

Effective Parts of *Gentiana straminea* Maxim Attenuates Hypoxia-Induced Oxidative Stress and Apoptosis

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Abstract

Hypoxia occurs in physiological situations and several pathological situations, inducing oxidative stress. *G. straminea* Maxim (*G.s* Maxim) is a traditional Tibetan medicine that exerts several biological effects. This study focused on the protective effects of *G.s* Maxim in hypoxia-induced oxidative stress and apoptosis. We found that *G.s* Maxim significantly increased survival and reduced oxidative stress in hypoxic mice. Various extraction parts of *G.s* Maxim showed antioxidant activity and significantly improved survival in hypoxia-injured PC12 cells. *G.s* Maxim reduced hypoxia-induced cell apoptosis and leakage of lactate dehydrogenase. Hypoxic cells had increased malondialdehyde levels but reduced superoxide dismutase activity and *G.s* Maxim reversed these effects. Moreover, *G.s* Maxim suppressed hypoxia-induced apoptosis by inducing protein expression of B cell leukemia/lymphoma-2 and reducing the expression of hypoxia-inducible factor-1 α , Bcl-2-associated X, and nuclear factor-k-gene binding. These findings suggest that *G.s* Maxim attenuates hypoxia-induced injury associated with oxidative stress and apoptosis.

Keywords

gentiana straminea maxim, hypoxia, oxidative stress, apoptosis

Introduction

Oxygen (O₂) is essential for aerobic organisms to produce energy.¹ Hypoxia occurs when the supply of O₂ does not meet cells' needs. The condition is defined as a decrease in the availability of oxygen that reaches tissues, leading to stress responses such as tachypnea, tachycardia, and higher blood pressure.² Hypoxia also occurs in physiological situations such as high altitude and pathological situations such as ischemia, stroke, neurodegenerative diseases, and cardiovascular injury.^{3,4} There is increasing evidence that hypoxia can have adverse effects on the brain.^{5,6} The brain consumes substantial amounts of O₂ and is rich in unsaturated fatty acids, and has low antioxidant capacity; for these reasons, the brain is exquisitely sensitive to hypoxic damage.⁷ Chronic exposure to hypoxia is associated with defective blood vessel formation and may limit the availability of O₂ to the brain, leading to neurological dysfunction.⁸

Oxidative stress is caused by excessive reactive oxygen species (ROS) production that cannot be eliminated by the antioxidant defense system, damaging cellular proteins, lipids,

and DNA, resulting in necrosis and apoptosis. Malondialdehyde (MDA) is the final lipid peroxidation product by oxygen free radicals and has significant cytotoxicity.⁹ Under physiological conditions, the small amount of ROS produced by cells can be inhibited by endogenous enzymes such as superoxide dismutase (SOD) and glutathione oxidase.¹⁰ By contrast, excess ROS can lead to the excessive conversion of lipid peroxides, resulting in elevated levels of oxidative stress.

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In hypoxia-induced damage, oxidative stress and apoptosis are significant factors.^{11,12} Hypoxia can induce excess production of ROS to cause cellular injury and death, thereby promoting oxidative stress.¹³ Excessive ROS, such as superoxide anion, hydrogen peroxide (H₂O₂), and hydroxyl radicals (•OH), are produced in mitochondria, causing cellular structure and function changes by attacking lipids, membranes, and proteins, and DNA, leading to apoptosis.^{14,15}

Gentiana straminea Maxim (*G.s* Maxim) is translated as “Jiejigabao” in Tibetan. *G.s* Maxim is found in alpine meadows, shrubs, forest margins, hillside grasslands, ravines, and river beaches at an altitude of 2000 to 5000 m in Tibet, Qinghai, Gansu, and other provinces in China.¹⁶ It is a traditional Tibetan medicine with a history of more than 2000 years.¹⁷ Pharmacological studies indicated that *G.s* Maxim mediates antipyretic, analgesic, anti-inflammatory, and antihypertensive effects; it also participates in central sedation, hepatoprotective, bile secretion, and immune regulation activities.¹⁸⁻²¹

Our previous work demonstrated the protective effect of ethanol extracts from *G.s* Maxim on lung and heart damage in rats at high altitude.^{22,23} This study investigated whether *G.s* Maxim can protect PC12 cells from hypoxia-induced oxidative stress and apoptosis and determined the underlying mechanisms.

Methods

Reagents

G.s Maxim was purchased from the Tibetan Medicine Company of Traditional Tibet Medical College. Soda lime was purchased from Beijing Deerli Soda Lime Factory (Beijing, China). Acetazolamide was purchased from Yuancheng Technology Co., Ltd (Wuhan, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma. Assay kits for hydroxyl free radicals, inhibition and produce superoxide anion, total antioxidant capacity (the FRAP method), lactate dehydrogenase (LDH), SOD, and MDA were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Sodium dithionite (Na₂S₂O₄) was purchased from Shanghai Sulfuric Acid Plant (Shanghai, China). H₂O₂ was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (Tianjin, China). Hoechst 33 258 was purchased from Beyotime (Shanghai, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from Boao Tuoda Technology Co., Ltd (Beijing, China). Analytical grade petroleum ether, ethyl acetate, and N-butanol were purchased from Tianjin Tianli Chemical Reagent Co., Ltd (Tianjin, China). Primary antibodies for B cell leukemia/lymphoma 2 (Bcl-2), Bcl-2-associated X (Bax), hypoxia-inducible factor-1 α (HIF-1 α), and nuclear factor kappa-B were purchased from ABclonal (Shanghai, China). β -actin was purchased from Cohesion Biosciences (Beijing, China). Cell

culture medium was purchased from Gibco™ Invitrogen (Thermo Fisher, New York).

