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Ethanol Extract of *Gentiana straminea* Maxim Displays Anti-Hypoxia Effects by Regulating Antioxidant Enzymes and Energy Metabolism

Dan Song[1,2], Ming-Juan Ge[3], Jie Li[1,2], Yi Jiang[1], Xiu-Mei Kong[1], Jiao-Jiao Xu[1], Xu Ji[1,2], Rui-Xin Shi[1] and Qin Zhao[1,2]*

[1]*Joint Laboratory for Research on Active Components and Pharmacological Mechanism of Tibetan Materia Medica of Tibetan Medical Research Center of Tibet, School of Medicine, Xizang Minzu University, Xianyang 712082, Shaanxi, China*
[2]*Engineering Research Center of Tibetan Medicine Detection Technology, Ministry of Education, School of Medicine, Xizang Minzu University, Xianyang 712082, Shaanxi, China*
[3]*Xianyang Hospital of Yan'an University, Xianyang 712000, Shaanxi, China*

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Key words
***Gentiana straminea* Maxim, Ethanol extract, Anti-hypoxia, Antioxidant, Energy metabolism**

ABSTRACT

Oxygen is an essential regulator for normal aerobic metabolism in humans and animals, oxidative stress and energy metabolism during hypoxia may be related to hypoxia-related diseases. *Gentiana straminea* Maxim (*G. straminea*), a natural Tibetan herb with exerts several biological effects, was used to study the anti-hypoxia effects of its ethanol extract. Three extract methods were employed to evaluate the best extraction methods. Male Kunming specific pathogen-free (SPF) mice were randomly divided into blank control, model (hypoxia), positive (propranolol 30 mg/kg + hypoxia), and three *G. straminea* ethanol extracts dose groups (10, 5 and 2.5 g/kg respectively + hypoxia), administered intragastrically once a day for 14 consecutive days. After that, multiple hypoxia experiments were conducted including soda lime normobaric hypoxia test, sodium nitrite poisoning test, isoproterenol poisoning test, and acute cerebral ischemic hypoxia test. Subsequently, the content of superoxide dismutase (SOD), malondialdehyde (MDA), and activity of total antioxidant capacity (T-AOC), catalase (CAT) in mice liver were measured; while SOD and MDA contents and activity of T-AOC, CAT, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, pyruvate kinase (PK) and phosphofructokinase (PFK) in mice brain were evaluated. In the results, *G. straminea* ethanol extract markedly enhanced hypoxia tolerance in mice. It attenuated hypoxia-induced oxidative stress by reducing MDA levels (in liver and brain), and elevating SOD (in liver and brain), T-AOC and CAT (in liver). Furthermore, pre-treatment with *G. straminea* ethanol extracts significantly increased ATP content, up-regulated the activity of Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, PK, and PFK in hypoxia mice brain. In conclusion, this research demonstrated the anti-hypoxia activity of ethanol extract of *G. straminea*, which may be related to increased energy metabolism. Our findings provide a basis for investigating hypoxia-related diseases and drug development.

INTRODUCTION

Oxygen plays a vital role in regulating aerobic metabolism by serving as a key regulator of cell energy production and enzyme activation (Lu *et al*., 2020). Hypoxia refers to abnormal changes in the morphology, metabolism, and function of tissues and organs caused by insufficient oxygen supply or oxygen dysfunction (Murray *et al*., 2018). Hypoxia is a central factor in acute and chronic altitude sickness (Avellanas, 2018), and can be caused by factors such as low oxygen content and pressure, impaired oxygen transport, and impaired cellular oxygen uptake or utilization (MacIntyre, 2014). Hypoxia is involved in the development of hypertension, cardiovascular and metabolic disorders, and respiratory diseases. The oxidative stress during hypoxia may be causally related to these diseases (McGarry *et al*., 2018).

