Cancer Treatment and Research Communications 25 (2020) 100242

Contents lists available at ScienceDirect

Cancer Treatment and Research Communications

journal homepage: www.sciencedirect.com/journal/cancer-treatment-and-research-communications

Emerging therapeutic modality enhancing the efficiency of chemotherapeutic agents against head and neck squamous cell carcinoma cell lines

Mohammed E. Grawish [a,b,*], Mohamed I. Mourad [c], Doaa AM Esmaeil [c], Rehab A Ahmed [d], Islam Mohamed Ateia [e], Eman Hany [a], Mazen Tharwat Abou Elkhier [a]

[a] Department of Oral Biology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt
[b] Department of Oral Biology, Faculty of Oral and Dental Medicine, Delta University for Science and Technology, Mansoura, Egypt
[c] Department of Oral Pathology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt
[d] Department of General Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[e] Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mansoura University, Mansoura, Egypt

ARTICLE INFO

Keywords:
Head and neck squamous cell carcinoma
Bee venom
Cisplatin
Chemotherapy
Apitoxin

ABSTRACT

The current work aimed to evaluate bee venom (BV) cytotoxic effect and its synergistic action when combined with cisplatin (CIS) against four types of head and neck squamous cell carcinoma (HNSCC) cell lines. 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability, reverse transcription-polymerase chain reaction (RT-PCR) for expression of BCL2 associated X (BAX), B-cell lymphoma 2 (BCL2) and epidermal growth factor receptor (EGFR) genes and, flow cytometry for cell cycle analysis were performed. MTT assay revealed that BV caused an approximately 50% cell death for UMSCC12, UMSCC29, UMSCC38 and, UMSCC47 cell lines after 72 hr with 54.809 μg/ml, 61.287 μg/ml, 71.328 μg/ml and, 61.045 μg/ml, respectively. RT-PCR demonstrated a significant up-regulation of BAX gene and a significant down-regulation of BCL2 and EGFR genes among single or combined treatments with CIS and BV as compared to vehicle-treated. The cell lines treated with both BV and CIS showed marked elevation of BAX and a notable drop of BCL2 and EGFR expressions than single-treated groups. Cell cycle analysis *via* flow cytometry revealed significantly increased cells in the G2/M phase in single or combined-treated cell lines with CIS and BV when compared with vehicle-treated. Moreover, a significant decrease in cells in S phases among all single and combined treatments when matched with vehicle-treated. Briefly, the findings of the present study suggest that BV can exert an anti-cancer effect on HNSCC and may have the potentiality for potentiation of CIS cytotoxic effects and reduction of its adverse effects.

Introduction

Head and neck squamous cell carcinoma (HNSCC) involves heterogeneous malignancies that arise from the squamous epithelium of the pharynx, larynx, and oral cavity [1]. The prognosis of HNSCC is often dismal, with substantial room for improvement over current therapy [2]. Numerous strategies for the treatment of HNSCC are readily available such as chemotherapy, surgery, immunotherapy, and radiation therapy [3]. Of the major factors in the failure of cancer treatment is the partial restriction of existing chemotherapeutic agents, as cisplatin (CIS), due to their adverse undesired toxic effects. CIS is an ancient, widely used, and powerful drug for the treatment of most cancers. It produces DNA adduct that leads to the initiation of cancer cells' apoptosis. Toxic side effects of CIS include hemolytic anemia, bone marrow suppression, nephrotoxicity, and neurotoxicity [4,5].

These unwanted effects happen because several cytotoxic drugs act on normal cells as well as cancer cells. Fast-growing cells are the most affected ones likely as cells that make up hair, skin, digestive tract, and blood. Other cells, like those in the nervous system and lungs, kidneys, heart, and liver organs can also be affected. Tumor therapy efficacy could be improved by the management of side effects and good prophylaxis [6]. Attention for the chemotherapeutic drug toxicity has principally contributed to the progressed use of natural and herbal products for the treatment of cancer. A combination of conventional

* Corresponding author.
E-mail address: grawish2005@yahoo.com (M.E. Grawish).

https://doi.org/10.1016/j.ctarc.2020.100242

Available online 21 November 2020

anticancer drugs with natural products is supposed to enhance the efficiency of cancer treatment due to their synergistic effects as well as it could lessen potentially adverse effects of chemotherapy. Nevertheless, such claimed benefits of natural products have not been established scientifically [7].

Lately, a combination of natural compounds possessing absorption improving activities with chemotherapeutic agents has extended great awareness for enhancement of the bioavailability of poorly bioavailable drugs. The enhancing effect of natural compounds such as quercetin, piperine, glycyrrhizin, sinomenine, genistein, lysergols, niazeridine, and naringin were evaluated along with modern medicines [8]. Apitoxin or bee venom (BV) contains a mixture of complex and efficient elements to shield bees from broad and variable predators. It has numerous peptides including mast cell degranulating peptide, melittin, adolapamin, and apamin. Also, it contains biologically active amines, non-peptide components, and enzymes [9]. BV had been utilized for the treatment of some inflammatory conditions like the relief of pain in oriental medicine and rheumatoid arthritis. Moreover, it has been used for cancer treatment modulation [10]. Up to date studies examined the anticancer effects of BV and CIS combinations against HNSCC cell lines were rare. We assumed that this treatment modality can accomplish dose reduction and synergistic effects. Hence, this work was carried out to evaluate the potential therapeutic effects of BV when utilized singly or in combination with CIS on the HNSCC cell lines.

Material and methods

Cytotoxic agents

Apitoxin was purchased in powder form (ApiHealth NZ Ltd, New Zealand) and stored at 20 °C in a dry dark place to protect it from moisture and light. The powder was dissolved within phosphate-buffered saline (PBS) and passed through a 0.2 μm filter to obtain a homogenous and sterile solution. The main stock solution was prepared with 1 mg BV/ 1 ml PBS and the required concentrations were achieved by dilution of the main solution and were prepared freshly for each assay. CIS (Sobhan Oncology Company of Iran) was purchased with 50 mg/ml concentration and stored for 2 days in a 4 °C dark situation. CIS efficiency was determined through its physical and chemical stabilities. The physical stability was determined by visual inspection in normal light as there was no change in color or any turbidity. The chemical one was measured utilizing a stability-indicating high-performance liquid chromatography.

Half maximal inhibitory concentration (IC50) for cytotoxic agents

Concentrations of CIS and BV that inhibit 50% of cells (IC50) were calculated by the use of XLfit5 software (IDBS) and expressed in μg/mL at 95% confidence intervals.

The combination index (CI)

Stock solutions of IC50 of CIS and BV were prepared. Combinations of IC50 fractions of the two reagents were applied as following (1/2:1/2, 1/3:2/3, 2/3:1/3, 1/5:4/5, 4/5:1/5, 1/6:5/6, and 5/6:1/6 from IC50 of each compound). The dose-effect of CIS and BV combinations was done using Chou-Talalay method. The type of interactions between the two ingredients was identified by the combination index (CI) using compusyn software with CI < 1 point to synergism between the tested components [11].