Preparation of *G.s* Maxim Extraction

100 g of *G.s* Maxim were soaked in 500 mL 95% ethanol for 24 h, then boiled with a heating reflux device and adjusted to a simmer for 1 h, followed by vacuum rotary evaporation to remove the ethanol; we added double-distilled water (ddH₂O) to achieve a volume of 100 mL.

Extraction proceeded according to the order of polarity of organic solvents. Ethanol from *G.s* Maxim was extracted using petroleum ether, ethyl acetate, and water-saturated N-butanol solution sequentially until the organic solvent was fully evaporated. After vacuum freeze-drying, the extract from each part was obtained and formulated with ddH₂O to 0.01 g/mL for further use.

Preparation of Acetazolamide

400 mg of acetazolamide were mixed with 20 mL preheated ddH₂O, and then ultrasonically mixed to generate a 20 mg/mL solution stored at 4°C. The solution was mixed well before use, and 0.1 mL/10 g body weight was administered via gavage.

Animals and Treatments

Specific pathogen-free Kunming mice were obtained from the Xian Jiaotong University Animal Center (Xian, China). Mice were housed in the Xizang Minzu University Laboratory Animal Center with a 12 h–12 h light–dark cycle and regular chow and water provided ad libitum.

Effective parts of *G.s* Maxim were extracted as described in section 2.2; acetazolamide was used as a positive control. Mice were randomly divided into 7 groups: (1) Control; (2) Hypoxia; (3) 200 mg/kg body weight Acetazolamide + Hypoxia; (4) 0.1 g/kg body weight petroleum ether extraction of *G.s* Maxim + Hypoxia; (5) 0.1 g/kg body weight ethyl acetate extraction of *G.s* Maxim + Hypoxia; (6) 0.1 g/kg body weight N-butanol extraction of *G.s* Maxim + Hypoxia; and (7) 0.1 g/kg body weight residual water extraction of *G.s* Maxim + Hypoxia.

Mice in control groups were under normal conditions; mice in the effective extraction parts of *G.s* Maxim groups were intragastrically administered various extraction parts of *G.s* Maxim for 14 consecutive days. Forty minutes after the final administration, all mice except those in the control group were challenged with Soda lime and survival times were recorded.

All procedures were conducted following the “Guiding Principles in the Care and Use of Animals” (China) and were approved by the Laboratory Animal Ethics Committee of Xizang Minzu University (20200-7).

