Mechanism of apoptosis induced by quinoxaline from the myxobacterium Stigmatella erecta WXNXJ-B in B16 mouse melanoma cell line

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The biological activities of quinoxaline, a novel small molecular substance isolated from the broth of the myxobacterium Stigmatella erecta WXNXJ-B, was investigated. This study was designed to determine the anti-proliferative, apoptotic property of quinoxaline, using B16 mouse melanoma cells as a model system. The results showed that quinoxaline has antitumor activity and can significantly inhibit the proliferation of B16 cells. The extent and the timing of apoptosis were strongly dependent on the dose. After treating with quinoxaline and staining with Hoechst 33342, B16 cells showed typical apoptotic morphological features such as chromatin condensation by fluorescent microscopy. DNA isolated from B16 cells cultured with quinoxaline showed a typical DNA ladder of apoptosis in agarose gel electrophoresis. Further investigation results showed that the apoptotic machinery of B16 induced by quinoxaline was associated with drop in mitochondrial membrane potential from 5.35% to 23.7%, up-regulation of Bax and down-regulation of Bcl-2 in a dose-dependent manner. And a significant increased activation of caspase-3. Our finding suggests that quinoxaline could suppress the growth of B16 cells and reduces cell survival via disturbing mitochondrial membrane potential and inducing apoptosis of tumor cells.
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ABSTRACT The biological activities of quinoxalone, a novel small molecular substance isolated from the broth of the myxobacterium *Stigmatella eracta* WXNXJ-B, was investigated. This study was designed to determine the anti-proliferative, apoptotic property of quinoxalone, using B16 mouse melanoma cells as a model system. The results showed that quinoxalone has antitumor activity and can significantly inhibit the proliferation of B16 cells. The extent and the timing of apoptosis were strongly dependent on the dose. After treating with quinoxalone and staining with Hoechst 33342, B16 cells showed typical apoptotic morphological features such as chromatin condensation by fluorescent microscopy. DNA isolated from B16 cells cultured with quinoxalone showed a typical DNA ladder of apoptosis in agarose gel electrophoresis. Further investigation results showed that the apoptotic machinery of B16 induced by quinoxalone was associated with drop in mitochondrial membrane potential from 5.35% to 23.7%, up-regulation of Bax and down-regulation of Bcl-2 in a dose-dependent manner. And a significant increased activation of caspase-3. Our finding suggests that quinoxalone could suppress the growth of B16 cells and reduces cell survival via disturbing mitochondrial membrane potential and inducing apoptosis of tumor cells.

Subjects: Toxicology, Pharmacology

Keywords: *Stigmatella eracta* WXNXJ-B, Quinoxalone, Apoptosis, Mitochondrial membrane potential, Bax, Caspase-3
INTRODUCTION

Myxobacteria are gram-negative unicellular rod shaped bacteria that move by gliding and travel in swarms, containing many cells kept together by intercellular molecular signals (Wioletta et al., 2016). They can be frequently isolated from soil, dung of herbivorous animals and other decaying organic material (Shimekets et al., 2006). They are unusual bacteria characterized by gliding behavior and forming fruiting body, and not obtained by the routine method due to their complicated life cycle (Velicer and Vos, 2009).

Myxobacteria are one of the important sources for natural microbial products besides actinomycetes and fungi (Gerth et al., 2003). Ambruticin, the first myxobacterial antibiotic, was isolated from a strain of Sorangium cellulosum (Connor et al., 1977). The first structure of myxothiazol was reported by Gerth et al. (1980). Within the last 30 years, the myxobacteria have emerged as a promising alternative source of bioactive molecules (Johnson et al., 2012; Schmitz et al., 2013; Plaza and Müller, 2014; Schaberle et al., 2014). Myxobacterial secondary metabolites do not commonly produce by other microbes, such as hybrids of polyketides and non-ribosomally peptides (Diez et al., 2012). The metabolites exhibit many unique structural features and novel modes action, making them attractive and promising sources for drug development. Many compounds from myxobacteria show quite different mechanisms of action. They inhibit the protein synthesis of prokaryotic and eukaryotic and stimulate potassium export from gram-positive bacteria. But, the mechanism of action of most compounds has not yet been elucidated (Weissman and Muller, 2010).