Mitochondria produce energy by using a variety

* Corresponding author: xyzhaoqin@126.com
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of energy sources. Energy is transferred between cells in the form of adenosine triphosphate (ATP) to support cell activity. Mitochondrial energy metabolism is highly regulated to continuously meet the energy demands of cells (Benard *et al*., 2010). Mitochondria are significant targets of hypoxic injury, which involves the production of reactive oxygen species (ROS) (Ham and Raju, 2017). ROS includes hydrogen peroxide, hydroxyl radicals, and superoxide anions essential for normal cell function (Finkel and Holbrook, 2000). The cellular antioxidant system typically removes ROS; however, ROS production overwhelms antioxidant capacity in hypoxic injury, leading to DNA damage, lipid peroxidation, and mitochondrial membrane depolarization (Bhat *et al*., 2015). These mechanisms lead to the release of cytochrome C and apoptosis (Lemasters *et al*., 2009; Murphy and Steenbergen, 2008; Wu and Bratton, 2013). Hypoxia causes insufficient oxygen supply to various organs, inhibits oxidation, promotes glycolysis, and leads to insufficient ATP production (Liu *et al*., 2020). As a result, tissues and organs undergo apoptosis due to a lack of ATP and energy with subsequent tissue damage (Bickler *et al*., 2017). Hypoxic damage can be substantially reduced if a cell's anti-hypoxia ability is enhanced, and energy metabolism can improve (Ferraresi *et al*., 2015). Inadequate oxygen supply reduces intracellular oxygen partial pressure, leading to mitochondrial dysfunction and affecting energy metabolism (Li *et al*., 2021).

Anoxia occurs at high altitude such as the Tibet Plateau where the acute altitude response has become a serious problem. In addition, physiological conditions such as ischemia, stroke, neurodegenerative disease, cardiovascular injury and other pathological conditions can also lead to hypoxia (Heinicke *et al*., 2003; Katayama *et al*., 2004; Savourey *et al*., 1996). In high altitude hypoxia environment, a series of stress reactions will occur in the body, resulting in organ hypoxia damage. Among them, oxidative stress damage, immune system damage and disturbance of cellular energy metabolism are the main mechanisms.

Under hypoxic environment, the antioxidant capacity of the body is disordered, and the brain and other organs may die due to insufficient energy supply (Jiao *et al*., 2019). Although drugs such as dexamethasone, acetazolamide, propranolol, and carbamazepine are used to treat hypoxic diseases, some of these have slow curative effects and are burdened by side effects. They are not suitable for long-term use (Khambatta *et al*., 1987; Reddy *et al*., 2013; Shimoda *et al*., 2021). Therefore, identifying natural, nontoxic and effective anti-hypoxia bioactive substances is vital and urgent.

Tibet's special climate and geographical environment have formed rich medicinal plants and mineral resources with unique curative effects. Using Tibet's unique plateau plant resources, developing high-quality and efficient anti-hypoxia drugs has become possible. *Gentiana straminea* Maxim (*G. straminea*), also called as "Jiejigabao" in Tibetan, is an important Tibetan medicine (Zhou *et al*., 2021). The chemical constituents of *G. straminea* are complex and diverse, including iridoids, triterpenes, flavonoids, alkaloids, steroids, and carbohydrates (Kakuda *et al*., 2001; Pan *et al*., 2016; Yang *et al*., 2014). *G. straminea* is mainly used for the treatment of rheumarthritis, icterepatitis, constipation, pain and hypertension (Tan *et al*., 1996; Yu *et al*., 2004). Pharmacological studies reported that *G. straminea* exhibits several pharmacological properties, including analgesic, anti-hypoxia, anti-inflammatory, anti-bacterial, antihypertensive, hepatoprotective, diuretic, antipyretic, immune regulation, and free radical scavenging properties (Song *et al*., 2022). However, few studies have reported the effect of *G. straminea* on energy metabolism.

This research demonstrated the effects of *G. straminea* on normobaric hypoxia using animal models, and provided an experimental basis for studying hypoxic diseases and novel drug development.

