Impact of bee venom and melittin on apoptosis and biotransformation in colorectal carcinoma cell lines

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This study provide data about anticancer properties of bee venom and its dominant compound, melittin on colorectal carcinoma cells (HCT-116 and SW-480) in regard to their proapoptotic activity and expression of genes involved in biotransformation process. Based on results, they are a strong cytotoxins, where the melittin showed also selectivity against cancer cells compared to normal, HaCat. They induce proapoptotic activity by affecting apoptosis signaling molecules (Fas receptors, caspase 9, and members of Bcl-2 family of proteins) and mainly suppress expression of genes involved in their biotransformation, suggesting their ability to prevent develop the resistance of colorectal cancer cells.

Keywords: animal products, apoptosis, cell death, honeybee, venom.

Introduction

Colorectal carcinoma most frequently occurs due to malignant transformation of epithelial cells of the gastrointestinal tract (Torre *et al*. 2015). At the molecular level, hereditary factors play a crucial role, followed by diet, obesity, smoking and physical activity (Antić 2014). Various genetic mutations of oncogenes and tumor suppressor genes, especially the *APC*, *KRAS*, and *p53* genes as most prominent and together with epigenetic mutations are crucial molecular mechanisms in colorectal carcinoma development (Guanti 2000).

The discovery and design of novel anticancer substances, especially of natural origin, that are less harmful than the commercially available cytostatics and development of targeted strategy to improve current therapy are desirable. One of the strategies could be induction of apoptosis due activation of the external, death receptor-mediated pathway, targeting the specific caspases, by affecting the mitochondria and regulators of mitochondria membranes permeability such as members of Bcl-2 protein family, etc. (Mayer and Oberbauer 2003). Although plants are considered the most common resource of bioactive substances, the accelerated research of new natural products includes animal products as well (Gomes *et al*. 2010). Bee venom is used in the treatment of different diseases like rheumatoid arthritis and inflammatory diseases due to its impact on various proinflammatory molecules (Son *et al*. 2007). Anticancer effects of bee venom were also investigated (Premratanachai *et al*. 2014). Their effectiveness is mainly mediated by the induction of apoptosis via activation of initiator and effector caspases and death receptor expression, such as Fas (*First apoptosis signal*) (Lee *et al*. 2015). Bee venom is a mixture of compounds that have diverse effects individually (Bogdanov, 2011). Melittin, as a dominant constituent of bee venom, induces apoptosis and eliminates tumor cells without initiating an immune response (Zheng *et al*. 2015). There is some literature data which confirm that melittin

leads to apoptosis by activating cell membrane death receptors on the human ovarian cancer cells SKOV3 and PA-1 (Jo *et al.* 2012) and significantly reduces the level of mitochondrial potential and induces apoptosis in MCF-7 cells (Ip *et al*. 2008).

One of the most common problems in cancer therapy is development of cancer cell resistance which occur on various mechanisms, like impossible enter of the anticancer substance into the cell, their biotransformation, increased efflux from the cell, ability of cancer cells to avoid apoptosis and many other (Adams and Cory 2007; Baguley, 2010). The main members of the biotransformation process of anticancer agents in the first phase are CYP enzymes (*CYP1A1* is expressed in the colon tissue), which contribute to the hydrolysis, reduction and oxidation of xenobiotics to create more soluble forms of xenobiotics (Guengerich 2007). Besides, transporters that are most often associated with the occurrence of resistance in anticancer therapy are *P-gp* (Multidrug resistance protein 1, P-glycoprotein 1), *MRP1* (Multidrug resistance-associated protein 1) and *BCRP* (Breast cancer resistance protein) and they eject a large spectrum of xenobiotics (Dinić *et al*. 2015).

In this study, potential anticancer effects of bee venom from *Apis mellifera* L. and its dominant component melittin were investigated. The potential anticancer properties were evaluated in colorectal carcinoma cell lines by determination of its cytotoxic activity, type of cell death, apoptotic mechanisms and effect on expression of genes involved in apoptosis (*Bax* and *Bcl-2*) and biotransformation (*CYP1A1* - cytochrome P450, family 1, member A1; *GSTP1* - glutathione S-transferase pi 1; *MRP-2* - multidrug resistance-associated protein 2). Normal human keratinocytes, HaCaT, were used for evaluation of cytotoxic effects on control non-cancerous cell line.

