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Original Article

First report on the isolation of melittin from Iranian honey bee venom and evaluation of its toxicity on gastric cancer AGS cells

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Abstract

Background: It has been previously reported that melittin, the main ingredient of honey bee venom, has anticancer properties. However, there appears to be no earlier study focusing on the isolation of melittin from Iranian honey bee venom (*Apis mellifera meda*), and evaluation of its effect on cancerous cells.
Methods: We isolated melittin using reversed-phase high performance liquid chromatography, and its potential toxicity on gastric cancer AGS cells was determined with an MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Furthermore, to ascertain whether melittin induces apoptosis or necrosis in these cells, morphological evaluation, DNA fragmentation assay, propidium podide and annexin-V-FITC dual staining, and flow cytometric analysis were also conducted.
Results: The results of our study suggested that melittin inhibited the proliferation of AGS cells in a dose and time-dependent trend. All of the above four distinct assays indicated that melittin induces necrosis in AGS cells at concentrations of $\geq$ 1 μg/mL.
Conclusion: The present study indicated that melittin has an anticancer effect on gastric cancer AGS cells and stimulates necrotic cell death in these cells.

Keywords: Apis mellifera meda; apoptosis; gastric cancer; melittin; necrosis

1. Introduction

Gastric cancer (GC) is one of the most common cancers in the world, in that ~1 million new cases of GC and > 700,000 deaths (12% of all cancer related deaths) are reported each year.[1] Unfortunately, current strategies in GC therapy are most of the time essentially ineffective.[2] Regarding this matter, there is an urgent need for the global medical community to conceive novel strategies to be used in the fight against this lethal disease. Various natural substances have been recognized as having anticancer properties.[3,4] One of these natural products is bee venom (BV) and its principal ingredient, melittin.[5]

Melittin ($C_{131}H_{229}N_{39}O_{31}$) is a cationic 26 amino acid residue long peptide and is reported to have strong hemolytic activity.[6] The first 20 residues (N-terminal) of the structure of melittin are predominantly hydrophobic amino acids, whereas the carboxyl-terminal of the peptide is mostly composed of hydrophilic residues.[7] This amphipathic entity allows the peptide to interact with phospholipid membranes.[8]

The effect of melittin on various cancer cell lines has been investigated broadly and its inhibitory effect on the proliferation of these cells has been demonstrated. In addition, it has been

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

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evaluated whether melittin exhibits apoptotic or necrotic cell death in cancerous cells. Induction of apoptosis by melittin or BV has been reported in human lung cancer,[9] rheumatoid arthritis,[10] hepatocellular carcinoma,[11] human leukemic cells,[12] human breast cancer,[13] prostate,[14] and ovarian cancer cells.[15] It seems that melittin-induced apoptosis is mediated through the activation of the caspase-dependent pathway.[4] However, other studies have shown that melittin exhibits necrotic cytotoxicity in rat thymocytes,[16] murine skeletal muscles,[17] erythrocytes[18], lymphocytes,[19] lymphoblastoid cells,[20] and Caco-2 cells.[21] Identification of the precise mechanism of the effect of melittin on cancerous cells plays an important role in the future therapeutic application of this interesting peptide.

Apis mellifera meda (A. m. meda) is smaller than the European honey bee, and produces less honey and is more aggressive.[22] European honey BV has been studied extensively.[23,24] However, there are no published reports regarding the antiproliferative activity of *A. m. meda* venom components, especially melittin, on human gastric adenocarcinoma cell line (AGS) cells. The present study aimed to isolate melittin from Iranian honey BV and to evaluate its inhibitory effect on the proliferation of the gastric cancer AGS cell line.

2. Methods

2.1. Chemicals

We purchased 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide, triflouroacetic acid (TFA), acetonitrile, and melittin from Sigma–Aldrich (St. Louis, MO, USA). Annexin V-FITC Apoptosis Detection Kit (K-101-100) was obtained from Bio Vision (Mountain View, CA, USA). The materials that were used in the cell culture included RPMI-1640, fetal bovine serum (FBS), and antibiotics, and were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Bees

A hive of Iranian honey bees were selected and checked for disease and race. This hive was located in a bee keeping farm in the Koohrang region, Chaharmahal and Bakhtiari, a South-West province of Iran.

2.3. Bee venom collection

Honey BV was collected according to the electrical stimulation method[25] with a slight modification. Briefly, the wired glass plate of the venom collector was placed inside (top) of the hive. When bees covered the surface of the wired glass plate, the wires were electrified and a mild shock was applied to the bees for 10 seconds and then the current stopped for 20 seconds. In this method, the bees sting the surface of the glass plate in response to the electrical stimulation. Secreted venom from the bees' sting dried rapidly exposed to the air. Dried venom was scraped off with a sharp scalpel and transferred to the laboratory.

2.4. Venom profiling and isolation of melittin

Collected venom was dissolved in 1 mL ultra-pure water and then centrifuged at 16,000*g* for 10 minutes at room temperature. Soluble supernatant was filtered through a 0.2 μm membrane filter and stored at −20°C. In order to facilitate venom profiling and melittin isolation a high performance liquid chromatography (HPLC) system (Dr. Ing. Herbert Knauer GmbH, Berlin, Germany) was used. The HPLC system consisted of the following instruments: pump K-1000, UV detector 2550, manual injector with 20 μL loop, a computer system with ChromGate software, and a C18 column (Knauer, Eurosfer-100 C18, 250 mm × 4.6 mm). TFA/Water (1:1000) and TFA/ACN (1:1000) were used as solvent A and B, respectively. Flow rate was established as 1 mL/min in all of the separation processes. A linear gradient of 0–60% solvent B was applied for 55 minutes and the column elute monitored at 214 nm wavelength. The retention time of the isolated melittin was compared with an external standard (Sigma-Aldrich). The purity of the isolated melittin was evaluated with the same HPLC method. The melittin fraction was manually collected and lyophilized in a freeze dryer (Christ 2 alpha, Osterode am Harz, Germany).