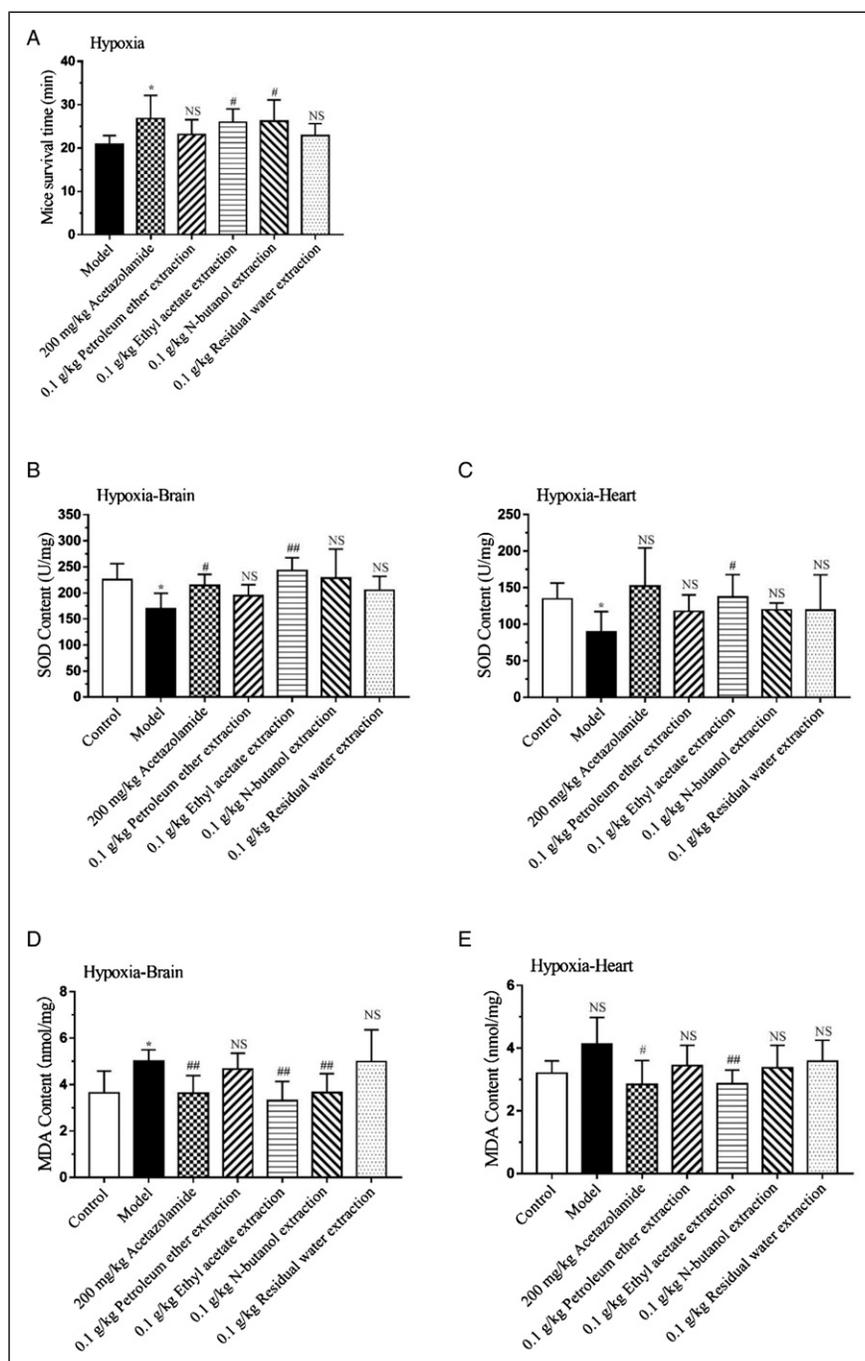


Figure 1. Protective effects of *G.s Maxim* against hypoxia-induced oxidative stress in mice. Effects of *G.s Maxim* on (A) mice survival rate; (B) brain SOD activity, (C) heart SOD activity, (D) brain MDA content, and (E) heart MDA content in mice challenged with hypoxia. * $P < 0.05$ vs. control group, NS, not significant, $^{\#}P < 0.05$ and $^{##}P < 0.01$ vs. hypoxia model group.

Cell Culture

PC12 cells were cultured at 37°C under 5% CO₂ in RPMI1640 medium containing 10% fetal bovine serum (Hyclone, South America) with 100 IU/mL streptomycin and 100 IU/mL penicillin (Amresco, USA).

Hypoxia Exposure

To generate the cell hypoxia injury model, PC12 cells were subjected to a hypoxic environment (H₂O₂, Na₂S₂O₄, or 1% O₂), and normoxic control cells were cultured at 37°C in a 95% O₂ and 5% CO₂ incubator. To determine the protective effect of *G.s*

Table 1. Antioxidant ability of different extraction parts of *G.s Maxim* ethanol extract. Assays were performed according to the manufacturer's protocols. (A) The scavenging effect on DPPH. (B) The scavenging effect on •OH. (C) Total antioxidant capacity assay.

A. Scavenging Effect of Each Extraction Part of *G.s Maxim* Ethanol Extract on DPPH

Group	Dose-response curve	R ²	IC ₅₀ (g/L)
Petroleum ether extraction	Y = 0.1608x + 0.0129	0.9912	14.125
Ethyl acetate extraction	Y = 0.6335x + 0.1468	0.9315	0.411
N-butanol extraction	Y = 0.3889x + 0.0807	0.9514	1.263
Residual water extraction	Y = 0.2022x + 0.0085	0.9774	5.882
Vitamin C	-	-	0.0059

B. Scavenging effect of each extraction part of *G.s Maxim* ethanol extract on OH

Petroleum ether extraction	-	-	-
Ethyl acetate extraction	y = 0.1584ln(x) + 0.5186	0.9946	0.798
N-butanol extraction	y = 0.1266ln(x) + 0.3906	0.9455	1.573
Residual water extraction	y = 0.041ln(x) + 0.1341	0.8366	22.147
Vitamin C	y = 0.1583ln(x) + 0.9667	0.9991	0.053

C. Total antioxidant capacity each extraction part of *G.s Maxim* ethanol extract

Group	Dose-response curve	R ²	μmol/g
Petroleum ether extraction	y = 0.0659x + 0.033	0.9399	215.28 ± 0.15
Ethyl acetate extraction	y = 1.4724x + 0.0027	0.997	1531.52 ± 0.12
N-butanol extraction	y = 0.5027x + 0.0356	0.9889	685.43 ± 0.13
Residual water extraction	y = 0.0264x + 0.8357	0.8078	343.50 ± 0.21
Vitamin C	y = 1.0842x - 0.0101	0.9957	12136.89 ± 1.17

Maxim against hypoxia-induced injury, PC12 cells were pre-incubated with various concentrations of *G.s Maxim* extraction before hypoxia exposure.

Cell Viability

Cell viability was measured using an MTT assay. In brief, PC12 cells were seeded in 96-well culture plates. Then concentrations and extraction parts of *G.s Maxim* were added to the wells. After treatment, 5 mg/mL MTT was added to each well, followed by incubation at 37°C for 4 h. Then, the supernatants with MTT were removed, and the formazan products were dissolved in dimethyl sulfoxide. The absorbance was measured at 490 nm. The results were expressed as the relative percentage of the control group.

Hoechst Staining

Hoechst staining was used to measure the protection of *G.s Maxim* in hypoxia-exposed PC12 cells. In brief, at the end of incubation, 5 μg/mL Hoechst 33258 staining solution was added and incubated with PC12 cells for 30 min at 37°C in the dark, followed by several washes in phosphate-buffered saline. Then, the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

LDH Leakage, MDA Content and SOD Activity

After treatment, culture supernatants from each dish were collected, and LDH activity in the medium was measured using LDH assay kits. PC12 cells were harvested and homogenized after two washes in cold phosphate-buffered saline. Total protein was measured using a BCA protein assay kit. MDA content and SOD activity were determined using appropriate assay kits.