MATERIALS AND METHODS

Materials, main reagents and animals

Gentiana straminea Maxim (*G. straminea*) was purchased from Tibetan Medicine Co., Ltd. (Lhasa, China). Soda lime was procured from Beijing Deerli Soda Lime Factory (Beijing, China). Propranolol was obtained from Shanxi Linfen Jianmin Pharmaceutical Factory Co., Ltd. (Shanxi, China).

The superoxide dismutase (SOD) kit, malondialdehyde (MDA) kit, ATP kit, ATPase kit, pyruvate kinase (PK) kit, phosphofructokinase (PFK) kit, total antioxidant capacity (T-AOC) kit, and catalase (CAT) kit were procured from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The cell counting kit-8 (CCK-8) was purchased from Sangon Biotech (Shanghai, China).

Specific pathogen-free male Kunming mice were obtained from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China). They were housed in a standard laboratory environment with a 12 h light-dark cycle, regular chow, and ad libitum water.

Preparation of G. straminea extracts

To evaluate the effect of different extraction methods on the anti-hypoxia properties, three extraction methods were used to prepare *G. straminea* extracts as described by Song *et al*. (2022), including water extraction, water extraction and alcohol precipitation, as well as ethanol extraction (Table I).

Table I. Preparation of three different extracts from *G. straminea*.

Extract method	Operation steps
Water extraction	100 g *G. straminea* were added to 500 mL ddH_2O at 100°C for 1 h twice, then filtered to obtain water extract. The filtrate was stored at 4°C for further use.
Water extraction and alcohol precipitation	100 g *G. straminea* were added to 1000 mL ddH_2O for 24 h, then boiled for 3 times, 0.5 h each time, and added 1000 mL ddH_2O each time. Water extraction were collected, and centrifuged at 2500 rpm for 0.5 h, take the supernatant and concentrated to 100 mL. To get the water extraction and alcohol precipitation, 95% ethanol were added to the concentrated, followed by vacuum rotary evaporation to evaporate the ethanol to reach a final volume of 100 mL.
Ethanol extraction	100 g *G. straminea* were added to 500 mL 95% ethanol for 24 h, then heated to reflux for 1.5 h, followed by vacuum rotary evaporation to evaporate the ethanol. Finally, to obtain the ethanol extract, ddH_2O was added to reach a final volume of 100 mL

Five groups were set up (n= 6 per group): model group (phosphate-buffered saline, PBS), propranolol group (30 mg/kg), and three *G. straminea* extract groups (10 g/kg water extraction, 10 g/kg water extraction and alcohol precipitation, 10 g/kg ethanol extraction. The mice were intragastrically administered their respective treatments doses once a day for 14 consecutive days. After 30 min of the last administration, all the mice were challenged with soda lime, and their survival time in each group was recorded.

Animal treatments

To further assess the anti-hypoxia effects of different concentrations of ethanol extract of *G. straminea*, thirty-six mice were randomly divided into 6 groups (n= 6 per group): blank control; model (hypoxia treatment only); positive groups (30 mg/kg propranolol for hypoxia treatment); and three *G. straminea* ethanol extract dose groups (10, 5, and 2.5 g/kg respectively for hypoxia treatment). Mice in control and model groups received PBS administration, while mice in positive group and *G. straminea* dose groups were administered intragastrically once a day for 14 consecutive days. After 30 min of the last administration, all mice in each group except the control were challenged with subsequently treatments.

Soda lime normobaric hypoxia test

Each mouse in model, positive and three *G. straminea* ethanol extract dose groups was placed in a 250-mL tank (1 mouse in each tank) containing 15 g of soda lime and tightly covered with vaseline around the neck. The survival time was recorded (Li *et al*., 2021; Yang *et al*., 2019).

Sodium nitrite ($NaNO_2$) poisoning test

Mice were intraperitoneally injected with 240 mg/kg $NaNO_2$, and their survival time was recorded accordingly (Li *et al*., 2021; Yang *et al*., 2019).