Cell culture

Four HNSCC cell lines were utilized: (UMSCC12, UMSCC29, UMSCC38, and UMSCC47). They were purchased from the American Type Culture Collection (ATCC, NY, USA). These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 10% (v/v) fetal bovine serum, 100 u/ml Penicillin and 100 mg/ml streptomycin (GIBCO, New York, USA) and 2% (v/v) L-glutamine (Invitrogen, New York, USA). Mycoplasma testing was done by Myco Alert Mycoplasma Testing Kit (Lonza, Rockland ME).

Table. 1
Primer sequences used in qPCR.

Gene	Forward primer(5′–3′)	Reverse primer (5′–3′)
Bax	CCTGTGCACCAAGGTGCCGGAACT	CCACCCTGGTCTTGGATCCAGCCC
BCL2	AGGAAGTGAACATTTCGGTGAC	GCTCAGTTCCAGGACCAGGC
EGFR	TGCCCATGAGAAATTTACAGG	ATGTTGCTGAGAAAGTCACTGC
GAPDH	GAG AAG GCT GGG GCT CAT TT	AGT GAT GGC ATG GAC TGT GG

Cell viability and MTT assay

Tumor cells were seeded in 96-well plates after their trypsinization with 0.25% (v/v) trypsin-EDTA (Gibco-Invitrogen) and cultured overnight to fully adhere to the plate (1×10^5/ well, 100μl/ well). After that, they were treated with different concentrations (ranging from 0 to 200 μg/ml) of the CIS and BV chemotherapeutic agents and further incubated for 72 hr. Then, a 10% solution of 5 mg/ml 3-(4,5-dimethylthiazol-2-Yl)−2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well and incubated at 37 °C for 2 hr. Formazan was dissolved by the addition of dimethylsulfoxide (DMSO, Sigma) to each well. The absorbance was measured at 570 nm wavelength by a spectrophotometer (Milton Roy-Spectronic 2I D- America). The viability percent was considered as follows:

Viability% = (optical density of experimental group/ optical density of control group) x100 [12].

Experimental design

The utilized cell lines (UMSCC12, UMSCC29, UMSCC38, and UMSCC47) were categorized into vehicle control (DMSO-treated), CIS treated and BV treated with doses equal to their IC50. Combined treatments were prepared as a non-constant ratio from fractions of IC50 of the two cytotoxic agents. All treatments were applied on UMSCC12, UMSCC29, UMSCC38, and UMSCC47 cell lines at 70– 80% confluence, and the cells were incubated in a CO_2 incubator for 24 hr. Then, cells were collected by trypsinization and immediately managed for RT-PCR and flow cytometric analysis.

Cell cycle analysis

After trypsinization, UMSCC12, UMSCC29, UMSCC38, and UMSCC47 cells were centrifuged at 4500 rpm for 5 min, washed twice, resuspended in warm PBS, fixed by ice-cold absolute ethanol, and then incubated at − 20 °C for 24 hr. After twice PBS washes, cells were resuspended in propidium iodide (PI) solution containing 100 μl (0.02 mg/ml) PI, 50 μl (0.2 mg/ml) RNase A, and 0.1% v/v Triton X-100 in PBS, incubated in darkness for 30–60 min at room temperature and then analyzed using Attune flow cytometer (Applied Bio-system, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from UMSCC12, UMSCC29, UMSCC38, and UMSCC47 cells using Gene JET RNA Purification Kit (Thermo Scientific, USA). RNA concentration and purity were determined by nanodrop (Q5000, Quawell, USA) and 1% gel electrophoresis. 5 μg of RNA was reverse transcribed using Quantiscript reverse transcriptase. The produced cDNA was used as a template to determine the relative expression of BCL2 associated X (BAX), B-cell lymphoma 2 (BCL2), Epidermal growth factor receptor (EGFR) genes using Step One Plus real-time PCR

Table. 2
IC50 for CIS and BV against the 4 investigated HNSCC cell lines.

Variables	UMSCC12	UMSCC29	UMSCC38	UMSCC47
CIS	38.539 μg/ml	20.879 μg/ml	37.339 μg/ml	28.159 μg/ml
BV	54.809 μg/ml	61.287 μg/ml	71.328 μg/ml	61.045 μg/ml

system (Applied Biosystem, USA) and specific primers (Table1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The selection criteria of GAPDH as an endogenous control were that it passes through all steps of analysis identically manner to the genes to be quantified and its constant transcription in epithelial cell types. All assays were performed in triplicate and the expression was calculated based on $2^{-\Delta\Delta Ct}$ method [13,14].

Statistical analysis

Statistical Package for the Social Sciences, version 21.0 (SPSS, IBM Corp., Armonk, NY, United States) was used for statistical evaluation of the tabulated raw data. Homogeneity of variance was tested using Levene's test and normality of the distribution was evaluated using the Shapiro–Wilk statistical test, One-way ANOVA was used to determine significant differences between the different groups, followed by LSD post hoc statistical test. *P*-value < 0.05 was considered significant.

Results

Table 2 representing IC50 values of CIS and BV which inhibit the proliferative response for UMSCC12, UMSCC29, UMSCC38, and UMSCC47 by 50%.

Effect of CIS and BV on the viability of cell lines

MTT assay displayed dose-dependent and significant anti-proliferative activity for CIS and BV on UMSCC12, UMSCC29, UMSCC38, and UMSCC47 when used in combinations or independently when compared to vehicle-treated cells. All applied non-constant combinations on cell lines caused significantly higher cytotoxicity on UMSCC12, UMSCC29, UMSCC38, and UM-SCC47 than singly treated (*P* = 000). Furthermore, the combination of 1/5 CIS: 4/5 BV demonstrated the highest inhibition for the viability of UMSCC12 and UMSCC38. Meanwhile, 1/4 CIS: 3/4 BV led to the highest inhibition for the viability of UMSCC29. The great reduction of UMSCC47 viability was found with 1/3CIS: 2/3 BV (Table 3).

Synergistic effect of BV and CIS against cell lines

The combination index (CI) values for all examined CIS and BV combinations were ˂ 1 (Table 4). Hence, the interaction between CIS and BV indicated strong synergistic effects in inhibition of UMSCC12, UMSCC29, UMSCC38, and UMSCC47 growth.