2.5. Hemolytic activity

A hemolytic activity assay was performed according to the previously described method with slight modification.[26] Briefly, 2% red blood cells (RBC) suspension were transferred to a 96-well plate (100 μL per well). The RBCs were treated with melittin at final concentrations of 8 μg/well, 4 μg/well, 2 μg/well, 1 μg/well, 0.5 μg/well, 0.25 μg/well, 0.125 μg/well, and 0.0625 μg/well. The amount of 100 μL of supernatant from each well were transferred gently to a new 96-well plate and the optical density of released hemoglobin was measured at 540 nm with a microplate spectrophotometer (Epoch, BioTek, Luzern, Switzerland). The data were compared of a positive control (100 μL RBC and 100 μL Triton X-100 1%) and a negative control (100 μL RBC and 100 μL phosphate buffered saline), and all experiments were performed in triplicate. The percent of hemolysis was calculated as follows:

$$\text{Hemolysis percent} = [\text{OD}(\text{optical density})_{540(\text{Sample})} - \text{OD}_{540(\text{Neg.control})}]/(\text{OD}_{540(\text{Pos.control})} - \text{OD}_{540(\text{Neg·control})}) \times 100$$

2.6. Cell culture

AGS cell line was purchased from the Pasteur Institute of Iran Cell Bank (Tehran, Iran). AGS cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin 100 unit/mL, and streptomycin 100 μg/mL. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

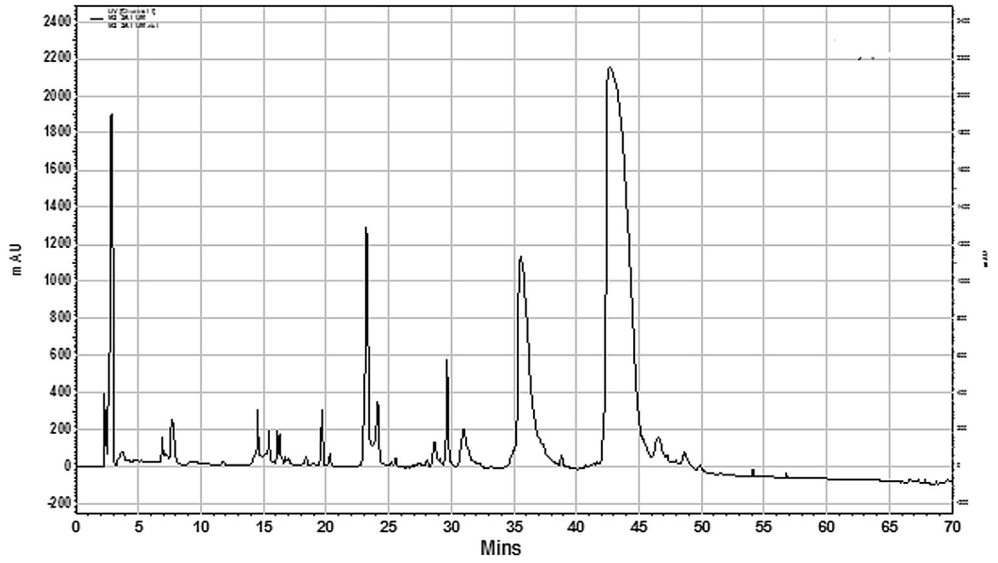

Fig. 1. Iranian honeybee venom chromatogram. A linear gradient method with 0–60% gradient of Solvent B for 55 minutes was applied and column eluates were monitored at 214 nm wavelength. Reverse phase-high performance liquid chromatography was performed using a C18 column (Knauer, Eurosfer-100 C18, 250 × 4.6 mm). Flow rate was 1 mL/min in the entire process.

