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Nisin induces apoptosis in cervical cancer cells via reactive oxygen species generation and mitochondrial membrane potential changes

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Abstract

Nisin, an antimicrobial peptide produced by *Lactococcus lactis*, is widely used as a safe food preservative and has been recently attracting the attention of many researchers as a potential anticancer agent. The cytotoxicity of nisin against HeLa, OVCAR-3, SK-OV-3, and HUVEC cells was evaluated using MTT assay. The apoptotic effect of nisin was identified by Annexin-V/propidium iodide assay, and then it was further confirmed by western blotting analysis, mitochondrial membrane potential ($\Delta\Psi m$) analysis, and reactive oxygen species (ROS) assay. The MTT assay showed concentration-dependent cytotoxicity of nisin towards cancer cell lines, with the IC_{50} values of 11.5-23 μM, but less toxicity against normal endothelial cells. Furthermore, treatment of cervical cancer cells with 12 μM nisin significantly ($P<0.05$) increased the Bax/Bcl-2 ratio (4.9-fold), reduced $\Delta\Psi m$ (70%), and elevated ROS levels (1.7-fold). These findings indicated that nisin might have anticancer and apoptogenic activities through mitochondrial dysfunction and oxidative stress damage in cervical cancer cells.

Keywords: Nisin, Apoptosis, Mitochondrial membrane potential, Reactive oxygen species, Uterine cervical neoplasms, Ovarian neoplasms

Introduction

Gynecologic cancers (*e.g.,* cervical and ovarian cancer) are common cancer with high mortality among women worldwide. Cervical cancer was the fourth most common cancer among women and was also the fourth leading cause of death due to cancer in women according to the Global Cancer Observatory 2018 database (Arbyn et al., 2020). In 2021, 21,410 new ovarian cancer cases will be diagnosed and an estimated 13,770 women will die from this cancer in the United States (Siegel et al., 2021). Acquired (*e.g.,* chronic human papillomavirus or HPV infection) and genetic (*e.g.,* family history) susceptibility factors are the major cause of cervical cancer (Tan and Ankathil, 2015, Chan et al., 2019). Recent studies have explored that vaginal dysbacteriosis or vaginal microbiota imbalance also plays an important role in the development of reproduction system cancers including cervical cancer in women (Brusselaers et al., 2019).

Different therapeutic approaches including surgery, radiation therapy, and pharmacotherapy are currently applied for the treatment of cervical cancer. However, most patients experience adverse effects which have long-term effects on their quality of life (Pfaendler et al., 2015). Therefore, the development of novel anticancer agents is still required to reduce side effects and improve the effectiveness of therapeutic approaches for the management of cervical cancer. Recent evidence has shown that probiotics and their metabolites (*e.g.,* antimicrobial peptides) can rebalance the host's microbiome, improve the immune system, and reduce inflammation (Zheng et al., 2020); therefore, these agents have potential preventive, therapeutic, or diagnostic applications in different malignancies including gynecologic cancers (Perisić et al., 2011, Cha et al., 2012, Linn et al., 2019).

Nisin is a cationic antimicrobial peptide produced by *Lactococcus lactis*, the first living transgenic bacteria used for the treatment of human disease (Braat et al., 2006). This

antimicrobial peptide is widely used as a food preservative and is generally regarded as safe (GRAS) for human usage even at high doses (2021). Nisin exhibits various pharmacological activities including antimicrobial/biofilm, immunomodulatory and antitumor properties (Shin et al., 2016). Anticancer and cytotoxic effects of nisin have been investigated in several cancer cell lines including head and neck squamous carcinoma cells (HNSCC) (Kamarajan et al., 2015), breast cancer cells (*e.g,* MCF-7) (Avand et al., 2018), skin cancer cells (*e.g.,* A375) (Lewies et al., 2018) and colorectal cancer cells (*e.g*., SW480) (Hosseini et al., 2020).

To the best of our knowledge, there is no report on the cytotoxic effect of nisin against ovarian and cervix cancer cell lines. In this study, we aimed to evaluate the anticancer effect of nisin against cervical and ovarian cancer cell lines (HeLa, OVCAR-3, and SK-OV-3) using the cell viability assay. We also performed flow cytometric analysis, western blot analysis, mitochondrial membrane potential analysis, and reactive oxygen species (ROS) assay to gain mechanistic insights into the nisin-induced cervical cancer cell death.