Effect of BV and CIS on the expression of BAX, BCL2, and EGFR genes

Cell lines treated singly with BV or CIS (IC50 concentrations) exhibited notable elevation of BAX and marked decrease of BCL2 and EGFR gene expressions. The cell lines treated with both BV and CIS showed marked elevation of BAX and a notable drop of BCL2 and EGFR expressions than single-treated groups. Among the different combinations (IC50 fractions), 1/5 CIS: 4/5 BV exhibited the highest BAX expression by UMSCC12 and UMSCC38. Meanwhile, the highest up-regulation of BAX by UMSCC29 was found with 1/4 CIS: 3/4 BV. Nevertheless, UMSCC47 revealed the highest BAX expression with 1/3CIS: 2/3BV. Regarding the regulation of BCL2 and EGFR genes, 1/5 CIS: 4/5 BV showed the maximal drop of their expressions by UMSCC12 and UMSCC47. Meanwhile, the lowest expression of these genes by UMSCC29 and UMSCC38 was detected with 1/4 CIS: 3/4 BV. Statistically, a significant down-regulation in expression levels of EGFR and BCL2 genes and a significant up-regulation in expression levels of BAX gene in CIS and BV combined- treated cell lines and CIS or BV single treated when compared with vehicle-treated UMSCC12, UMSCC29, UMSCC38, and UMSCC47. Also, cell lines treated with both BV and CIS

Table. 4
Statistical results of one-way ANOVA and LSD post-hoc tests for non-constant combinations of IC50 fractions (μg/ml) of CIS and BV against the 4 utilized HNSCC cell lines.

Combinations	Mean ± SD UMSCC12	UMSCC29	UMSCC38	UMSCC47
IC50/2	85.32±0.07	83.12±0.02	87.82±0.02	86.41±0.02
1/3:2/3	83.77±0.18	86.74±0.03	85.44±0.03	88.33±0.02
2/3:1/3	84.15±0.05	79.33±0.04	82.35±0.03	85.37±0.02
1/4:3/4	81.16±0.02	87.23±0.04	86.32±0.02	87.48±0.02
3/4:1/4	85.82±0.08	83.70±0.03	83.72±0.03	85.61±0.02
1/5:4/5	88.85±0.04	84.81±0.01	89.21±0.02	87.80±0.03
4/5:1/5	87.15±0.09	81.89±0.02	84.42±0.02	84.65±0.03
1/6:5/6	86.15±0.02	82.51±0.01	85.22±0.02	86.39±0.02
5/6:1/6	83.70±0.05	80.72±0.01	81.32±0.02	85.91±0.02
One-way ANOVA (F; *P* value)	(3466.839; 0.000)	(34,706.230; 0.000)	(37,505.055; 0.000)	(9840.991; 0.000)

Table. 3
Combination index data for non-constant combinations of IC50 fractions (μg/ml) of CIS and BV against the 4 utilized HNSCC cell lines.

Cell lines		Non constant combinations of IC50 fractions for CIS and BV (CIS:BV) IC50/2	1/3:2/3	2/3:1/3	1/4:3/4	3/4:1/4	1/5:4/5	4/5:1/5	1/6:5/6	5/6:1/6
	CIS dose	19.27	12.85	25.69	9.64	28.91	7.71	30.81	6.42	32.12
UMSCC12	**BV dose**	27.4	36.54	18.27	41.11	13.70	43.84	10.96	45.68	9.14
	% of cell death	85.41	83.97	84.18	81.17	85.89	**88.82**	87.19	86.17	83,72
	CI	0.30	0.359	0.343	0.372	0.316	0.261	0.285	0.294	0.352
	CIS dose	10.44	6.96	13.92	5.22	15.66	4.18	16.72	3.48	17.40
UMSCC29	**BV dose**	30.65	20.43	40.86	15.32	45.96	12.26	49.03	10.22	51.08
	% of cell death	83.12	86.77	79.33	**87.20**	83.70	84.83	81.93	82.54	80.72
	CI	0.32	0.361	0.302	0.264	0.298	0.289	0.301	0.296	0.322
	CIS dose	18.76	12.45	24.9	9.34	28.01	7.47	29.87	6.22	31.12
UMSCC38	**BV dose**	35.67	23.78	47.55	17.58	52.57	14.27	57.06	11.89	59.44
	% of cell death	87.82	85.42	82.36	86.32	83.72	**89.21**	84.45	85.22	81.34
	CI	0.248	0.299	0.316	0.263	0.311	0.203	0.271	0.318	0.339
	CIS dose	14.08	9.37	18.77	7.04	21.12	5.63	22.53	4.69	23.47
UMSCC47	**BV dose**	30.53	20.35	40.7	15.26	45.79	12.21	48.84	10.18	50.90
	% of cell death	86.42	**88.35**	85.39	87.52	85.62	87.81	84.69	86.39	85.92
	CI	0.279	0.213	0.323	0.319	0.315	0.283	0.307	0.278	0.291

Table. 5
Statistical results of one-way ANOVA test for CIS, BV and their combinations on the expression of BAX, BCL2 and EGFR genes in 4 utilized HNSCC cell lines.

Combinations	Mean ± SD											
		UMSCC12			UMSCC29			UMSCC38			UMSCC47	
	BAX	BCL2	EGFR	BAX	BCL2	EGFR	BAX	BCL2	EGFR	BAX	BCL2	EGFR
Control	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02	1.01±0.02
CIS treated	4.29±0.01	0.28±0.01	0.42±0.00	5.19±0.00	0.30±0.01	0.40±0.00	4.29±0.00	0.27±0.00	0.42±0.00	5.14±0.05	0.26±0.00	0.41±0.00
BV treated	3.77±0.01	0.41±0.00	0.54±0.00	4.78±0.00	0.41±0.00	0.48±0.00	3.78±0.00	0.41±0.00	0.54±0.00	3.78±0.00	0.42±0.00	0.34±0.00
IC50/2	7.66±0.01	0.14±0.00	0.07±0.00	8.14±0.00	0.08±0.00	0.04±0.00	9.88±0.00	0.04±0.00	0.01±0.00	10.20±0.00	0.08±0.00	0.06±0.00
1/3:2/3	6.60±0.01	0.24±0.01	0.12±0.00	9.82±0.00	0.02±0.00	0.03±0.00	8.20±0.00	0.08±0.00	0.05±0.00	14.80±0.00	0.02±0.00	0.02±0.00
2/3:1/3	7.45±0.01	0.17±0.01	0.08±0.00	6.63±0.00	0.24±0.00	0.11±0.00	6.63±0.00	0.24±0.00	0.14±0.00	5.63±0.00	0.21±0.00	0.21±0.00
1/4:3/4	5.97±0.00	0.30±0.01	0.27±0.00	10.87±0.00	0.01±0.00	0.02±0.00	8.73±0.00	0.01±0.00	0.01±0.00	12.30±0.00	0.05±0.00	0.04±0.00
3/4:1/4	8.24±0.00	0.08±0.01	0.05±0.00	6.13±0.00	0.24±0.00	0.18±0.00	6.16±0.00	0.25±0.00	0.12±0.00	6.16±0.00	0.24±0.00	0.18±0.00
1/5:4/5	9.87±0.00	0.01±0.00	0.01±0.00	8.36±0.00	0.05±0.00	0.03±0.00	11.87±0.00	0.05±0.00	0.03±0.00	13.31±0.00	0.01±0.00	0.01±0.00
4/5:1/5	9.31±0.05	0.04±0.00	0.02±0.00	5.97±0.00	0.30±0.00	0.26±0.00	7.41±0.00	0.16±0.01	0.08±0.00	4.98±0.00	0.27±0.00	0.27±0.00
1/6:5/6	8.37±0.01	0.05±0.00	0.04±0.00	7.87±0.01	0.13±0.00	0.06±0.00	7.87±0.01	0.14±0.00	0.24±0.01	8.66±0.00	0.14±0.00	0.12±0.02
5/6:1/6	6.16±0.01	0.26±0.00	0.19±0.01	7.45±0.00	0.16±0.00	0.08±0.00	5.97±0.00	0.30±0.00	0.26±0.00	7.45±0.00	0.16±0.00	0.14±0.00
One-way ANOVA (F; *P* value)	(81,266.320 ; 0.000)	(3125.854; 0.000)	(4292.185; 0.000)	(385,911.08 2; 0.000)	(4641.277; 0.000)	(7035.973; 0.000)	(601,212.64 7; 0.000)	(4740.364; 0.000)	(38.193; 0.000)	(273,452.95 9; 0.000)	(5953.741; 0.000)	(1724.731; 0.000)

