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Original Article
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Cytotoxic Effect of Bee (*A. mellifera*) Venom on Cancer Cell Lines

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Objectives: Nowadays cancer treatment is an important challenge in the medical world that needs better therapies. Many active secretions produced by insects such as honey bees used to discover new anticancer drugs. Bee venom (BV) has a potent anti inflammatory, anti cancer and tumor effects. The aim of present study is evaluation of anticancer effects induced by *Apis mellifera* venom (AmV) on cell Lines.
Methods: AmV was selected for study on cancer cell lines. Total protein, molecular weight and LD_{50} of crude venom were determined. Then, cells were grown in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum and 1% antibiotics. The A_{549}, HeLa and MDA-MB-231 cell Lines were exposed by different concentration of AmV. The morphology of cells was determined and cell viability was studed by MTT assay. Evaluation of cell death was determined by and DNA fragmentation.
Results: The results from MTT assay showed that 3.125 μg/mL of A_{549}, 12.5 for HeLa and 6.25 μg/mL of MDA-MB-231 killed 50% of cells ($p < 0.05$). Morphological analysis and the results from hoescht staining and DNA fragmentation indicated that cell death induced by AmV was significantly apoptosis.
Conclusion: The data showed that using lower dosage of AmV during treatment period cause inhibition of proliferation in time and dose dependant manner. Findings indicated that some ingredients of AmV have anticancer effects and with further investigation it can be used in production of anticancer drugs.

Keywords: cancer, bee venom, cell lines, MTT, apoptosis

INTRODUCTION

Cancer is one of the diseases that is associated with abnormal cell growth and in most cases can spread to other parts of the body and remains as one of the leading causes of mortality worldwide [1]. Estimates are that in 2018, 18.1 million new cases of cancer and 9.6 million deaths occured globally. According to the world health organization (WHO) reports, the number of new cancer cases will increase rapidly in the coming years [2].

Various therapies have been used for treating cancer such as chemotherapy, radiotherapy, immunotherapy and gene therapy [3]. In recent years, biotoxins such as snakes, scorpions and bee venoms have been used as cancer therapeutic agents [4]. In the meantime, bee venom (BV) are very important. BV, like many other complementary medicine approaches, has been used for thousands of years attempting to alleviate a range of diseases [5]. BV have been widely used in traditional medicine to relieve pain and to treat inflammatory diseases. Other potential bee venom-related treatments are currently under investigation [6].

Recently, BV is widely used to treat various cancers [7]. This venom contains various active protein compounds [8, 9]. BV is a mixture of biologically active compounds such as enzymes, peptides and amines [10]. The main enzyme present is phospholipase A_2 and the main peptide is mellitin. Approximately

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50% of BV is comprised of melittin, which has a profound neurohormonal and immunological effect on the body [11].

Peptides in BV containing melittin have attracted considerable attention for their potential use in cancer therapy. It seems that melittin, a powerful anticancer peptide might be the better choice than whole BV [12].

Melittin displays strong membrane-perturbing activity, which is responsible for its anti-microbial [13], antiviral [14], antifungal [15], and anticancer [16] activities. When several melittin molecules penetrate into the cell membrane, they break down the phospholipids leading to cell lysis. In fact, this peptide disintegrates the phospholipid and synthetic bilayers [17, 18]. It has been reported that it can induce apoptosis and has antitumor effects. Melittin can target different cancer cell types such as kidney, liver, lung, prostate, and bladder cancers, as well as breast carcinoma and leukemia [9].

Antitumor compounds induce apoptosis in cancer cells; therefore, apoptosis plays an important role in preventing tumor progression. Consequently, BV can help treat cancer through inducing apoptosis in some cell lines [18].

Due to the disadvantages of the existing therapeutic methods, it is felt more than ever to use new compounds and methods, especially natural compounds, to control and treat cancer. That is why in the present research, cytotoxic effect of AmV on cancer cell lines was investigated.

MATERIALS AND METHODS

1. Collection and preparation of AmV

A. mellifera venom (AmV) was obtained from a beekeeping farm in Ardestan, Isfahan province, Iran. The extracted venom was lyophilized and stored in –20℃ until use. In order to prepare a stock solution of the venom, 1 mg of AmV was dissolved in 1 mL of phosphate-buffered saline (PBS). After vertex, centrifugation (15,000 g, 5 min) was performed at 25℃. The supernatant filtered through a 0.2 μ membrane filter and stored in darkness at –40℃.

