Moraea sisyrinchium inhibits proliferation, cell cycle, and migration of cancerous cells and decreases angiogenesis in chick chorioallantoic membrane

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Abstract

Objective

Experimental studies reported that some plants in the genus of *Moraea* (*Iridaceae* family) show anticancer multiform and HepG2 liver cancer cells.

Methods

The cells were incubated potential. This study aimed to evaluate the effects of *Moraea sisyrinchium* on U87 Glioblastoma for 24 with hydro alcoholic extract of the stem, flower, and bulb of *Moraea sisyrinchium*. The cell proliferation, cell cycle, and migration were determined by MTT assay, propidium iodide staining, and scratch assay, respectively. Oxidative stress was evaluated by determining the levels of reactive oxygen species and lipid peroxidation. The activity of matrix metalloproteinases (MMPs), the expression of Bax and Bcl-2 proteins, and angiogenesis were evaluated by the gelatin zymography, Western blotting, and model of chick embryo chorioallantoic membrane, respectively.

Results

The extracts of the flower, stem, and bulb significantly decreased the proliferation of HepG2 and U87 cells. This effect was more for U87 than for HepG2 and for bulb and stem than for flower. In U87 cells, the bulb extract increased oxidative stress, cell cycle arrest, and the Bax/Bcl-2 ratio. Also, this extract suppressed the migration ability of HepG2 and U87 cells, which was associated with the inhibition of MMP2 activity. In addition, it significantly reduced the number and diameter of vessels in the chorioallantoic membrane. Liquid chromatography-mass spectrometry revealed the presence of xanthones (bellidifolin, mangiferin), flavonoids (quercetin, luteolin), isoflavones (iridin,tectorigenin),and phytosterols (e.g., stigmasterol) in the bulb extract.

Conclusion

The extract of *Moraea sisyrinchium* bulb decreased the proliferation and survival of cancer cells by inducing oxidative stress. The extract also reduced the migration ability of the cells and inhibited angiogenesis.

Introduction

Plant-derived compounds are good candidates for finding new anticancer drugs(1, 2). In the last decades, the anticancer activity of many phytochemicals and plant extracts has been proposed by experimental and clinical studies (1, 3, 4). The in vitro investigations showed that some secondary metabolites of the plants suppress cancer cells through oxidative DNA damage and induction of apoptosis(3).Also, several

phytochemicals have been shown to prevent the invasion of cancer cells through inhibiting matrix metalloproteinases (MMPs) activities and modulating different signaling pathways such as the PI3k/Akt pathway, VEGF pathway, and MAPK pathway(5–7).

Previous studies reported that some plants in the family of *Iridaceae* such as *Moraea polystachya* show cytotoxic activity against cancer cells (8, 9). *Moraea sisyrinchium*(synonym: *Gynandriris sisyrinchium* and *Iris sisyrinchium*) is one of the plants in this genus that is distributed in many countries including Egypt, Turkey, Jordan, and Iran. (10–12). Pharmacological effectsof this plant have not been well determined so far. It has been suggested that its leaves and bulbs have antimicrobial activities(13). In an in vitro study,Al-Qudah et al. showed that is flavones isolated from this plant reducedthe viability of human promyelocytic leukemia cells (12).As far as we know, no other study has examined the anticancer activity of *Moraea sisyrinchium*. The present work aimed to examine the cytotoxic effect of *Moraea sisyrinchium*againstHepG2 liver cancer and U87 Glioblastoma multiform cells.

Hepatocellular carcinoma, the most common type of primary liver cancer, is one of the leading causes of cancer-related death in the world. The incidence rate of liver cancers has increased over the last decades and is expected to rise in future years (14). Glioblastoma is one of the most common malignant tumors of the brain and accounts for approximately 15% of all primary brain tumors and 46% of primary malignant brain tumors (15). Despite advances in surgery, radiotherapy, and chemotherapy, the survival of patients with hepatocellular carcinoma and glioblastoma multiform is low (16, 17).Therefore, many studies are designed to find novel anti-cancer substances against these cancers.

Materials And Methods

Preparation of Moraea sisyrinchium **extract**

Moraea sisyrinchium was collected from Dargazmountains (Northeast Iran) and identified by Dr. Mohammad Sadegh Amiri at the herbarium of Dargaz Payame Noor University (voucher specimen number:712). The flowers and stems were ground separately to fine powders, while the bulbs were used fresh. A sample (10 g) of each part of the plant was soaked in 70% ethanol (200 mL) for3days at 40°C with occasional shaking. The obtained extracts were filtered (pore size 250 µm) and then centrifuged for 5 min at 500 g to remove sediment particles. The supernatants were dried in an oven at 40°C and the dried extracts were kept frozen at less than − 18°C until use. The solid residue of the extracts of the flower, stem, and bulb were 40%, 18%, and 5%, respectively.

Cell Culture And Treatment

The HepG2, U87, and L929 (mouse fibroblast as nonmalignant control) cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), and 1% penicillin/streptomycin solution (Sigma, St Louis, MO, USA). For cell proliferation assay and reactive oxygen species (ROS) evaluation, the cells were seeded in

96-well plates (5×10^3 cells/well) and then treated for 24 hr with the extracts (25–400 µg/mL). For cell cycle analysis, the cells were seeded in 12-well plates (5×10^5 cells/well) and treated for 24 hr with the extracts.

Cell proliferation assay

The cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) reagent (Sigma, St Louis, MO, USA), which is reduced to purpleformazan crystals in mitochondria of living cells (18). The working solution of this reagent was added to the culture medium at a final concentration of 0.05%. After 4 hr, the medium was replaced by dimethyl sulfoxide (200 µL per well) to dissolve the formazan crystals. Then, the optical absorbance of each cell was measured at 570 and 620 nm (background).

Cell Cycle Analysis

After incubation with the extracts, the U87 cells were trypsinized, centrifuged (5 min at 500 *g*), and re-suspended in a buffer containing propidium iodide reagent(Sigma, St Louis, MO, USA). This buffer also consisted of0.1% sodium citrate and 0.1% Triton-X 100, which makes the cell membrane permeable to stain nucleic acids. After 30 min, the cell cycle was evaluated using a FACSCALIBUR™ flow cytometer (Becton Dickinson, Mountain View, CA, USA). The analysis of flow cytometry data was performed by FlowJo ® vX.0.7 software (Tree Star, Ashland, OR, USA).

Measurement Of Intracellular Reactive Oxygen Species (Ros)

The level of ROS in U87 cells was determined using dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma, St Louis, MO, USA), which is converted to highly fluorescent dichlorofluorescein in the presence of ROS. At the end of treatment, the cells were washed with PBS and incubated for 30 min with20 µM of H2DCF-DA at 37°C. Then, the fluorescence intensity was determinedusingan FLUO-star galaxy fluorescence plate reader (Perkin Elmer 2030, Multilabel reader, Finland) at an excitation/emission wavelength of 485/530 nm.

Lipid Peroxidation Assay

Lipid peroxidation in U87 cells was evaluated by determining the malondialdehyde level, the end-product of the peroxidation reaction. At the end of treatment (24 hr),the cells were trypsinized and centrifuged for 5 min at500*g*. Then, the cells were homogenized in an ice-cold KCl solution (1.15%) and centrifuged at 2000 *g* for 30 min at 4°C.Finally, 500 µL of the supernatant was incubated with 500 µL of trichloroacetic acid (15%) and 800 µL of thiobarbituric acid (0.7%). The mixture was put in boiling water for 60 min and after cooling, it was centrifuged for 10 min in 1000*g* at 4ºC. The fluorescence intensity of the supernatant was determinedat an excitation/emission of 530/550 nm.