showed significant difference as compared to single treated ($P < 0.001$) (**Table 5**, **Fig. 1**).

Effect of BV and CIS on cell cycle

The influence of combined or single treatment by BV and CIS on UMSCC12, UMSCC29, UMSCC38, and UMSCC47 cell cycle exhibited a significant elevation in numbers of UMSCC12, UMSCC29, UMSCC38, and UMSCC47 cells in G2/ M phase in combined or single treated cell lines, when compared with vehicle-treated ($P < 0.001$). The highest G2/ M phase numbers were observed in the following combinations: 1/5 CIS: 4/5 BV for UMSCC12 and UMSCC38, 1/4 CIS: 3/4 BV for UMSCC29, and 1/3CIS: 2/3BV for UMSCC47. Moreover, all combined and singly treated cells were decreased significantly in the S phase when compared with vehicle-treated. Statistically, significant difference was found between all cell lines treated with both BV and CIS and single-treated groups concerning cell cycle phases ($P < 0.001$) (**Table 6**, **Fig 2**).

Discussion

The currently used chemotherapeutic drugs are non-selective and associated with adverse toxic effects [15]. These complications connected with radiation and chemotherapy in the treatment of cancer might lower the effectiveness of such medication. Therefore, using naturalistic yields in cancer treatment has become an important matter [9]. Biological response modifiers were approaches or agents which adapt the relations between neoplasms/other diseases, treatment, and host through the modification of the host's biological response with subsequent therapeutic effects [16]. BV has been suggested to be considered as a therapeutic tool for various diseases including cancer [9].

MTT assay of the present study revealed that CIS and BV alone or in combinations have a cytotoxic effect on UMSCC12, UMSCC29, UMSCC38, and UMSCC47 cell lines, and they can reduce the cell viability in a dose-dependent manner. By increasing CIS and BV concentrations, cell viability has been reduced. Also, a dose-dependent significant reduction of cell viability was detected among the single and combinations treated groups as compared with the control one. These are following other studies which showed the BV inhibitory effects on variable cancers including liver [17], lung [18], cervical [19], breast [20], ovarian cancer [21], prostate [22], renal [23], colon cancer cells as well as leukemic cells [24].

Besides, Abd-Elhakim et al. [25] reported that BV peptides especially melittin, BV protein component, had a considerable consideration for their probable cytotoxicity against cancer. Similarly, Orsolić [19] mentioned that numerous cancer cells such as lung, renal, prostate, liver, and bladder as well as leukemic and mammary cancer cells could be targets for BV peptides such as phospholipase A2 and melittin. Hu et al. [26] stated that BV showed a cytostatic impact, prevented proliferation, and produced apoptosis of SMMC-7721 human hepatoma cells. Interestingly, Oršolić et al. [27] reported that intravenous injection of BV has frustrated mammary carcinoma in murine when contrasted to control murine. Also, the tumor size was reduced after intratumorally administration of venom, and murine survived longer than control. Gülmez et al. [28] examined the mechanism of BV action, that results in genomic DNA cleavage and cell migration inhibition, representing the induction of apoptosis. Their immunohistochemical studies verified that BV reduced P16 and BCL-2 expressions. Furthermore, Saleh [29] indicated that melittin links to some tumor cells at a higher affinity than to healthy cells which is an interesting feature. Also, Oršolić [27] suggested that the critical mechanism of BV cytotoxic effects by melittin was through the activation of PLA2. Meanwhile, Saleh [29] explained that BV could induce lysis of the tumor cell membrane, inhibit tumor cell proliferation, and activate cancer cell apoptosis. Besides, Hong et al. [10] attributed the DNA fragmentation to the ability of BV to promote up-regulation of caspases 8 and 9 through activation of caspase-3. Other

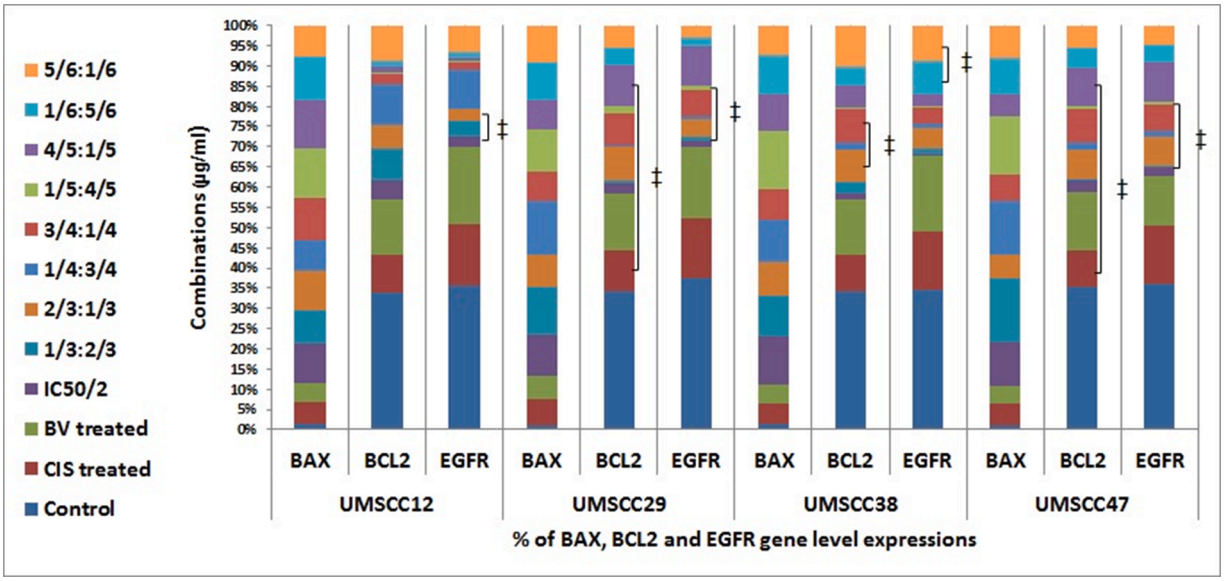

Fig. 1. 100% stacked column for the effect of CIS, BV, and their combinations on the expression of BAX, BCL2, and EGFR genes in 4 utilized HNSCC cell lines. The symbol (‡) represents non-significant difference between two different fractions among the tested gene using LSD post-hoc test.

studies showed that BV comprises many substances that display anti-proliferative activities against different tumor cells [28].