Isoproterenol poisoning test

Mice were intraperitoneally injected with 15 mg/kg isoproterenol. After 15 min, the mice were placed in a 250-mL tank (1 mouse in each tank) containing 15 g of soda lime, and the neck was tightly covered with petroleum jelly. The time of death was then recorded (Cai *et al*., 2011).

Acute cerebral ischemic hypoxia test

Mice were decapitated. The time from decapitation to cessation of wheezing were recorded (Li *et al*., 2021).

Tissues collection and indicators determination

At the end of the above soda lime normobaric hypoxia test, all the mice including control group were sacrificed. Their liver and brain tissues were washed twice with precooled PBS, dried and weighed for tissue homogenate. Using the ratio of tissue weight (g) to PBS (mL) of 1:9, liver and brain tissues were homogenized in an ice bath and centrifuged at 4°C, 3000 rpm for 10 min to separate the supernatants. The concentrations of SOD, MDA, T-AOC and CAT in the liver, and the SOD, MDA, ATP, Na^+-K^+-ATPase, Ca^{2+}-Mg^{2+}-ATPase, PK and PFK content in the brain were determined using the commercial kits.

Statistical analysis

The experiments were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for multiple comparisons, and differences with P <0.05 were considered statistically significant.

RESULTS

Anti-hypoxia effects of three different extraction methods of G. straminea *in mice*

The anti-hypoxia effects of three different extraction methods of *G. straminea* on mice were compared by

measuring the survival time. As shown in Figure 1, no significant difference in the survival time of mice among the water extraction group, water extraction and alcohol precipitation group, and model group under normobaric hypoxia tests. However, compared with model group, the survival time of mice in *G. straminea* ethanol extract group was significantly extended, indicating that ethanol extraction method was effective, and therefore had a significant anti-hypoxia effect on mice compared to other two methods.

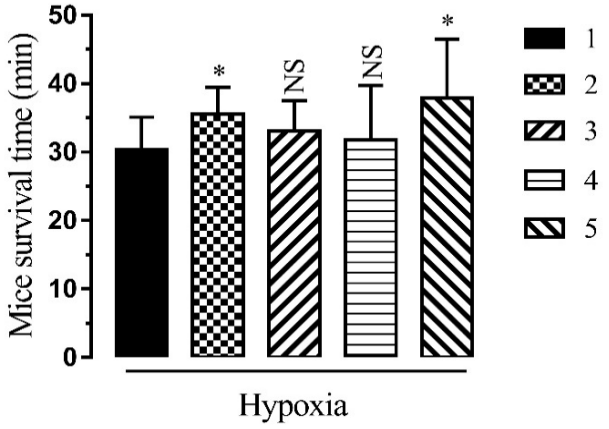

Fig. 1. Effects of *G. straminea* extracts obtained by three extraction methods on the survival time of soda lime normobaric hypoxia model mice.
Notes: n= 6; **P* <0.05 *vs.* model group, NS, not significant. **1**: model; **2**: 30 mg/kg propranolol; **3**: 10 g/kg water extraction; **4**: 10g/kg water extraction and alcohol precipitation; **5**: 10 g/kg ethanol extraction.

Anti-hypoxia effect of different concentrations of G. straminea *ethanol extract*

To investigate the anti-hypoxia activity of *G. straminea* ethanol extract, we conducted various hypoxia experiments, including soda lime normobaric hypoxia test, $NaNO_2$ poisoning, isoproterenol hypoxia, and an acute cerebral ischemic hypoxia test. Compared with the hypoxia-only group, the ethanol extract prolonged the survival time of mice under various hypoxia experiments, confirming its anti-hypoxia effect (Fig. 2). These results indicated that *G. straminea* ethanol extract increased hypoxia tolerance.