2. Determination of protein concentrations and molecular weight

Protein concentration of AmV was determined according to the method described recently [19]. The approximate molecular weight of the AmV was determined by 12% SDS-PAGE and subsequent staining with coomassie blue [20].

3. Determination of lethal dose

An approximate lethal dose 50 (LD_{50}) of the AmV was determined in 30 NIH mice (18 to 20 g). LD_{50} of AmV was determined according to the method described recently [19]. Five doses (1, 1.5, 2, 2.5 and 3 mg/kg) were injected into two groups of mice for the determination of LD_{50} of the combination starting from 0% to 100% mortality. The percentage of mortality was calculated using the method of Spearman-Karber [21].

4. Cell culture

The A_{549} (ATCC: CCL-185^{TM}), HeLa (ATCC: CCL-2^{TM}) and MDA-MB-231 (ATCC: HTB-26^{TM}) cell Lines were prepared. Cells were cultured in Dulbecco's Modified Eagle (DMEM) medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and kanamycin/neomycin (100 U/mL). Cell cultures were then maintained at 37℃ in a 95% humidified with 5% CO_2 [22].

5. Cell viability assay

To determine the cell number, The A_{549}, HeLa and MDA-MB-231 cells were plated in 16-well plates (5×10^4 cells/well), and subconfluent cells were subsequently treated with AmV (1, 5, and 10 mg/mL) or melittin (0.5, 1, and 2.5 mg/mL) for 24, 48, and 72 h. After treatment, cells were trypsinized and pelleted by centrifugation for 5 min at 200 g, resuspended in PBS (10 mL), and 0.2% trypan blue (0.1 mL) was added to the cancer cell suspension in each of the solutions (0.9 mL each). Subsequently, a drop of suspension was placed into a Neubauer chamber and the living cancer cells were counted. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable. Each assay was carried out in triplicate [22].

6. Cytotoxic effect

AmV cytotoxic effect on A_{549}, HeLa and MDA-MB-231 cell Lines, were determined according to MTT assay [9]. The cells were plated in 96-well plates at a density of 40,000 cells/well in 10% FBS supplemented DMEM. The plates were incubated at 37℃, 5% CO_2 and 95% humidity for 12 h. The medium

was then carefully removed and venom concentrations (1.56, 3.12, 6.25, 12.5, 25 and 50 μg/mL) was added. Incubation was performed for 24 and 48 h. After incubation, 100 μL of MTT reagent (5 mg/mL in physiological solution) added to each well in the dark condition and incubation continued for 4 h. After this time, the supernatant was removed and DMSO (100 μL) was added to each well to dissolve formazan salt. Absorbance measured at 570 nm and viability Percent calculated as follows:

Viability of cells = test absorbance / control absorbance × 100

7. Morphological and apoptosis analysis

The cells were seeded at density of 5×10^5 cells/well in 12-well plates containing RPMI-1640 supplemented with 10% FBS and incubated for 12 h. Then the medium was removed, treated with 1, 1.8 and 4 μg/mL AmV (diluted in FBS free medium), and incubation continued for 12 h. The cells were observed by an invert microscope (INV100-FL, BEL-Italy) equipped with phase-contrast lens and finally morphological events compared with untreated cells [9]. Detection of apoptosis was done as described elsewhere [22, 23].

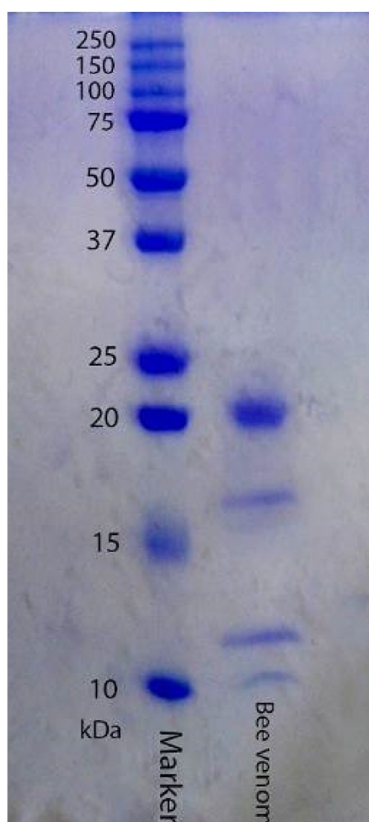

Figure 1. SDS-PAGE of BV.