The philosophy of the present therapeutic strategy is to attain a synergistic therapeutic effect and to lessen dose and toxicity. Results of the current study revealed synergistic potentiation with CI < 1 with better efficacy of combined treatment than single treatment with best results of combination between the BV and CIS. This could be supported by Alizadehnohi et al. [12]; Alonezi et al. [30] demonstrated that BV and its main component mellitin showed synergistic effects when combined with CIS on ovarian cancerous CIS resistant A2780cp cells. Also, they are agreed with other previous studies that reported a synergistic effect between BV and anticancer drugs such as Orsolić [19] who explored the possible growth-inhibiting impacts of BV exercised singly or in conjunction with bleomycin, a cytotoxic drug, on HeLa and V79 cells in vitro. He observed that the adjuvant medication caused a dose-dependent decline in cell survival due to DNA damage, indicating that BV may find a therapeutic benefit in enhancing cytotoxicity of the anticancer factor bleomycin.

However, BV and CIS concentrations of the present experiment that inhibited growth cell lines by 50% after three days were different from other previous studies in other cancerous cell lines. For instance, Sisakhta et al. [31] assessed the effects of BV on activity and expression of matrix metalloproteinase-2 as well as apoptogenic and cytological toxicity properties of BV on glioblastoma cells reported 28.5 µg/ml as BV IC50 value. The present study IC50 concentration of CIS was 38.539 µg/ml for UMSCC12, 20.879 µg/ml for UMSCC29, 37.339 µg/ml for UMSCC38 and 28.159 µg/ml for UMSCC47. While IC50 concentration of BV was 54.809 µg/ml for UMSCC12, 61.287 µg/ml µg/ml for UMSCC29, 71.328 µg/ml for UMSCC38 and 61.045 µg/ml for UMSCC47. This might be interpreted by He et al. [32] who explained that IC50 differs in cancerous cell lines depending on types of cell lines, time, and dose-dependent manner. For example, Alizadehnohi et al. [12] found that BV IC50 was 8 µg/ml after 24 hr on human ovarian cancer cell line A2780cp while Shiassi Arani et al. [33] reported 6 µg/ml on 4T1 invasive mammary carcinoma cell lines after 24 hr. Also, Jang et al. [18] who determined the viabilities of human lung cancer (NCI- H1299) cells incubated with BV at a concentration of 1 µg/ml for 6, 12, 24, and 48 h were 84.96%, 80.57%, 75.53%, and 52.55% of the control value, respectively. Meanwhile, the viabilities of cells incubated with BV at a concentration of 10 µg/ml for 6, 12, 24, and 48 h were 70.82%, 50.57%, 47.09%, and 33.94% of the control value, respectively.

Two of the major genes responsible for regulating the mitochondrial apoptosis pathways are anti-apoptotic BCL2 and pro-apoptotic BAX genes [34]. RT-PCR has become a commonly used method for precise determination of gene expression [35]. Most types of apoptotic cell death are happened *via* BCL2 inhibition, denoting a familiar lethality mechanism. BCL2 is restricted to intracellular sites of oxygen free radical generations including endoplasmic reticula, nuclear membranes, and mitochondria. It secured cells from menadione and H2O2-induced oxidative deaths. Subsequently to apoptotic signal, cells sustained progressive lipid peroxidation. BCL2 upregulation can lead to complete suppression of lipid peroxidation, and it proposes a model in which BCL2 adjusts the anti-oxidant pathways at sites of free radical generation. BCL2 family members are proteins that are characterized by their capability to form complexes of homo-dimers with itself and hetero-dimers with BAX [36]. Thus, BAX/BCL2 ratio is supposed to decide the susceptibility of cell apoptosis.

In the present study, SCC cell lines treated with CIS and/or BV showed reduced cell viability by inducing apoptosis. RT- PCR revealed a significant downregulation of the anti-apoptotic BCL2 and EGFR genes and a significant upregulation of the pro-apoptotic BAX. This is accordant with Hur and Song [37] who stated that BV exerts anti-cancer action as an effective sensitizer of death receptors (DR.)-mediated apoptosis through up-regulation of pro-apoptotic caspases - 8, 9, −3 through increased BAX/BCL-2 ratio and overexpression of death receptors 3–6 and FAS. Also, Alizadehnohi et al. [12] emphasized that BV up-regulates p53, Fas, BAX, p21, and caspases-8, 9 but down-regulates BCL-2 expression. They explained that BV caused the discharge of endonuclease G and apoptosis-inducing factors from mitochondria that were directed to apoptosis through the caspase-independent pathway in Ca Ski cells. Moreover, other cell lines such as U937 cells [38] and human breast cancer MCF7 cells [20] confirmed that BV and melittin cause apoptosis of prostate carcinoma DU145, LNCaP, and PC-3 cells through caspase-3 activation *via* inactivation of NFκB both in vivo and in

Table. 6
Statistical results of one-way ANOVA test for CIS, BV and their combinations on G0/G1, S and G2/M cell cycle phases in 4 utilized HNSCC cell lines.

