agent to suppress specific oncogenic activity in breast cancer. However, neither bee venom nor melittin affected EGFR expression as well as cisplatin. MCF10 cells derived from fibrocystic tissue of breast showed increased expression of EGFR after bee venom and melittin whereas it was downregulated by cisplatin treatment.

The effects of melittin and bee venom have been previously studied in breast cancer [10][26][27][28]. The mechanisms of anti-breast cancer action were shown by inhibiting either growth receptor activation [28], SDF-1α/CXCR4 Signaling Pathway [27], or NF-κB and AP-1-dependent MMP-9 expression [29] whereas by upregulating Mfn1 and Drp1 expression [26]. However the expression profiles of *BRMS1*, *DRG1* and *CD82* genes have not been elucidated in terms of anti-metastatic function of melittin and bee venom in breast cancer. These genes/proteins examined in this study are associated with each other by negative or positive regulation feedback[14] **(Figure 7, graphical abstract)**.

Conclusions

This study investigated whether the anti-metastatic effect of bee venom and melittin on metastatic breast cancer cells was regulated by upregulation of anti-metastatic *BRMS1*, *DRG1* and *CD82/KAI1)* genes and downregulation of pro-metastatic *WNT7* and *EGFR* genes. Bee venom induced overexpression of *CD82/KAI1* and *DRG1* while melittin induced overexpression of *DRG1* and *BRMS1* in MDA-MB-231 cells. But EGFR was upregulated after treatments. This suggests that bee venom or melittin may be applied with an *EGFR* inhibitor to enhance the selective activity.

Experimental Section

Cell culture

The metastatic breast cancer cell line, MDA-MB-231 (ATCC, CRM-HTB-26), and epithelial breast cells, MCF-10A (ATCC code CRL-10317), were cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). This should be noted that MDA-MB-231 cells were cultured in media including 20% right after thawing. All cells were cultured at 37°C with 5% CO_2 humidification. Passage 10 and passage 14 were used for MDA-MB-231 and MCF10A cells respectively.

Bee venom, melittin and cisplatin treatments

Bee venom was obtained and prepared as previously [30]. For MTT, cells were treated with 8, 12, 25, 50 or 100 µg/mL of final concentration of bee venom in the media for 24 h. For wound healing experiments, final concentrations of bee venom were used as 1, 2, 4 and 8 µg/mL in MCF10A cells

while a group of cells was left untreated. However, in MDA-MB-231 cells, 1, 2 and 4 µg/mL were used, because 8 µg/mL was the IC50 value. Cells were incubated for 6, 24, 30, 48, 54, 72 and 96 hours during wound healing. For MTT after melittin (Sigma-Aldrich, U.S., Cat. No. M2272) cells were treated with 0.375, 0.75, 1.5, 3, 6 and 12 µg/ml of final concentration of melittin for 24 h. Counterpart cells were left untreated. For wound healing, cells were treated with melittin as 0.5, 1, 1.5 or 2 µg/ml for 6, 24, 30, 48, 54, 72 and 96 hours. Cisplatin (Koçak Pharma, TURKIYE, Cat. No. 19111614) was treated similar to bee venom as MTT concentrations (8, 12, 25, 50 or 100 µg/mL for 24 h) and wound healing (1, 2, 4 and 8 µg/mL for 6, 24, 30, 48, 54, 72 and 96 hours).

MTT cytotoxicity assay

Cytotoxicity profiles of MDA-MB-231 and MCF10A cells were measured by MTT assay. The protocol briefly is as follows: 1) media was removed from the cells in 96-well plate after 24 hours of treatment, 2) media including 20% (v/v) MTT dye (Sigma, Cat. No. M2128) was added to each well, and incubated for 2.5 hours at 37°C, 3) After incubation, media with MTT was removed, 4) 150 microliters of DMSO was added to each well for 1 hour incubation in the dark on a shaker to visualize color development from purple to yellow. (Purple indicates live, yellow indicates dead cells, and the degree of colors are associated with cell viability), 5) Formazan crystals were checked under the inverted microscope (Zeiss, Germany) whether these were dissolved in the wells, 6) Absorbances at 570 nm were read using a spectrophotometer. The percentages of cell viability were calculated from absorbances. The absorbances measured for untreated control cells were set as 100% viable, and viability of treated cells was proportionally calculated. IC50 values were calculated as previously described [31][32]. Selective indexes for each agent were calculated by the formula: normal cells IC50 / cancer cells IC50.