Epothilones from Sorangium cellulosum and their analogues have demonstrated antitumor
activity towards multidrug resistant tumor cells (Altman et al., 2009; Gong et al., 2014). These compounds target the eukaryotic cytoskeleton, interference with microtubuli in the cell disabling the assembly of functional mitotic spindles required for cell proliferation and thus resulting in apoptosis. One such analog, known as Ixabepilone is a FDA-approved chemotherapy agent for the treatment of metastasis breast cancer (Wioletta et al., 2016). Several other metabolites are currently being evaluated in preclinical studies (Kim et al., 2013). Due to their extraordinary ability to produce novel classes of secondary metabolites, myxobacteria represent a very promising source for the discovery of new lead structures and novel natural products (Wenzel and Muller, 2009).

Due to their potent biological activities, which results in various applications in the pharmaceutical and agrochemical industry, many research groups have tried to identify novel groups of natural product producers over the last decade. In continuing effort to find novel bioactive metabolites from myxobacteria, the researchers in our lab obtained five myxobacteria which showed strong antitumor bioactivity in vitro (Guo and Tao, 2008). Quinoxalone, isolated from the myxobacterium Stigmatella eracta WXXNJ-B and stored in our lab, is a novel bioactive metabolite (Fiuger 1). Our previous study showed that quinoxalone exhibited significant effect of anti-proliferation on the tumor cells in vitro, however, the mechanism of anti-proliferative has yet not been elucidated clearly (Wang et al., 2014). In the present report, we investigated the quinoxalone antitumor activity by evaluating its effects on the B16 mouse melanoma cell line and its possible apoptosis mechanism.
MATERIALS AND METHODS

Microorganism and culture conditions

The strain myxobacteria *Stigmatella eracta* WJXN-4 was used in this study. Medium for slant was CY medium. Medium composition for seed and fermentation cultures was as described by Wang et al. (2014). In the fermentation medium, about 20 g/L XAD-16 adsorbent resins (Rohm & Haas, USA) were added to adsorb the bioactive metabolites. *S. eracta* WJXN-4 was grown on CY medium at 30°C for 5 days, then inoculated in seed medium for flask culture at 30°C with shaking at 150 rev/min. After 2 days, the seed broth was transferred to fermentation medium and fermented at 30°C with shaking at 150 rev/min for 7 days.

Preparation of quinoxaline

After fermentation, the XAD-16 adsorbent resins were separated. The resins were extracted with methanol for 6 h. The extract was concentrated at 45°C and further purified by partition between water and chloroform. The chloroform extract was isolated by chromatography using a Sephadex LH-20 column, with two gradients of 80% and 90% methanol at flow rate 2 mL/min. The fraction eluted with 90% methanol was purified using a preparative RP-HPLC using a Sephadex C18 column (5 µm × 10 mm × 150 mm, with mobile phase 80% methanol at flow rate 3 mL/min). Quinoxaline was obtained.

Cell lines

B16 mouse melanoma cell line, CT-26 murine colon carcinoma cell line MDA-MB231 and MCF-7 human breast cancer cell line, HepG2 human liver hepatocellular cell line were provided by college of Medicine and Pharmaceutics, Jiangnan University, China. All cells were cultured in...
RPMI-1640 medium (Gibco, USA) with 10% inactivated fetal bovine serum (Gibco, USA), streptomycin (100 µg/mL) and penicillin (100 U/mL) at 37°C in a 5% CO₂ incubator. Epothilone B and Paclitaxel were purchased from Sigma-Aldrich Co.

Evaluation of quinoxalone in vitro (MTT assay)

B16, CT-26, HepG2, DMA-MB231 and MCF-7 cells were used to evaluate the antitumor effects of quinoxalone. Cells were harvested, counted, diluted and seeded into 96-well plates at a density of approximately 7000 cells/well. After incubating for 24 h, 200 µL medium with different concentration quinoxalone which was dissolved into dimethylsulfoxide (DMSO) was added into per well. To avoid the influence of DMSO, medium containing 0.5% DMSO was used as a control. Incubation was carried out for another 48 h. The cell viability was assessed by MTT (colorimetric 3-[4,5--2-Yl]-2,5-diphenyl tetrazolium bromide) assay. Twenty microlitre of MTT solution (5 mg/mL) was added into each well and incubated at 37°C for additional 4 h. The formazan product was dissolved by adding 200 µL DMSO and shaked for 5 min. Then, the absorption was measured at 570 nm with a microplate reader. The inhabitation rate was calculated as follows: inhabitation rate= (1-OD<sub>treated</sub>/OD<sub>control</sub>)×100%. Data were obtained from six repeat experiments.