Materials and Methods

Chemicals and reagents

High glucose Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI), fetal bovine serum (FBS), penicillin-streptomycin were obtained from Bioidea (Iran). Nisin, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were provided from Sigma-Aldrich (USA). Mouse anti-Bcl-2 (sc-7382), mouse anti-β-actin (sc-47778), mouse anti-Bax (sc-20067), and goat anti-mouse IgG-HRP (sc-2031) were provided from Santa Cruz Biotechnology (USA). All other reagents were purchased from Sigma Aldrich (USA) and were of analytical grade.

Cell lines

The human cervical cancer cell line (HeLa), human ovarian carcinoma cell lines (OVCAR-3 and SK-OV-3), and human umbilical vein endothelial cells (HUVEC) were purchased from the Pasture Institute (Tehran, Iran). The cells were incubated in a humidified atmosphere containing 5 % CO_2 at 37 ºC. HeLa, OVCAR-3 and SK-OV-3 were fed with RPMI supplemented with 10 % FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. HUVEC cells were cultured in high glucose DMEM supplemented with 10 % FBS and penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively).

Cell viability assay

To evaluate the cytotoxic effect of nisin on the OVCAR-3, SK-OV-3, HeLa, and HUVEC cells, the MTT assay was performed as described previously (Safaeian et al., 2016). Briefly, the cells were seeded at a density of 1×10^4cells/well in 96-well plates and incubated overnight. Based on the IC_{50} values reported in the previous studies (Avand et al., 2018, Rana et al., 2019), the 0.75-75 µM concentration range was chosen for the present work. The cells were incubated with different concentrations of nisin (0.75–75 µM). Every plate had four wells with cells treated with phosphate buffered saline (PBS) as the negative control. After 48 h of incubation, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37 °C for 4 h. Then, the medium was removed and 150 µl DMSO was added to each well to dissolve insoluble formazan crystals. Finally, the absorbance values were measured at 570 nm by a plate reader (BioTek Instruments, USA). The percentage of cell viability was determined according to the following equation:

% Cell viability = (Absorbance of treated wells − Absorbance of blank) / (Absorbance of negative control – Absorbance of blank) × 100

Apoptosis assay via Annexin-V/PI staining

Induction of apoptosis by nisin was evaluated using Apoptosis Detection Kit (Annexin V-FLUOS Staining kit, Roach, USA). HeLa cells (2×10^5 cells/well) were seeded in a 12-well plate and incubated with various concentrations of nisin (3, 6, 12 and 24 μM) and the negative control for 16 and 24 h. Then, the cells were harvested and washed with PBS by centrifugation at 500 ×*g* for 5 min at 4 °C. Next, the cells were co-stained with FITC conjugated Annexin V (Annexin-V) and propidium iodide (PI) according to the manufacturer's instruction and analyzed by flow cytometry (BD Biosciences, USA).

Western blot analysis

To evaluate the effect of nisin on the expression of apoptotic and anti-apoptotic proteins, western blot analysis was performed as described previously (Shahrestanaki et al., 2019). Briefly, HeLa cells were grown on cell culture dishes and treated with different concentrations of nisin (6, 12, and 24 μM) and the negative control for 48 h. The cells were harvested using a sterile cell scraper and resuspended in RIPA buffer containing protease inhibitors (0.5 mM PMSF, and 0.5% protease and phosphatase inhibitor cocktails) and then subjected to probe-sonication (Hielscher, Germany) on ice to disrupt cells. The cell debris was removed by centrifugation at 12,000 ×*g* for 10 min at 4 °C and the total protein content of the supernatant was determined using the Bradford method. An equal amount of each protein sample was separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) 2 h at 25 °C and then probed with anti-human Bax, Bcl-2, and β-actin antibodies at 4 °C overnight. After three washes, the membrane was incubated with a peroxidase-conjugated secondary antibody for 2 h at 25 °C. Then, the membrane was washed three times

with TBST and the blots were visualized on an X-ray film using an ECL kit (Cell signaling, USA). The density of each band was quantified by ImageJ software (version 1.52, National Institutes of Health, Bethesda, USA).

