Pharmacogn. Mag.
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ORIGINAL ARTICLE

Effects of Drought Stress on the Quality of Licorice

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Submitted: 16-Feb-2022 Revised: 13-Mar-2022 Accepted: 03-Aug-2022 Published: 23-Nov-2022

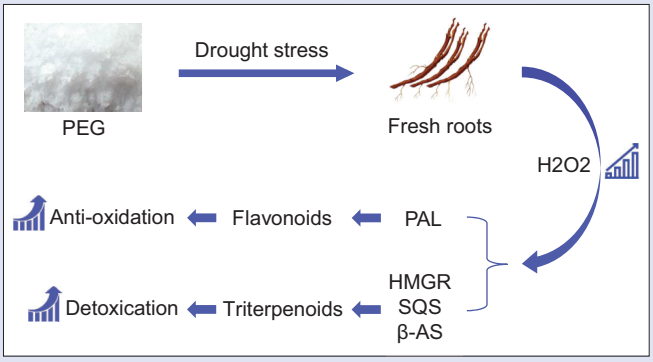
ABSTRACT

Background: Licorice, one of the most commonly used phytomedicines in Asia, has detoxication and anti-oxidation properties. Due to the exhausted wild resources, licorice is obtained mainly from cultivation, and the suitable conditions under cultivation lead to a serious decline in quality. **Objectives:** To establish a method to improve the quality of cultivated licorice by environmental stress. **Materials and Methods:** The fresh roots of *Glycyrrhiza uralensis* were soaked in 5%, 10%, and 20% polyethylene glycol-6000 (PEG) for 4 days to construct a physiological state of drought. The changes in hydrogen peroxide (H_2O_2) and enzymes related to secondary metabolism in the treated licorice were observed, and their detoxication and anti-oxidation were verified by reducing aconite-induced cardiotoxicity and free radical scavenging rate experiments. **Results:** The H_2O_2 content notably boosted, and gene expression and allosterism of enzymes related to secondary metabolism such as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR), squalene synthase (SQS), β-amyrin synthase (β-AS), and phenylalanine ammonia-lyase (PAL) were promoted, and enhance their activities. Under a drought stress of 10% PEG, secondary metabolites significantly increased, and the effectiveness of the drug also intensified. For the myocardial injury caused by aconite, compared with the untreated, creatine kinase (CK), cardiac troponin-T (cTn-T), and lactate dehydrogenase (LDH) decreased by 9.3%, 14.6%, and 6.3% in the 10% PEG. Meanwhile, the clearance rates of 1,1-diphenyl-2-picryl hydrazyl radical (DPPH·) and hydroxyl radical (OH·) heightened by 7.5% and 13.1%, separately. **Conclusion:** Drought stress can greatly increase the secondary metabolites of licorice and enhance its detoxication and anti-oxidation.

Key words: Anti-oxidation, detoxication, drought stress, flavonoids, licorice, triterpenoids

SUMMARY

- The fresh roots of licorice were treated with 5%, 10%, and 20% PEG-6000 to simulate drought stress
- After treatment, the H_2O_2 content raised significantly, and the contents of 10 kinds of secondary metabolites increased greatly
- Detoxication and anti-oxidation of licorice were enhanced, and the increase in secondary metabolites contents was achieved through the H_2O_2 pathway, thereby enhancing the efficacy.



Abbreviations used: PEG: Polyethylene glycol-6000; H_2O_2: hydrogen peroxide; HMGR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; SQS: squalene synthase; β-AS: β-amyrin synthase; PAL: phenylalanine ammonia-lyase; CK: creatine kinase; cTn-T: cardiac troponin-T; LDH: lactate dehydrogenase; DPPH·: 1,1-diphenyl-2-picryl hydrazyl radical; OH·: hydroxyl radical; -OH: phenolic hydroxyl; ROS: reactive oxygen species; $O_2^{\bullet}$: superoxide radical; MVA: mevalonate; ChP: Chinese Pharmacopoeia; RT-qPCR: quantitative real-time PCR.