Fluorescence microscope observation of B16 cells

To observe the change in nuclear structure, B16 cells were plated onto glass cover slips in 6-well plates and treated with 5 and 10 µg/mL quinoxalone for 48 h. Then, cells were washed twice with PBS, fixed with 1% glutaraldehyde, stained with Hoechst 33342 (Sigma, USA) for 15 min at room temperature. Nuclear morphology was examined by fluorescence microscope (Olympus, Tokyo, Japan).
DNA fragmentation assay

B16 cells were treated with quinoxaline at concentrations of 0, 2.5, 5 and 10 μg/mL for 48 h. Following with centrifugation at 600 g for 5 min, the harvested cells were lysed in a DNA extraction buffer (containing 20 mmol/L EDTA, 100 mmol/L Tris, 0.8% (w/v) SDS) and incubated at 37 °C for 30 min. After centrifugation at 10000 g for 10 min, 10 μL RNase A (500 U/mL) was added into the lysate of cells at 50 °C for 90 min, followed by treatment with 10 μL proteinase K (500 μg/mL) at 50°C for 90 min. The supernatant was extracted using phenol: chloroform: isoamyl: alcohol (25:24:1) and centrifugated at 12000 g for 10 min. The supernatant was precipitated with ice-cold ethanol for 24 h. The precipitated DNA was dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA) and electrophoresis containing 1% agarose gel and 0.5 μg/mL ethidium bromide was then performed. DNA ladders were visualized after staining with bromophenol blue.

Measurement of mitochondrial transmembrane potential

The inner mitochondrial transmembrane potential of B16 cells was analyzed using a FACScan flow cytometer according to the reported method (Sun, et al., 2006). B16 cells were treated with different concentrations of quinoxaline for 48 h. After trypsinization, B16 cells were washed twice with PBS, and then the concentration of cell suspension was adjusted to 1×10^7 cells/mL. One hundred microliter Rhodamine 123 solutions (Rh123, 20 μg/mL) was added to the harvested cells and incubated at 37°C in the dark for 30 min. Then, the cells were washed with PBS again and stained with propidium iodide solution (PI, 100 μg/mL), rinsed with PBS twice, and checked immediately with flow cytometer. All data were collected and analyzed with Cellfit Analysis Software.
The analysis of expressions levels of Bcl-2, Bax and P53

After treating with different concentrations of quinoxaline (0, 5 and 10 μg/mL), the expressions levels of Bcl-2, Bax and P53 proteins in B16 cells were checked by flow cytometer (Aggarwal and Gupta, 1998). B16 cells were collected, washed and sequentially fixed with 2% paraformaldehyde for 10 min. The cells were treated with 75% ethanol for 1h at 4°C. After washing, the cells were respectively incubated with anti-Bcl-2, anti-Bax and anti-53 (Beyotime Biotechnology Inc., China) for 30 min at 37°C. The cells were incubated with FITC-conjugated goat anti-mouse IgG (Beyotime Biotechnology Inc., China) for 30 min at 37°C. Then, the cells were washed twice with PBS, and checked with FACScan flow cytometer. The percentage of positive cells expressing fluorescence intensity of Bcl-2, Bax and P53 was measured by mean fluorescence channel number.

Caspase-3 activity

Caspase-3 is an important molecular in the regulation of apoptosis. Activity of caspase-3 was detected by using a Caspase-3 colorimetric assay kit (Biovision Inc., USA) according to the manufacturer’s protocol. B16 cells were incubated for different time (12, 24 and 48 h) in the absence (control) or presence of various concentrations of quinoxaline (5, 10 μg/mL). The cells were collected, washed twice, resuspended in 50 μL of chilled cell lysis buffer and incubated on ice for 10min. The lysate was centrifuged at 10000 g at 4°C for 10 min. Then, 50 μL of reaction buffer (containing 10 mM DTT) was added to each sample. After incubation at 37°C for 1.5 h with 5 μLDEVD-pNA substrate (200 μM final concentration), the absorbance was measured at the wavelength of 405 nm in a microtiter plate reader. Results were expressed as the fold relative to control in absorbance.
159  **Statistical analysis**

160  Data were represented as mean±SD. Statistical differences were determined by Student’s t-test.

161  Samples with P values of p<0.05 were considered statistically different.
RESULTS

Antitumor evaluation of quinoxalone on different tumor cells

In this study, B16, DMA-MB231, MCF-7, HepG2 and CT-26 cell lines were used to evaluate the antitumor bioactivity of quinoxalone in vitro. The five cell lines were treated by different concentration quinoxalone for 48 h. Paclitaxel and Epothilone B were the positive controls. As shown in Figure 2, quinoxalone showed strong cytotoxicity to HepG2, B16, MCF-7, CT-26 and DMA-MB231 tumor cell lines, which the value of IC50 were 2.42, 2.2, 6.73, 2.05 and 3.04 µg/mL, respectively. CT-26 cell was more sensitive to quinoxalone than the others. Comparing with the positive controls, the cytotoxicity of quinoxalone was similar with Paclitaxel. The results suggested that quinoxalone showed broad spectrum activity to tumor cells.