Combinations	Mean ± SD											
		UMSCC12			UMSCC29			UMSCC38			UMSCC47	
	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	0.60±0.00	0.22±0.00	0.15±0.00	0.57±0.00	0.24±0.00	0.16±0.00	0.64±0.00	0.21±0.00	0.14±0.00	0.62±0.00	0.20±0.00	0.15±0.00
CIS treated	0.51±0.00	0.11±0.00	0.38±0.00	0.46±0.00	0.13±0.00	0.40±0.00	0.54±0.00	0.11±0.00	0.34±0.00	0.54±0.00	0.11±0.00	0.34±0.00
BV treated	0.52±0.00	0.12±0.00	0.35±0.00	0.48±0.00	0.11±0.00	0.38±0.00	0.50±0.00	0.13±0.00	0.34±0.00	0.52±0.00	0.13±0.00	0.33±0.00
IC50/2	0.30±0.00	0.12±0.00	0.56±0.00	0.30±0.00	0.13±0.00	0.55±0.00	0.30±0.00	0.15±0.00	0.54±0.00	0.21±0.00	0.16±0.00	0.61±0.00
1/3:2/3	0.35±0.00	0.13±0.00	0.50±0.00	0.22±0.00	0.10±0.00	0.65±0.00	0.43±0.00	0.13±0.00	0.41±0.00	0.17±0.00	0.11±0.00	0.70±0.00
2/3:1/3	0.38±0.00	0.15±0.00	0.45±0.00	0.40±0.00	0.13±0.00	0.45±0.00	0.36±0.00	0.10±0.00	0.51±0.00	0.26±0.00	0.14±0.00	0.58±0.00
1/4:3/4	0.42±0.00	0.13±0.00	0.44±0.00	0.18±0.00	0.10±0.00	0.71±0.00	0.32±0.00	0.17±0.00	0.48±0.00	0.34±0.00	0.12±0.00	0.51±0.00
3/4:1/4	0.26±0.00	0.15±0.00	0.57±0.00	0.28±0.00	0.15±0.00	0.55±0.00	0.24±0.00	0.16±0.00	0.57±0.00	0.36±0.00	0.15±0.00	0.46±0.00
1/5:4/5	0.18±0.00	0.11±0.00	0.70±0.00	0.25±0.00	0.13±0.00	0.60±0.00	0.15±0.00	0.10±0.00	0.73±0.00	0.31±0.00	0.13±0.00	0.63±0.00
4/5:1/5	0.22±0.00	0.12±0.00	0.65±0.00	0.36±0.00	0.12±0.00	0.47±0.00	0.26±0.00	0.12±0.00	0.60±0.00	0.38±0.00	0.11±0.00	0.48±0.00
1/6:5/6	0.24±0.00	0.13±0.00	0.61±0.00	0.34±0.00	0.14±0.00	0.50±0.00	0.37±0.00	0.15±0.00	0.45±0.00	0.34±0.00	0.10±0.00	0.54±0.00
5/6:1/6	0.41±0.00	0.14±0.00	0.44±0.00	0.38±0.00	0.13±0.00	0.46±0.00	0.39±0.00	0.13±0.00	0.46±0.00	0.35±0.00	0.12±0.00	0.50±0.00
One-way ANOVA (F; *P* value)	(1828.295; 0.000)	(100.607; 0.000)	(2955.711; 0.000)	(2205.995; 0.000)	(208.205; 0.000)	(2829.057 ; 0.000)	(3158.359 ; 0.000)	(133.281; 0.000)	(3066.591; 0.000)	(3054.462 ; 0.000)	(131.749; 0.000)	(3002.356; 0.000)

vitro. Other previous reports like Hong et al. [10], Ip SW et al. [20] explored that BV elevates reactive oxygen species and cytoplasmic Ca2+ and decreases mitochondrial membrane potential, that enhances the levels of PARP, caspase-3, p53, FAS, BAX and p21, and inhibits BCL-2 level. Moreover, Moon et al. [38] stated that melittin alone induced apoptosis in human leukemic U937 cells *via* reduction of NF-kB, BCL-2, and increase of caspase-3.

Treatment of cell lines of the present study with BV and/or CIS singly or in combination revealed a significant reduction in EGFR expression level with the lowest levels among combination-treated groups. These are agreed with Jeong et al. [39] BV and melittin inhibit significantly EGF-induced migration and invasion of breast cancer cells. Moreover, Jeong et al. [40] found that BV reduced EGF-induced F- actin cell invasion and reorganization, and repressed EGF-induced epithelial-mesenchymal transitions which are processes related to metastasis in lung cancer. The possible explanation was that melittin and BV inhibited the EGF-stimulated F-actin reorganization at the leading edge with no apamin effect. Also, they reduced the EGF-induced MMP-9 expression by blocking the NF-κB and PI3K/Akt/mTOR pathway. Moreover, melittin significantly repressed the EGF-induced FAK phosphorylation through inhibition of the mTOR/p70S6K/4E-BP1 pathway [39].

Cell cycle checkpoints, in eukaryotic cells, perform an important and significant role in the regulation of cell cycle transition, and aberrations of these checkpoints regulation frequently occur in cancerous cells. Cell cycle checkpoints regulate the arrest of the cell cycle following DNA damage. Irreversible damage of cells might activate cell death *via* apoptotic pathways. In the response to DNA damage, two apical checkpoint kinases (ATM and ATR) directly phosphorylate the checkpoint transducer kinases CHK2 and CHK1, respectively. ATM-CHK2 activates the p53– p21 pathway, p21 being an inhibitor of cyclins/CDKs that regulates cell cycle arrest, Thus, when cells undergo G2/M phase arrest, the expression of cyclin B1/CDK1 is reduced [41].

In the present study, cell cycle analysis *via* flow cytometry revealed significantly increased numbers of cells in the G2/M phase in single or combined- treated cell lines with CIS and BV when compared with vehicle-treated. Moreover, a significant decrease in cells in S phases among all single and combined treatments when matched with vehicle-treated. Another investigation carried out by El-Fiky et al. [42] aimed to in vitro estimation of the anti-cancer potentials of BV in addition to evaluation of the synergetic potential of dates extract to BV against lung cancer (A549). Cell cycle analysis revealed that BV, dates, and their mix induced cell cycle arrest at the G2/M phase and pre G1 apoptosis.

Conclusions

The present study findings indicated that BV has inhibitory cytotoxic effects against HNSCC cell lines depending on its concentration. Furthermore, CIS cytotoxic effects have promoted and become more eligible when combined with nonlethal doses of BV. Therefore, this study evolves a new strategy for developing potential antineoplastic agents with low adverse effects and high cytotoxicity.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Mohammed E. Grawish: Writing - review & editing. **Mohamed I. Mourad:** Conceptualization, Methodology. **Doaa AM Esmaeil:** Validation. **Rehab A Ahmed:** Formal analysis. **Islam Mohamed Ateia:** Supervision. **Eman Hany:** Investigation, Resources. **Mazen Tharwat Abou Elkhier:** Data curation, Writing - original draft.