Materials and methods

Chemicals

Phosphate buffered saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from GIBCO, Invitrogen, USA. Ethidium bromide (EB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), absolute ethanol and chloroform (molecular biology grade) were obtained from SERVA, Germany. Acridine Orange (AO) was obtained from Acros organics, New Jersey, USA. Kit for qPCR Kit (Luna Universal qPCR Master Mix Kit) were from BioLabs, New England. Nuclease-free water and TRIzol were from Ambion, USA. The secondary antibody conjugated with Cy3, Diamidino-2-phenylindole (DAPI) and kit for translating RNA into complementary DNA (High-Capacity cDNA Reverse Transcription Kits) from Thermo Scientific, USA. Polyvinyl alcohol mounting medium was obtained from Fluka Analytical, Switzerland. All solvents and chemicals were of analytical grade. Melittin (dry powder) was used from Sigma Aldrich.

Bee venom collection and storage

Bee venom was obtained from *Apis mellifera* in the Smederevska Palanka territory, Serbia (44° 21′ 34″ N, 20° 57′ 28″ E). After collection from honeybees, venom was dissolved in distilled water, filtered using 0.2 μm membrane filters to remove impurities and then dried. Bee venom was dissolved in 1% PBS immediately before use for assays, after which appropriate dilutions with DMEM were made. Melittin was dissolved and stored at +4°C.

SDS-PAGE gel electrophoresis for bee venom protein content analysis

SDS PAGE electrophoresis on 16% gel was used to examine the protein content of dried bee venom (Laemmli, 1970). Densitometry was used to estimate quantity of major proteins in the venom using the ImageJ program. The protein detection parameters are mwm - molecular weight markers, masses are expressed in kilodaltons.

Cell lines and culturing

The human colorectal carcinoma cells, HCT-116 and SW-480 obtained from ATCC - American Type Culture Collection, USA, and normal human keratinocytes, HaCaT cell lines were obtained from CLS - Cell Lines Service, Eppelheim, Germany. Standard maintaining conditions were previously published (Milutinović *et al.* 2015), and studies were done with cells at 70-80% confluence.

Cell viability assay (MTT assay)

The effects of bee venom and melittin on HCT-116 and SW-480 colorectal carcinoma and normal HaCaT cell viability were determined by MTT assay (Mosman 1983). The cells were seeded in a 96-well plate (10^4 cells/well). After pre-incubation (24 h), the cells were treated with 100 µL of different concentrations of bee venom (0.1-10 µg/ml) and melittin (0.1-10 µg/ml) while untreated cells served as a control. The assay was performed 24 and 72 h after treatment.

Fluorescence microscopic analysis of cell death

Acridine orange/ethidium bromide (AO/EB) double staining assay was performed for cell death type determination on HCT-116 and SW-480 cells (Baskić *et al*. 2006). The cells were seeded in the same way as for MTT assay and treated with 1 and 5 µg/ml of bee venom and melittin. The assay was performed using inverted fluorescent microscope (Nikon Ti-Eclipse) at 400x magnification, 24 h after treatment. The cells were calculated to determine percentages of viable cells (V), apoptosis (early, EA, and late stage, LA) and necrosis (N) in relation to the total cell number per sample. The minimum of 300 cells was counted in each sample.

Immunofluorescence staining for determination of Fas receptor and caspase 9 protein expression

Protein expression of Fas receptor and caspase 9 in HCT-116 and SW-480 cells was detected by immunofluorescence (Higuchi *et al*. 2001). The cells were seeded in a 6-well plate (5 x 10^4 cells/well), incubated for 24 h and treated with 2 ml of bee venom and melittin at a concentration of 1 and 5 µg/ml. The cells were visualized using inverted fluorescent microscope (Nikon Ti-Eclipse) at 600x magnification, 24 h after treatment.

Quantitative mRNA analysis by qPCR method

Total RNA was isolated from HCT-116 and SW-480 cells (control and treated) by published method (Chomczynski and Sacchi, 1987). RNA concentration in all samples was measured on a biophotometer (Eppendorf BioPhotometer plus). The samples were stored at -80 °C until analysis. The cells were seeded in T25 cm^2 flask and treated with 5 ml of bee venom and melittin in concentration of 1 and 5 µg/ml. The analysis was performed 24 h after treatments.

For transcribing single-stranded RNA in the cDNA (complementary DNA), the High-Capacity cDNA Reverse Transcription Kits from Thermo Scientific, USA, were used.