Western Blot

Cell lysates were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and electro-transferred to polyvinylidene difluoride membranes. The membranes were then blocked for 1 h at room temperature with 5% bovine serum albumin. Specific proteins were then detected using appropriate primary antibodies incubated overnight at 4 °C, followed by incubation with specific secondary antibodies for 1 h at room temperature.

RNA Extraction and qPCR Analysis

Total RNA was extracted with Trizol Reagent (Invitrogen, USA) and 1 μg total RNA was reverse-transcribed. qPCR was performed using the CFX96 connect instrument

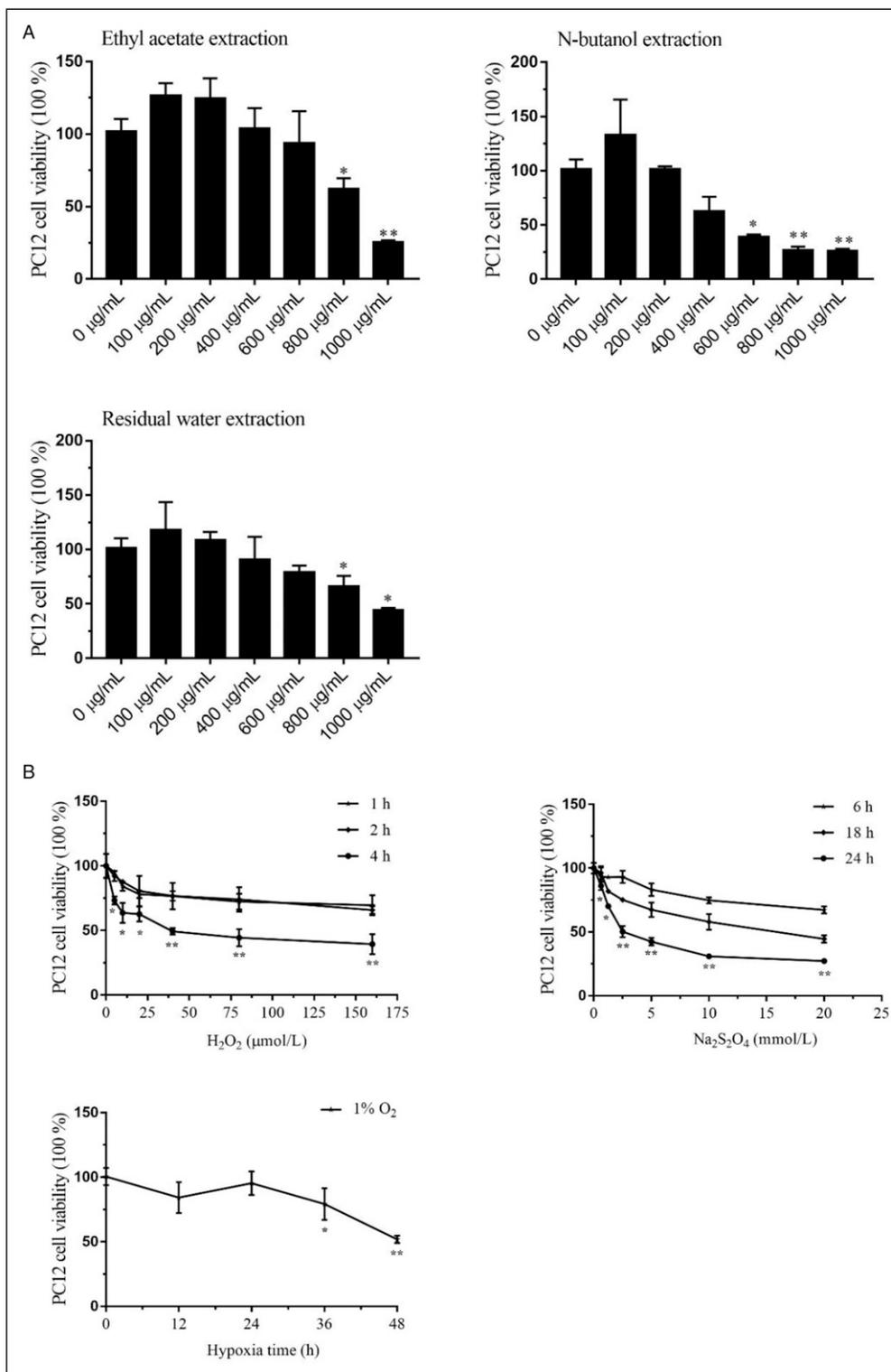


Figure 2. *G.s Maxim* improves the survival of hypoxia-injured PC12 cells. (A) PC12 cells were treated with different extraction parts of *G.s Maxim*, respectively, cell viability was determined by MTT. (B) To construct hypoxia-injured PC12 cells models, (1) cells were treated with 0, 5, 10, 20, 40, 80, or 160 µmol/L H₂O₂ for 1, 2, or 3 h, (2) cells were treated with 0, .625, 1.25, 2.5, 5, 10, or 20 mmol/L Na₂S₂O₄ for 6, 18, or 24 h, (3) cells were incubated under 1% O₂, 94% N₂ and 5% CO₂ for 12, 24, 36, or 48 h. Cell viability were determined by MTT. (C) To evaluate the protective effect of *G.s Maxim* against hypoxia-induced injury, PC12 cells were pre-incubated with different concentrations of *G.s Maxim* extraction before hypoxia treatment. (D) The efficacy of ethyl acetate extraction of *G.s Maxim*. **P* < 0.05 and ***P* < 0.01 vs. control group, NS, not significant, #*P* < 0.05 and ###*P* < 0.01 vs. hypoxia model group.