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DOI: 10.4103/pm.pm_84_22

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INTRODUCTION

Licorice, the root of *Glycyrrhiza uralensis* Fisch.,[1] has been used for more than 2,000 years[2] and has various pharmacological effects such as detoxication, anti-oxidation, bacteriostasis, anti-inflammatory, anti-virus, and anti-depression.[3-5] Among them, detoxication is the most popular in the recipes, known as "ten prescriptions, nine licorices",[6] and even can alleviate the cardiotoxicity caused by aconite, a highly toxic phytomedicine.[7] Flavonoids in licorice with phenolic hydroxyl (–OH) can react with oxygen radicals to generate stable semiquinone radicals and terminate the radical chain reaction and have strong anti-oxidation.[8] Due to its remarkable efficacy and characteristics of mild and non-toxic, licorice is widely used, with an annual requirement of 10 million tons.[9]

G. uralensis grows mostly in arid areas, and drought is not only the main ecological stress that affects the growth and development but also the environmental factor that affects the quality of herbs.[10,11] The ecological role of secondary metabolites, that is, medicinal compositions, is to improve the adaptability of plants under stress.[12,13] Secondary metabolites will be heavily synthesized only under stress and vary with the adversity level.[14] Therefore, the growing environment has an important influence on the plant's secondary metabolism.[15] The secondary metabolites of licorice are complex, mainly containing triterpenoids and flavonoids.[16] Triterpenoids such as glycyrrhizic acid and glycyrrhetinic acid are synthesized through the pathway of mevalonate (MVA),[17] involving 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR),

Cite this article as: Fan J, Shen Y, He LW, Deng DQ, Meng XC. Effects of drought stress on the quality of licorice. Phcog Mag 2022;18:976-84.

squalene synthase (SQS), and β-amyrin synthase (β-AS). Flavonoids, for instance, liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, isoliquiritin apioside, echinatin, and licochalcone B, are biosynthesized through the pathway of phenylpropanoid under the regulation of phenylalanine ammonia-lyase (PAL).[18] Drought or dehydration will stress plants to synthesize large amounts of triterpenoids,[11] or flavonoids,[10] in a short time. The scarcity of wild resources and the huge market demand lead to cultivated licorice becoming popular, but the good cultivation environment results in a serious decline in the quality of phytomedicine.[19,20] Of the two most important medicinal compositions in the cultivated licorice, glycyrrhizic acid is lower than 20.0 $mg{\cdot}g^{-1}$, liquiritin is less than 5.0 $mg{\cdot}g^{-1}$,[21,22] far below that of the wild (glycyrrhizic acid is 62.8 $mg{\cdot}g^{-1}$ and liquiritin is 30.3 $mg{\cdot}g^{-1}$),[23] even fail to meet the standard of Chinese Pharmacopoeia (ChP, 2020). The decreased secondary metabolites will greatly reduce the medicinal effect of the licorice.[24]

The serious decline in quality due to cultivation has threatened the healthy and steady development of Chinese medicine, and how to improve the quality of cultivated medicinal materials is the key and difficulty. The aqueous solution of polyethylene glycol-6000 (PEG), a polymer that can reduce the water potential of the solution, is often used to create drought stress.[25,26] Plant cells all have a complete secondary metabolic system. The fresh roots of *G. uralensis* directly exposed to PEG can avoid the influence of growth metabolism and may present better results. Based on it, a method to enhance licorice's quality will be explored and evaluated.

MATERIALS AND METHODS

Plant roots

Three-year-old *G. uralensis* fresh roots, identified by Prof. Xiang-Cai Meng, were collected from the medicinal botanical garden of Heilongjiang University of Chinese Medicine, China, on October 25, 2020. Some uniformly cylindrical roots were selected and cleaned. All roots were equally divided into 4 parts, 3 of which were soaked in 5.0%, 10.0%, and 20.0% PEG, severally, placed at 20 to 25°C for 4 days, and the remaining 1 part was directly sampled without any treatment. For each part, about 30 g of fresh roots were dried at 55°C for 48 h and then crushed to determine secondary metabolites contents. 0.3 g ×3 of phloem was collected to determine gene expressions and activities of HMGR, SQS, β-AS, and PAL.