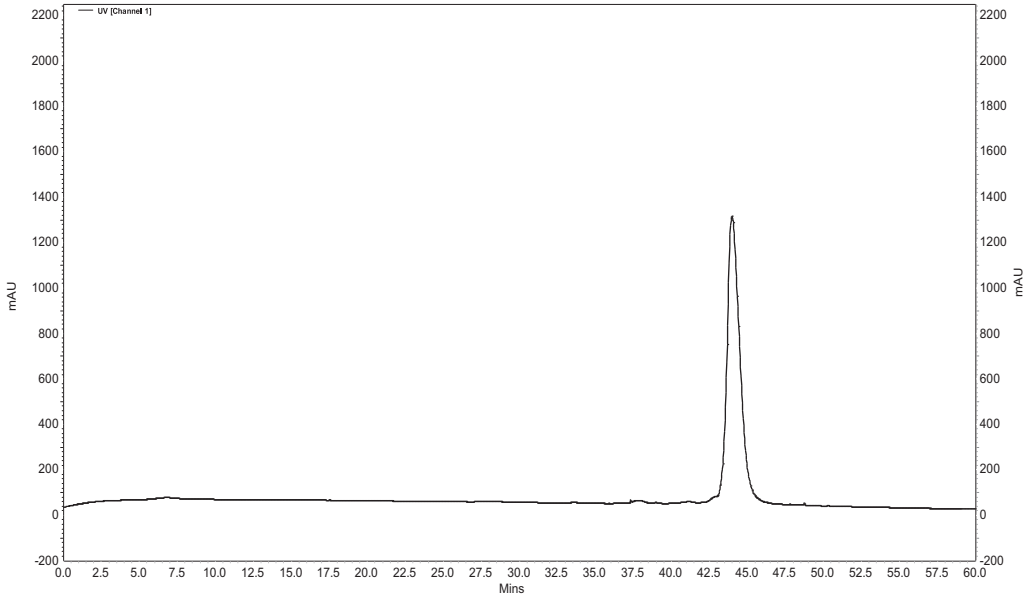

Fig. 2. Reverse phase-high performance liquid chromatography of mixed melittin from Iranian honey bee venom and a standard (Sigma) which shows similar retention times. This experiment was done to demonstrate that the purified melittin in this study contains the same retention time as a standard one.

2.7. Cytotoxicity assay

AGS cells were plated overnight in 96-well plates at a density of 4.5×10^4 cells/well in 200 μL FBS supplemented medium. Then, the cells were treated with melittin (diluted in FBS free medium) at final concentrations of 8 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL, 0.5 μg/mL, and 0.25 μg/mL and incubated for 6 hours, 12 hours, and 24 hours. Four hours before ending the incubation time, 20 μL MTT salt (2.5 mg/mL) were added to each well and plates were returned to the incubator for completion of incubation time. At the end, plates were centrifuged (1500*g*, 10 minutes) and the supernatants were then removed. Thereafter, 100 μL dimethyl sulfoxide was added to each well in order to dissolve the formazan salt. Plates were shaken and the optical density of each well was measured at 570 nm with a microplate spectrophotometer (Epoch, BioTek, Luzern, Switzerland). Untreated cells were considered as the control group. The percentage of viability percent was calculated as follows:

$$\text{Viability percent} = \left[\left(OD_{570(\text{Melittin treated sample})} / OD_{570(\text{control})}\right)\right] \times 100.$$

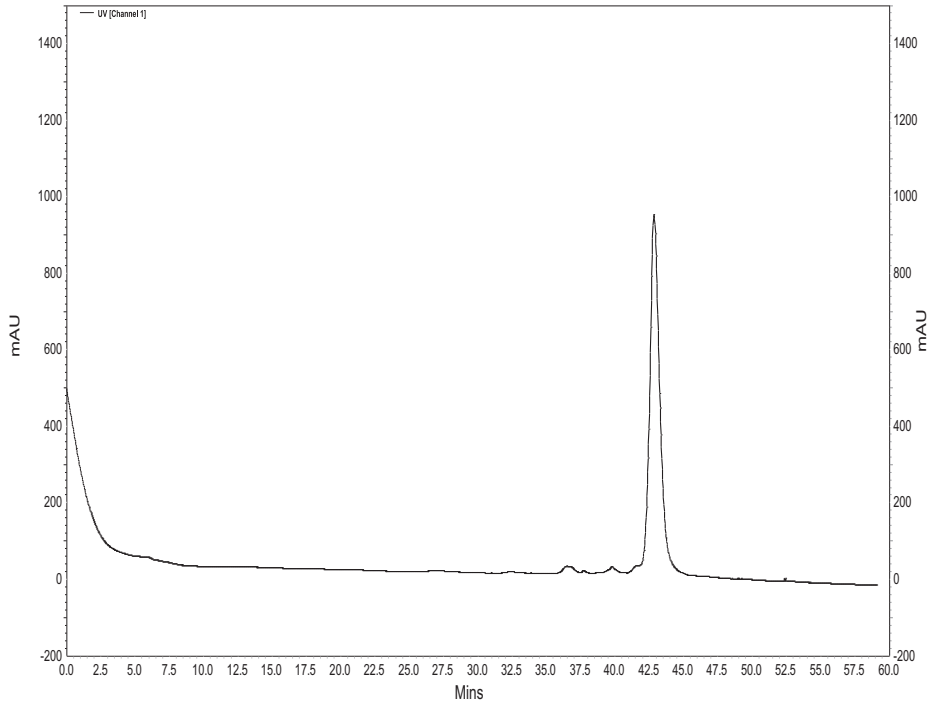

Fig. 3. Chromatogram for controlling the purity of isolated melittin. Isolated melittin freeze-dried and subjected to reverse phase-high performance liquid chromatography with the same method as Fig. 2. Retention time is shown at 42 minutes, the same as the external standard.

2.8. Morphological evaluation

In order to characterize melittin-induced cell death in AGS cells, treated cells with 2 μg/mL, 1 μg/mL, and 0.5 μg/mL melittin for 12 hours were observed using an inverted microscope with phase contrast objectives (INV100-FLBEL Engineering srl, Monza, Italy). Morphology of the treated cells was then compared with untreated cells.