8. Data analysis

Data were analyzed using SPSS software (Version 21). Data are presented as mean ± SE. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

1. Protein concentration, SDS-PAGE of AmV

The amount of protein in crude AmV solution was 1.48 mg/mL. The molecular weight of AmV proteins was estimated at a range of 10-22 kDa. Crude AmV is composed four protein bands with molecular weights of 10, 12, 16 and 21 kDa, respectively (Fig. 1).

2. Crude AmV LD_{50}

LD_{50} values using the Probit method was measured (Fig. 2). The AmV LD_{50} was found to be 208 μg/mouse.

3. The cytotoxic effect of AmV using MTT method

A: A_{549} cells: Cell viability decreased with increasing doses of AmV. This venom inhibited the proliferation of A_{549}, MDA-MB-231 and HeLa cell lines in a different concentration of AmV. IC_{50} (The half maximal inhibitory concentration of AmV) in 24, and 48 h were 3.125 and 2.5 μg/mL (A_{549}), 6.25 and 3.125 μg/mL (MDA-MB-231) and 12.5 and 6.25 μg/mL (HeLa) re-

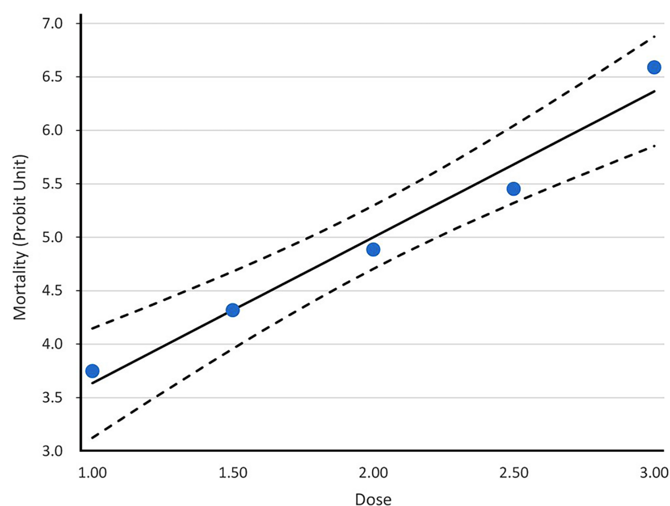

Figure 2. LD_{50} values using the Probit method.

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spectively. The results of cytotoxic effect for A_{549}, MDA-MB-231 and HeLa cell lines are shown in Table 1 and Fig. 3.

4. Morphological and apoptosis analysis

A significant difference in morphology between A_{549}, MDA-MB-231 and HeLa cells treated with AmV and control cells was observed (Figs. 4-9). Cell shrinkage, irregularity in cellular shape, cellular detachment and serious damage to cell membrane were observed in the AmV-treated cultures, but not in the control.

DISCUSSION

Old texts show that for years physicians used animal venom to treat diseases. Extensive studies on inhibition of tumor cell proliferation and metastasis as well as induction of apoptosis by BV give promise of more effective treatment of human tumor types. The results of the present study showed that treatment of three cell lines with different concentrations of BV inhibited proliferation of the cells and induced apoptosis in a dose- and time-dependent manner. This dependence means that increasing melittin concentration and treatment duration can improve inhibition of cell growth [24].

The highest death cell was observed in A_{549} cell line treated with BV, while the lowest one was occurred with HeLa cell line. The results of this study were consistent with El-Bassiony et al [25]. Thier results indicated that the studied BV can be used as effective anticancer agent.

Morphological examination of cells with an inverted microscope revealed a major feature of apoptotic cells, *i.e.* the condensed nucleus, and flow cytometry proved that BV induced cell death through apoptosis. In agreement with these results, Jang et al. reported that BV was capable of inducing apoptosis and inhibiting cyclooxygenase (COX-2) expression in the human lung cancer cell line NCI-H1299. They showed that a certain concentration of BV was able to fragment DNA, following activation of endonucleases, and to induce apoptosis-related morphological changes [26].

Mohseni et al. examined the effects of BV on normal human lymphocytes and the human HL-60 leukemia cell line. They reported that whole BV had selective cytotoxic effects on normal and cancer cells. Using this compound in a dose-dependent manner for up to 24 hours reduced the survival of HL-60 cells. Similar effects were observed in the present study [27].