Animals

A total of 72 Sprague Dawley rats, with a body mass of 160 to 200 g, were purchased from the Center for Safety Evaluation of Drugs at Heilongjiang University of Chinese Medicine (Harbin, China). The production license number of experimental animals is SCXK (Hei) 2013-0004. Animals were raised in the animal laboratory where the temperature was 18 to 22°C and the relative humidity was 55% to 70%.

Reagents

Liquiritigenin (578-86-9), isoliquiritigenin (961-29-5), liquiritin apioside (74639-14-8), isoliquiritin apioside (120926-46-7), isoliquiritin (5041-81-6), and glycyrrhetinic acid (471-53-4) were obtained from Chengdu Purechem-standard Co., Ltd. (Chengdu, China). Glycyrrhizic acid (1405-86-3) and liquiritin (551-15-5) were obtained from Shanghai Jinsui Biological Technology Co., Ltd. (Shanghai, China). Licochalcone B (58749-23-8) was obtained from Chengdu Lemeitian Pharmaceutical Technology Co., Ltd. (Chengdu, China). Echinatin (34221-41-5) was obtained from Nanjing Yuanzhi Biological Technology Co., Ltd. (Nanjing, China).

Kits of hydrogen peroxide (H_2O_2, 20210320), creatine kinase (CK, 20211201), lactate dehydrogenase (LDH, 20211203), and PAL (20210914) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Kits of cardiac troponin-T (cTn-T, 20211208) were obtained from Bio-Swamp Life Science (Shanghai, China). Kits of HMGR (20210510), SQS (20210604), and β-AS (20210623) were obtained from Meimian Industrial Co., Ltd. (Wuhan, China). The UNIQ-10 column TRIzol total RNA isolation kit (B511321) was obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

H_2O_2 content in fresh roots

The content of H_2O_2 was determined using the kits, and there was a characteristic absorption at 405 nm.[27] The content is calculated using the following equation: H_2O_2 $(mmol{\cdot}g^{-1}prot) = [(A_{sample}/A_{standard}) \times C_{standard}]/C_{sample's\ protein}$, where A is the absorbance, and C is the concentration.

Gene expressions of HMGR, SQS, β-AS, and PAL

Total RNA was extracted according to the instructions of the kit, and cDNA was synthesized by reverse transcription. The mRNA of HMGR, SQS, β-AS, and PAL were amplified and quantified with quantitative real-time PCR (RT-qPCR). The sequences of all primers are shown in Table 1. The amplification conditions were pre-denaturation at 95°C for 3 min, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and 45 cycles. Using actin as the internal reference gene, the expression of each treatment relative to the untreated was calculated in $2^{-\Delta\Delta Ct}$.

Activities of HMGR, SQS, β-AS, and PAL

The activities of HMGR, SQS, and β-AS were determined using the kits. The absorbance was measured at 450 nm, and the activity of each sample was calculated according to the linear regression equation of the standard. PAL activity was determined by counting the change in value at 290 nm.[28] One unit of PAL was defined as the absorbance increase of 0.01 per min. The above enzymes' activities in the samples (0.3 g) before and after drought treatment were measured in these directions.

A total of 0.3 g of *G. uralensis* fresh root was ground into a homogenate, fixed capacity up to 10 mL with precooled phosphate buffer

Table 1: Primer sequence for biosynthetic enzymes related to secondary metabolic pathways in *G. uralensis*

Name	Sequence oligos (5′→3′)	Amplicon length (bp)	Genbank accession No.
actin	forward, TGAATTGCGTGTTGCTCCTGAGG reverse, ACATGGCAGGCACATTGAAGGTC	163	EU190972
HMGR	forward, TGGCTGGTGCTCTTGGTG reverse, CTTGCCATCATTCACTGCTTC	140	GQ845405
SQS	forward, CACTATGTGGCAGGACTTGTTG reverse, TACCTGAAGAAACAGACCCATTG	114	MN567154
β-AS	forward, TGGATCAGCAATTCAGGCAC reverse, GCGAACCAAGAACCGTAAGT	174	MN567155
PAL	forward, CCAAGTTGCCAAGAGGACAC reverse, CTTTTGCATCAATGGGTACGT	154	MK341789

solution (pH = 7.8), and then centrifuged to get the supernatant. A total of 100 μL of H_2O_2 with different concentrations (including 0.0, 0.0001, 0.001, 0.01, 0.1, and 1.0 μM) were directly added to 400 μL of the supernatant, separately. After 20 min (25°C), the activities of HMGR, SQS, β-AS, and PAL were determined according to the above-related methods.