Scratch Assay

The migration capability of HepG2 and U87 cells was evaluated by scratch wound healing assay, which determines their expansion rate on culture surfaces. The cells were cultured in 12-well flat-bottom plates until grown to a confluence cell monolayer. Then, a linear scratch was applied in the monolayer using a sterile pipette tip and the cellular debris was washed with PBS. Then, the cells were incubated in the presence of the bulb extract(1–6 µg/mL) for 24 hr at 37°C with 5% CO_2. The experiment was performed in triplicate and images were analyzed using Image J software.

Gelatin Zymography

The activities of MMP-2 and MMP-9 were evaluated by the gelatin zymography method. The U87 cells were seeded in 12-well plates (2×10^5 cells/well) and treated with the bulb extract. After24hr,the supernatant of each well was centrifuged at 300 *g* for 10 minand 30 µg of the supernatant was electrophoresed on a 10% polyacrylamide gel containing gelatin as substrate (10 mg/mL). The gel was washed with 2% Triton X-100 and incubated in a developing buffer (50 mM Tris-HCl, 5 mM CaCl2, 0.2 M NaCl, and 1.0 µM ZnCl2) for 36 hr at 37°C. After incubation, the gel was stained with Coomassie Brilliant Blue R-250 and de-stained using a solution composed of 25% ethanol and 10% acetic acid in dH2O. Finally, the gel was photographed by aGS-800 calibrated densitometer (Bio-Rad, HC, USA) and analyzed using Image J software.

Western Blot Analysis

The U87 cells were treated for 24 hr with the bulb extract. Then, they were lysed using RIPA buffer, centrifuged, and the supernatants were subjected toprotein concentration assay by a BSA kit. The proteins were separated by 7.5–15% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (GEHealthcare Lifescience, Germany). After blocking, the membrane was incubated for 3 hr with primary antibodies against Bax, Bcl-2, and β-actin proteins. Then, the horseradish peroxidase-conjugated secondary antibody was added and the protein bands were made visible using an enhanced chemiluminescence system. The relative expression was determined using Image J software and compared to the β-actin protein.

Angiogenesis Assay

The effect of the bulb extract onangiogenesis was evaluated using the chicken chorioallantoic membrane model. The fertilized chicken eggs were put in an incubator with a temperature of 37°C and humidity of 70%. After 8 days, a window (1.5-2 cm) was opened on each eggshell and the extract(25 or 50 µg/egg)or PBS (50 µL, as control) was injected into the chorioallantoic sac. The windows were closed with sterile parafilm and the eggs were returned to the incubator. On day 12, the chorioallantoic membrane

vasculatures were photographed using a stereo microscope equipped with a digital camera (Canon EOS 40D). Analysis of the vessels was performed by Image J software.

Liquid Chromatography-mass Spectrometry

High-performance liquid chromatography-mass spectrometry (LC-MS) was performed in an Agilent 1200 series liquid chromatography coupled with Agilent 6410 triple quadrupole Mass Spectrometer. Liquid chromatography separation was performed on an Agilent Eclipse Plus C18 (2.1×100mm×3.5 µm) column. The flow rate was set at 0.4 mL/min and the mobile phase consisted of (A) water + 0.1% formic acid and (B) methanol + 0.1% formic acid. The gradient programs were as follows: 0.0–1.0 min 10% B, 1.0–40 min from 10–100% (B), 40.0–42.0 min 100% (B), and 42.0–50 min from 100–10% (B). The mass spectra were acquired in a range of 100 to 1000 within the 50 min scan time. The positive electrospray ionization (ESI) mode was applied for the mass spectrometer. Mass feature extraction of the acquired LC-MS data and maximum detection of peaks was done using the MZmine analysis software package, version 2.3.

Statistical analysis

Comparison of data between control and concentrations of the extracts or reference drugs (doxorubicinand, temozolomide) was made by one-way analysis of variance (ANOVA) followed by the Dunnett post hoc test. The $p < 0.05$ was considered statistically significant and results are presented as mean ± SEM.

Results

Effects of Moraea sisyrinchium **on cell proliferation**

As shown in Fig. 1, the extracts of the flower, stem, and bulb of *Moraea sisyrinchium* significantly decreased the proliferation of HepG2 cells at concentrations of ≥ 100 µg/mL after 24 hr ($p < 0.05$). Doxorubicin as a reference drug showed anti proliferative activity at much lower concentrations than the plant extracts (3 and 6 µg/mL, $p < 0.001$).

Similarly, incubation of U87 cells for 24 hr with the extract of flower, stem, or bulb of *Moraea sisyrinchium*, significantly ($p < 0.001$, at ≥ 25 µg/mL) decreased their proliferation (Fig. 2). Temozolomide as a reference drug for U87 cells inhibited proliferation at concentrations of ≥ 6 µg/mL ($p < 0.05$). The effect of temozolomide at concentrations of 12 and 24 µg/mL was approximately comparable to the effect of bulb extract at 25 µg/mL. The extract of the bulb at concentrations of 25 and 50 µg/mL could increase the antiproliferative activity of temozolomide (1.5–12 µg/mL).

The tested extracts had no antiproliferative effect on nonmalignant L929 cells at 24 hr. However, after 48 hr the extracts of stem and bulb decreased L929 proliferation at 400 µg/mL(Fig. 3).

Effects of Moraea sisyrinchium on oxidative stress

Incubation of U87 cells for 24 hr with the extract of stem or bulb, at concentrations of 25 µg/mL and above, significantly ($p < 0.001$) increased the ROS content (Fig. 4A). However, the extract of flower could increase the ROS level only at high concentrations(200 and 400 µg/mL).

An increase in ROS content can lead to peroxidation of cellular lipids, which can be assessed by evaluatingthe level of malondialdehyde. As shown in Fig. 4B, all the tested extracts significantly increased the level of malondialdehyde in U87 cells.

Effects of Moraea sisyrinchium on sub-G1 cell population

As shown in Fig. 5, the culture of U87 cells in the presence of extracts of stem or bulb significantly increased the percentage of cells in the sub-G1 stage compared to untreated cells ($p < 0.05$). This pro-apoptotic effect was concentration-dependent for both stem and bulb extracts.

Effects of Moraea sisyrinchium on cell migration

Representative photographs of HepG2 cells treated with non-cytotoxic concentrations of the bulb extract are shown in Fig. 6. Scratch migration assay showed that the distance between the cells disappeared after 24 hr in the untreated group. The bulb extract at concentrations of 6, 12.5 and 25µg/mL significantly decrease the migration rate ($p < 0.05$). Similarly, the extract significantly inhibited the migration U87 cells at non-toxic concentrations (1.5 µg/mL and 3 µg/mL, Fig. 7).

Effect of Moraea sisyrinchium on the activity of MMPs

As shown in Fig. 8, the activity of MMP-2 in U87 cells significantly decreased in the presence of 3 µg/mL of the bulb extract($p < 0.01$). However, treatment with the extract had no significant effect on the MMP-9activity.

Effect of Moraea sisyrinchium on Bax and Bcl-2 expression

The analysis of Western blotting showed that the bulb extract at a concentration of 25 µg/mL significantly increased the expression of Bax protein in U87 cells ($p < 0.05$, Fig. 9). On the other hand, the expression of Bcl-2 was down-regulated under exposure to 12.5or 25 µg/mL of the extract($p < 0.01$).

Effect of Moraea sisyrinchium on angiogenesis

The eggs that were treated with 25 or 50 µg of the bulb extract had fewer vessels in their chorioallantoic membrane than the control group ($p < 0.05$, Fig. 10). Also, the diameter of vessels formed in the membrane significantly decreased in groups treated with the extract($p < 0.05$ and $p < 0.05$).