Moreover, in a separate experiment, the cells were stained with propidium iodide (PI) and AnnexinV-FITC (AnV-FITC) and observed using an inverted fluorescence microscope (INV100-FL, BEL Engineering). The cells were plated in a 24-well plate at a density of 1×10^5 and treated with 2 μg/mL, 1 μg/mL, and 0.5 μg/mL concentrations of melittin for 12 hours. AGS cells were harvested and stained with equal volumes of PI and AnV-FITC and incubated for 5 minutes at room temperature in the dark. Then the cells were placed on a

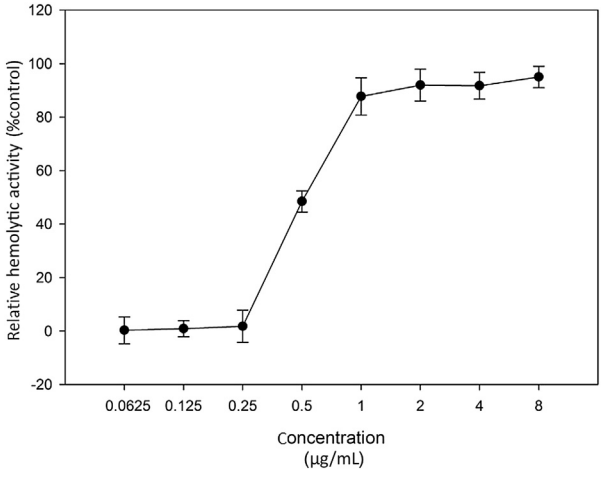

Fig. 4. Hemolytic activity assay. The percentage of hemolytic activity of melittin was determined in comparison with positive control (Triton X-100). Results are presented as mean ± standard deviation. Experiments were done in triplicate and the significance was determined using the Student *t* test ($p < 0.05$ vs. untreated control).

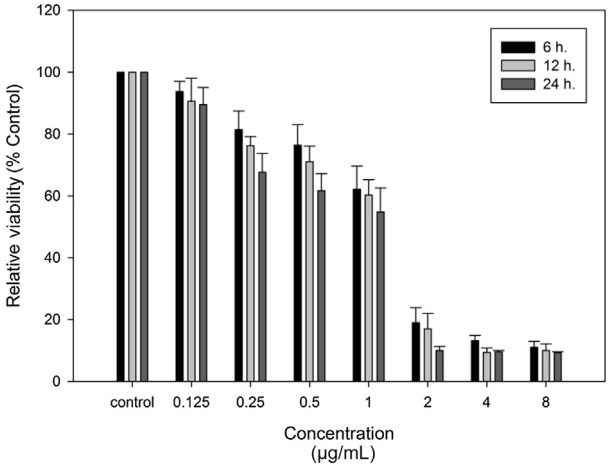

Fig. 5. Cytotoxicity of melittin on AGS cell line was determined via MTT assay. Results are presented as mean ± standard deviation. Experiments were done in triplicate and significance was determined using the Student *t* test ($p < 0.05$ vs. untreated control).

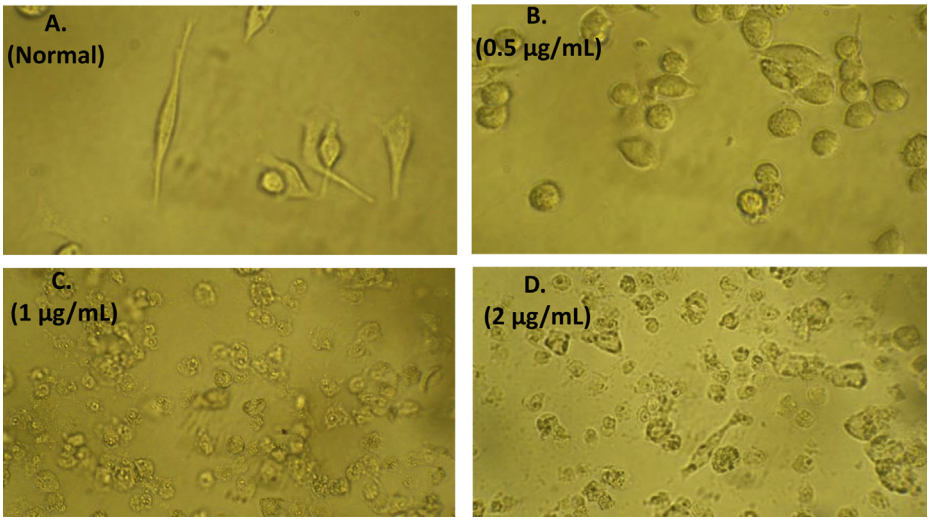

Fig. 6. Effect of isolated melittin from Iranian honey bee venom on morphology of AGS cell line. (A) Control; (B) cells were treated with 0.5 µg/mL melittin; (C) 1 µg/mL 1 melittin; and (D) 2 µg/mL melittin and observed using a phase-contrast microscope. Cell shrinkage, irregularity in cellular shape, cellular detachment and serious damage to cell membrane were observed in the melittin-treated cultures, but not in the control.

glass slide and covered with a cover slip. Finally, the samples were observed under an inverted fluorescence microscope using a green filter set for FITC and a blue filter set for PI.

2.9. DNA fragmentation assay

In order to detect apoptotic DNA cleavage, DNA fragmentation assay was performed. At first, 1×10^6 AGS cells were plated in each well of a 6-well plate and treated with 2 µg/mL, 1 µg/mL, and 0.5 µg/mL melittin for 12 hours. Then, the cells were harvested and lysed with lysis buffer (Tris 10mM, ethylenediaminetetraacetic acid 1mM, and *sodium dodecyl sulfate* 1%) and the lysates were incubated with 10 µL RNase A at 37°C for 30 minutes followed by treatment with 10 µL proteinase K (2 mg/mL) at 56°C for 1 hour.