Secondary metabolite contents

A total of 0.5 g of herbal powder was placed in a 50 mL volumetric flask, added with 70% ethanol, ultrasonically extracted for 30 min, and then filtered with a microporous membrane (0.45 μm). HPLC analysis used the Diamonsil C_{18} column (250 × 4.6 mm, 5 μm). The injection volume was 10 μL. The flow rate was 0.8 mL·min^{-1}. The column temperature was 40°C. The peaks of liquiritigenin, liquiritin, liquiritin apioside, glycyrrhizic acid, and glycyrrhetinic acid were obtained at 250 nm. The detection wavelengths of isoliquiritigenin, isoliquiritin, isoliquiritin apioside, echinatin, and licochalcone B were obtained at 360 nm. The mobile phases consisted of ultrapure water with 0.05% phosphoric acid (A) and acetonitrile (B). The elution gradient was as follows: 0 to 10 min, 86% A→77% A; 10 to 24 min, 77% A→70% A; 24 to 30 min, 70% A→66% A; 30 to 35 min, 66% A → 64% A; 35 to 42 min, 64% A→58% A; 42 to 48 min, 58% A→49% A; 48 to 53 min, 49% A→30% A; 53 to 75 min, 30% A→10% A.

Detoxication

Aconite (50.0 g) was taken, added with 10 times the amount of water, soaked for 12 h, decocted for 1 h, then concentrated under low pressure to 50.0 mL, and a single decoction of aconite was obtained. A combined decoction of licorice and aconite was obtained in the same way, and the compatibility ratio was 1 to 1.

72 rats were randomly divided into the control (distilled water), model (single decoction of aconite), untreated (combined decoction of untreated licorice and aconite), 5% PEG (combined decoction of 5% PEG-treated licorice and aconite), 10% PEG (combined decoction of 10% PEG-treated licorice and aconite), and 20% PEG (combined decoction of 20% PEG-treated licorice and aconite), with 12 rats in each group. Samples were taken after 28 days of continuous intragastric administration (10 mL·kg^{-1}).

Sampling was carried out as follows: (1) According to body weight, the rats in each group were anesthetized by intraperitoneal injection of the mixture of ketamine (50 mg·kg^{-1}) and xylazine (5 mg·kg^{-1}). (2) The abdominal cavity was opened, and 5 mL of blood was drawn from the aorta using a disposable vacuum blood collection tube without the anticoagulant. After standing for 45 min, blood was centrifuged at 4 °C and 3000 r·min^{-1} for 15 min. The supernatant was stored in a refrigerator at −80 °C. At the end of the experiment, the animals were euthanized with a high dose of pentobarbital sodium.

The supernatant was used to determine the CK, LDH, and cTn-T via the kits.[29,30]

Anti-oxidation

A total of 0.5 g of licorice powder was placed in a 25 mL volumetric flask, added with anhydrous ethanol up to the tick mark, and ultrasound-assisted extraction for 30 min. After centrifugation, the supernatant was taken as the sample solution.

Clearance rate of the 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical: [31] The same volume of DPPH (0.6 mmol·L^{-1}) was added to 3 mL of the sample, mixed well, and reacted at 25°C (light-sheltered) for 30 min, and then the absorbance was measured at 517 nm, which was denoted as A_1. DPPH was replaced with anhydrous ethanol, and the absorbance was recorded as A_2. The sample was replaced with anhydrous ethanol, and the absorbance was recorded as A_3. The ability for scavenging DPPH ·was calculated as $[1 - (A_1 - A_2)/A_3] \times 100\%$.