The LC-MS analysis of Moraea sisyrinchium hydroalcoholic extract

In total, 31 compounds were identified in the hydro-ethanol extract of *Moraea sisyrinchium* bulb which is mainly a rich source of xanthones, flavonoid-glycosides, is flavones, and other derivatives. Data concerning the identification of the compounds are shown in Table 1. The total ion chromatogram of the extract is shown in Fig. 11. The MS spectral data were compared with the reported compounds in some previous literature. Some examples of extracted ion chromatograms from the total ion chromatogram and its related mass are shown in Supplementary Figure S1-S5. Most of the compounds detected in the hydroalcoholic extract have been previously reported in *Iris* species. Some xanthones including iriflophenone 4-O-hexoside, mangiferin, 7-O-methyl-mangiferin, iriflophenone 4-O-(6"-acetyl)-hexoside, polygalaxanthone, bellidifolin, and iriflophenone were detected in the extract. Some flavonoids including luteolin 8-hexoside, apigenin 6-C-hexoside, quercetin 3-O-glucoside, and irisdichotin B were also identified. The compounds tectoridin, iristectorin B and A, iristectorigenin A-O-hexuronide, iridin, irifloside, tectorigenin, iristectorigenin A, irigenin, irilone, iriflogenin and irisolidone were characterized as isoflavones. Therefore, it seems thatthe hydroalcoholic extract *Moraea sisyrinchium* bulb is a good source of xanthones, flavonoids, and isoflavones.

Table 1
Peak assignment of metabolites in the hydro-ethanol extract of *Moraea sisyrinchium* using LC-MS in the positive mode.

Peak No.	Compound	t_R (min)	[M + 1] (*m/z*)	Ref.
1	Iriflophenone 4-O-hexoside	31.0	409.3	(26, 27)
2	Mangiferin	40.1	423.3	(26, 28, 29)
3	7-O-Methyl-mangiferin	39.8	437.3	(26, 27)
4	Iriflophenone 4-O-(6"-acetyl)-hexoside	41.3	451.3	(26, 27)
5	Polygalaxanthone III	39.6	569.2	(26, 27)
6	Bellidifolin	27.5	274.4	(26, 27, 30)
7	Luteolin 8-hexoside	40.9	449.3	(26, 27)
8	Apigenin 6-C-hexoside	43.8	433.3	(26, 27)
9	4'-O-Methyl-apigenin-Chexoside	40.1	447.3	(26, 27)
10	Quercetin 3-O-glucoside	41.0	465.3	(26, 27, 30)
11	Irisdichotin B	40.9	495.3	(26, 27)
12	Tectoridin	40.3	463.2	(26, 27)
13	Iristectorin B	42.0	493.2	(26, 27)
14	Iristectorigenin A 7-O-hexuronide	37.1	507.2	(26, 27)
15	Iristectorin A	42.1	493.3	(26, 27, 30)
16	Iridin	37.1	523.1	(26, 27, 30)
17	7-O-Methyl-tectorigenin 4'-O-(6"-hexosyl)-hexoside	42.0	639.2	(26, 27)
18	Irifloside	34.4	491.1	(26, 27)
19	Irisolidone 7-O-hexoside	41.7	477.3	(26, 27)
20	Tectorigenin	22.6	3012	(26, 27, 30)
21	Dichotomitin 3'-O-hexoside	43.0	521.2	(26, 27)
22	Irilone 4'-O-[6"-(3-hydroxy-3-methylglutaryl)]-hexoside	44.2	605.2	(26, 27)

Peak No.	Compound	t_R (min)	[M + 1] (*m/z*)	Ref.
23	Iristectorigenin A	41.3	331.4	(26, 30)
24	Irigenin	25.3	361.2	(26, 27)
25	Irilone	30.2	299.3	(26, 30)
26	Iriflogenin	40.5	329.4	(26, 30)
27	Irisolidone	34.0	315.3	(26, 30)
29	Iriflophenone	14.4	247.1	(26, 30)
30	Pallasone B Dihydroirisquinone	38.1	377.4	(30)
31	Stigmasterol	40.5	413.3	(30)
32	Unknown	25.7	365.1	-
33	Unknown	18.1	325.4	-
34	Unknown	34.9	319.4	-
35	Unknown	38.8	353.4	-
36	Unknown	43.2	615.3	-

Discussion

The present study showed that different parts of *Moraea sisyrinchium* decrease the proliferation and migration of liver cancer and glioblastoma cells *in vitro*. The inhibitory effect of all the parts (flower, stem, bulb) of *Moraea sisyrinchium* on cell proliferation was in a concentration-dependent manner. The anticancer effect of the stem and bulb was higher than the flower for both HepG2 and U87 cells. Since the extract of stem considerably decreased the proliferation of non-malignant L929 control cells at a concentration of 400 µg/mL, we focused on the bulb extract (which seems to be safer) for the next experiments. Also, for cell cycle evaluation and oxidative stress assessment, we mainly focused on U87 cells because the effect of bulb extract (at 25 µg/mL) was almost comparable to that of temozolomide (at 12 and 24 µg/mL), while the plant extracts failed to show antiproliferative activity on HepG2 at concentrations close to that of doxorubicin.

Doxorubicin, an antibiotic-derived compound, is used for the treatment of different cancers. The mechanisms responsible for its anticancer effect include disruption of topoisomerase-II-mediated DNA repair and increase of free radicals generation, which damage cellular membranes, proteins, and DNA (19). In the present study, doxorubicin was used as a reference drug for HepG2 cells and showed concentration-dependent antiproliferative activity. A similar activity was observed for temozolomide (≥ 6 µg/mL), as a reference drug against U87 cells.The bulb extract could increase the antiproliferative activity

of temozolomide even at concentrations of this drug that did not have a significant action (1.5 and 3 µg/mL). Therefore, this extract may be a candidate to be used in combination with chemotherapy drugs for glioblastoma. The extract alsoenhanced the generation of ROS and lipid peroxidation in U87 cells. These effects were associated with an increase in the percent of sub-G1 cells, suggesting the occurrence of cell cycle arrest or apoptosis due to oxidative stress. In support of this possibility, the bulb extract increased the expression of the pro-apoptotic protein Bax and decreased the anti-apoptotic protein Bcl-2.

One of the biggest problems in the treatment of cancer is the migration of malignant cells to nearby tissue and distant organs.(20). In the present work, we tested whether *Moraea sisyrinchium* could decrease the migration capability of glioblastoma and liver cancer cells. It was observed that the bulb extract at non-cytotoxic concentrations decreased the migration ability of both HepG2 and U87 cells. This effect was accompanied by a decrease in the activity of the MMP-2 enzyme. The MMP-2 was shown to modulate glioma cell migrationby disrupting the composition of the extracellular matrix andaltering the expression of cell surface adhesion receptors(21).Also, theMMP-2 can regulate vascular growth and therefore survival and invasion of glioblastoma(22).The model of chick embryo chorioallantoic membrane showed that the bulb extract inhibitedvascular growth as the number and diameter of vessels decreased.

Some types of plant-derived compounds including polyphenols, alkaloids, and terpenes have been found to inhibit cancer initiationand invasion.(1, 5).Only limited works have been done to characterize active compounds in *Moraea sisyrinchium.* Shabrawy et al. foundsome flavonoids including apigenin, luteolin, orientin, saponarin, and isovitexin in this plant(11).Al-Qudah et al. reported that *Moraea sisyrinchium* containsβ-sitosterol, hispidulin, galangustin,ursolic acid, andladanetin. In the present study, liquid chromatography-mass spectrometry revealed the presence of several compounds including xanthones (e.g., bellidifolin and mangiferin), flavonoids (e.g., quercetin and luteolin), isoflavones (e.g., iridin andtectorigenin), and phytosterols (e.g.,stigmasterol)in the bulb extract. Although it is currently not possible to suggest exactly which compound is responsible for the anticancer effects of *Moraea sisyrinchium*, previous studies have shown cytotoxic activity for tectorigen in, stigmasterol,mangiferin,tectoridin, iristectorin B, and bellidifolin against different cancerous cells includingglioblastoma (tectorigenin) and HepG2 liver cancer (tectorigenin, stigmasterol)(23–25).