Wound healing assay and analyses of wound area

MDA-MB-231 and MCF10-A cells were seeded into a 6-well plate with 200.000 cells in each plate, with a total of 2 ml of media, and incubated in a 37°C incubator humidified with 5% CO_2 until they reach at full confluency. A one-way wound was created on the cells covering the plate surface using a sterile 200 µl pipette tip. After wound formations, photos were captured by microscope (Axiovert, Zeiss) followed by treatment with agents. Treated cells were observed after 6, 24, 30, 48, 54, 72 and 96 hours until at least a wound (in any 6-wells) was healed at any concentrations for the treatments.

Wound areas were measured using the select tool of ImageJ software **(Figure 8)**, and the calculated areas were statistically compared by SPSS software.

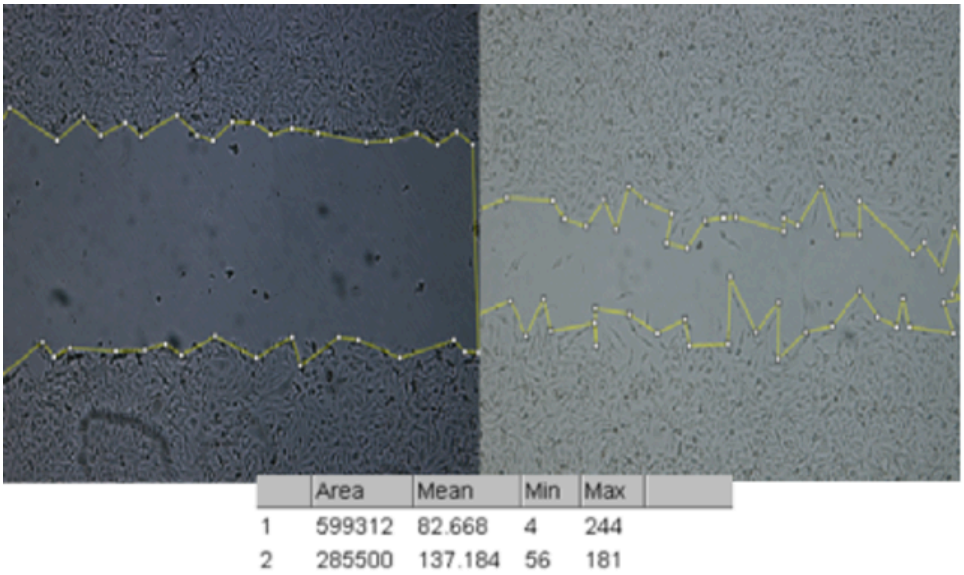

Figure 8. Representative image for wound area analyses by Image J software. Left and right panels represent wide and narrow wounds, respectively.

RNA isolation and cDNA conversion

Total RNA isolation was performed to determine the expression level of the genes using RNA isolation kit (Catalog number MG-RNA-01) from HIBRIGEN Biotechnology (Kocaeli, TURKIYE). The protocol steps are as follows: 1) sample digestion and homogenization by isothiocyanate (for protection from RNases). 2) Ethanol addition to the sample right after homogenization. 3) Samples transfer to filtered tubes for RNA attachment. 4) Washing the samples, and 5) Collection of pure total RNA with DEPC-treated water, 6) Concentration of purity measurement of the isolated RNA using NanoDrop, and 7) sample storage at -80°C until use. RNA samples were then converted to cDNA using cDNA Synthesis Kit (Catalog number C03-01-05) from A.B.T. ® Laboratory Industry (Ankara, TURKIYE). The cDNA kit includes a reverse transcription reaction including reaction buffer (10X), dNTP mix (2.5 mM each), random hexamer (50µM), reverse transcriptase (200 U/µl), RNase inhibitor, RNase free water and RNA sample. The reaction conditions were 10 min at 25°C, 120 min at 37°C and 5 min at 85°C for one cycle. After conversion, cDNA samples were kept at 4°C until use.