Clearance rate of the hydroxyl radical (OH·):[32] 1 mL of ferrous sulfate (6.0 mmol·L^{-1}) and 1 mL of H_2O_2 (6.0 mmol·L^{-1}) were added to 1 mL of the sample, mixed well, stood for 10 min, then added with 1 mL of salicylic acid (6.0 mmol·L^{-1}), and reacted at 37°C for 30 min; the absorbance was measured at 510 nm, which was recorded as A_4. Salicylic acid was replaced with ultrapure water, and the absorbance was denoted as A_5. The sample was replaced with ultrapure water, and the absorbance was recorded as A_6. The calculation formula was as follows: Clearance rate (OH·) = $[1 - (A_4 - A_5)/A_6] \times 100\%$.

Statistical analysis

The SPSS version 23.0 statistical software package was used for statistical analysis, and significant differences were detected by analysis of variance (ANOVA) using LSD. All pictures were obtained using Origin 2021. Ten kinds of secondary metabolites of licorice in different treatments were analyzed by OPLS-DA with SIMCA version 14.1.

RESULTS

Effect of drought stress on H_2O_2 contents

Figure 1 shows that the H_2O_2 contents in fresh roots of *G. uralensis* were heavily boosted under drought stress, and the 5%, 10%, and 20% PEG raised by 99.7%, 59.8%, and 93.3%, separately.

Effect of drought stress on gene expressions and activities of HMGR, SQS, β-AS, and PAL

Figure 2 and Figure 3 show that drought stress can greatly improve the gene expressions and activities of HMGR, SQS, β-AS, and PAL in fresh roots of *G. uralensis*. Compared with the untreated, 10% PEG showed the greatest rise, its activities increased by 107.9%, 53.2%, 50.5%, and 42.3%, respectively. The order of influence on the gene expressions of the above indexes was 10%, 20%, and 5% PEG, and the enhancement of the 10% PEG was about 1.3 times that of 5% PEG (or 20% PEG) for the HMGR and PAL.

Effect of exogenous H_2O_2 on activities of HMGR, SQS, β-AS, and PAL

With the rise of exogenous H_2O_2, the activities of HMGR, SQS, β-AS, and PAL showed a trend of increase first and then decrease in the grinding

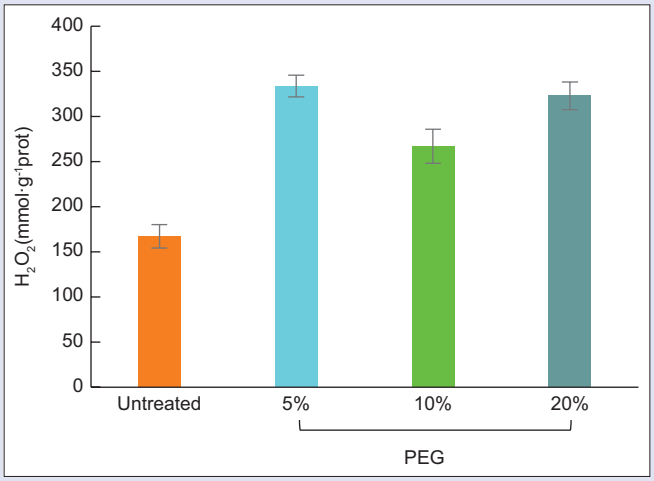

Figure 1: Effects of drought stress on H_2O_2

solution of fresh roots of *G. uralensis*, as displayed in Figure 4. For the activities of various enzymes, 0.01 μM H_2O_2 had the greatest effect, in the same trends, and HMGR, SQS, β-AS, and PAL increased by 42.4%, 40.2%, 36.4%, and 38.6%, separately, compared with 0.0 μM H_2O_2.

Contents of secondary metabolites

Figure 5 presents that secondary metabolites of licorice significantly increased after treatment. Compared with the untreated, 5% PEG increased liquiritigenin by 69.7%, 10% PEG raised liquiritin apioside, licochalcone B, isoliquiritigenin, isoliquiritin, glycyrrhizic acid, isoliquiritin apioside, and liquiritin by 34.0%, 35.9%, 54.1%, 57.0%, 59.1%, 50.5%, and 35.7%, respectively, 20% PEG raised echinatin and glycyrrhetinic acid by 34.2% and 66.4%. The OPLS-DA analysis showed that the most notable of all was 10% PEG, followed by 5% PEG [Figure 6].