In conclusion, the extracts of the bulb, stem, and flower of *Moraea sisyrinchium* decreased the proliferation and cell cycle of cancerous cells by inducing oxidative stress. The bulb extract also inhibited angiogenesis and diminished the migration capability of the cells by reducing the activity of MMP-2. Therefore, *Moraea sisyrinchium* may be a candidate for complementary treatment of glioblastoma in future studies.

Declarations

Ethics approval

The questionnaire and methodology for this study was approved by the Human Research Ethics committee of Mashhad University of Medical Sciences (Ethics approval number: IR.MUMS.MEDICAL.REC.1398.418).

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Figures

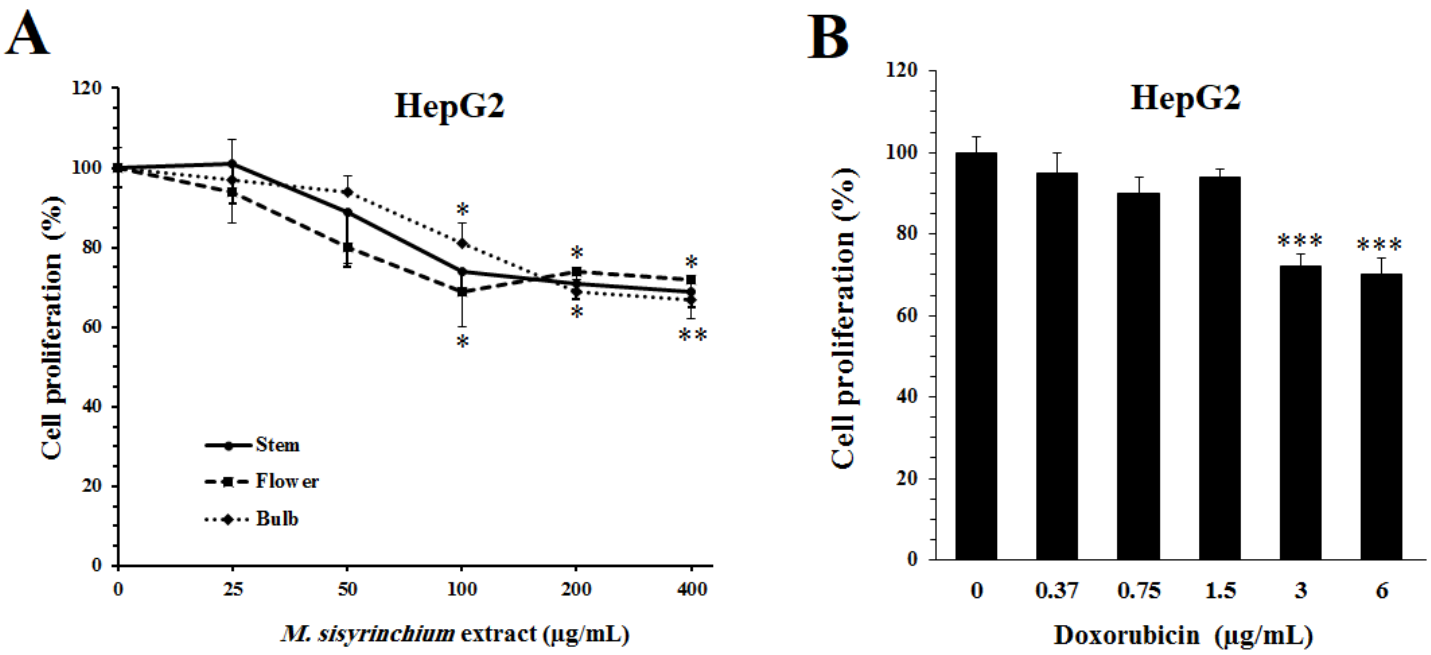

Figure 1

Effects of *Moraea sisyrinchium*(A) and doxorubicin (B) on the proliferation of liver cancer (HepG2) cells. The cells were incubated for 24 hr. Data are presented as mean ± SEM (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 compared to untreated cells (concentration of 0)

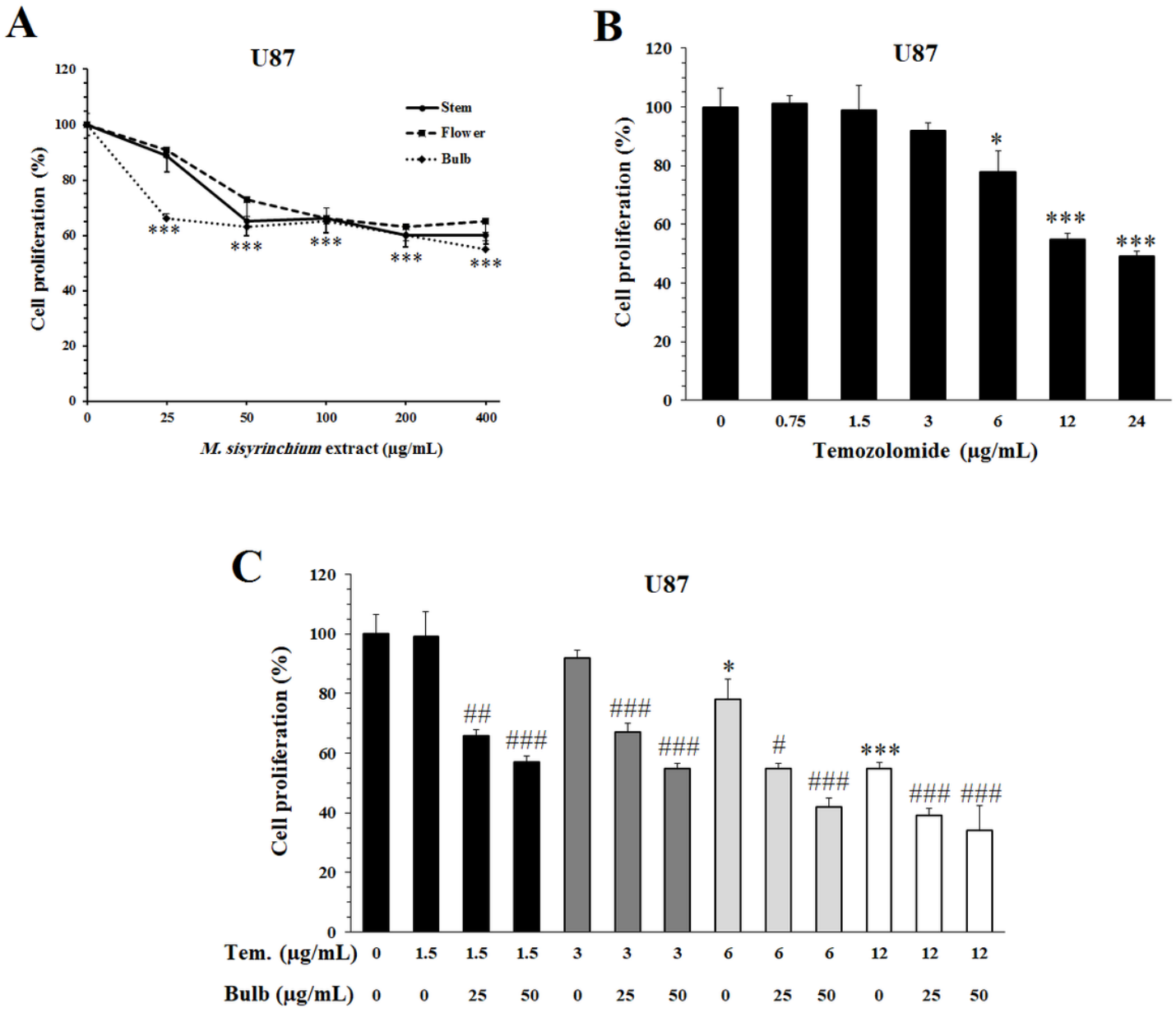

Figure 2

Effects of *Moraea sisyrinchium* and temozolomide(Tem) on the proliferation of glioblastoma multiforme (U87) cells. The cells were incubated for 24 hr with hydroalcoholic extract of the stem, flower, and bulb of *Moraea sisyrinchium* (A), Tem, or a combination of the bulb extract and Tem (C). Data are presented as mean ± SEM (n = 4). *p < 0.05, ***p < 0.001 compared to untreated cells (concentration of 0); $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 compared to only Tem-treated cells (corresponding concentration)