Tu et al. investigated the effects of BV on the human melanoma cell line A2058. They acknowledged that BV, in a calcium-dependent and caspase-independent pathway, induced apoptosis in this cell line, whereas it did not have such effect on normal Detroit 551 human fibroblast cells [28].

In study of Liu et al., the IC_{50} for melittin on MHCC97L cells, MHCC97H cells, Rac1-DA-transfected MHCC97H cells and Rac1-DN-transfected MHCC97H cells were 9.24, 4.06, 3.83 and 25.69 μg/mL respectively [29]. These studies reported the toxicity of melittin on higher concentration but in the present study, MTT assay showed that melittin kills cancerous cells at IC_{50} of 3.125 and 2.5 μg/mL (A_{549}), 6.25 and 3.125 μg/mL (MDA-MB-231) and 12.5 and 6.25 μg/mL (HeLa) during 24, and 48 h respectively. Morphological effects of melittin on HeLa cells observed at IC_{50} concentration. Morphological alterations increased with increasing in melittin concentration and were in accordance to MTT results.

It is noteworthy that some studies have proved that melittin has hemolytic activity [9, 30]. Therefore, one can say that he-

Table 1. A_{549} viability with different concentrations of AmV using MTT method

Cell lines	Concentrations of AmV (μg/mL)	Cell viability (%), 24 h	Cell viability (%), 48 h
A_{549}	Control	100 ± 0	100 ± 0
	1.56	82.8 ± 0.015	59.1 ± 0.005
	3.125	56.0 ± 0.015	42.7 ± 0.004
	6.25	37.5 ± 0.011	23.0 ± 0.004
	12.5	23.1 ± 0.009	17.3 ± 0.004
	25	17.1 ± 0.004	14.5 ± 0.004
	50	14.8 ± 0	11.2 ± 0.007
MDA-MB-231 cells	Control	100 ± 0	100 ± 0
	1.56	78.0 ± 0.039	62.0 ± 0.006
	3.125	65.8 ± 0.016	52.2 ± 0.010
	6.25	49.6 ± 0.019	39.2 ± 0.010
	12.5	35.4 ± 0.019	26.1 ± 0.010
	25	30.3 ± 0.012	22.8 ± 0.006
	50	21.9 ± 0.007	13.7 ± 0.006
HeLa	Control	100 ± 0	100 ± 0
	1.56	86.3 ± 0.015	70.0 ± 0.005
	3.125	71.7 ± 0.015	62.3 ± 0.004
	6.25	63.2 ± 0.011	46.1 ± 0.004
	12.5	41.8 ± 0.009	35.0 ± 0.004
	25	30.7 ± 0.004	27.3 ± 0.004
	50	18.8 ± 0	18.8 ± 0.007

Figure 3. A_{549}, MDA-MB-231 and HeLa viability with different concentrations of AmV using MTT method (Mean ± SEM, ***p < 0.001).

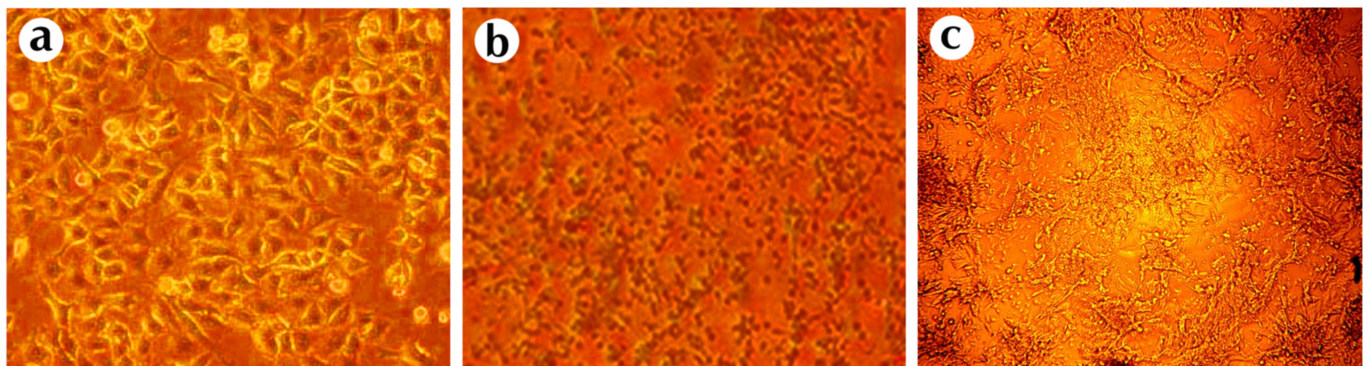

Figure 4. A_{549} cell morphology treated with AmV. (A) Control; (B) 3.125; (C) 12.5 μg/mL of AmV.