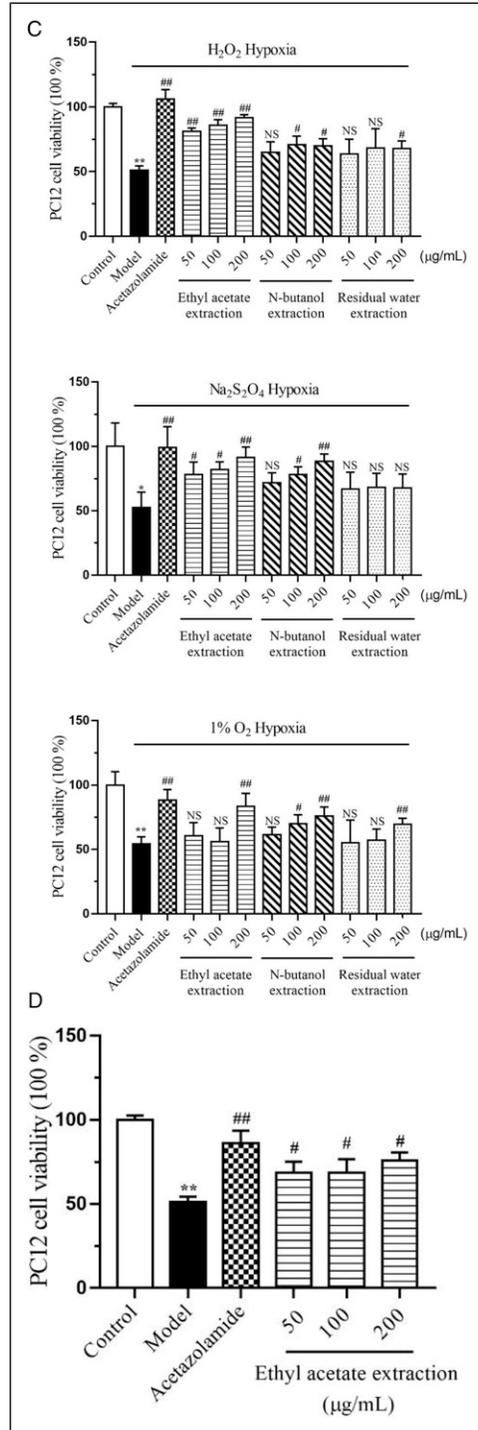


Figure 2. Continued.

(Bio-Rad, USA) and a reaction mixture that consisted of SYBR Green PCR Master Mix (Toyobo, Japan), cDNA template, and primers. qPCR was performed according to the manufacturer’s protocols.

Statistical Analysis

The data were expressed as mean ± S.D. *P* < 0.05 was considered statistically significant.