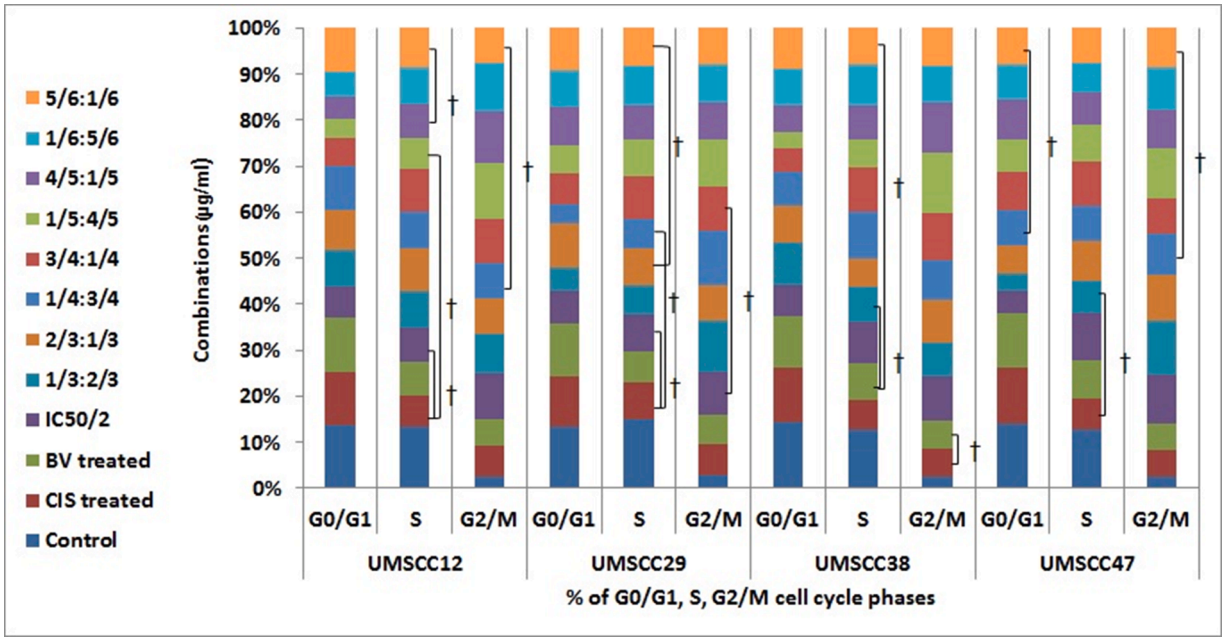

Fig. 2. 100% stacked column for the effect of CIS, BV, and their combinations on G0/G1, S, and G2/M cell cycle phases in 4 utilized HNSCC cell lines. The symbol (†) represents non-significant difference between two different fractions among the cell cycle phase using LSD post-hoc test.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. All authors deny any conflict of interest

References

[1] S.I. Pai, W.H. Westra, Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment, Annu. Rev. Pathol 4 (2009) 49–70.

[2] C. Lo Nigro, N. Denaro, A. Merlotti, M Merlano, Head and neck cancer: improving outcomes with a multidisciplinary approach, Cancer Manag. Res 9 (2017) 363–371.

[3] A.M. De Costa, M.R Young, Immunotherapy for head and neck cancer: advances and deficiencies, Anticancer Drugs 22 (2011) 674–681.

[4] R. Bayat Mokhtari, T.S. Homayouni, N. Baluch, E. Morgatskaya, S. Kumar, B. Das, H Yeger, Combination therapy in combating cancer, Oncotarget 8 (2017) 38022–38043.

[5] R.K. Mehmood, Review of cisplatin and oxaliplatin in current immunogenic and monoclonal antibody treatments, Oncol. Rev 8 (2014) 256.

[6] K. Nurgali, R.T. Jagoe, R Abalo, Editorial: adverse effects of cancer chemotherapy: anything new to improve tolerance and reduce sequelae? Front Pharmacol 9 (2018) 245.

[7] E. Rajesh, L.S. Sankari, L. Malathi, J.R Krupaa, Naturally occurring products in cancer therapy, J. Pharm. Bioallied Sci 7 (Suppl 1) (2015) S181–S183.

[8] G.K. Randhawa, J.S. Kullar, Rajkumar, Bioenhancers from mother nature and their applicability in modern medicine, Int J. Appl. Basic Med. Res 1 (2011) 5–10.

[9] R. Wehbe, J. Frangieh, M. Rima, D. El Obeid, J.M. Sabatier, Z Fajloun, Bee Venom: overview of main compounds and bioactivities for therapeutic interests, Molecules 24 (2019) pii: E2997.

[10] S.J. Hong, G.S. Rim, H.I. Yang, C.S. Yin, H.G. Koh, M.H. Jang, C.J. Kim, B.K. Choe, J.H Chung, Bee venom induces apoptosis through caspase-3 activation in synovial fibroblasts of patients with rheumatoid arthritis, Toxicon 46 (2005) 39–45.

[11] T.C. Chou, Drug combination studies and their synergy quantification using the Chou-Talalay method, Cancer Res 70 (2010) 440–446.

[12] S.P. Angeline Kirubha, M. Anburajan, B. Venkataraman, R. Akila, D. Sharath, B Raj, Evaluation of mammary cancer in 7,12-dimethylbenz(a)anthracene-induced Wister rats by asymmetrical temperature distribution analysis using thermography: a comparison with serum CEA levels and histopathology, J. Biomed. Biotechnol 2012 (2012), 786417.

[13] M.A. El-Magd, W.S. Abdo, M. El-Maddaway, N.M. Nasr, R.A. Gaber, E.S. El-Shetry, A.A. Saleh, F.A.A. Alzahrani, D.H Abdelhady, High doses of S-methylcysteine cause hypoxia-induced cardiomyocyte apoptosis accompanied by engulfment of mitochondaria by nucleus, Biomed. Pharmacother 94 (2017) 589–597.

[14] M.A. El-Magd, S.F. Khalifa, F.A. A Alzahrani, A.A. Badawy, E.S. El-Shetry, L. M. Dawood, M.M. Alruwaili, H.A. Alrawaili, E.F. Risha, F.M. El-Taweel, H.E Marei, Incensole acetate prevents beta-amyloid-induced neurotoxicity in human olfactory bulb neural stem cells, Biomed. Pharmacother 105 (2018) 813–823.

[15] Y. Wen, J.R. Grandis, Emerging drugs for head and neck cancer, Expert Opin. Emerg. Drugs 20 (2015) 313–329.

[16] H. Wang, Y. Cai, Y. Zheng, Q. Bai, D. Xie, J Yu, Efficacy of biological response modifier lentinan with chemotherapy for advanced cancer: a meta-analysis, Cancer Med 6 (2017) 2222–2233.

[17] S. Liu, M. Yu, Y. He, L. Xiao, F. Wang, C. Song, S. Sun, C. Ling, Z Xu, Melittin prevents liver cancer cell metastasis through inhibition of the Rac1-dependent pathway, Hepatology 47 (2008) 1964–1973.

[18] M.H. Jang, M.C. Shin, S. Lim, S.M. Han, H.J. Park, I. Shin, J.S. Lee, K.A. Kim, E. H. Kim, C.J Kim, Bee venom induces apoptosis and inhibits expression of cyclooxygenase-2 mRNA in human lung cancer cell line NCI-H1299, J Pharmacol Sci 91 (2003) 95–104.

[19] N. Orsolić, Potentiation of bleomycin lethality in HeLa and V79 cells by bee venom, Arh Hig Rada Toksikol 60 (2009) 317–326.