Mitochondrial membrane potential analysis

The mitochondrial membrane potential ($\Delta\Psi m$) was determined using a mitochondrial probe, JC-1, as described previously (Shahali et al., 2018). Briefly, HeLa cells (1×10^3 cells/well) were seeded in a 384 well plate and were treated with nisin (6, 12, 24 µM) and the negative control for 24 h. The medium was replaced with 100 µl per well of HEPES buffer and incubated for 30 min at 37 °C. The plate was washed once more with 100 µl per well of HEPES buffer and then cells were stained with 2.5 mM JC-1 in the dark. Fluorescence was measured for each well at every 15 min for 2 h at excitation/emission 485/530 nm (green) and excitation/emission 530/580 nm (red) using a microplate reader (Synergy H1 Hybrid Multi-Mode; Bio-Tek). Changes in the ratio of red to green fluorescence were determined and then normalized to untreated control cells.

Reactive oxygen species (ROS) assay

The generation of ROS was determined using a Live Cell Fluorescent Reactive Oxygen Species detection kit (Marker Gene Technologies, USA) as described previously (Shahali et al., 2018). In brief, HeLa cells were seeded in a 384-well plate at 1×10^3 cells per well overnight, and incubated with nisin (6, 12, 24 µM) for 48 h. The medium was removed and cells were stained with 20 µM 2′,7′-dichlorofluorescein diacetate (DCFDA) at 25 °C for 1 h in the dark. Fluorescence was determined for each well at excitation/emission 485/528 nm using a microplate reader (Synergy H1 Hybrid Multi-Mode; Bio-Tek). The ROS levels were expressed as a fold of change to the control.

Statistical analysis

147 The IC_{50} was calculated with the Prism 9 software (GraphPad Prism Software Inc., San Diego,
148 CA). Data from at least three independent experiments were reported as mean ± standard
149 deviation (SD). Significant differences between groups were determined by analysis of variance
150 (ANOVA) using SPSS (Statistical Package for the Social Sciences) software program (Chicago,
151 IL, USA), version 20 followed by a Tukey post hoc test. The *p* values <0.05 were considered
152 significant.

153 **Results**

154 *In vitro cytotoxicity of nisin*

155 To determine the cytotoxicity of nisin, HUVCE, HeLa, OVCAR-3, and SK-OV-3 cell lines were
156 incubated with nisin at different concentrations for 48 h and an MTT assay was performed to
157 evaluate the cell viability. As presented in Figure 1, nisin revealed a low level of cytotoxicity for
158 non-cancerous cells, HUVEC; cell viability was slightly reduced at higher concentrations of
159 nisin (6 and 12 µM) and the IC_{50} value was 63.2 µM. In contrast, nisin exhibited significant
160 cytotoxicity (P<0.05) on HeLa and OVCAR-3 cells, and the IC_{50} value was 11.5 µM and 14.6
161 µM, respectively. Interestingly, SK-OV-3, a more invasive ovarian cancer cell line, was less
162 affected by nisin and the IC_{50} was obtained 22.9 µM. Our findings indicate that the HeLa cell
163 line was the most sensitive to nisin. Therefore, we decide to proceed with HeLa cells in the
164 following experiments.

165 *Nisin induces apoptosis in HeLa cells*

166 Annexin-V/PI staining was performed to evaluate the effect of nisin on the induction of
167 apoptosis in HeLa cells. In flow cytometric analysis, Annexin-V/PI-negative cells indicate live
168 cells; Annexin-V-positive and PI-negative cells refer to early apoptotic cells, Annexin-V-
169 negative and PI-positive cells indicate primary necrotic cells and Annexin-V/PI-positive cells

show late apoptotic cells or secondary necrosis. As shown in Figure 2 (A and B), with enhancing nisin concentration both early apoptotic and late apoptotic or necrotic cells were increased in a concentration-dependent manner. A significant increase ($P<0.001$) in early apoptosis and late apoptosis were observed after treatment with nisin (12 and 24 μM) for 16 and 24 h when compared to the control (Figure 2.C). Incubation of HeLa cells with nisin at a concentration near IC_{50} (12 μM) for 16 h led to 19.33±3.05%, 20±3.46%, and 3.39±0.43% of early apoptosis, late apoptosis or secondary stage of necrosis and primary necrotic stage, respectively. In comparison, longer incubation time (24 h) with 12 μM of nisin showed 11.97±3.63%, 40.64±4.27%, and 6.20±0.35% of early apoptotic cells, the late apoptotic or secondary necrotic cells and the primary necrotic cells, respectively.

Nisin increases Bax/Bcl-2 ratio in HeLa cells

Western blotting analysis was performed to evaluate the effect of nisin on the expression of a pro-apoptotic protein, Bax, and an anti-apoptotic protein, Bcl-2. As represented in Figure 3.A, the expression of Bax was increased while the expression of Bcl-2 was decreased after treatment with nisin in a concentration-dependent manner. Quantification of the intensity of bands by ImageJ densitometer showed (Figure 3.B) that following treatment of cells with 6, 12 and 24 μM of nisin, the Bax/Bcl-2 ratio was remarkably enhanced (3, 4.9, and 9.9 times, respectively) as compared with the control group (untreated cells).