The quantification of mRNA expression was performed by using an Applied Biosystems, Quantitative Real-Time system (Applied Biosystems 7500/7500 Real-Time PCR Software v2.0). All amplifications were done using a Luna Universal qPCR Master Mix Kit (BioLabs). Each sample contained 10 µl Luna Universal qPCR Master Mix, 0.5 µl of forward and reverse primer (5 pmoL/µl) and 1 µl cDNA and 8 µl nuclease-free water. The negative control contained nuclease-free water and 2RT reaction instead of cDNA. To analyze the qPCR results, we used $2^{\wedge(-\Delta\Delta Ct)}$ method (Schmittgen and Livak, 2008). *β-actin* was employed as a housekeeping-reference gene. Gene specific primers are shown in Table 1 in Supplementary material.

Statistical analysis

The data are expressed as mean ± standard error (SE). Biological activity was examined in three individual experiments, performed in triplicate for each dose. Statistical significance was determined using the Student's t-test or the one-way ANOVA test for multiple comparisons. A p value < 0.05 was considered significant. The IC_{50} values were calculated from the dose curves by a computer program (CalcuSyn). The relative intensity of fluorescence in control and treated cells, and densitometry analysis were measured by ImageJ program (Wayne Rasband, ImageJ, http://rsb.info.nih.gov/ij/).

Results

The protein content of bee venom

The percentage of proteins in the dried bee venom analyzed by SDS-PAGE gel electrophoresis, shows that melittin was the most dominant component of bee venom, followed by phospholipase A and hyaluronidase (Table 1, Figure 1 in Supplementary material). The abundance of undefined proteins was below 1% of analyzed material (Table 1).

Cytotoxic effects of bee venom and melittin

The effect of bee venom and melittin on HCT-116 and SW-480 colorectal carcinoma and normal HaCaT keratinocytes viability was investigated by MTT assay. The obtained results indicate that the bee venom and melittin significantly reduced the cell viability of the investigated colorectal carcinoma cell lines (Figure 2 in Supplementary material). Normal keratinocytes showed less sensitivity to the treatment of melittin compared to cancer cells.

The cytotoxic activity was calculated and expressed as an IC_{50} value (μg/ml) - concentration that killed 50% of cells (Table 2). The IC_{50} values obtained indicate considerable cytotoxicity of bee venom on HCT-116 and SW-480 cells. However, it showed non-selective effects, due to their significant cytotoxicity on normal HaCaT keratinocytes. Based on IC_{50} values, melittin has a

selective effect, since it does not have cytotoxic effects on normal keratinocytes in the range of concentrations used to treat cancer cells. IC_{50} values shows that bee venom has a stronger cytotoxic effect than melittin.

Type of cell death

The fluorescence and morphological changes of AO/EB double stained HCT-116 and SW-480 control cells and cells treated by bee venom and melittin were observed. The micrographs of treated cells clearly show the changes in cell morphology, such as chromatin condensation (light green fluorescence), characteristic for early apoptosis, highly condensed chromatin with dark green to orange fluorescence characteristic for late apoptosis and red fluorescence that indicate necrosis (Figure 1). The treatment significantly induced early and late apoptosis, while necrosis appeared in a smaller percentage in all tested samples (Table 3).

Fas receptor and caspase 9 protein expression

Apoptosis induced by proapoptotic substances required evaluation of signaling pathways involved in this process, by monitoring numerous activated or inhibited signaling molecules. The protein expression of the Fas receptor on the cell membrane, as well as the caspase 9, were increased in bee venom and melittin (1 and 5 μg/ml) treated HCT-116 and SW-480 cells compared to control (Figure 2 and 3).

The fluorescence quantification by the ImageJ program shows more pronounced protein expression of Fas receptors and caspase 9 in SW-480 cells compared to HCT-116 (Figure 2 and 3).

The impact of bee venom and melittin on mRNA expression of genes involved in apoptosis and biotransformation

The results for mRNA expression of genes involved in the process of apoptosis (*Bax* and *Bcl-2*) and biotransformation (*CYP1A1, GSTP1* and *MRP-2*), under the influence of bee venom and melittin, are shown in Table 4. Bee venom decreased expression of mRNA of all tested genes in SW- 480 cells. In HCT-116 cells, increased the expression of mRNA of the *MRP-2* gene and the proapoptotic *Bax*, while other investigated genes were inhibited. The observed Ct values (Table 2 in Supplementary material) indicate that the bee venom affects the expression of the constituent *β-actin* gene, so other endogenous controls (*18S rRNA*, *GAPDH*) were used in this case to determine the most stable endogenous control and to normalize the results. Normalization is done via *β-actin* because it is the most stable endogenous control. This is not the case with melittin, where the *β-actin* is stable under the treatment. Melittin acts similar to bee venom, a decrease in the mRNA expression of the tested genes was observed in SW-480 cells. In HCT-116 cells it was noticed an increase in expression of mRNA of the *MRP-2* gene, while other investigated genes were reduced.