Ethanol extraction of G. straminea *alleviates liver oxidative stress*

To determine the antioxidant activity of the ethanol extract of *G. straminea*, mice were intragastrically administered with PBS, propranolol and various doses of ethanol extract (10, 5, 2.5 g/kg) for 14 days, respectively. Subsequently, the mice were challenged with soda lime normobaric hypoxia, whereas the blank control group gavaged with PBS only. Hepatic SOD activity was markedly lower in the normobaric hypoxia model group compared with the control group. However, pre-treatment with the ethanol extract of *G. straminea* (5 and 10 g/kg) significantly improved SOD activity in hypoxia mice (Fig. 3A). MDA content obviously increased in the normobaric hypoxia model group compare to the control group; ethanol extract of *G. straminea* administration decreased hepatic MDA content compared to the model group, although the difference was not statistically significant (Fig. 3B).

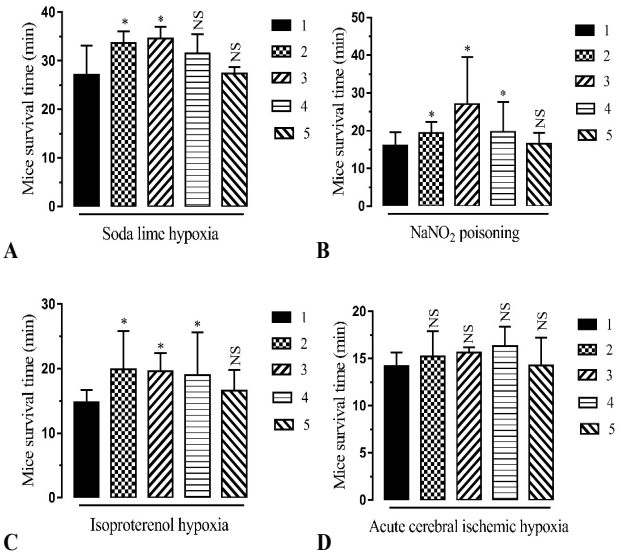

Fig. 2. Effects of different concentrations of *G. straminea* ethanol extract on survival time in (A) soda lime normobaric hypoxia test, (B) $NaNO_2$ poisoning test, (C) isoproterenol hypoxia test, (D) and acute cerebral ischemic hypoxia test.
Notes: **P* <0.05 *vs.* model group, NS, not significant. N=6 in each treatment. **1**: model; **2**: 30 mg/kg propranolol; **3**: 10 g/kg ethanol extraction; **4**: 5 g/kg ethanol extraction; **5**: 2.5 g/kg ethanol extraction..

The influence of ethanol extract of *G. straminea* on hepatic T-AOC and CAT in hypoxic mice was further explored. Normobaric hypoxia attenuated T-AOC and CAT activity, while pre-treatment with ethanol extract of *G. straminea* increased enzymatic activity in hypoxia mice groups (Fig. 3C, D). Thus, these results suggest that the anti-hypoxic effect of *G. straminea* ethanol extract was associated with its antioxidant and oxidase-regulating activities.

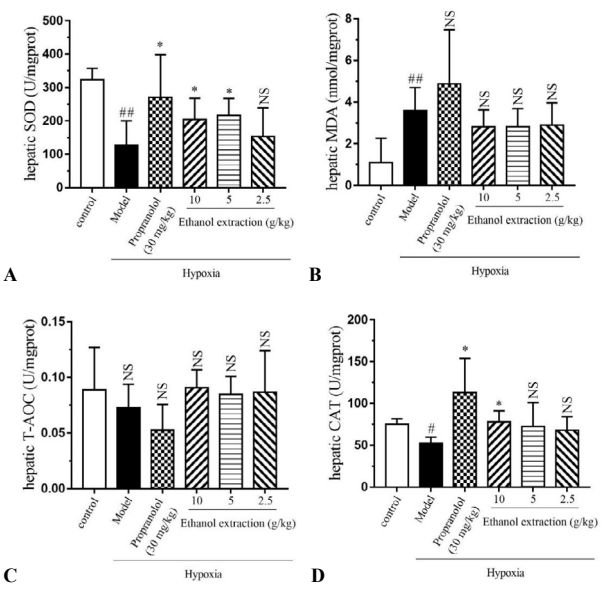

Fig. 3. Effects of *G. straminea* ethanol extract on oxidative stress parameters in the liver of mice under normobaric hypoxia.
Notes: (A) SOD, superoxide dismutase; (B) MDA, malondialdehyde; (C) T-AOC, total antioxidant capacity; (D) CAT, catalase. $^{\#}$ P <0.05 and $^{\#\#}$ P <0.01 *vs.* control group, NS, not significant; * P <0.05 *vs.* hypoxia model group.