Effect of quinoxalone on the morphology of B16 cells

In order to check the influence of quinoxalone on B16 cells, the cells were treated with 0, 5, 10 µg/mL quinoxalone for 48 h. As we can see from Figure 3, it could significantly inhibit the growth of B16 cells. When the dose were 10 and 20 µg/mL, the inhibit rate was about 60% and 80%, respectively, and, some cells turned round and floated comparing with the control. The nuclear morphology of B16 cells treated with quinoxalone for 48 h was observated under a fluorescence microscope by Hoescht 33342 staining (Figure 4). The nuclei of the treated B16 cells have nuclear shrinkage and condensed chromatin.

Effect of quinoxalone on DNA of B16 cells

Externalization of phosphatidylserine and cleavage of DNA, the hallmarks of apoptosis, were also recently found in alternative types of programmed cell death (Jakopec et al., 2006). To determine whether the processes were induced because of quinoxalone, B16 cells were treated with different concentration of quinoxalone (0, 2.5, 5, 10 and 20 µg/mL) for 48 h. As shown in Figure 5, the results of agarose gel electrophoresis showed that DNA fragmentation about 180–200 bp called “DNA ladders” were detected. The concentration of DNA ladders increased
following the concentration of quinoxalone. This observation suggested that quinoxalone could induce apoptosis of B16 cells in the concentration dependent manner.

Effect of quinoxalone on mitochondrial transmembrane potential in B16 cells

Mitochondria is an important organelle in cell. It is very sensitive to around environment and plays an important role in the propagation of apoptosis. The mitochondrial transmembrane potential decreases in apoptosis cell because the permeability of mitochondrial membrane happens to change (Zhang and Huang, 2006). In this study, B16 cells were used to check the effect of quinoxalone on the mitochondrial membrane potential and plasma membrane integrity with PI and Rh123 double-staining method. As we can see from Figure 6, after treatment with 0, 2.5, 5 and 10 µg/mL quinoxalone for 48 h, the percentages of were PI negative and strongly stained by Rh123(Rh123-PI$, normal cells $) decreased insignificantly from 93.0% to 65.9%. The PI negative and low-staining by Rh123 (Rh123-PI$, early apoptosis cells $) group increased markedly from 5.35% to 23.7% in a dose-dependent manner. But, there is no significantly difference between the experimental groups and the control in the cell death group of Rh123-PI$^+$ and Rh123$^+$PI$^+$. These results indicated that quinoxalone target the mitochondria in treatment-induced apoptosis in B16 cells.

Effect of quinoxalone on expression of relative gene-proteins p53, bcl-2 and bax in B16 cell

Many factors influence the process of apoptosis, including of p53, bax and bcl-2 genes. In these genes, bcl-2 and p53 are anti-apoptosis genes. But, bax is promoting-apoptosis gene(Kenji et al.,2003). Based on the apoptosis analysis on cell arrest, p53, bax and bcl-2 genes were checked after treated with different concentrations of quinoxalone, and the expressions levels of P53, Bax and Bcl-2 proteins were analyzed by flow cytometry. As shown in Figure 7, quinoxalone significantly increased the expression level of Bax protein, but significantly decreased the expression of Bcl-2 protein. P53 protein expression level changed slightly after treating with quinoxalone. The results suggested that quinoxalone can efficiently induce B16 cells apoptosis, which is correlated with up-regulating bax expression and down-regulating bcl-2 expression.