Discussion

Beside plants, mushrooms and lichens, as the most investigated sources of active substances delivered from nature, the animal products were also examined, including the bee venom. Bee venom is mainly composed of protein-related components, where the most common is melittin, a lytic peptide with confirmed anticancer properties (Gajski and Garaj-Vrhovac 2013), including its proapoptotic effects on human colorectal cancer cells (Zheng *et al*. 2015). In our experimental conditions, bee venom significantly reduced the viability of colorectal carcinoma HCT-116 and SW-480 cells, but anticancer selectivity was not observed due to its cytotoxicity on normal HaCaT cells (Table 2). Our data were in accordance with other authors (Zheng *et al*. 2015) who confirmed the cytotoxicity of bee venom on colorectal carcinoma cells. The cytotoxic activity

can be attributed largely to melittin, whose cytotoxic activity was also notable. Contrary to bee venom, melittin showed selectivity against cancer cells, considering the lower effects on normal cells in investigated concentrations.

The analysis of induced cell death indicates that bee venom and melittin cytotoxicity are dominantly mediated by apoptosis, with minimal necrosis (less than 1%). Apoptosis is most noticeable in SW-480 cells under the influence of treatments, suggesting higher sensitivity to treatments, compared with HCT-116 cells (Table 3, Figure 1). Cell apoptosis occurs through different signaling pathways, either external or internal. Various molecules and ligands could initiate apoptosis by binding death complex with membrane death receptors like the Fas, thus triggering external apoptotic pathway. Protein expression of the Fas receptors are increased after bee venom and melittin treatments in both HCT-116 and SW-480 cell lines (Figure 2 and 3), which is in accordance with the results obtained by Western blot analysis on the same cell lines (Zheng *et al*. 2015). The most pronounced increase of Fas receptors expression and potential trigger of external pathway in SW-480 cells, observed in the treatment by concentration of 1 and 5 μg/ml, is in accordance to highest percentage of apoptosis in this cell line compared to HCT-116. Increased expression of proapoptotic *Bax* gene by bee venom in combination with decrease of antiapoptotic *Bcl-2* gene support this result and explain that HCT-116 cells suggests activation of mitochondrial apoptosis signaling pathway in these cells. In addition, the increase of caspase 9 in both tested cells also suggest activation of mitochondrial apoptosis signaling pathway in tested cells (Figure 2 and 3). Namely, the HCT-116 cells belong to type II, where the apoptosis is dependent on mitochondria and downstream activated caspase 9. Contrary, the caspase 9 is an optional solution in SW-480 cells, otherwise, when it is blocked, the apoptosis occurs through a death receptor pathway (Ozoren and El-Deiry 2002).

The evaluation of mRNA expression of genes whose protein products are involved in biotransformation process provides us information about metabolism of bee venom and melittin in colorectal cancer cells (Table 4). Otherwise, overexpression of these metabolic genes and drug transporters in tumors, demonstrated by several studies, are responsible for accumulation, distribution and efflux of many chemotherapeutic drugs (Akhdar *et al*. 2012). Our results suggest reduction of *CYP1A1*, *GSTP1* and *MRP-2* gene expression in SW-480 cells under the influence of bee venom and melittin. On HCT-116 cells, results for expression of genes included in process of biotransformation are similar for bee venom and melittin, because both treatments cause reduction of *CYP1A1* and *GSTP1* gene expression and increase of *MRP-2* gene. This indicates the good effect of the bee venom and melittin, since an increase in these genes would indicate its active metabolism in tested cancer cells and therefore the possibility of developing resistance. In SW-480 cells, reduction of *MRP-2* gene expression may suggest a retention of treatments in cells and thereby the higher cytotoxicity. This is not the case with HCT-116 cells because they significantly increase the expression of the *MRP-2* gene under the influence of bee venom and melittin. MRP-2 as an export pump acts by eliminating agents such as cisplatin exclusively in the presence of glutathione (Evers et al., 2000). In cells which they do not have enough glutathione, only some organic anions can be eliminated by MRP-2 export pump. MRP-1 is a more efficient transporter for glutathione conjugates, while MRP-2 is more effective in secreting pharmacological organic anions such as antibiotics and diuretics (Bakos et al., 2000). Bee venom and this components like melittin, are an organic substance, and in this case, it can be seen as a substrate for MRP-2 in HCT-116 cells.