Primer design and RT-QPCR

Primers were designed using the Primer3 online tool, with approximately 20 bases and annealing temperature around 59°-60°C **(Figure 9)**. Real-time polymerase chain reaction was performed from cDNA samples to examine the expression levels of the determined genes. qPCR was performed using the Eva-Green Master Mix kit (with ROX) (A.B.T. ®, Catalog number Q02-02-02). Components and PCR cycles were prepared in accordance with the protocol specified in the kit. The reactions were run by CFX96 qPCR instrument (BioRad).

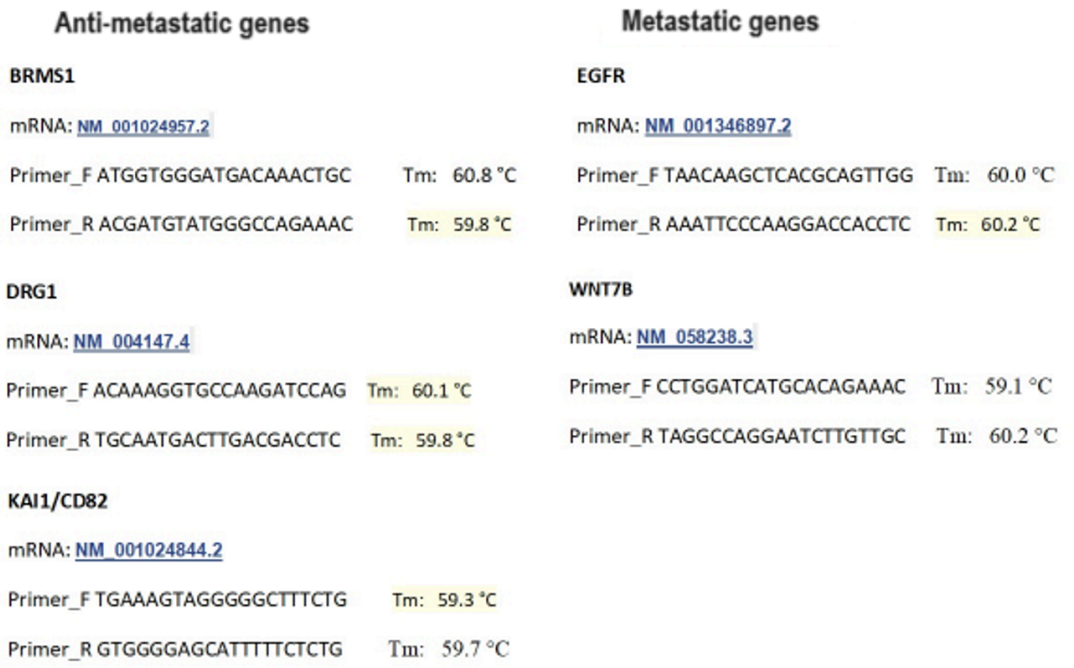

Figure 9. Primers used in the study.

Statistical analyses

Wound area before and after treatments was calculated by ImageJ program followed by statistical comparison by UNIANOVA test of SPSS program (Version 13). Experiments were performed as three independent repeats, and *p* values less than 0.05 were considered as significant.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

Statements and Declarations

Acknowledgements

This study was granted by Karadeniz Technical University (Project no: FBA-2021-9803).

Author Contribution Statement

NSS performed wound healing, RNA isolation, cDNA conversion and QPCR, ST performed MTT assays, SK provided bee venom and performed its analysis previously, SCU designed the study, was funded, wrote the manuscript.

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Declarations

Funding: This study was granted by Karadeniz Technical University (Project no: FBA-2021-9803).

Potential competing interests: No potential competing interests to declare.