Nisin decreases ΔΨm levels in HeLa cells

The changes in ΔΨm levels in HeLa cells treated with nisin were evaluated using JC-1 fluorescent dye which selectively internalized by mitochondria. When cells have an intact mitochondrial membrane and high ΔΨm level, JC-1 polymerizes to J-aggregates and shows red fluorescence. In the case of mitochondrial depolarization, it remains as a JC-1 monomer and

produces green fluorescence. The red/green fluorescence intensity ratio was used to determine the level of ΔΨm (Figure 4). Our finding showed that in comparison with untreated control cells, ΔΨm levels decrease 44%, 70% and 82% after treatment with 6, 12 and 24 μM of nisin, respectively (P<0.05).

Nisin increases ROS levels in HeLa cells

To investigate the effect of nisin on induction of oxidative stress in HeLa cells, DCFDA fluorogenic dye was applied, which produces green fluorescence after oxidation. As shown in Figure 5, treatment with 6, 12 and 24 of nisin led to an increase in the intracellular ROS of about 1.36, 1.70, and 2.06 fold, respectively, compared to the untreated control (P<0.05).

Discussion

Chorionic vaginal dysbacteriosis and outgrowth of some groups of anaerobic bacteria releasing toxic metabolites (*e.g.*, nitrous acid) can promote gynecological malignancies including ovarian and cervical cancers (Sowjanya et al., 2016). Probiotic microorganisms and their metabolites including bacteriocins can recover the vaginal microbiome balance, reduce the HPV-induced cytological alterations and inhibit the proliferation of cervical cancer cells (Wang et al., 2018, Verhoeven et al., 2013). Nisin, an FDA-approved bacteriocin, has been shown to have anti-tumor and apoptotic effects on several cancer cell lines. Here, we found that nisin exhibits significant cytotoxic effects on ovarian and cervical cancer cell lines and induces apoptotic cell death mainly via mitochondrial membrane potential disruption and ROS generation.

Our results showed that nisin remarkably inhibited the growth of three cancer cell lines with IC_{50} values of 9-21 μM. Another research group reported that nisin showed the cytotoxic effect on A431 human skin carcinoma cell line with IC_{50} at 9.5 μM (Rana et al., 2019). In another investigation, nisin displayed significant cytotoxicity to the intestinal epithelial cells, HT29 and

Caco-2, with IC_{50} values of 89.9 and 115.0 μM, respectively, which is much higher than that of the present study (Maher and McClean, 2006). Differences in the sensitivity of different cell lines to nisin can be explained based on membrane variations. The main interactions between nisin and cancer cell membrane components are electrostatic interactions. Based on our previous study, the cytotoxic activity of nisin as a cationic peptide can be improved by decreasing the pH (Avand et al., 2018). Furthermore, nisin is more soluble and stable at low pH which is compatible with the pH of vaginal and cervical mucosa. Therefore, nisin might have better activity in the genital tract. However, more *in vitro* and *in vivo* investigations are still required to evaluate its efficacy and safety in gynecologic cancers.

Notably, nisin exhibited much less cytotoxicity against normal cells, HUVEC. Our findings are consistent with previous works reporting the high selectivity of nisin for malignant HNSCC and A375 cells without affecting normal keratinocytes (Lewies et al., 2018, Kamarajan et al., 2015). The plasma membrane of tumor cells is composed of more negatively charged molecules (*e.g.*, anionic phospholipids) and thereby can more interact electrostatically with cationic peptides such as nisin (Ran et al., 2002). Furthermore, the surface of cancer cells contains many more microvilli than that of normal cells which may provide more surface area for interaction with nisin (Chan et al., 1998).

Annexin-V/PI flow cytometry analysis showed that nisin can induce significant apoptotic cell death in the cervical cancer cell line, HeLa, in a concentration-dependent manner. A reduction in ΔΨm and enhanced levels of the Bax/Bcl-2 ratio further confirm that the cytotoxic effect of nisin towards cervical cancer cells is mainly due to the activation of an apoptotic pathway.