Based on these results, it can be concluded that further detailed studies are necessary regarding the mechanism of action of the bee venom and its most common component, melittin. They are a

strong cytotoxins with a favorable proapoptotic effect and less likely to develop the resistance of colorectal cancer cells to investigated treatments, considering the effects on biotransformation genes.

Acknowledgments

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Declaration of interest statement

There is no conflict of interests.

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Figure captions

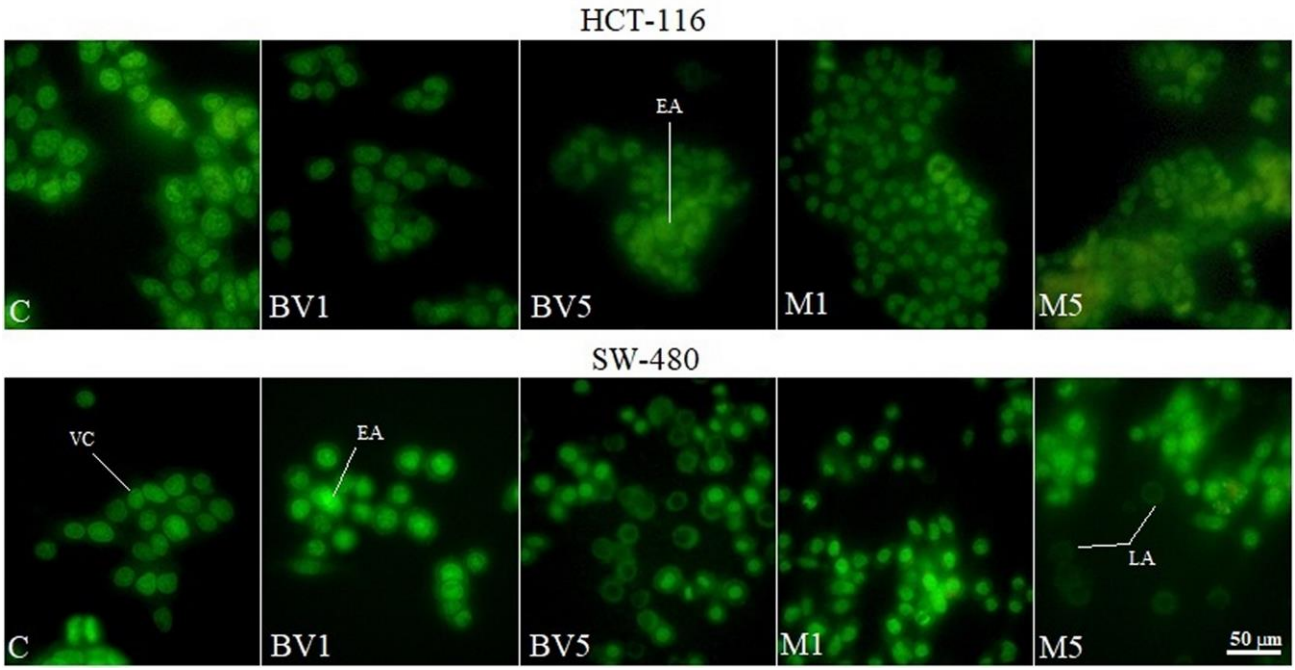


Figure 1. Morphology of HCT-116 and SW-480 control (C) and cells treated by bee venom (BV) and melittin (M) in concentration of 1 and 5 μg/ml, detected by AO/EB double staining, 24 h after treatments.