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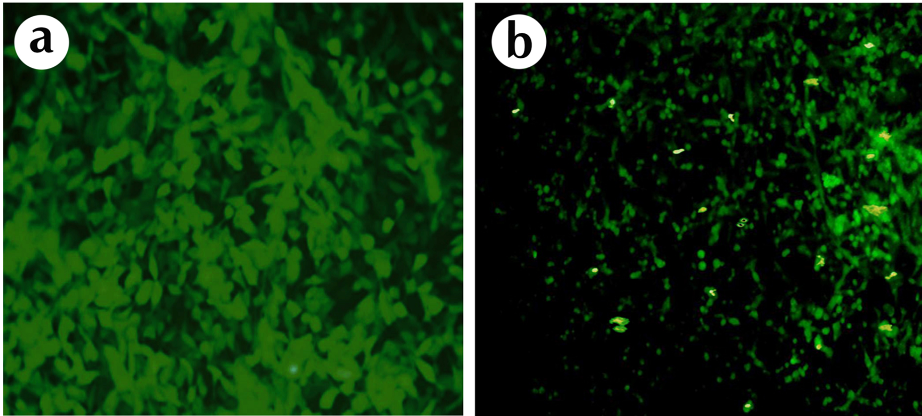

Figure 5. A_{549} cell morphology treated with AmV. (A) Control; (B) 3.125 μg/mL of AmV.

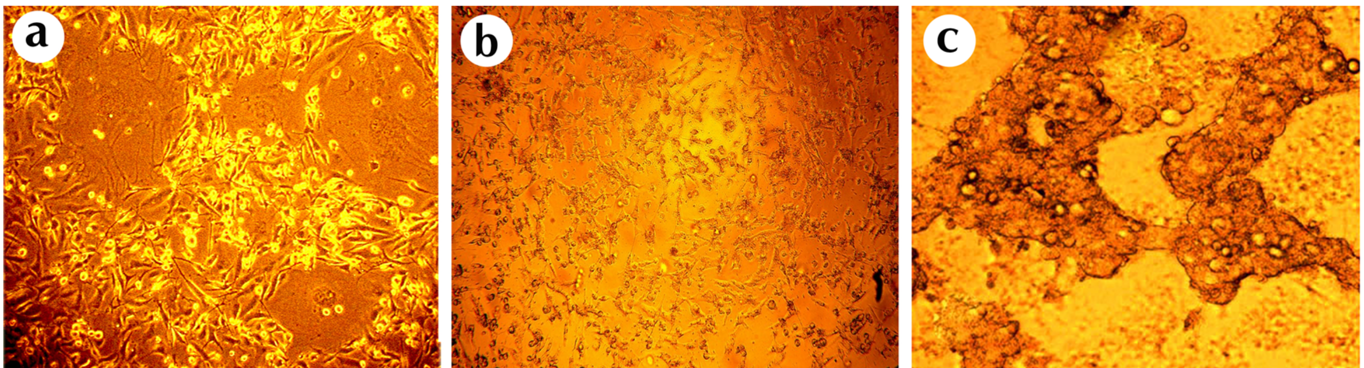

Figure 6. MDA-MB-231 cell morphology treated with AmV. (A) Control; (B) 6.25; (C) 12.5 μg/mL of AmV.