Effect of G. straminea *ethanol extract on lipid peroxidation and energy metabolism in hypoxia model mice brain*

The SOD and MDA levels in brain tissues were measured to determine the degree of lipid peroxidation in mice brain. Compared with the control group, the content of SOD in rice brain was obviously lower, while MDA level was significantly higher in normobaric hypoxia model mice. Pre-treatment with *G. straminea* ethanol extract for 14 days significantly increased SOD level and slightly decreased MDA content compared to the model group (Fig. 4A, B).

ATP plays a critical role in cellular metabolism by storing and transferring chemical energy, serving as the primary energy source for living organisms. Compared with model mice under normobaric hypoxia, after 14 days of *G. straminea* ethanol extract administration, ATP content, and activities of Na^{+}-K^{+}-ATPase and Ca^{+}-Mg^{+}-ATPase in mice brain were restored (Fig. 4C, E). PK and PFK are essential enzymes involved in energy metabolism via glycolysis. Under normal circumstances, PK and PFK exhibit lower activity levels. However, PK and PFK were induced by hypoxia due to the consumption of ATP (Fig. 4F, G). PK and PFK activities were enhanced by *G. straminea* ethanol extract pre-treatment. The results showed that when the mice were in a hypoxic state, the hypoxic environment activated PF and PFK. Anaerobic glycolysis provides essential energy sources, thereby improves the ability to resist hypoxia, and prolongs survival. These findings suggest that the anti-hypoxia activity of *G. straminea* ethanol extract might be based on its regulation of ATP and energy metabolism.