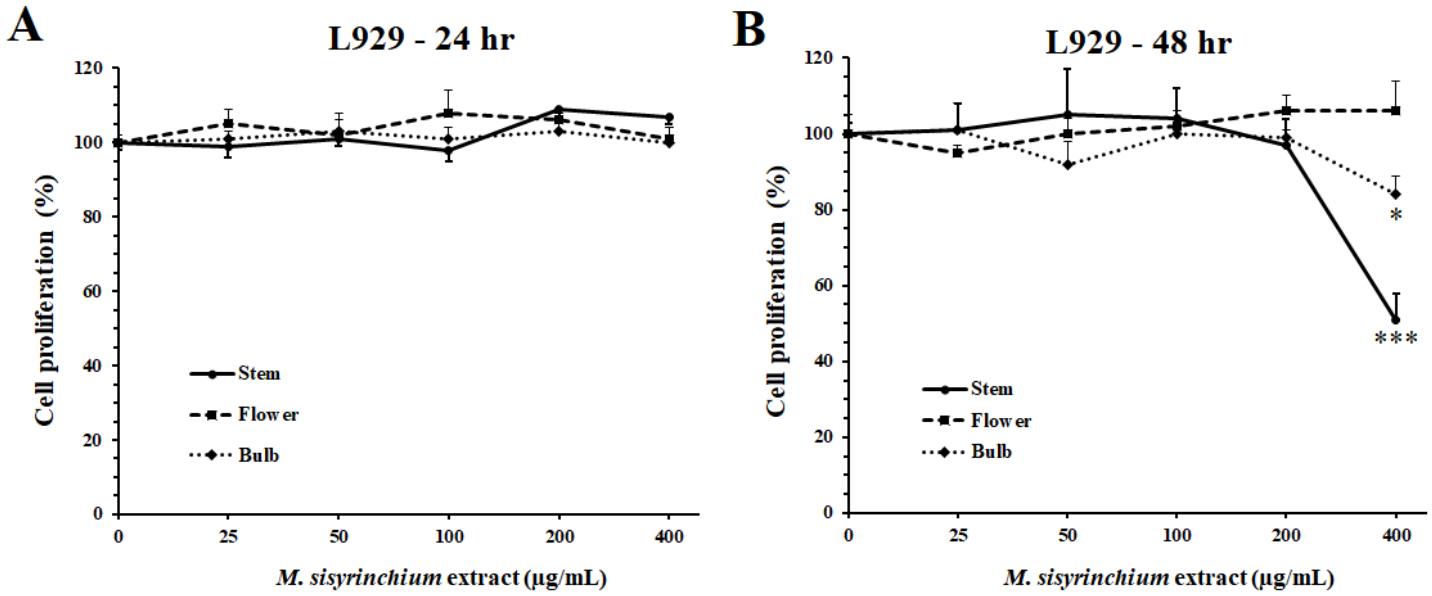

Figure 3

Effects of *Moraea sisyrinchium*on the proliferation of mouse non-malignant fibroblasts (L929). The cells were incubated for 24 hr (A) or 48 hr (B) with hydroalcoholic extract of the stem, flower, and bulb of *Moraea sisyrinchium.*Data are presented as mean ± SEM (n = 4). *p < 0.05, ***p < 0.001 compared to untreated cells (concentration of 0).

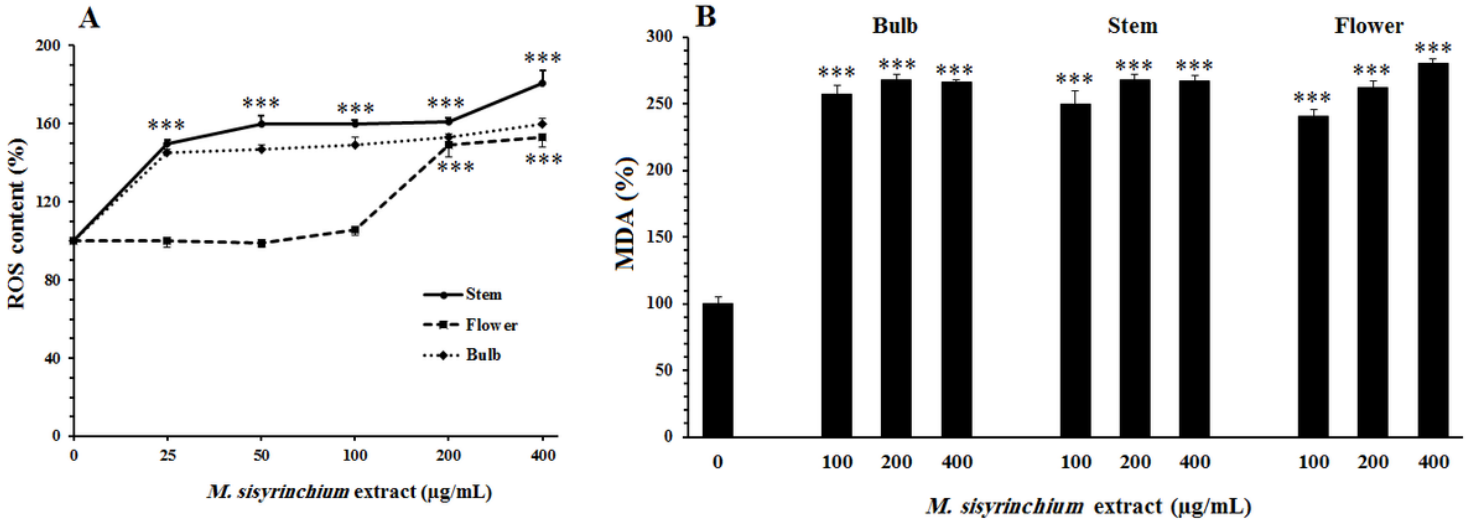

Figure 4

Effects of *Moraea sisyrinchium*on the content of reactive oxygen species (ROS) (A) and malondialdehyde (B) in glioblastoma multiforme (U87) cells. The cells were incubated for 24 hr with hydroalcoholic extract of the stem, flower, and bulb of *Moraea sisyrinchium.*Data are presented as mean ± SEM (n = 6). ***p < 0.001 compared to untreated cells (concentration of 0)