BCL-2 family plays a critical role in the regulation of the mitochondrial apoptotic pathway. The ratio of pro- to anti-apoptotic members of the BCL-2 family significantly influences the

susceptibility of cells to undergo apoptotic death (Hata et al., 2015). In agreement with our findings, it was reported that 30-60 μM of nisin up-regulated Bax expression and down-regulated the expression of Bcl-2, which led to elevated Bax/Bcl-2 ratio both at mRNA and protein levels in SW480 colon cancer cells (Ahmadi et al., 2017).

Recent evidence indicates that nisin interaction can modify the tumor cell membrane properties such as fluidity and dipole potential resulting in the death of malignant cells (Kamarajan et al., 2015, Prince et al., 2019). Nisin contains a pore-forming domain which may lead to a reduction in $\Delta\Psi m$. Similar to our results, another work reported that nisin can depolarize the mitochondrial membrane of melanoma cancer cells which negatively affects glycolysis metabolism and mitochondrion respiration (Lewies et al., 2018).

Intracellular ROS plays an important role in oxidative stress and can affect cellular metabolism, differentiation and survival (Zhang et al., 2016). Mitochondria are one of the main sources of cellular ROS generation. The excess levels of ROS can result in cell death via promoting cell signaling pathways triggering apoptosis. Cancer cells usually have lower levels of antioxidants than healthy cells, and thus are more susceptible to ROS-induced cell death (Zaidieh et al., 2019). In the present study, treatment of HeLa cells with nisin led to increased intracellular ROS levels, suggesting its role in apoptosis induction. Furthermore, ROS generation can indirectly induce apoptosis through more disruption of mitochondrial membrane and subsequently complete mitochondrial depolarization as reported in this work. Similarly, another study showed the involvement of mitochondrial dysfunction and ROS overproduction in the apoptosis induced by nisin in melanoma cells (Lewies et al., 2018).

Conclusion

In summary, here we have shown that nisin exhibits great cytotoxicity HeLa, OVCAR-3 and SK-OV-3 cell lines and low toxic effect against normal endothelial cells. Nisin induces apoptosis in cervical cancer cells through regulation of BCL-2 family proteins, depletion of mitochondrial potential and ROS accumulation. Our findings provide preliminary evidence for the potential application of nisin alone or in combination with other anticancer agents for the treatment of cervical malignancies. However, further investigations are still required to confirm anti-tumor effectiveness and safety of nisin in experimental *in vivo* cervical cancer models.

Competing interests

The authors declare there are no competing interests.

Author contribution

V.A. is the principal investigator of the project; M.A. and H.S. planned for the project; H.S., M.A and V.A. performed the required experiments; V.A. wrote the manuscript in consultation with M.A. and H.S. All authors discussed the results and contributed to the final form of the manuscript.

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Figure 1. Cytotoxicity of nisin on the HUVEC, SK-OV-3, OVCAR-3 and HeLa cell lines. Cells were incubated different concentrations of nisin for 48 h and the cell viability was determined by MTT assay. (mean±SD, n=3)

Figure 2. Representative flow cytometric analysis for apoptosis in HeLa cells after treatments with nisin (3, 6, 12 and 24 μM) for 16 h (A) and 24 h (B). The percentages indicated in the lower left, the lower right, the upper right and the upper left quadrants refer to viable cells, early apoptotic cells, late apoptotic cells or secondary necrotic cells and primary necrotic, respectively. C) The results are shown as bar chart. (mean±SD, n=3)

Figure 3. A) Representative western blot analysis of Bax and Bcl-2 expression levels in HeLa cells after treatment with nisin (6, 12 and 24 μM). B) The graph showing the level of Bax and Bcl-2 compared with the untreated control group.Quantification of the intensity of bands was performed using ImageJ software and the protein level of Bax and Bcl-2 were normalized to the β-actin level. (mean±SD, n=3)

Figure 4. The effect of nisin on mitochondrial membrane potential (ΔΨm) of HeLa cells. Cells were treated with nisin (6, 12 and 24 μM) and then stained with JC-1 probe. The red/green fluorescence intensity ratio or ΔΨm was measured by spectrofluorometry and normalized to untreated control cells (mean±SD, n=3). Asterisks indicate the means which were significantly different ($P < 0.05$) from the control.

Figure 5. The effect of nisin on intracellular ROS generation of HeLa cells. Cells were treated with nisin (6, 12 and 24 μM) and then loaded with DCFDA. The fluorescence intensity or ROS was determined by spectrofluorometry and normalized to untreated control cells (mean±SD, n=3). Asterisks indicate the means which were significantly different ($P < 0.05$) from the control.