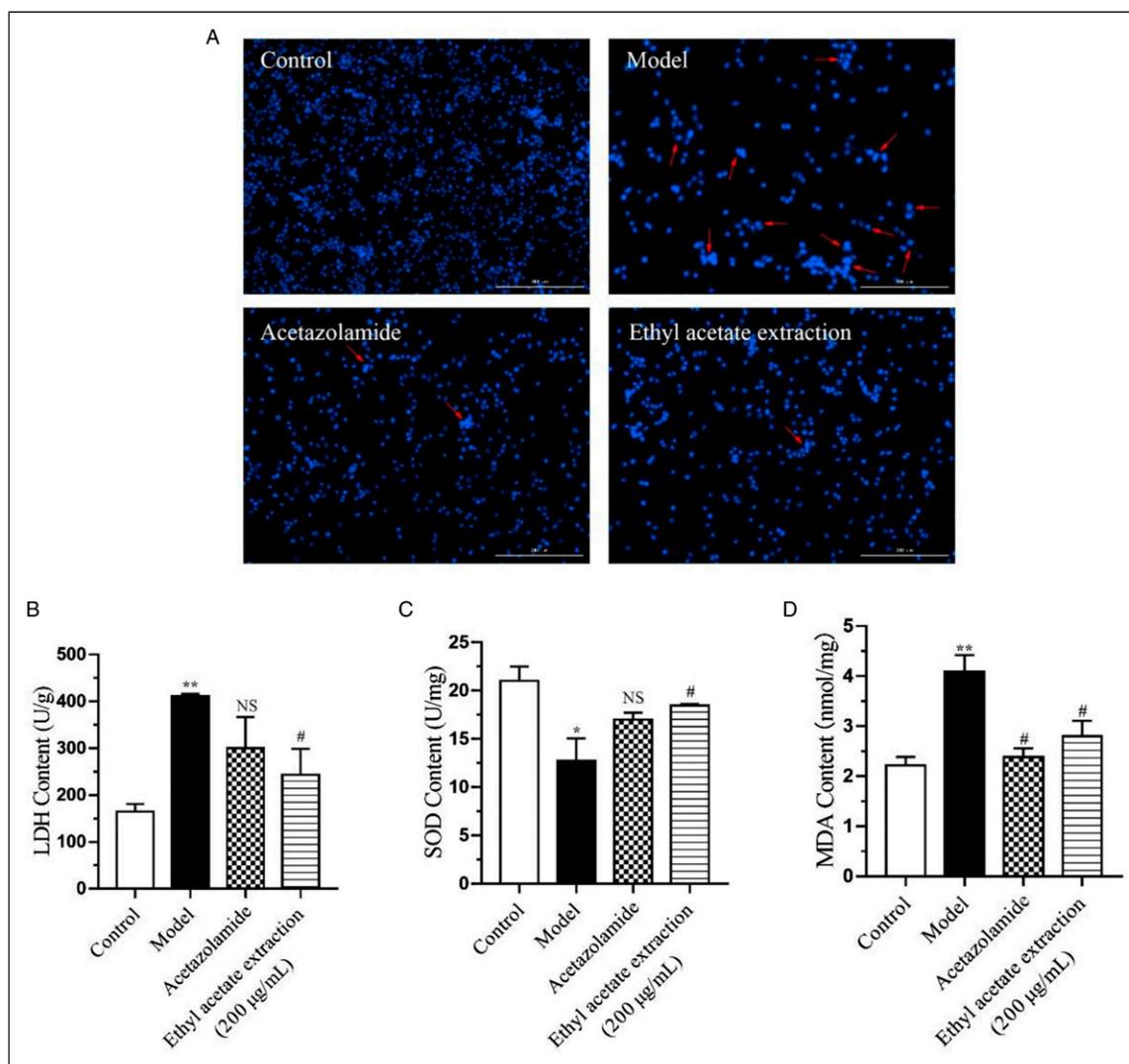


Figure 3. Ethyl acetate extraction of *G.s Maxim* ethanol extract inhibits hypoxia-induced oxidative stress. (A) Hoechst staining of PC12 cells. Scale bars, 200 µm. (B) LDH leakage (C) SOD activity and (D) MDA content were determined. * $P < 0.05$ and ** $P < 0.01$ vs. control group, NS, not significant, [#] $P < 0.05$ vs. hypoxia model group.

Results

Protective Effects of *G.s Maxim* Against Hypoxia-Induced Oxidative Stress in Mice

The effects of each extraction part of *G.s Maxim* ethanol extract on survival are presented in Figure 1A. *G.s Maxim* treatments prolonged survival; ethyl acetate extraction and N-butanol extraction parts differed significantly. Figures 1B-1C shows that brain and heart SOD activity were reduced in the hypoxia group. *G.s Maxim* administration appeared to improve SOD activity, and the ethyl acetate extraction showed the best effect. We measured MDA levels to evaluate the degree of brain and heart lipid peroxidation. We found that MDA levels were much higher in the hypoxia group, those of the *G.s Maxim* groups were lower, and the

ethyl acetate extraction showed the best effect (Figures 1D-1E).

Antioxidant Ability of Different Extraction Parts of *G.s Maxim* Ethanol Extract

Because *G.s Maxim* exhibits antioxidant capacity *in vivo*, we explored its mechanism of action. We first examined the antioxidant effect of *G.s Maxim* *in vitro*. The petroleum ether extraction, ethyl acetate extraction, N-butanol extraction and residual water extraction of *G.s Maxim* ethanol extract were weighed and ddH₂O was added to make up to the concentration gradient of 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL Vitamin C as a positive control. As shown in Table 1A, the DPPH scavenging rate of each