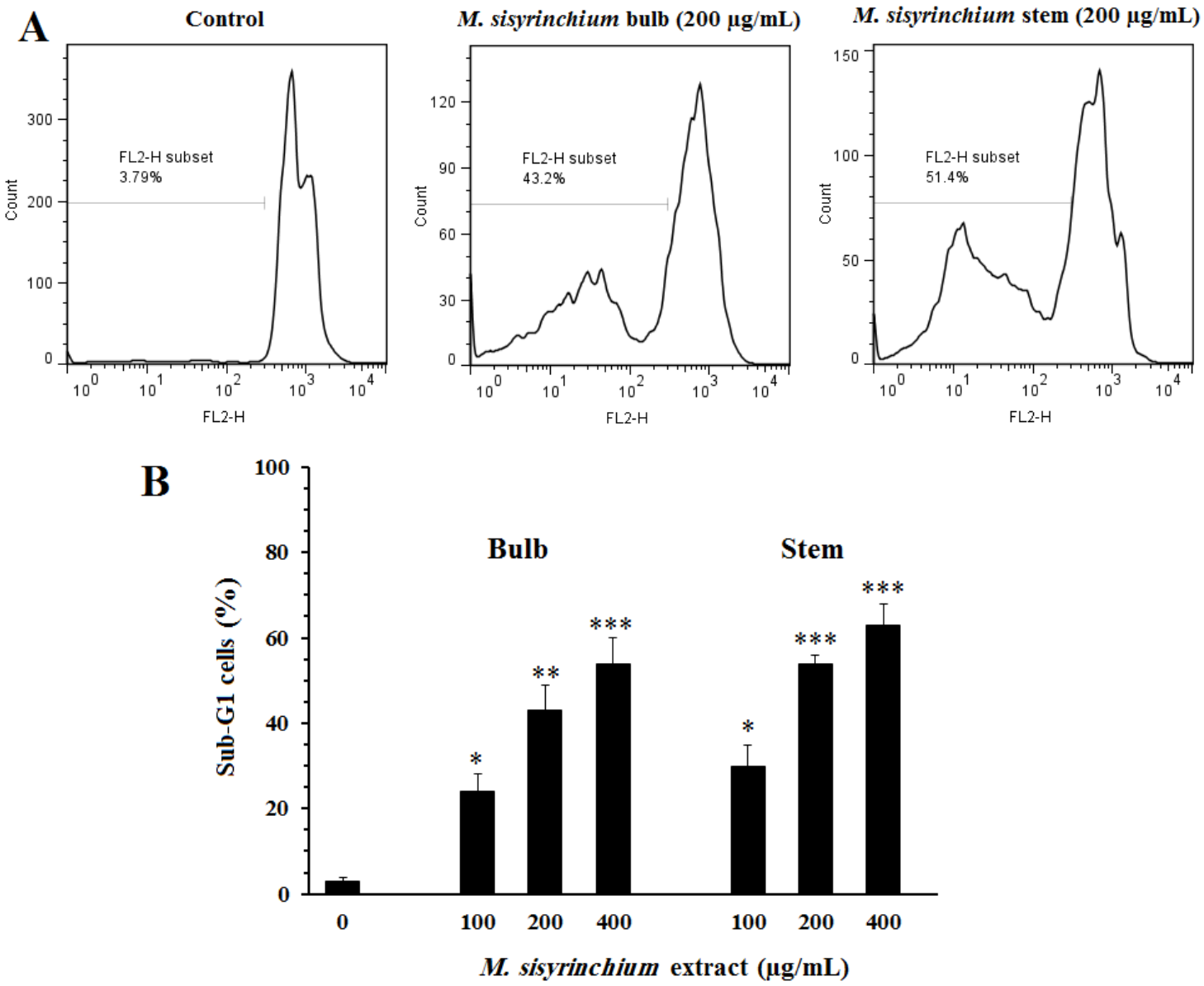

Figure 5

Effects of *Moraea sisyrinchium*on the cell cycle of glioblastoma multiforme (U87) cells. A: Representative histogram of propidium iodide-stainedU87 cells treated for 24 hr with hydroalcoholic extract of the bulb and stem of *Moraea sisyrinchium*.B: Quantitative analysis of cell population in the sub-G1 stage following treatment with *Moraea sisyrinchium* extracts.Data are presented as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to untreated cells (concentration of 0)

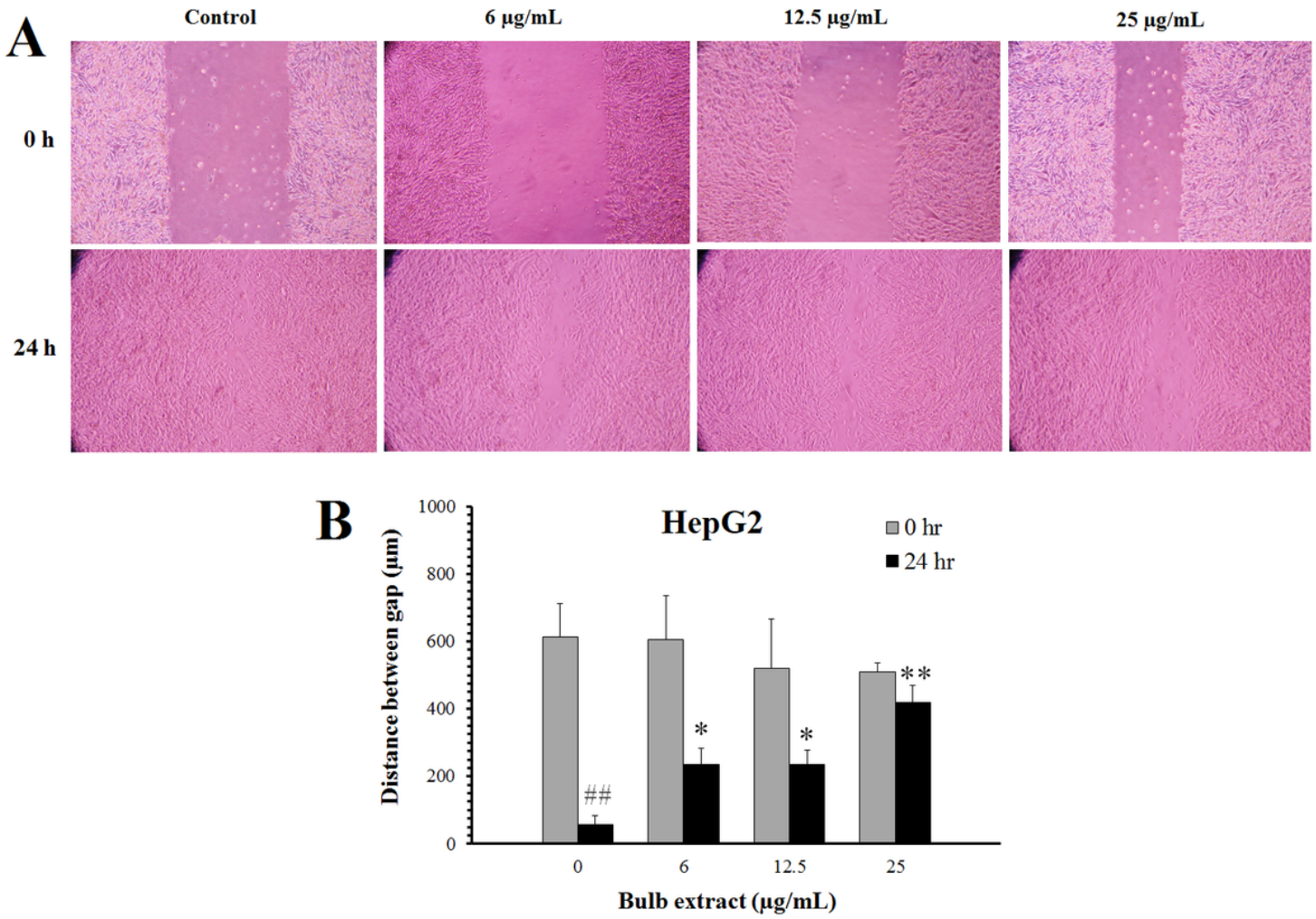

Figure 6

Effects of *Moraea sisyrinchium* bulb on the migration of liver cancer (HepG2) cells. A: Representing photomicrographs of the cells immediately and 24 hr following scratching culture plates. B: Quantitative analysis of the distance between scratched gap areas. Data are presented as mean ± SEM (n = 3). $^{\#\#}p < 0.01$ compared totime 0 (corresponding concentration); *p < 0.05 and **p < 0.01 compared to untreated cells (concentration of 0) at time 24 hr