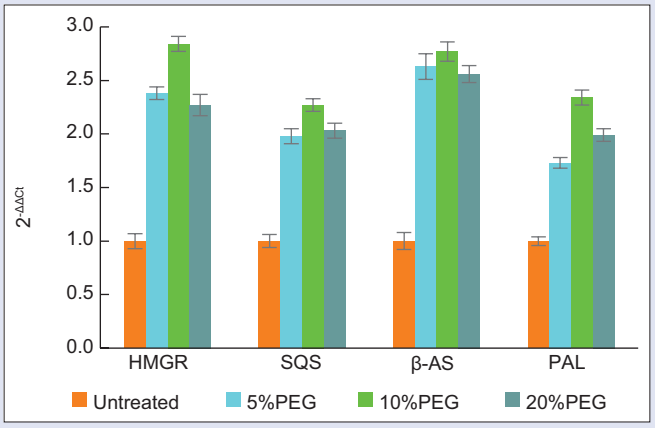

Figure 2: Effects of drought stress on the gene expressions of HMGR, SQS, β-AS, and PAL

Activities of CK and LDH, contents of cTn-T

Figure 7 presents that compared with the model, each treatment reduced CK and LDH activities and cTn-T contents. Compared with the untreated, 10% PEG had the best detoxication, which reduced CK, LDH, and cTn-T by 9.3% ($P < 0.01$), 6.3% ($P < 0.05$), and 14.6% ($P < 0.01$). The detoxication of 5% PEG was slightly better than that of the 20% PEG, and its CK, LDH, and cTn-T decreased by 6.6% ($P < 0.05$), 5.2% ($P < 0.05$), and 7.8% ($P < 0.01$), respectively.

Clearance rates of DPPH· and OH·

Figure 8 shows that compared with the untreated, each treatment could improve the clearance rates of DPPH· and OH·, especially 10% PEG, the clearance rates enhanced by 7.5% ($P < 0.01$) and 13.1% ($P < 0.01$), 5% PEG increased by 6.1% ($P < 0.05$) and 10.2% ($P < 0.01$), slightly better than 20% PEG.

DISCUSSION

Effects of drought stress on the quality of licorice

H_2O_2 in fresh roots

The essence of stress damage to plants is reactive oxygen species (ROS) which includes many types.[33] Superoxide radical ($O_2^{\bullet-}$) is the first generated ROS, with strong activity but a short life cycle, and easily converted into H_2O_2.[34] H_2O_2 has high stability and a relatively long survival cycle, easily penetrates biofilms, and is transported over long distances in cells. H_2O_2 can regulate the structure of enzymes and therefore exhibit a stronger ability to regulate the metabolic process.[35,36]

Figure 1 exhibits that under drought stress, H_2O_2 is greatly enhanced. The improvement of 10% PEG was less than that of 5% or 20% PEG, probably because 10% PEG had the greatest enhancement on the flavonoids [Figure 5 and Figure 6], especially, in the flavonoids with 3-OH, the content of isoliquiritigenin (-OH located at C-5, C-7, and C-4′) being about 1.9 times that of 20% PEG, and the content of licochalcone