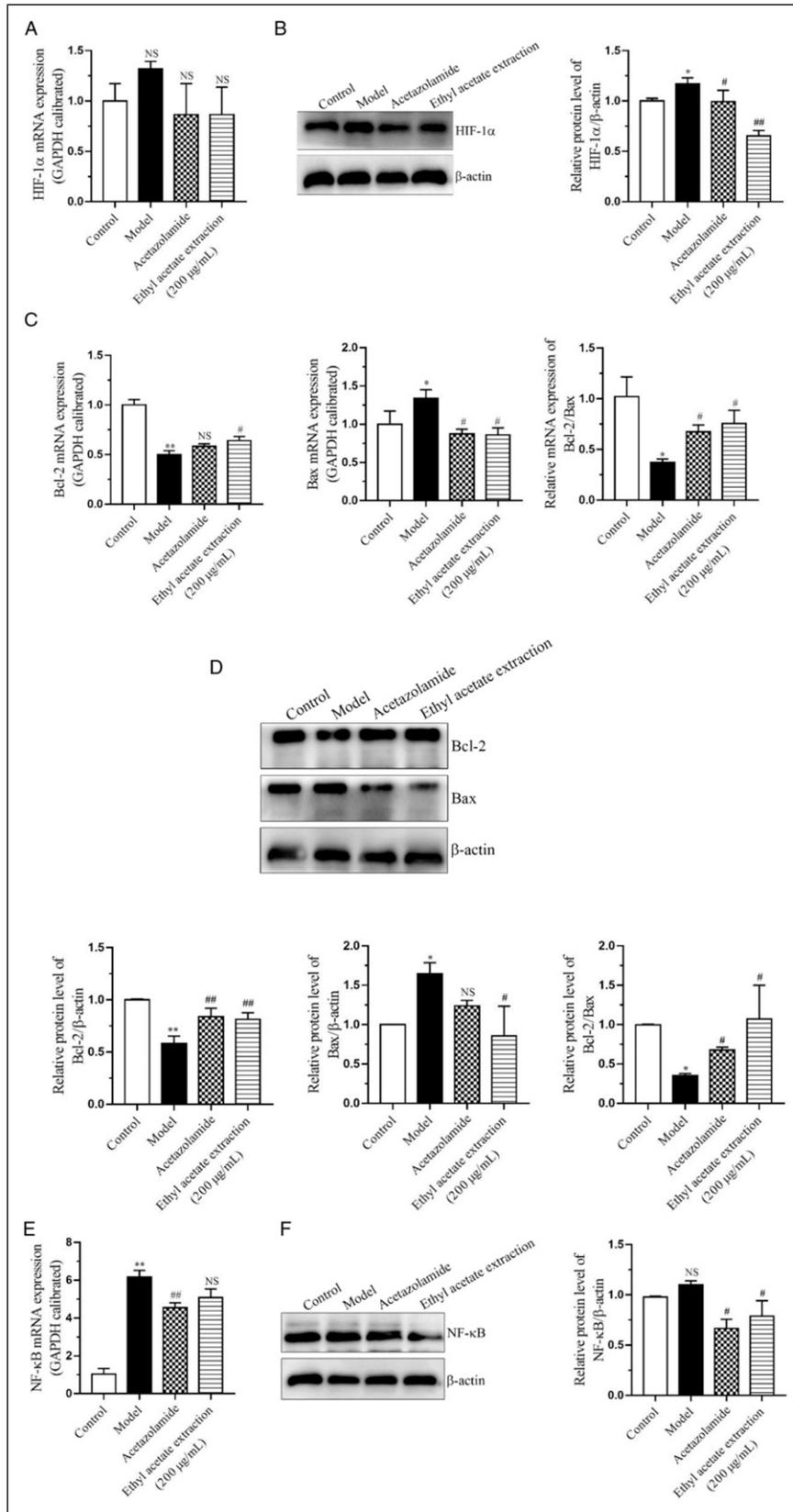


Figure 4. Ethyl acetate extraction of *G.s Maxim* ethanol extract suppresses hypoxia-induced apoptosis. The mRNA and protein expressions of HIF-1 α , Bcl-2, Bax and NF- κ B were detected using qPCR and western blot analysis. * $P < 0.05$ and ** $P < 0.01$ vs. control group, NS, not significant, # $P < 0.05$ and ## $P < 0.01$ vs. hypoxia model group.

extraction part increased in a linear, concentration-dependent manner. The ethyl acetate extraction's 50% inhibitory concentration (IC_{50}) was 0.411 g/L, the best antioxidant effect. The IC_{50} of the ethyl acetate extraction to clear $\bullet OH$ was 0.798 g/L (the best effect), and other extraction parts showed a good logarithmic relationship in scavenging $\bullet OH$ except petroleum ether extraction (Table 1B). The total antioxidant capacity assay was determined using a FRAP assay. The total antioxidant capacity of each extraction varied greatly, with ethyl acetate extraction showing the best total antioxidant capacity, and the petroleum ether extraction was the worst (Table 1C). These results suggest that *G.s Maxim* has antioxidant activity in vitro, and ethyl acetate extraction has the best effect.

G.s Maxim Improves the Survival of Hypoxia-Injured PC12 Cells

To measure the proliferative activity of *G.s Maxim*, cytotoxicity analysis was determined using an MTT assay. Cell viability significantly decreased when the ethyl acetate extraction was 800 $\mu g/mL$, the concentration of N-butanol extraction was 600 $\mu g/mL$, and the concentration of residual water extraction was 800 $\mu g/mL$ (Figure 2A). This finding suggests that the safe ranges of ethyl acetate extraction, N-butanol extraction, and residual water extraction are 0-600 $\mu g/mL$, 0-400 $\mu g/mL$, and 0-600 $\mu g/mL$, respectively, in PC12 cells.

We used several approaches to generate hypoxia-induced damage models in PC12 cells (Figure 2B). Significant hypoxic injury in PC12 cells was observed at 40 $\mu mol/L$ H_2O_2 for 4 h, 2.5 mmol/L $Na_2S_2O_4$ for 24 h, and <1% O_2 for 48 h; cell viability decreased by about 50%. We then investigated the protective effects of different extraction parts isolated from *G.s Maxim* ethanol extract on hypoxia-exposed PC12 cells. The MTT assay revealed that compared with the control group, different extraction parts of *G.s Maxim* protected PC12 cells injured by H_2O_2 , or $Na_2S_2O_4$, or 1% O_2 content-induced hypoxia; the ethyl acetate extraction showed the best protective effect (Figure 2C). Because the ethyl acetate extraction showed excellent anti-hypoxia ability, we measured its cellular efficacy and found that 200 $\mu g/mL$ showed the best effect. We selected this concentration for subsequent experiments (Figure 2D).