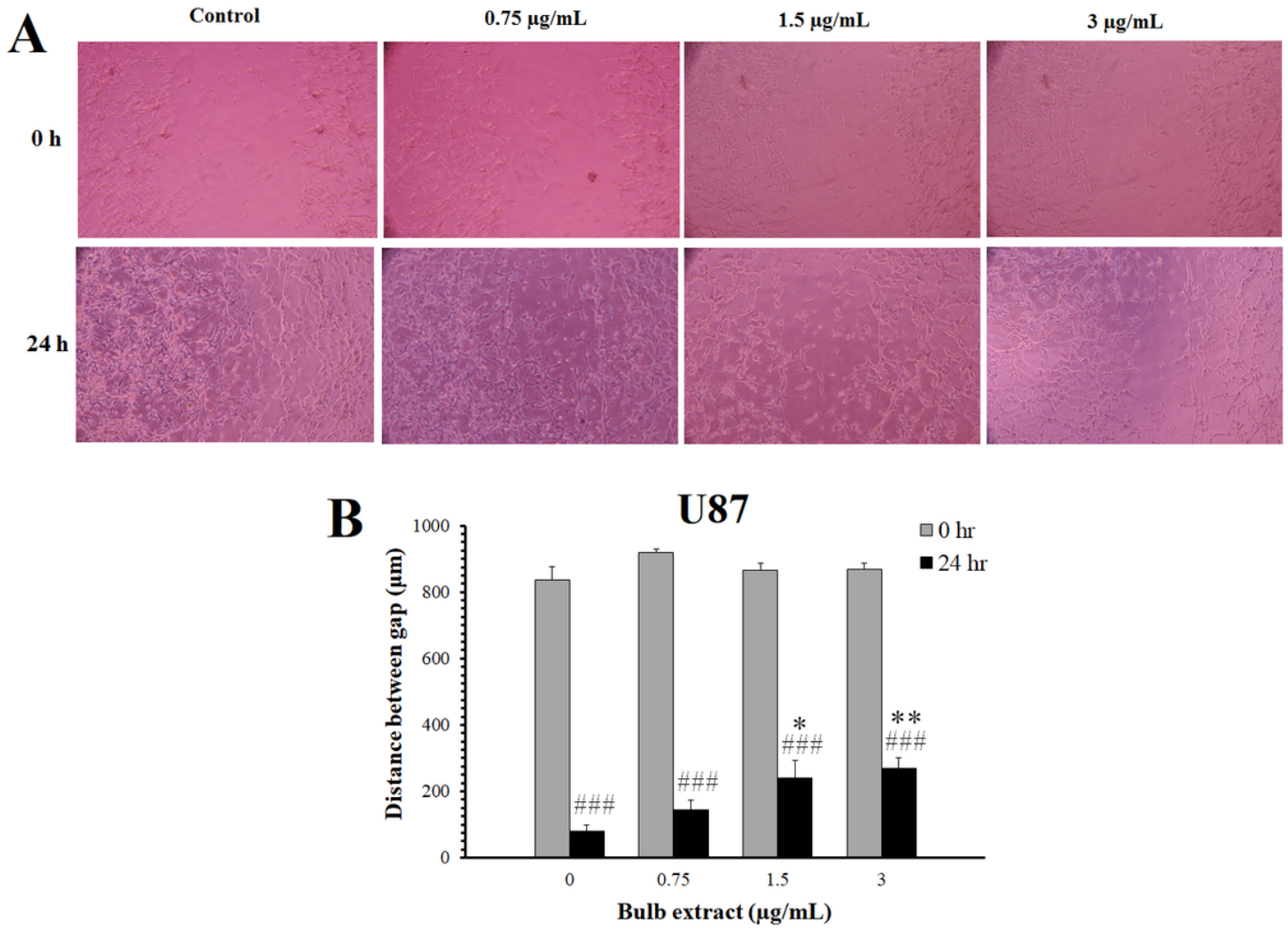

Figure 7

Effects of *Moraea sisyrinchium* bulb extract on the migration of glioblastoma multiforme (U87) cells. A: Representing photomicrographs of the cells immediately and 24 hr following scratching culture plates. B: Quantitative analysis of the distance between scratched gap areas. Data are presented as mean ± SEM (n = 3). $^{###}p < 0.001$ compared to time 0 (corresponding concentration); *p < 0.05 and **p < 0.01comparedto untreated cells (concentration of 0) at time 24 hr

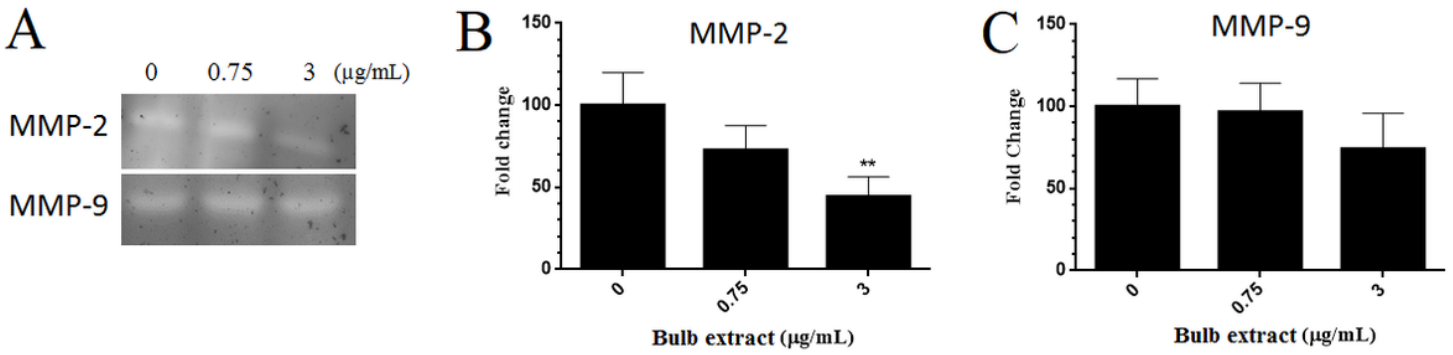

Figure 8

Effects of *Moraea sisyrinchium* bulb extract on the activity of matrix metalloproteinases (MMPs) in U87 cells. Extracellular activities of MMP-2 and MMP-9 were evaluated by the gelatin zymography method. The data are presented as the mean ± SEM (n = 3). **p < 0.01 compared to untreated cells (concentration of 0)

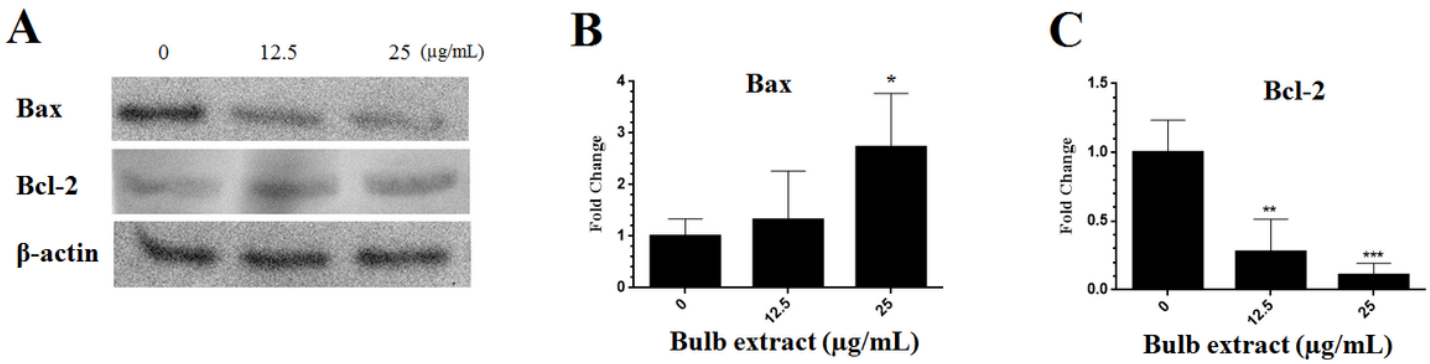

Figure 9

Effects of *Moraea sisyrinchium*on the expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 in U87 cells. The cells were treated with the bulb extract for 24 hr and then subjected to Western blot analysis. *p<0.05, **p<0.01 and ***P<0.001 compared to untreated cells (concentration of 0)