DNA was precipitated by adding 5 µL potassium acetate and 30 µL ice cold absolute ethanol and then centrifuged (1500*g*, 10 minutes). Precipitated DNA was then washed in 70% ethanol and diluted in 50 µL Tris-EDTA buffer. Finally, the extracted DNA was analyzed with agarose gel electrophoresis (1.2%, containing ethidium bromide) at 5 V/cm for 1 hour. At the end, gel was visualized and photographed in a gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

2.10. Flow cytometric analysis

As morphological evaluation is a qualitative method, to analyze quantitatively the rates of necrosis and apoptosis induced by melittin, we performed flow cytometric analysis. Flow cytometric analysis was performed using an AnV-FITC Apoptosis Detection Kit (K-101-100, Bio Vision), according to the manufacturer's instruction. Briefly, cells were plated in 6-well plates at a density of 1×10^6 cells/well and treated with 0.5 µg/mL, 1 µg/mL, and 2 µg/mL melittin concentrations for 12 hours. Then, the cells were harvested and resuspended in 500 µL binding buffer in 1.5 mL micro tube. A total of 5 µL PI and 5 µL AnV-FITC were added to each well and placed at room temperature (RT) for 5 minutes in the dark. Finally, the samples were analyzed using a flow cytometer (CyFlowSL, Partec GmbH, Munster, Germany) at 488 nm. Histograms were plotted using Flomax software (Partec GmbH). The populations of viable (AnV-FITC$^-$, PI$^-$), apoptotic (AnV-FITC$^+$, PI$^-$), and necrotic (AnV-FITC$^-$; PI$^+$, and AnV-FITC$^+$; PI$^+$) cells are represented in the lower left, lower right and upper right, and upper left quadrants, respectively.

3. Results

3.1. Reverse phase-HPLC

In the current study, a reverse phase (RP)-HPLC system with a C18 column was used to facilitate venom profiling and isolation of melittin from Iranian honey BV. The column elutes were monitored at 214 nm wavelength, and a chromatogram of *A. m. meda* venom is presented in Fig. 1. In this chromatogram, > 100 different peaks are detectable; however, < 20 major peaks are obvious. Melittin eluted 42 minutes after injection of venom at 40% acetonitrile, which was close to the retention time of the external standard (melittin, sigma; Figs. 2 and 3). To control the purity of the isolated melittin, it was subjected to RP-HPLC using the same method (Fig. 3). The fraction of melittin was manually collected, lyophilized, and stored at 20°C to be used in subsequent biological assays.

3.2. Hemolytic activity

Hemolytic activity assay indicated that the purified melittin from *A. m. meda* venom showed a strong hemolytic activity. According to the results (Fig. 4), isolated melittin exhibited complete hemolytic activity at concentrations above 1 µg/mL. Melittin did not show considerable toxic effect at very low

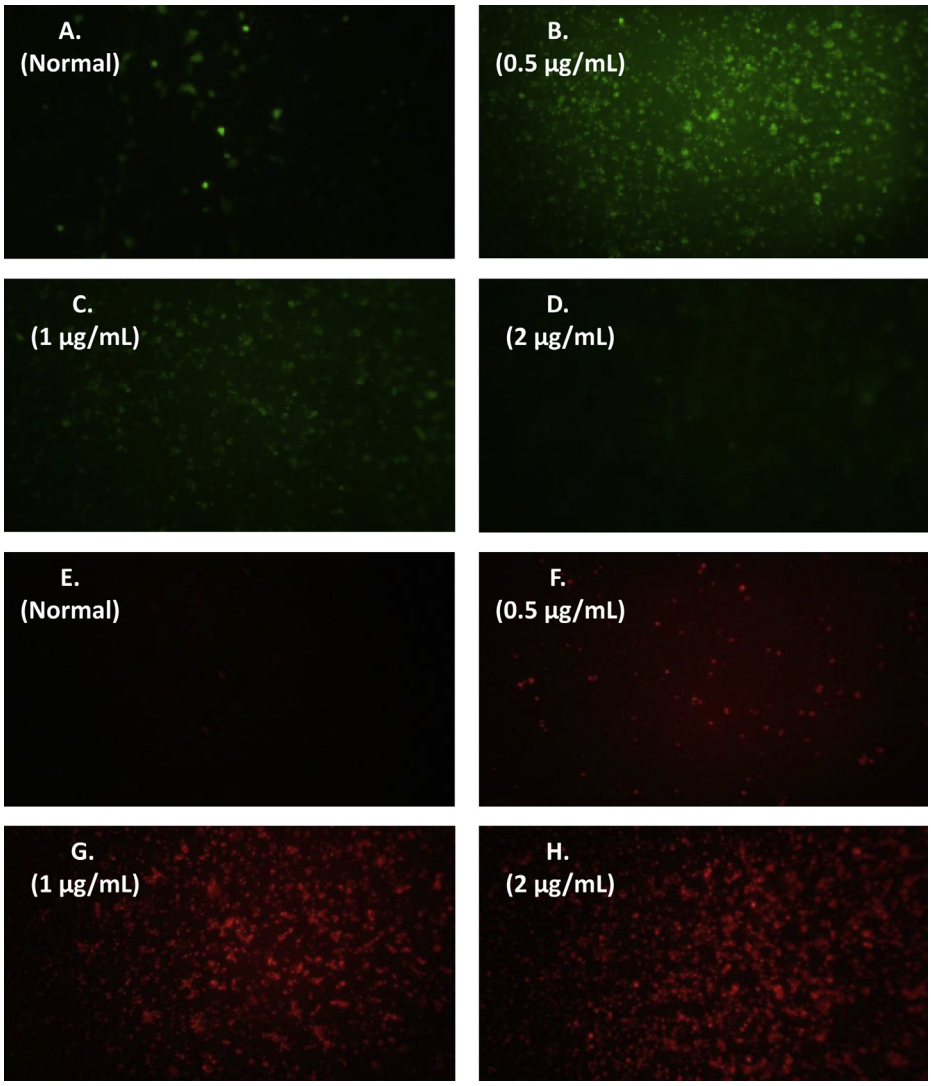

Fig. 7. Characterization of melittin induced cell death in AGS cells. (A) Normal cells; (B,F) cells were treated with 0.5 µg/mL melittin; (C,G) 1 µg/mL melittin; (E,H) 2µg/mL melittin and stained with annexin-V-FITC and propidium iodide-A. A fluorescent-invert microscope was used to observe the stained cells. No significant increase was observed in annexin-V-FITC stained cells (B–D) by increasing melittin concentration in comparison with control cells (A). Propidium iodide-A intensity was increased in higher melittin concentrations (F–H).