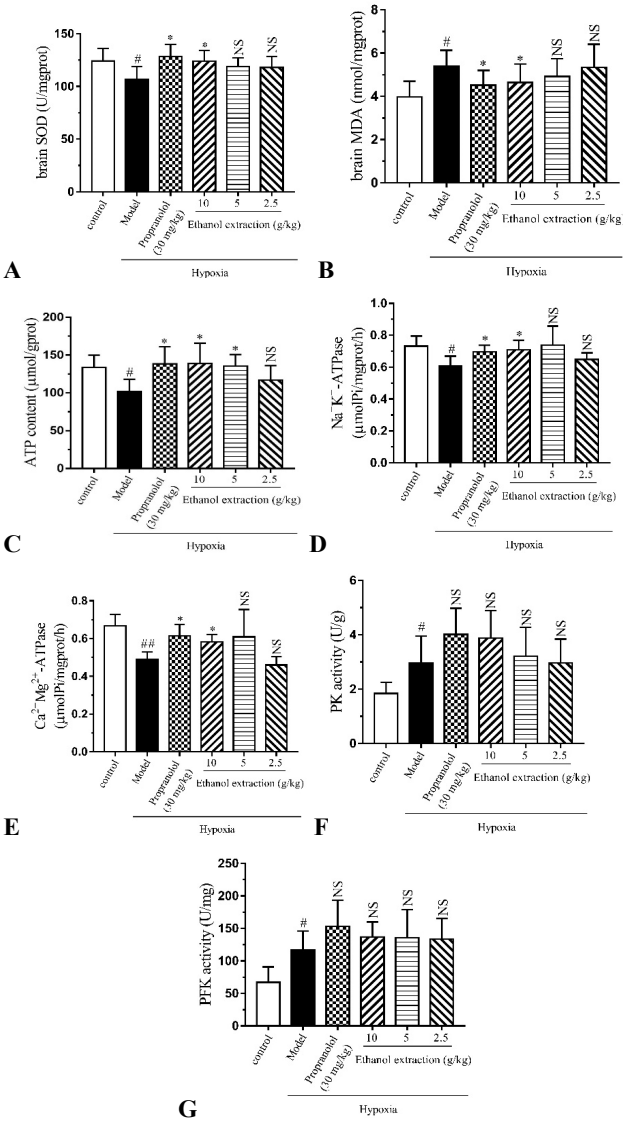

Fig. 4. Regulation of lipid peroxidation and energy metabolism in hypoxic mice brain by *G. straminea* ethanol extract.
Notes: (A) SOD; (B) MDA; (C) ATP, adenosine triphosphate; (D) Na^{+}-K^{+}-ATPase; (E) Ca^{2+}-Mg^{2+}-ATPase; (F) PK, pyruvate kinase; (G) PFK, phosphofructokinase. $^{\#}$ P <0.05 and $^{\#\#}$ P <0.01 *vs.* control group, NS, not significant; * P <0.05 *vs.* hypoxia model group.

DISCUSSION

The brain consumes one quarter of the body's oxygen, and insufficient oxygen supply can lead to brain damage or even brain death (Ferrer, 1973; Lenart, 2017). To investigate the potential protective effects of *G. straminea* extract, both liver and brain tissues were collected for the present study. Our results demonstrated that ethanol extract of *G. straminea* markedly enhanced mice's anti-hypoxia capability through ameliorating their anti-oxidative ability and energy metabolism. Breathing time can be used as an important indicator to evaluate the effect of hypoxia protection. In this work, the ethanol extract of *G. straminea* intervention significantly prolonged the respite time of hypoxic mice, indicating its potential anti-hypoxia effects. During hypoxia, the body undergoes anaerobic respiration, resulting in the accumulation of incomplete oxidation products, consequently leading to increased oxidative damage in mice tissues. Generally, the contents of SOD and MDA are essential indicator for lipid peroxidation. The results showed ethanol extract of *G. straminea* significantly induced SOD content and reduced MDA content in liver and brain. Furthermore, it significantly improved hepatic T-AOC and CAT activities, decreased free radical accumulation and accelerated lipid peroxides elimination.

Hypoxia disrupts normal oxidative decomposition function, and glycolysis is the primary short-term energy source (Fernie *et al*., 2004). ATP molecules serve as the direct substrate for energy, providing energy for cellular metabolism, and responsible for the storage and transmission of chemical energy (Miao *et al*., 2014). Our findings suggested that *G. straminea* ethanol extract pre-treatment significantly increased ATP content, Na^+-K^+-ATPase and Ca^+-Mg^+-ATPase activities, and enhanced PK and PFK activity, indicating that the mechanism of *G. straminea* ethanol extract may be related to increased energy metabolism. This study implies that the anti-hypoxia activity of *G. straminea* depends on its regulation of energy metabolism. Further research is needed to explore its in-depth mechanisms of the anti-hypoxic effect and its potential as a therapeutic agent.

CONCLUSION

In this study, we demonstrated the anti-hypoxia effects of *G. straminea* ethanol extract *in vivo* on mice under various hypoxia conditions. Our results showed that the ethanol extract of *G. straminea* significantly improved the survival time and increased their tolerance to hypoxia. Moreover, the anti-hypoxic effect is associated with the regulation of antioxidant enzyme activities and energy metabolism. These findings provide evidence and a molecular insight that *G. straminea* can serve as a potential natural anti-hypoxia agent and be used for prevention and treatment of hypoxic diseases, as well as in the design of anti-hypoxia drugs.

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IRB approval

This study did not involve humans; therefore, no IRB approval is needed.

Ethical statement

Research experiments conducted in this article with animals were approved by the Laboratory Animal Ethics Committee of Xizang Minzu University (Certify No.: 20200-7) following all guidelines, regulations, legal, and ethical standards as required for animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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