Micrographs were taken on a fluorescence microscope, magnification 400x. VC – viable cells, EA – Early Apoptosis; LA -Late Apoptosis; N – Necrosis

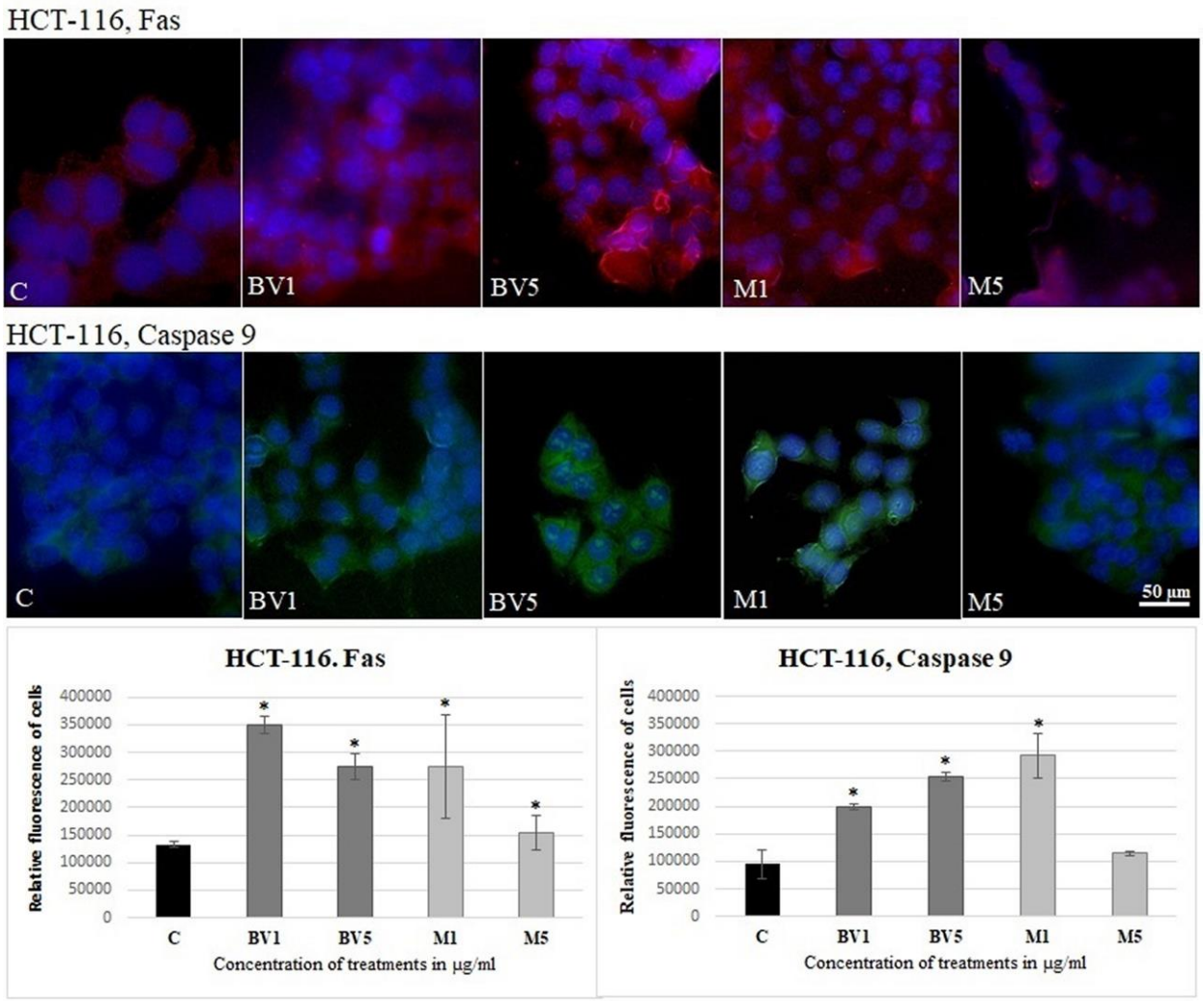


Figure 2. Protein expression of Fas receptor and Caspase 9 on HCT-116 control (C) and cells treated with bee venom (BV) and melittin (M) in concentration of 1 and 5 µg/ml, 24 h after treatments.

The cells are visualized using inverted fluorescent microscope (Nikon Ti-Eclipse) at 600x magnification. The nuclei are colored blue with DAPI color, Fas receptors are red (secondary antibody conjugated to Cy3) and caspase 9 are green (secondary antibody conjugated to

Alexa488). The relative intensity of fluorescence in control and treated cells was measured by ImageJ program. *Statistically significant difference ($p < 0.05$) in relation to control values.