[20] S.W. Ip, S.S. Liao, S.Y. Lin, J.P. Lin, J.S. Yang, M.L. Lin, G.W. Chen, H.F. Lu, M. W. Lin, S.M. Han, J.G Chung, The role of mitochondria in bee venom-induced apoptosis in human breast cancer MCF7 cells, In Vivo (Brooklyn) 22 (2008) 237–245.

[21] M. Alizadehnohi, M. Nabiuni, Z. Nazari, Z. Safaeinejad, S Irian, The synergistic cytotoxic effect of cisplatin and honey bee venom on human ovarian cancer cell line A2780cp, J. Venom Res 3 (2012) 22–27.

[22] M.H. Park, M.S. Choi, D.H. Kwak, K.W. Oh, D.Y. Yoon, S.B. Han, H.S. Song, M. J. Song, J.T Hong, Anti-cancer effect of bee venom in prostate cancer cells through activation of caspase pathway via inactivation of NF-κB, Prostate 71 (2011) 801–812.

[23] T. Putz, R. Ramoner, H. Gander, A. Rahm, G. Bartsch, M Thurnher, Antitumor action and immune activation through cooperation of bee venom secretory phospholipase A2 and phosphatidylinositol-(3,4)-bisphosphate, Cancer Immunol. Immunother 55 (2006) 1374–1383.

[24] J. Slaninová, V. Mlsová, H. Kroupová, L. Alán, T. Tůmová, L. Monincová, L. Borovičková, V. Fučík, V Cerovský, Toxicity study of antimicrobial peptides from wild bee venom and their analogs toward mammalian normal and cancer cells, Peptides 33 (2012) 18–26.

[25] Y.M. Abd-Elhakim, S.R. Khalil, A. Awad, L.Y Al-Ayadhi, Combined cytogenotoxic effects of bee venom and bleomycin on rat lymphocytes: an in vitro study, Biomed Res. Int 2014 (2014), 173903.

[26] H. Hu, D. Chen, Y. Li, X Zhang, Effect of polypeptides in bee venom on growth inhibition and apoptosis induction of the human hepatoma cell line SMMC-7721 in-vitro and Balb/c nude mice in-vivo, J. Pharm. Pharmacol 58 (2006) 83–89.

[27] N. Orsolić, L. Sver, S. Verstovsek, S. Terzić, I Basić, Inhibition of mammary carcinoma cell proliferation in vitro and tumor growth in vivo by bee venom, Toxicon 4 (2003) 861–870.

[28] Y. Gülmez, A. Aydın, I. Can, S. Tekin, E. Cacan, Cellular toxicity and biological activities of honey bee (Apis mellifera L.) venom, Marmara Pharm J 21 (2017) 51–260.

[29] R. Saleh, Bee venom therapy for cancer: a literature review, IJSER 8 (2017) 590–597.

[30] S. Alonezi, J. Tusiimire, J. Wallace, M.J. Dufton, J.A. Parkinson, L.C. Young, C. J. Clements, J.K. Park, J.W. Jeon, V.A. Ferro, D.G Watson, Metabolomic profiling of the synergistic effects of melittin in combination with cisplatin on ovarian cancer cells, Metabolites 7 (2017) 14.

[31] M. Sisakht, B. Mashkani, A. Bazi, H. Ostadi, M. Zare, F.Z. Avval, H.R. Sadeghnia, M. Mojarad, M. Nadri, A. Ghorbani, M. Soukhtanloo, Bee venom induces apoptosis and suppresses matrix metaloprotease-2 expression in human glioblastoma cells, Rev. Bras. Farmacogn. 27 (2017) 324–328.
[32] Y. He, Q. Zhu, M. Chen, Q. Huang, W. Wang, Q. Li, Y. Huang, W Di, The changing 50% inhibitory concentration (IC50) of cisplatin: a pilot study on the artifacts of the MTT assay and the precise measurement of density-dependent chemoresistance in ovarian cancer, Oncotarget 7 (2016) 70803–70821.
[33] F. Shiassi Arani, L. Karimzadeh, S.M. Ghafoori, M Nabiuni, Antimutagenic and synergistic cytotoxic effect of cisplatin and honey bee venom on 4T1 invasive mammary carcinoma cell line, Adv Pharmacol Sci 2019 (2019), 7581318.
[34] Q.S. Tong, L.D. Zheng, L. Wang, J. Liu, W Qian, BAK overexpression mediates p53-independent apoptosis inducing effects on human gastric cancer cells, BMC Cancer 4 (2004) 33.
[35] R.D. Canales, Y. Luo, J.C. Willey, B. Austermiller, C.C. Barbacioru, C. Boysen, K. Hunkapiller, R.V. Jensen, C.R. Knight, K.Y. Lee, Y. Ma, B. Maqsodi, A. Papallo, E. H. Peters, K. Poulter, P.L. Ruppel, R.R. Samaha, L. Shi, W. Yang, L. Zhang, F. M Goodsaid, Evaluation of DNA microarray results with quantitative gene expression platforms, Nat. Biotechnol 24 (2006) 1115–11122.
[36] J.C. Reed, Double identity for proteins of the Bcl-2 family, Nature 387 (1997) 773–776.
[37] K.Y. Hur, H.S. Song, Inhibitory effect of bee venom toxin on lung cancer NCI H460 cells growth through induction of apoptosis via death receptor expressions, Acupuncture 31 (2012) 121–130.
[38] D.O. Moon, S.Y. Park, Y.H. Choi, N.D. Kim, C. Lee, G.Y Kim, Melittin induces Bcl-2 and caspase-3-dependent apoptosis through downregulation of Akt phosphorylation in human leukemic U937 cells, Toxicon 51 (2008) 112–120.
[39] Y.J. Jeong, Y. Choi, J.M. Shin, H.J. Cho, J.H. Kang, K.K. Park, J.Y. Choe, Y.S. Bae, S.M. Han, C.H. Kim, H.W. Chang, Y.C Chang, Melittin suppresses EGF-induced cell motility and invasion by inhibiting PI3K/Akt/mTOR signaling pathway in breast cancer cells, Food Chem. Toxicol 68 (2014) 218–225.
[40] Y.J. Jeong, Y.Y. Park, K.K. Park, Y.H. Choi, C.H. Kim, Y.C Chang, Bee venom suppresses EGF-induced epithelial-mesenchymal transition and tumor invasion in lung cancer cells, Am. J. Chin. Med 47 (2019) 1869–1883.
[41] J. Zhang, X. Zhu, H. Li, B. Li, L. Sun, T. Xie, T. Zhu, H. Zhou, Z Ye, Piperine inhibits proliferation of human osteosarcoma cells via G2/M phase arrest and metastasis by suppressing MMP-2/-9 expression, Int. Immunopharmacol 24 (2015) 50–58.
[42] A.A. El-fiky, G. Gehan Hammad, M.G. SaadEldin, O.H Elhiatmy, Therapeutic effects of Apis mellifera and Pandinus Imperator venom on lung cancer: an In vitro study, Biosci. Res. 16 (2019) 2624–2640.