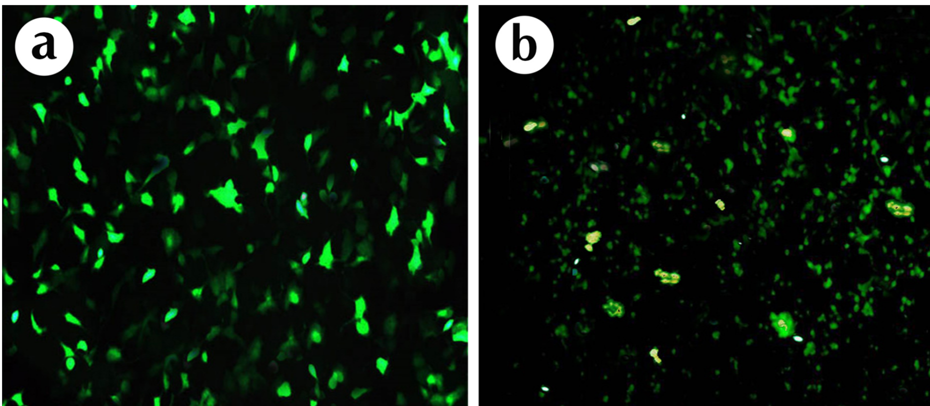

Figure 7. MDA-MB-231 cell morphology treated with AmV. (A) Control; (B) 6.25 μg/mL of AmV.

molytic activity of melittin is the main obstacle to its use in cancer treatment. Consequently, when designing cancer treatment using melittin, it should be taken into consideration that introduction of melittin into the bloodstream can damage red blood cells. Therefore, it may be more appropriate to treat cancer using melittin through gene therapy (transfer of the melittin gene to cancer cells) or by specific delivery of melittin to cancer cells (for example, use of liposomes as a melittin delivery vehicle).

Most of the drugs used in chemotherapy induce apoptosis in cancer cells and, according to the above reports, BV (and especially melittin) seems to be an appropriate option for cancer treatment. Finally, it must be said that the use of BV, which has fewer side effects than chemical drugs, will cause a revolution in the human struggle against cancer.

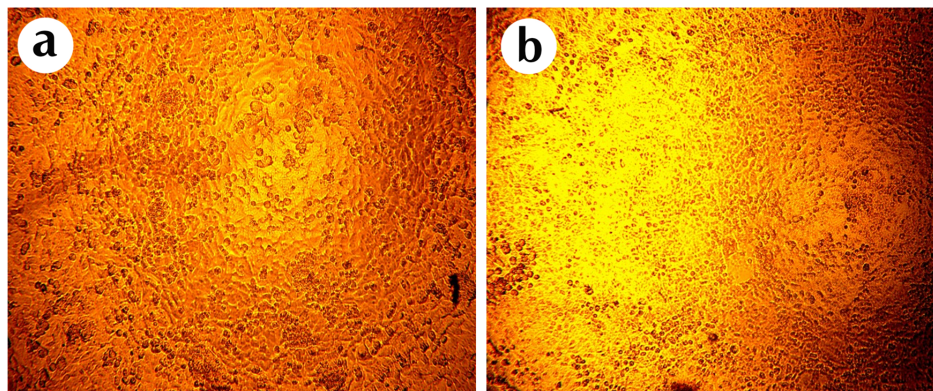

Figure 8. HeLa cell morphology treated with AmV. (A) Control; (B) 12.5 μg/mL of AmV.

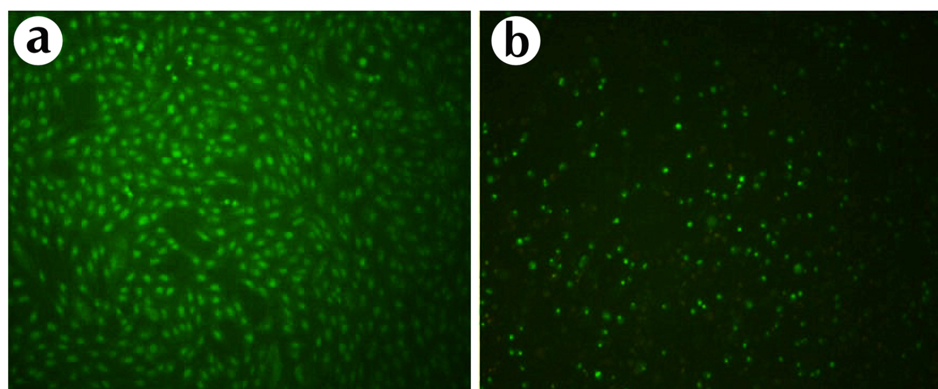

Figure 9. HeLa cell morphology treated with AmV. (A) Control; (B) 12.5 μg/mL of AmV.

CONCLUSION

Our results provide evidence that BV and its main constituents, especially melittin, may be developed as potential therapeutic agents for cancer metastasis. We assessed that BV as a natural product has cytotoxic effects on the A_{549}, HeLa and MDA-MB-231 cell Lines. It will be an excellent perspective to innovate approaches to prevent and treat some features of cancer.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

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