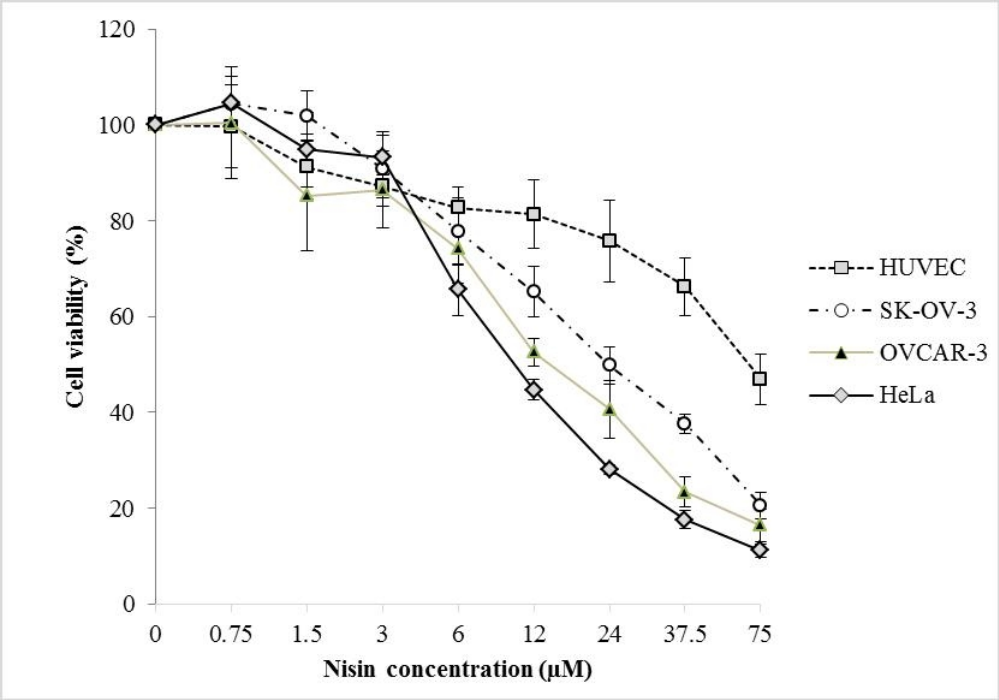

Figure 1. Cytotoxicity of nisin on the HUVEC, SK-OV-3, OVCAR-3 and HeLa cell lines. Cells were incubated different concentrations of nisin for 48 h and the cell viability was determined by MTT assay. (mean±SD, n=3)

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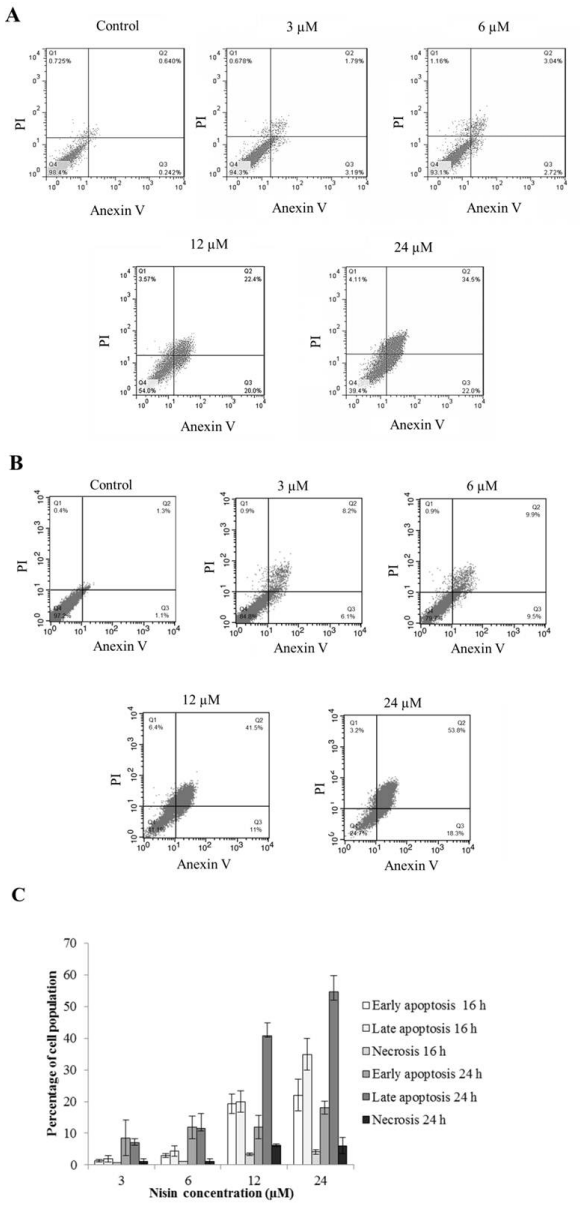