Ethyl Acetate Extraction of G.s Maxim Ethanol Extract Inhibits Hypoxia-Induced Oxidative Stress

Apoptotic PC12 cells were marked by strong blue fluorescence in the Hoechst staining test compared to the normal cells, which displayed weak blue fluorescence. Compared with the control cells, more apoptotic cells were observed under 1% O_2 content-induced hypoxia (Figure 3A). However, ethyl acetate extraction of *G.s Maxim* ethanol extract treatment decreased the number of apoptotic PC12 cells. The protective effect of ethyl acetate extraction was confirmed by LDH leakage; LDH levels in cell

supernatants were significantly increased under 1% O_2 content-induced hypoxia. Pretreatment with ethyl acetate extraction dramatically decreased LDH leakage, suggesting that ethyl acetate extraction restored the integrity of the cell membrane (Figure 3B). Excessive lipid peroxidation is a significant indicator of oxidative stress, and antioxidant enzymes such as SOD are the primary defense systems against oxidative stress. Compared to control cells, PC12 cells subjected to hypoxia lost almost 50% of their SOD activity, and ethyl acetate extraction prevented this reduction (Figure 3C). Hypoxia significantly increased MDA content, but MDA was inhibited in PC12 cells pretreated with ethyl acetate extraction (Figure 3D). These results suggest that ethyl acetate extraction of *G.s Maxim* ethanol extract protects PC12 cells against hypoxia-induced oxidative stress.

Ethyl Acetate Extraction of G.s Maxim Ethanol Extract Suppresses Hypoxia-Induced Apoptosis

HIF-1 α was measured using qPCR and western blot. Compared to the control group, HIF-1 α mRNA expression was higher, and protein expression was significantly greater in the hypoxia group; however, pretreatment of ethyl acetate extraction downregulated HIF-1 α mRNA and protein expression (Figures 4A-4B). To further confirm the anti-apoptotic effect and mechanism of ethyl acetate extraction against hypoxia-induced injury, the expression of apoptosis-related proteins, including Bcl-2 and Bax, were measured. As shown in Figures 4C-4D, 1% O_2 content-induced hypoxia significantly decreased mRNA and protein expression of Bcl-2 and increased Bax protein expression levels in PC12 cells. In contrast, ethyl acetate extraction of *G.s Maxim* ethanol extract pretreatment reversed these effects. Furthermore, hypoxia exposure upregulated mRNA and protein levels of nuclear factor kappa-B related to the inflammation response, which was also blocked by ethyl acetate extraction of *G.s Maxim* ethanol extract pretreatment (Figures 4E-4F).

Discussion

Humans often live in oxygen-deficient environments. Strenuous exercise, high altitude, certain diseases, and other factors may give rise to hypoxia. The brain is more susceptible to hypoxia than other organs due to high levels of oxygen consumption and reduced activity of antioxidant enzymes.²⁴ The literature suggests that various antioxidants inhibit the decrease in cell viability, scavenge ROS, increase antioxidant enzyme activity, and attenuate the elevation of intracellular free Ca^{2+} in oxygen-stressed neuronal cells.²⁵⁻²⁷ Much attention has been paid to identifying novel neuroprotective agents from natural products with excellent activity and few side effects.²⁸

G.s Maxim has various biological effects; however, there are few studies on its antioxidant activity. In the present study, we measured the protective effects of *G.s Maxim* on oxidative stress and apoptosis. Several lines of evidence suggest that hypoxic exposure causes cytotoxicity in many cell types.²⁹⁻³¹

We found that *G.s Maxim* significantly improved the survival and reduced oxidative stress of hypoxic mice. Hypoxia exposure caused substantial apoptosis, and *G.s Maxim* pretreatment improved survival in PC12 cells. LDH is an intracellular cytoplasmic enzyme released into cell culture supernatants when the cell membrane is damaged.³² Increased LDH activity in the cell supernatant suggests damaged plasma membranes. PC12 cells pretreated with *G.s Maxim* decreased the leakage of LDH, suggesting that *G.s Maxim* attenuated hypoxia-induced cell membrane damage.

Oxidative stress plays a crucial role in hypoxic injury.^{33,34} In the present study, MDA content and SOD activity were used to evaluate the protective effect of *G.s Maxim* on hypoxia-induced oxidative stress. We found that *G.s Maxim* pretreatment markedly inhibited MDA production and restored SOD activity, suggesting that *G.s Maxim* protected against hypoxia-induced oxidative damage.

Hypoxia induces apoptosis, and mitochondrial structure and function abnormalities are associated with apoptosis.³⁵ Hypoxia induces apoptosis by increasing the production of ROS, increasing the expression of HIF-1 α , and activating the mitochondrial pathway.³⁶⁻³⁸ We found that *G.s Maxim* attenuated hypoxia-induced upregulation of HIF-1 α . To determine whether *G.s Maxim* reduced apoptosis through the mitochondrial pathway, relative protein expression levels of Bcl-2 and Bax were measured. Bcl-2 family proteins such as the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax are critical regulators of apoptosis.³⁹ We found that hypoxia resulted in the downregulation of Bcl-2 and upregulation of Bax mRNA and protein expression; however, *G.s Maxim* pretreatment significantly reversed these changes.

Conclusion

Our findings suggest that *G.s Maxim* protects against hypoxia-induced oxidative stress and apoptosis by reducing MDA content and LDH leakage, maintaining antioxidant enzyme activity, and inhibiting the mitochondrial apoptosis pathway.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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