Effect of quinoxalone on caspase-3 activity in B16 cell
Caspase-3 plays a critical role in apoptosis and its activity has been suggested as an index of apoptosis (Cohen, 1997). To examine the role of caspases in the apoptosis induced by quinoxalone, its activation was measured using fluorometric detection. As shown in Figure 8, caspase-3 activity assay showed an enhancement of enzymic activity at all experimental time, and reached a peak after 48 h of exposure to quinoxalone. Caspase-3 activity increased following the enhancement of quinoxalone concentration (from 5 µg/mL to 10 µg/mL). The results suggested that quinoxalone activated caspase-3 in a time and dose dependent manner.
DISCUSSION

Tumor is a disease state characterized by proliferation disorder and apoptosis obstacle. Its key characteristics are uncontrolled cellular growth and proliferation. So, the efficient methods to treat tumor are to inhibit cell proliferation and induce apoptosis. Apoptosis is a regulated process characterized by cell shrinkage, nuclear disintegration, selective degradation of DNA, and formation of apoptotic bodies with a relatively intact plasma membrane (Cui et al., 2007). Many natural products inhibit the proliferation of some tumor cell via the apoptosis, for example, a flavone nitroderivative caused murine mammary adenocarcinoma cells death by apoptosis (Mariano et al., 2009). The ability to induce cell apoptosis is an important property of the candidate anti-cancer drugs.

Cytotoxicity determining, a common method to evaluate the biology activity of nature products, is helpful to confirm whether nature products have potential anti-tumor properties (Bruna et al., 1999; Kim et al., 2005). In the previous study, we reported that quinoxalone, a novel compound isolated from the broth of myxobacteria Stigmatella eracta WXNXJ-B, showed significant cytotoxic effect and the proliferation inhibition on different tumor cells. The elucidation of the type of cell death induced by quinoxalone and the role of apoptosis/necrosis is very important for understanding the bioactivity of quinoxalone. With the purpose of investigating the importance of programmed cell death in the cytotoxicity of quinoxalone, we used some different methods, which enabled us to know the mode of cell death and the process of apoptosis induced by quinoxalone. B16 cells were examined for biochemical hallmarks of apoptosis such as morphological changes, DNA fragmentation, sub-G1 cell population (apoptotic cell), mitochondrial transmembrane potential, expression of relative gene-proteins (p53, bcl-2 and bax), and caspase-3 activity.

In this study, we have found that B16, CT-26, HepG2, DMA-MB231 and MCF-7 cells exhibited markedly different sensitivity to quinoxalone. The cytotoxicity of quinoxalone was similar to that of taxal. Using agarose gel electrophoresis, flow cytometry and fluorescence microscopy, we have demonstrated that
Quinoxaline can cause B16 cells apoptosis. DNA fragmentation is very typical characteristic of the apoptotic process, with generation of a series of multiplets of a 180-200 bp subunit. In present study, typical DNA ladder of apoptosis in B16 cells after treated with quinoxaline was detected at every concentration in B16 after treatment, which further indicated that quinoxaline could induce the apoptosis of B16 cells.

Mitochondrial membrane potential plays an essential role in mediating apoptosis (Desagher and Martinou, 2000; Guo and Tao, 2008). In our research, the change of mitochondrial membrane potential was investigated with double-staining experiment (PI and Rh123) by flow cytometry. The experiments showed that the mitochondrial membrane potential decreased following the increase of concentration of quinoxaline. When the cells were treated with quinoxaline at the concentration of 10 µg/mL, we observed that the percentages of PI-Rh123+ decreased from 93.6% to 65.9%, the percentages of PI-Rh123+ increased from 5.35% to 28.7%. The results suggested that the mitochondrial apoptotic death-signal pathway plays a critical role in quinoxaline-induced apoptosis in B16 cells.

Apoptosis is a tightly regulated process and its mechanisms involve in mainly two signaling pathways, including cell death receptor pathway and mitochondrial pathway (Reed, 2001). Apoptosis is a cell death process that plays a critical role in mammalian development and tissue homeostasis. It has now become clear that apoptosis is also the mechanism of tumor cell death in response to a variety of chemotherapeutic agents. The Bcl-2 family of proteins plays a key role in the regulation of apoptosis. Some members of this family, including Bax, Bak, Bid, and Bik, function as proapoptotic factors, and others, including Bcl-2, Bcl-xL, Mcl-1 and A1, function as antiapoptotic proteins (Marc and Mark, 2015). In this study, quinoxaline increased the expression level of Bax protein, but significantly decreased the expression of Bcl-2 protein in B16 cells.

P53, an anti-oncogene, is related to cancer development and progression by its regulation of the tumor cell cycle when DNA is damaged or stressed. P53 functions primarily as a transcription factor, which exerts its downstream functions by activating or repressing a large number of genes that mainly initiate one of three programs of cell cycle arrest, DNA repair or apoptosis (Shu et al., 2007). In this study, we found that P53 protein expression level changed.
slightly after treating with quinoxaline.