concentrations (0.0625 µg/mL and 0.25 µg/mL). The HD50 value, which is the concentration of melittin which shows 50% hemolytic activity of the positive control, was determined to be 0.5µg/mL.

3.3. Cytotoxicity assay

In order to determine the potential toxic effect of melittin on an AGS cell line, MTT assay was performed. Cells were plated in 96-plates and treated with melittin at final concentrations of 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, and 8 µg/mL for 6 hours, 12 hours, and 24 hours. The viability of AGS cells treated with melittin at the above-mentioned concentrations for 6 hours was 93.74 ± 3.29, 81.42 ± 5.98, 76.42 ± 6.64, l62.13 ± 7.49, 18.80 ± 4.85, 13.17 ± 1.72, and 11.05 ± 1.90, respectively. The viability of AGS cells treated with melittin at the above concentrations for 12 hours were 90.60 ± 7.39, 76.23 ± 3.00, 71.10 ± 5.00, 60.24 ± 4.60, 26.30 ± 5.00, 9.39 ± 1.37, and 10.02 ± 2.10, respectively. The viability of AGS cells treated with melittin at the above concentrations for 24 hours was 89.47 ± 5.56, 67.61 ± 6.09, 61.70 ± 5.44, 54.82 ± 7.70, 10 ± 1.29, 9.55 ± 0.44, and 9.32 ± 0.29, respectively.

These results showed that isolated melittin from *A. m. meda* venom has a cytotoxic effect on AGS cell lines (Fig. 5). Based on the results of the MTT assay, doses, and treatment times of melittin for use in subsequent experiments was set at 0.5 µg/mL, 1 µg/mL, and 2 µg/mL for 12 hours.

3.4. Morphological evaluations

To indicate the effect of melittin on AGS cells morphology, cells were treated with various concentrations of melittin

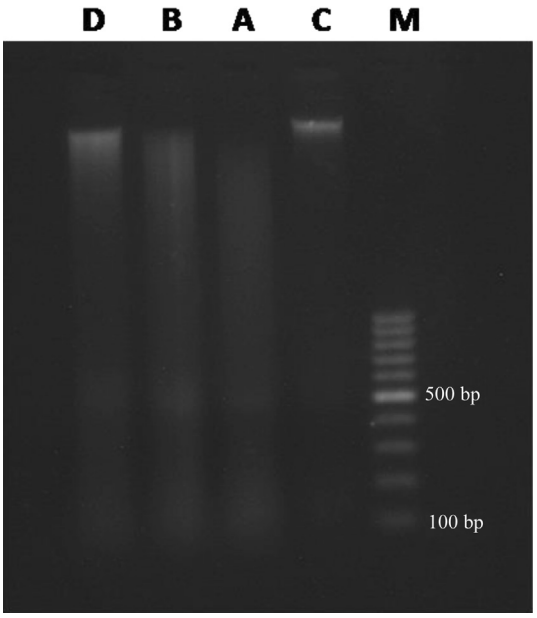

Fig. 8. DNA fragmentation assay. AGS cells were incubated for 12 hours without melittin and with 0.5 μg/mL, 1 μg/mL, and 2 μg/mL of melittin. Genomic DNA was extracted and analyzed with electrophoresis on a 1.2% agarose gel containing ethidium bromide. Melittin treated AGS cells did not show apoptotic DNA fragmentation pattern. A = 0.5 μg/mL; B = 1 μg/mL; C = control; D = 2 μg/mL of melittin; M = marker.

(0.5 μg/mL, 1 μg/mL, and 2 μg/mL) for 12 hours. After the desired treatment time (12 hours), for morphology, the images were taken with a microscope under 20 × magnification. As shown in Fig. 6, necrotic cell death hallmarks including loss of membrane integrity is obvious in the cells which are treated with 1 μg/mL and 2 μg/mL melittin. However, these signs were absent in the control group or less in the low treated (0.5 μg/mL) cells. These results suggest that melittin causes serious damage to AGS cell membranes leading to necrosis. Furthermore, in order to gather more precise information about the kind of melittin that induced death in AGS cells, PI/AnV-FITC staining was performed. Cells were treated with melittin (0.5 μg/mL, 1 μg/mL, and 2 μg/mL for 12 hours), and stained with PI and AnV-FITC dyes. Stained cells were observed with a fluorescent invert microscope.

As presented in Figs. 7B−7D, there is no significant increase in the intensity of AnV-FITC fluorescent in the cells treated with melittin. This means that translocation of the phosphatidylserine (an apoptotic hallmark) did not happen by treating the cells with melittin, significantly. By contrast, PI stained cells increased by an increase in the concentration of melittin (0.5 μg/mL, 1 μg/mL, and 2 μg/mL; Figs. 7F−7H). Normal cells are shown in Fig. 7A.