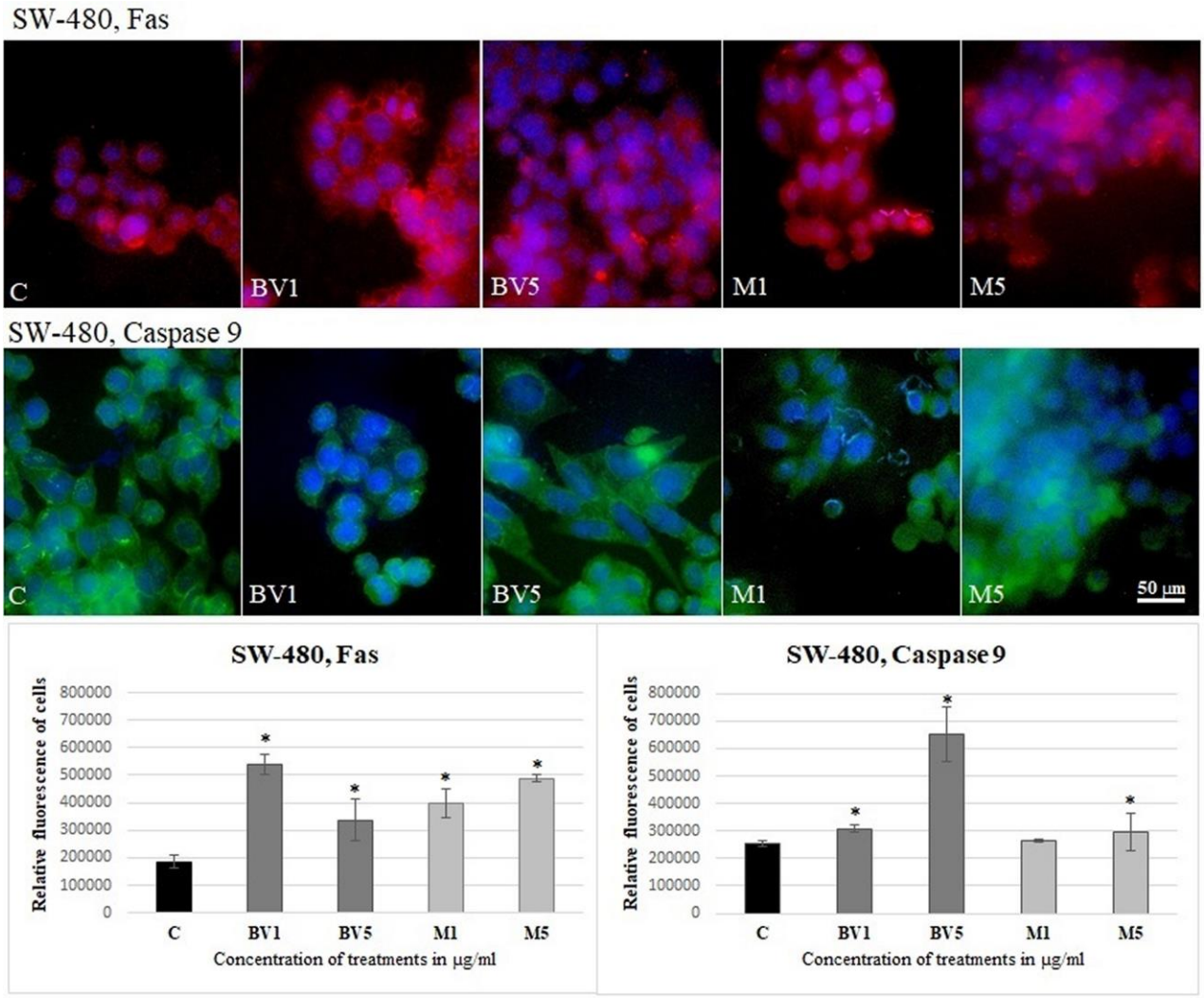


Figure 3. Protein expression of Fas receptor and Caspase 9 on SW-480 control (C) and cells treated with bee venom (BV) and melittin (M) in concentration of 1 and 5 µg/ml, 24 h after treatments.

The cells are visualized using inverted fluorescent microscope (Nikon Ti-Eclipse) at 600x magnification. The nuclei are colored blue with DAPI color, Fas receptors are red (secondary

antibody conjugated to Cy3) and caspase 9 are green (secondary antibody conjugated to Alexa488). The relative intensity of fluorescence in control and treated cells was measured by ImageJ program. *Statistically significant difference ($p < 0.05$) in relation to control values.

Supplementary material

Table 1. Specific human gene primers.