Apoptosis is caused by activation of intracellular proteases, known as caspases, which are responsible directly or indirectly for the morphological and biochemical events that characterize the apoptotic cell. Related references revealed that caspase-3, is essential for DNA fragmentation, the morphological change associated with apoptosis, and its activation represents a key and irreversible point in the development of apoptosis (Janicke et al., 1998; Eva et al., 2014). To study further insight into the quinoxaline bioactivity, we checked the activity of caspase-3 in B16 cells. Results showed that quinoxaline enhanced caspase-3 enzymic activity in a time and concentration dependent manner. This result suggested that the mechanism of quinoxaline-induced apoptosis in B16 cells probably involved caspase-3 activation.
CONCLUSION

In this study, we have confirmed that quinoxaline has potent anti-tumor bioactivity, arrest cell cycle and induce apoptosis in B16 cells. The induction of apoptosis was associated with the increase of mitochondrial transmembrane potential, Bax and caspase-3 expression level, the decrease of Bcl-2 expression level. Nevertheless, further studies are needed to clarify the cellular signaling process which quinoxaline induces apoptosis in B16 cell. This makes quinoxaline interesting for further investigations as a potential anti-cancer drug.
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**Competing Interests**

The authors declare there are no competing interests.

**Author Contributions**

Dahong Wang analyzed the data and wrote the paper.

Lanlan Wei performed the experiments, prepared figures.

Shuaiying Zhang performed the experiments.

**Data Availability**

The following information was supplied regarding data availability:

The raw data in the study has been supplied as a Supplemental File.
References


Marc K, Mark GH. 2015. The Bcl-2 family: structures, interactions and targets for drug


Figure 1: The structure of quinalone
Figure 2: Cytotoxicity of quinoxaline to different tumor cell lines. B16, CT-26, HepG2, MCF-7 and DMA-MB231 cells were incubated for 24 h and were treated with different concentrations of quinoxaline for another 48 h. Taxol and Epothilone B were the positive controls. The inhabitation rate and IC$_{50}$ value were calculated. Data were obtained from three repeat experiments.
Figure 3 The influence of quinoxaline on B16 cells observed by inverted microscope (×100).

B16 cells were plated onto glass cover slips in 6-well plates and treated with quinoxaline (A: control, B: 5 μg/mL, C: 10 μg/mL) for 48 h. Then, cells were washed twice with PBS, fixed with 1% glutaraldehyde, stained with Hoechst 33342 for 15 min at room temperature. Nuclear morphology was examined by fluorescence microscope.
Figure 4 Fluorescence micrographs of B16 cells stained with Hoechst 33342. B16 cells were treated without (A) and with quinoxaline (B: 5 μg/mL, C: 10 μg/mL) for 48 h. White arrow were the normal cells in A. White arrow were the apoptosis cells in B and C.
Figure 5 Effect of quinoxaline on DNA of B16 cells. B16 cells were treated with different dose of quinoxaline for 48h. Isolated DNA was analysed in agarose gel electrophoresis as described in Material and Methods. 200 bp DNA ladder marker (novoprotein, China) was used as marker (M) of DNA fragment size. Lane 1: Control; lane 2: 2.5 µg/mL, lane 3: 5 µg/mL; lane 4: 10 µg/mL; M: marker.
Figure 6 Effect of quinoxaline on mitochondrial transmembrane potential in B16 cells. After treatment without (A, control) and with 2.5 µg/mL (B), 5 µg/mL (C), 10 µg/mL (D) quinoxaline for 48 h, the cells were double-stained with Rhodamine-123 and PI for 30 min, respectively. The percentages of PI negative and low-staining (Rh123 PI-) group represent the apoptotic cell group.
Figure 7 Effect of quinoxaline on the expression of Bax, Bcl-2 and P53 protein in B16 cells. Cells were treated without (control) and with 5, 10 μg/mL quinoxaline for 48 h. After washing with 75% ethanol, the cells were respectively incubated with anti-Bcl-2 antibody, anti-Bax antibody and anti-P53 antibody. Then, the cells were incubated with FITC-conjugated secondary goat anti-mouse IgG. Bax (A), Bcl-2 (B) and P53 (C) levels were checked by flow cytometry.
Figure 8 Effect of quinoxaline on the activation of Caspase-3 in B16 cells. Cells were treated with 5 and 10 µg/mL quinoxaline for 12, 24 and 48 h. The levels of caspase-3 activities were evaluated using specific fluorogenic substrates. Data are means ± SD of three repeat experiments. Significant differences with control were designated as *p < 0.05, **p < 0.01.