3.5. DNA fragmentation

To assert the induction of necrotic death with melittin in an AGS cell line, DNA fragmentation assay was performed. The genomic DNA was isolated from treated cells with melittin and untreated cells (as negative control) and analyzed with agarose gel electrophoresis. As shown in Fig. 8, treatment of AGS cells with melittin (0.5 μg/mL, 1 μg/mL, and 2 μg/mL for 12 hours) did not result in fragmentation of DNA. From the DNA fragmentation assay it seems that melittin exhibits necrotic cytotoxicity on AGS cells. However, additional studies need to be undertaken to provide additional evidence of the melittin's necrotic cytotoxicity in wider and more diverse patient populations.

3.6. Flow cytometric analysis

To gain a quantitative analysis of melittin-induced cell death in an AGS cell line, PI/An V-FITC dual staining was performed and analyzed using a flow cytometer. For this purpose untreated cells were used as negative control. As shown in Fig. 9A, 98% of the control population is viable (PI/An V-FITC negative) and an insignificant percent is apoptotic or dead (a total of 1.44%).

After treatment of AGS cells with 0.5 μg/mL melittin for 12 hours, the population divided into four different portions: 79.26% viable, 5.66% apoptotic, 2.58% late apoptotic or necrotic, and 12.51% necrotic cells (Fig. 9B). In treated cells with 1 μg/mL in comparison with previous concentrations, a significant increase in the percent of necrotic cells (30.45%) and in necrotic or late apoptotic cells (18.83%) were observed and viable cells decreased to 45% (Fig. 9C). However, no significant change was observed in the percent of apoptotic cells (5%). Finally, the percent of necrotic cells in 2 μg/mL melittin treated AGS cells increased to 58%. The percent of apoptotic cells in this concentration was just 0.62% (Fig. 9D). These data showed that melittin exhibits necrotic toxicity at 0.5 μg/mL, 1 μg/mL, and 2 μg/mL concentrations on AGS cells.

4. Discussion

GC is the fourth most common cancer in the world and is a disease with a prohibitively high death rate.[27,28] After lung cancer, it's the second leading cause of cancer-related deaths.[29] Surgery, chemotherapy, or radiation can be used in the treatment of GC. Sometimes during surgical intervention, the main treatment strategy is to remove the entire stomach in order to prevent the tumor from spreading. In fact, surgeons may remove a part of the intestine or pancreas in late diagnosed cases. Unfortunately, stomach cancer may respond very poorly to chemotherapy. Subsequent to surgery, if chemotherapy is used, it is often just to reduce the tumor size and symptoms. As there is no reliable therapeutic strategy, it is necessary to find new and more efficient approaches in GC therapy. During the past several years, melittin has been considered as an ideal candidate to be used in cancer therapy.[12] Melittin is a powerful cationic antimicrobial peptide.[30] It comprises 40−50% of the dried venom and causes disruption of normal cellular activity and cell lysis.[31]

BV has been used as a pain killer and antirheumatoid agent in oriental medicine.[4,10] Also it has been reported that BV and its main ingredient (melittin) shows anticancer properties.[4]