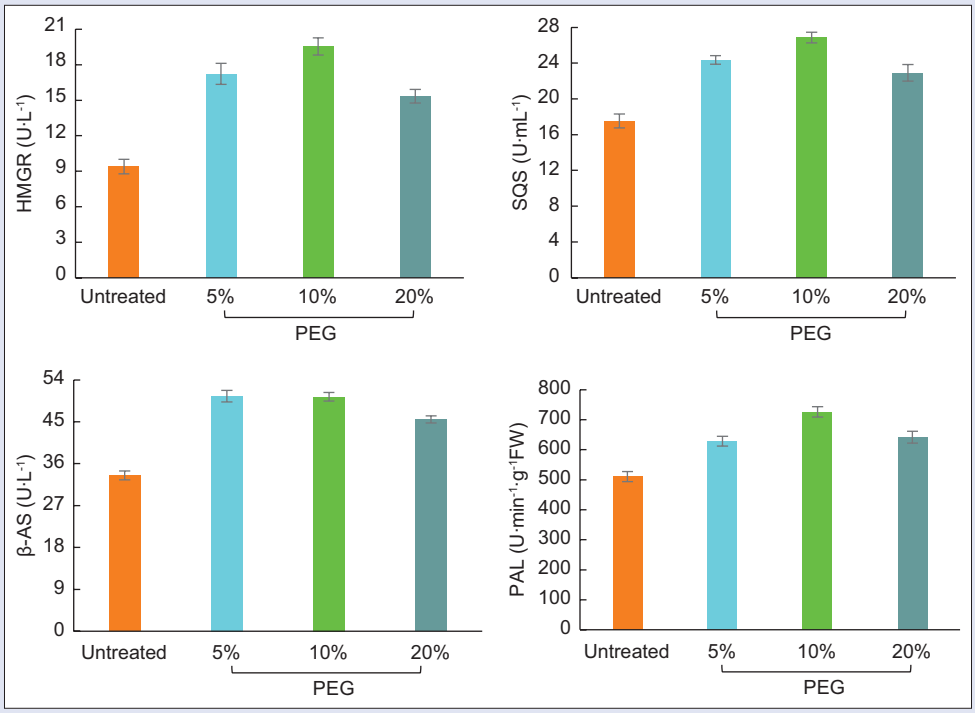

Figure 3: Effects of drought stress on the activities of HMGR, SQS, β-AS, and PAL

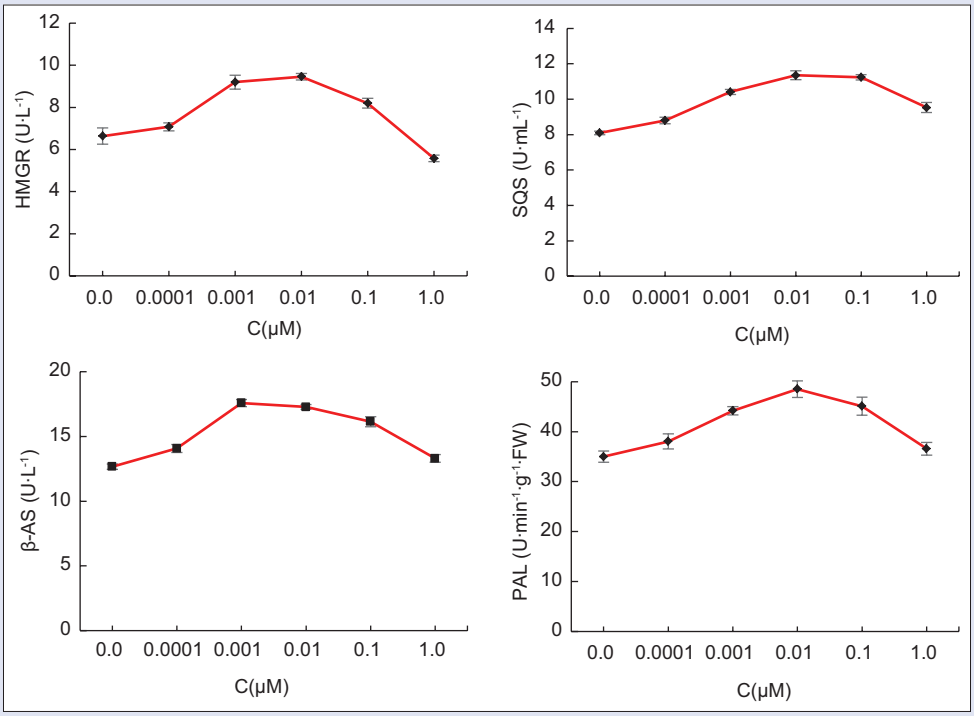

Figure 4: Effects of exogenous H_2O_2 on the activities of HMGR, SQS, β-AS, and PAL