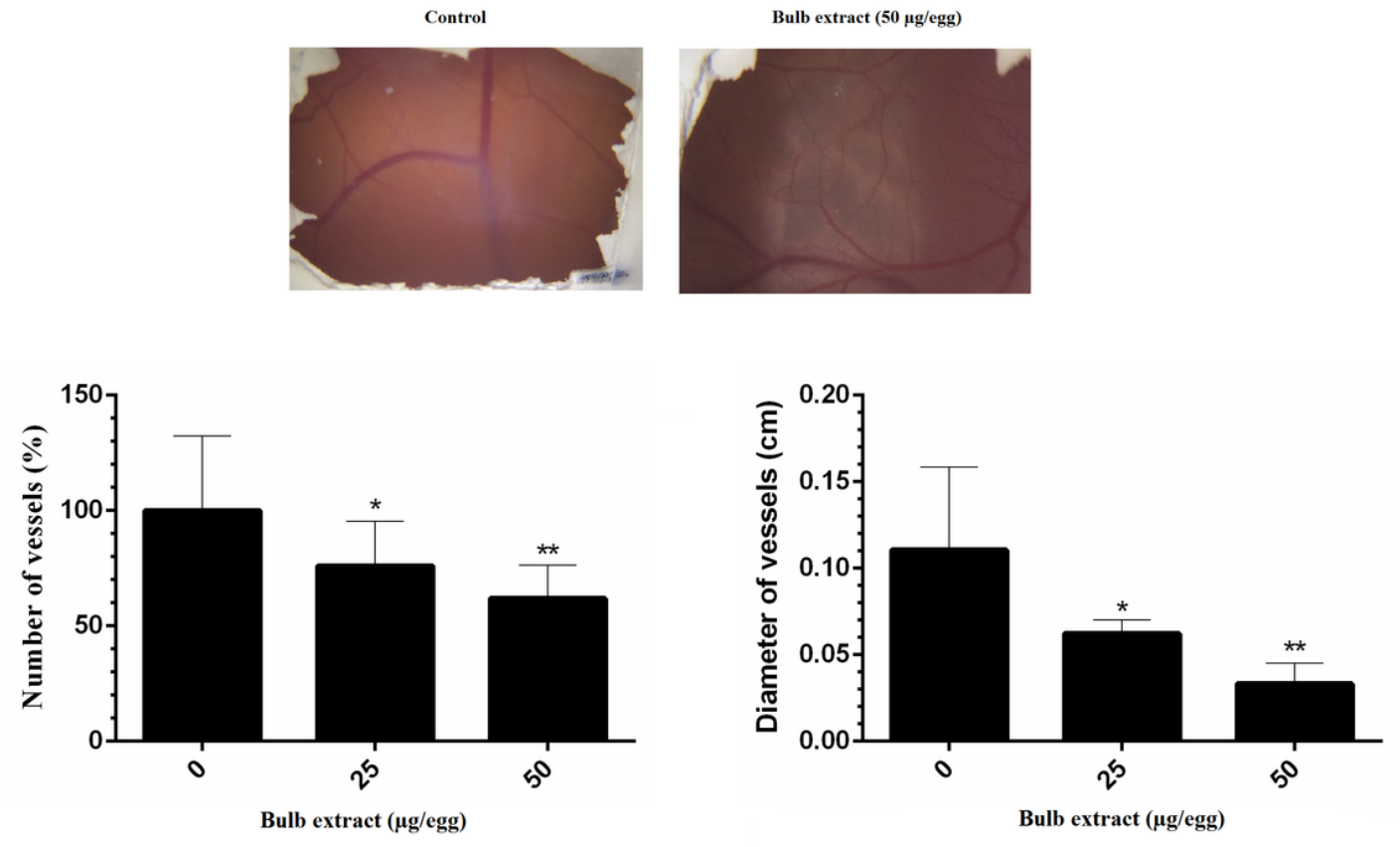

Figure 10

Effects of *Moraea sisyrinchium* bulb extract on angiogenesis in a chicken chorioallantoic membrane model. Representative photographs of the chorioallantoic membrane are shown for control andtreated eggs (A). B & C: Quantitative analysis for the number and diameter of vessels.*p < 0.05 and **p < 0.01 compared to untreated cells (concentration of 0)

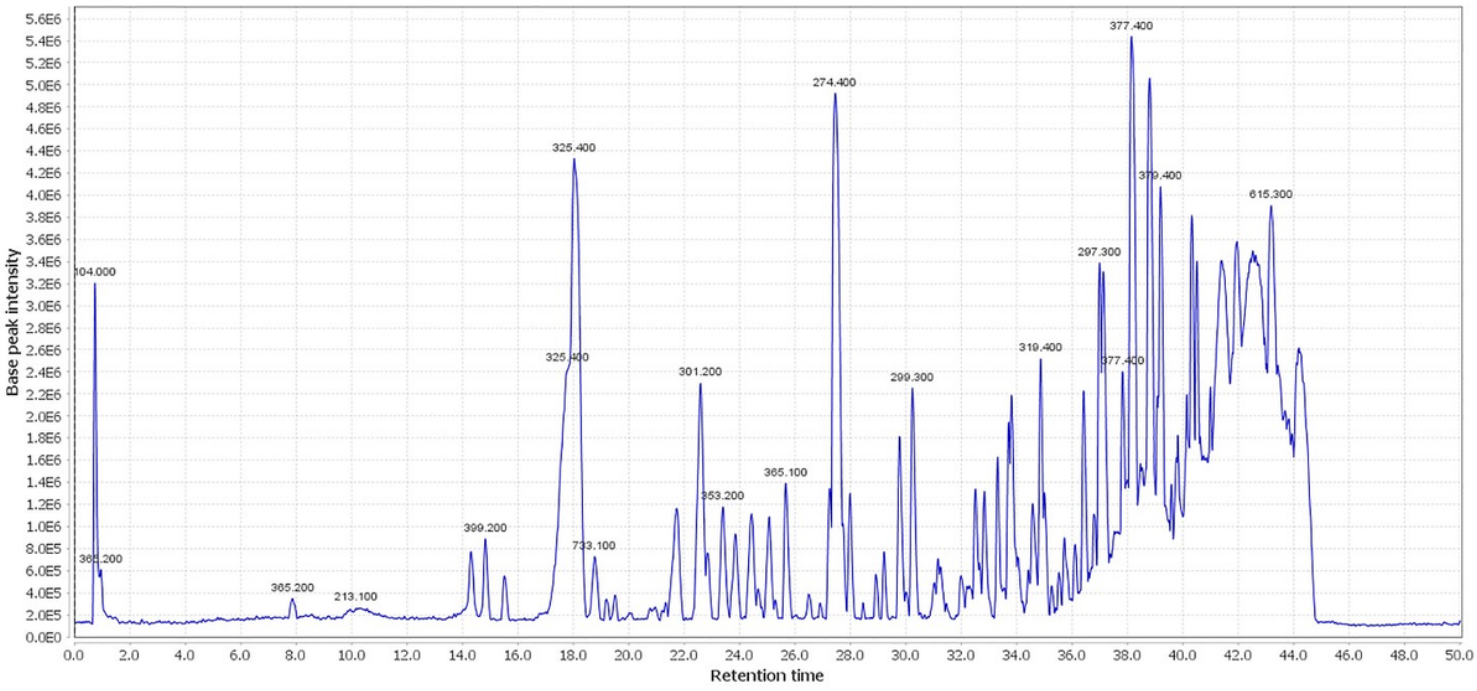

Figure 11

The total ion chromatogram of *Moraea sisyrinchium* bulb extract.

Supplementary Files

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- SupplementaryFigureS1S5.docx