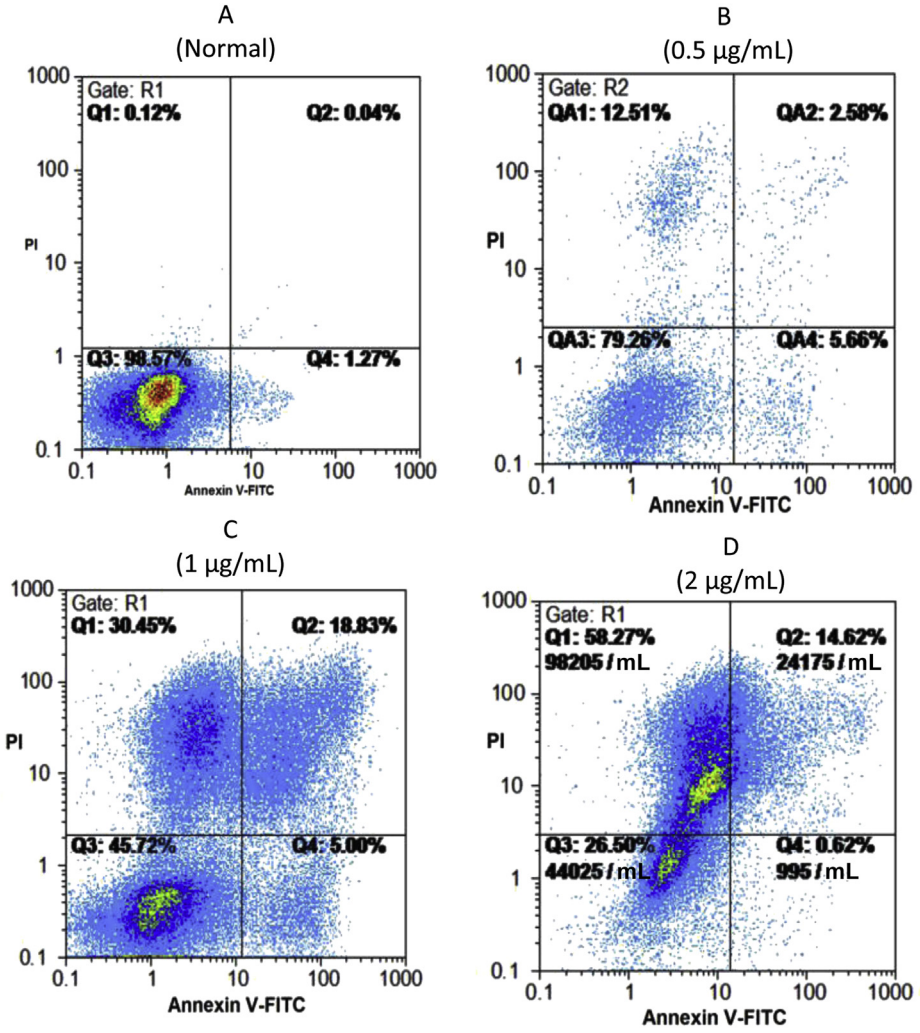

Fig. 9. Melittin shows necrotic toxicity on AGS cell line. (A) Untreated cells; (B) cells were treated with 0.5 µg/mL melittin; (C) 1 µg/mL melittin; and (D) 2 µg/mL of melittin for 12 hours and compared with untreated AGS cells, as a negative control. Cells were stained with propidium iodide-A and annexin-V-FITC and analyzed with a flow cytometer (CyFlowSL, Partec GmbH, Munster, Germany).

The inhibitory effect of BV and melittin on the proliferation of cancerous cells has been investigated extensively.[4] However, the mechanism of BV or melittin-induced cell death in these cells is not completely understood. It is speculated that the cytotoxic effect of melittin is mediated through the formation of channels on cell membranes. Furthermore, there is another hypothesis that proposes a detergent-like model interaction of melittin and membranes. The present study is the first report about the isolation of melittin from Iranian honey BV and the evaluation of its effect on gastric cancer AGS cells. It is believed that melittin can be used to arrest proliferation of GC cells, and have a profound effect by facilitating melittin-induced death in gastric cancer AGS cells.

In order to isolate melittin from the BV, RP-HPLC was performed and the resulting chromatogram showed that melittin constituted almost 50% of Iranian honey BV: this result was consistent with the reports about European honey BV components.[32] Purified melittin showed the same retention time with standard melittin in HPLC. The biological activity of purified melittin was subjected to a hemolytic activity assay. The results of the hemolytic activity assay exhibited high hemolytic activity of isolated melittin (HD_{50} = 0.5 µg/mL). The toxicity of melittin on an AGS cell line was assessed with MTT assay at various concentrations of melittin (8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, and 0.125 µg/mL) for 6 hours, 12 hours, and 24 hours. The MTT assay showed that melittin is able to arrest the proliferation of AGS cells at a very low concentration (0.5 µg/mL). Also, this experiment showed that melittin has a dose and time dependent toxicity on AGS cells.

Numerous studies concerning the cytotoxic effects of BV and melittin were performed from 1980 to 2000. However, the mechanisms of this cytotoxicity were investigated in the past 10 years.[4] The latest studies have focused on the mechanism of BV and melittin-induced cell death. It has been reported that BV or melittin induce apoptosis or necrosis in some of the cells. It would appear that melittin-induced death is dependent on cell type and the amount of the toxin. Many chemotherapeutic

agents induce apoptosis in cancer cells.[32,33] Usually these agents act in a similar manner; for example, many of them activate p53. In cancer therapy, apoptosis is preferable to necrosis, because apoptosis does not produce inflammation and damage to surrounding normal tissue.[34] Melittin has anticancer effects on GC AGS cells and stimulates necrotic cell death. However we think that necrosis can be useful to induce a more powerful immune system response against the cancer.

A morphological evaluation of treated AGS cells showed a looseness of cell membrane integrity in the presence of 1 μg/mL and 2 μg/mL melittin, which was a sign of necrotic cell death. Furthermore, DNA fragmentation assay indicated that melittin does not stimulate caspases-activated DNA digestion, one of the apoptotic hallmarks. This regular enzymatic cleavage produces 180−200 bp nucleosomal fragments,[35] which were not observed in this study. Finally, the effect of melittin on translocation of the phosphatidylserine and cell membrane integrity was evaluated using PI/An V-FITC dual staining with flow cytometry and fluorescence microscopy. The results of PI/An V-FITC dual staining demonstrated that melittin acts as a necrotic agent in AGS cell lines.

The best anticancer drug is a highly toxic agent against cancer cells that does not cause excessive damage to normal cells. Nowadays, an important aspect of medical research is focused on the synthesis, finding, and/or modification of different substances in order to introduce a useful anticancer drug. Natural products have a special importance in these studies. Melittin is one of these natural products, which could be an ideal candidate against cancer. The big obstacle in the clinical usage of melittin as an anticancer drug, is the extent of its toxicity on normal cells. In spite of this undesirable property of melittin, it can be used as a considerable anti-inflammatory, antibacterial, antifungal, and antitumor peptide. In order to decrease the toxic effect of melittin on normal cells, different strategies including gene therapy, encapsulation of melittin, and conjugation with antibodies have been suggested.[36] These strategies may be used in the near future to selectively control tumor cells by the introduction of melittin.

In the current study, in three distinct assays, morphological evaluation, DNA fragmentation, and flow cytometric analysis, no evidence of apoptosis was observed. The used concentrations of melittin in these tests were selected based on the results of the MTT assay. These concentrations ranged from a low toxic concentration (0.5 μg/mL) to a high toxic concentration (2 μg/mL).

In conclusion, our study has demonstrated that melittin inhibits the proliferation of GC AGS cells in a dose and time dependent trend. Additionally, it would appear that melittin stimulated necrosis in lethal cancer cells. We believe that these findings are noteworthy and represent an important achievement in the use of melittin to create better and more effective treatments for GC.

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