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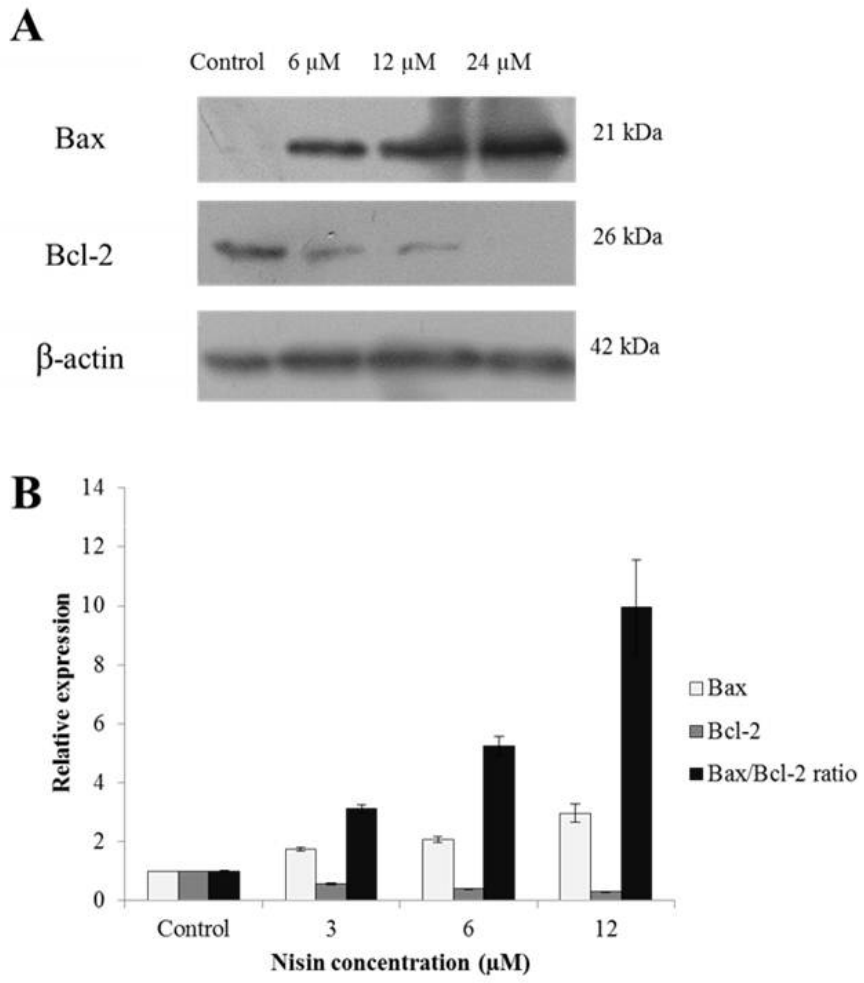