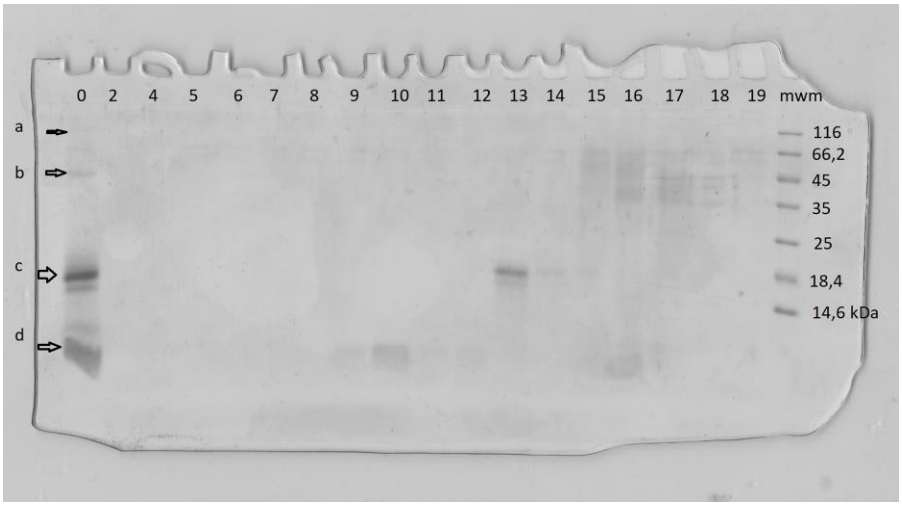
Genes	Forward sequence	Reverse sequence
β-actin	5'-AAGCAGGAGTATGACGAGTCCG-3'	5'-GCCTTCATACATCTCAAGTTGG-3'
18S rRNA	5'-TGAGAAACGGCTACCACATC-3'	5'-TTACAGGGCCTCGAAAGAGT-3'
GAPDH	5'-CTTCCCGTTCTCAGCCTTGA-3'	5'-GCTGAGACACCATGGGGAAG-3'
Bax	5'-GGACGAACTGGACAGTAACATGG-3'	5'-GCAAAGTAGAAAAGGGCGACAAC-3'
Bcl-2	5'-CTACGAGTGGGATGCGGGAGATG-3'	5'-GGTTCAGGTACTCAGTCATCCACAG-3'
CYP1A1	5-TAGACACTGATCTGGCTGCAG-3'	5'-GGGAAGGCTCCATCAGCATC-3'
GSTP1	5'-TCAAAGCCTCCTGCCTATAC -3'	5'-AGGTGACGCAGGATGGTATT-3'
MRP2	5'-ATACCAATCCAAGCCTCTAC-3'	5'-GAATTGTCACCCTGTAAGAG-3'

Table 2. Ct values for mRNA expression of tested genes in HCT-116 and SW-480 cells treated with bee venom (BV).

				HCT-116				
BV (μg/ml)	***β-actin***	***18S RNA***	***GAPDH***	***Bax***	***Bcl-2***	***CYP1A1***	***GSTP1***	***MRP-2***
0	22.23±0.38	16.38±0.41	24.93±0.01	28.60±0.55	29.37±0.56	27.37±0.57	25.42±0.52	31.39±0.90
1	19.78±0.85	12.14±0.20	18.69±0.25	25.78±0.84	28.90±1.04	26.58±0.71	23.47±1.28	26.66±1.28
5	17.56±0.30	9.75±0.19	16.01±0.23	24.10±0.28	27.93±2.04	25.31±1.33	21.82±0.59	24.38±0.34
				SW-480				
0	24.27±0.83	19.85±1.05	25.78±0.27	28.61±0.53	28.79±0.85	26.75±0.53	26.98±0.90	32.99±0.05
1	16.19±0.40	9.74±0.10	16.22±0.27	22.28±0.40	27.67±2.15	24.73±1.17	20.98±0.74	30.48±0.55
5	19.13±0.25	16.73±0.26	18.66±0.64	26.17±0.50	28.82±0.90	26.18±0.70	24.47±0.47	31.97±0.71

The results are presented as the mean of two independent experiments ± standard error.

Figure 1. Electrophoresis of fractions obtained after separation on a C5 column (16% gel).



0-initial sample, 2-19 fractions obtained after separation, mwm- molecular weight markers, masses are in kilodaltons.

Figure 2. The effects of bee venom and melittin on HCT-116, SW-480 and HaCaT cell viabilities. The results are presented as the mean of three independent experiments ± standard error.