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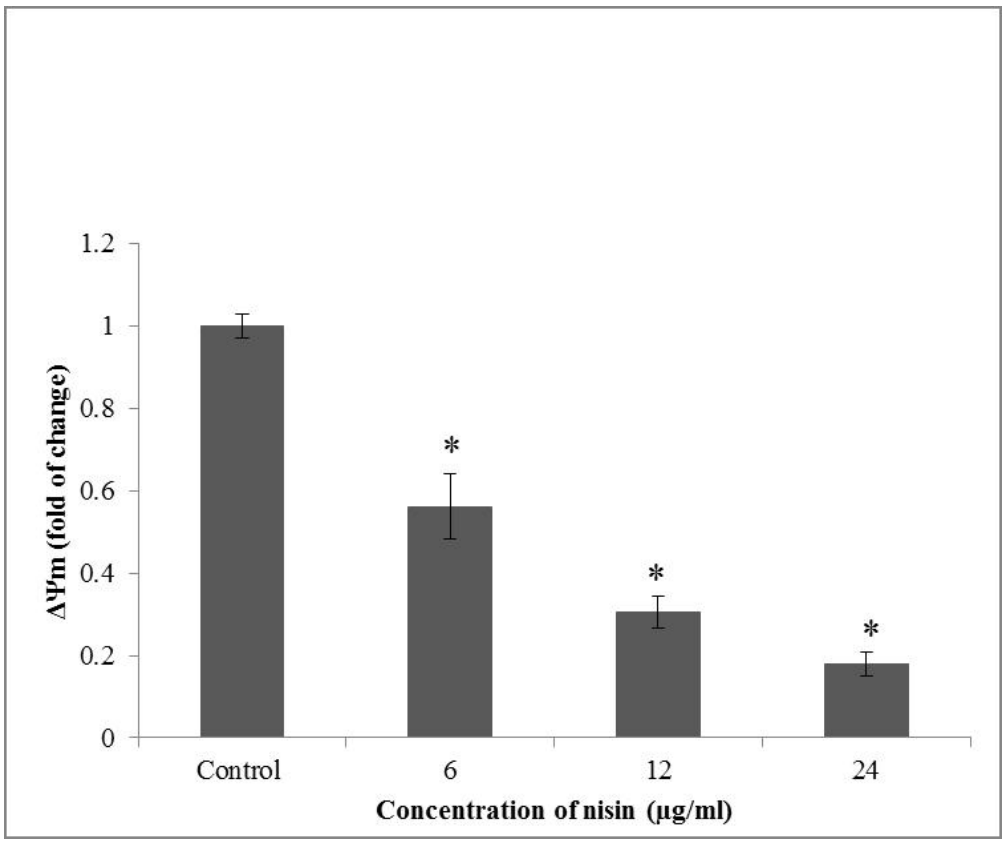

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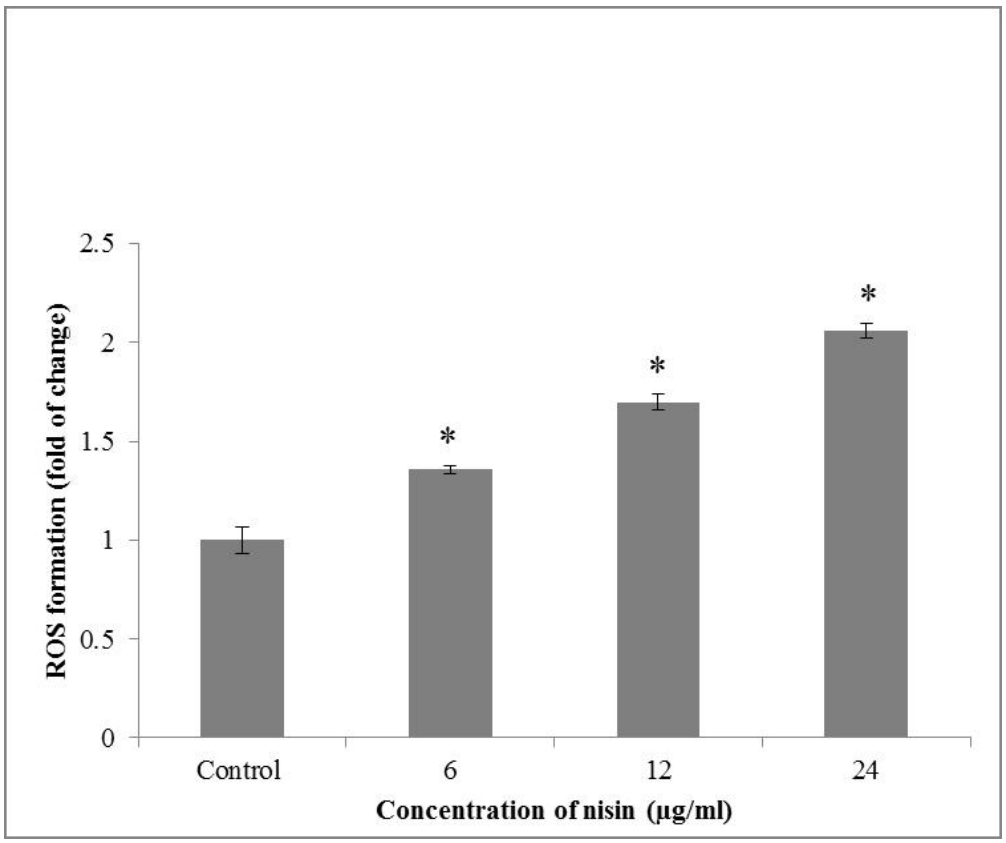

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