B (-OH located at C-7, C-3′, and C-4′) about 1.6 times that of 5% PEG. Flavonoids with -OH can directly scavenge ROS and alleviate oxidative damage.[37,38]

Enzymes related to the secondary metabolic pathway

HMGR, the first rate-limiting enzyme in the MVA pathway, determines the rate of triterpenoid biosynthesis.[39] SQS can catalyze farnesyl pyrophosphate to squalene which is a precursor for the synthesis of triterpenoids.[40] β-AS can catalyze 2,3-oxidosqualene to β-amyrin which is a basic skeleton of glycyrrhizic acid and glycyrrhetinic acid.[41] PAL, the first critical and rate-limiting enzyme regulating the downstream signaling pathway,[42,43] is responsible for catalyzing the deamination of phenylalanine to trans-cinnamic acid that is a precursor for the synthesis of flavonoids.[44] Therefore, HMGR, SQS, β-AS, and PAL are indispensable for the synthesis and accumulation of triterpenoids and flavonoids in *G. uralensis*.

Figure 2 shows that under drought stress, the gene expressions of HMGR, SQS, β-AS, and PAL in fresh roots of *G. uralensis* were heavily enhanced. Of these, 10% PEG had the most significant effect, and it was about 1.3 times that of 5% PEG (or 20% PEG). Meanwhile, the activities also notably improved [Figure 3], indicating that drought stress could improve the activities of enzymes related to secondary metabolic pathways. Enzymes are mainly proteins with high catalytic efficiency produced by living cells. Gene expression is just the enhancement of protein synthesis under the guidance of genes, and their final activities also depend on the tertiary or quaternary structure, so it is difficult to reflect the activity of enzymes only by gene expression. The enzyme has at least two sites in space, that is, the regulatory site and catalytic site. Some substances can interact with the regulatory site to change the conformation of the enzyme and affect the catalytic effect.[45] Exogenous H_2O_2 was directly added to the grinding solution of fresh roots, and it was found that H_2O_2 had a regulation on the above enzymes [Figure 4], 0.01 μM H_2O_2 showed the greatest enhancement, and it was about 1.4 times that of 0.0 μM H_2O_2. However, with the increase of H_2O_2, the activity of the enzyme showed a downward trend, probably because enzymes are proteins containing multiple hydrogen bonds which are easily oxidized, and excessive H_2O_2 will cause great damage to the structure of the enzymes. Based on the above, it can be concluded that H_2O_2 produced by drought stress on the activities of enzymes was achieved through the dual effects of gene expression and allosterism.

Secondary metabolites change

The 10 kinds of secondary metabolite contents in licorice hugely improved under drought stress [Figure 5 and Figure 6]. In particular, 10% PEG raised glycyrrhizic acid and liquiritin by 59.1% and 35.7%, exceeding the quality standards stipulated in ChP, especially, the aglycones such as liquiritigenin, isoliquiritigenin, licochalcone B, echinatin, and glycyrrhetinic acid, which have higher bioactivity and bioavailability due to more -OH and lack of glycosyl affecting absorption[46,47] also increased by 49.9%, 54.1%, 36.0%, 22.8%, and 58.3%, respectively. Also, 10% PEG also heightened isoliquiritin, liquiritin apioside, and isoliquiritin apioside by 57.0%, 34.0%, and 50.5%. The increase of 20% PEG was lower than that of 10% PEG, which may be because excessive H_2O_2 produced under drought stress seriously interfered with the metabolic process.[11]

In summary, drought stress increased H_2O_2, and an appropriate amount of H_2O_2 could heavily promote the synthesis of triterpenoids and flavonoids by increasing the gene expression and activities of related enzymes in the pathway of MVA and phenylpropanoid and improve the quality of cultivated licorice.

Quality evaluation of drought-stress licorice

The secondary metabolites in phytomedicine are complex, and the contents and effectiveness of each compound vary.[46] The increase of secondary metabolites cannot fully ensure the enhancement of pharmacological actions. Therefore, it is very necessary to evaluate the quality of licorice with pharmacological effects.

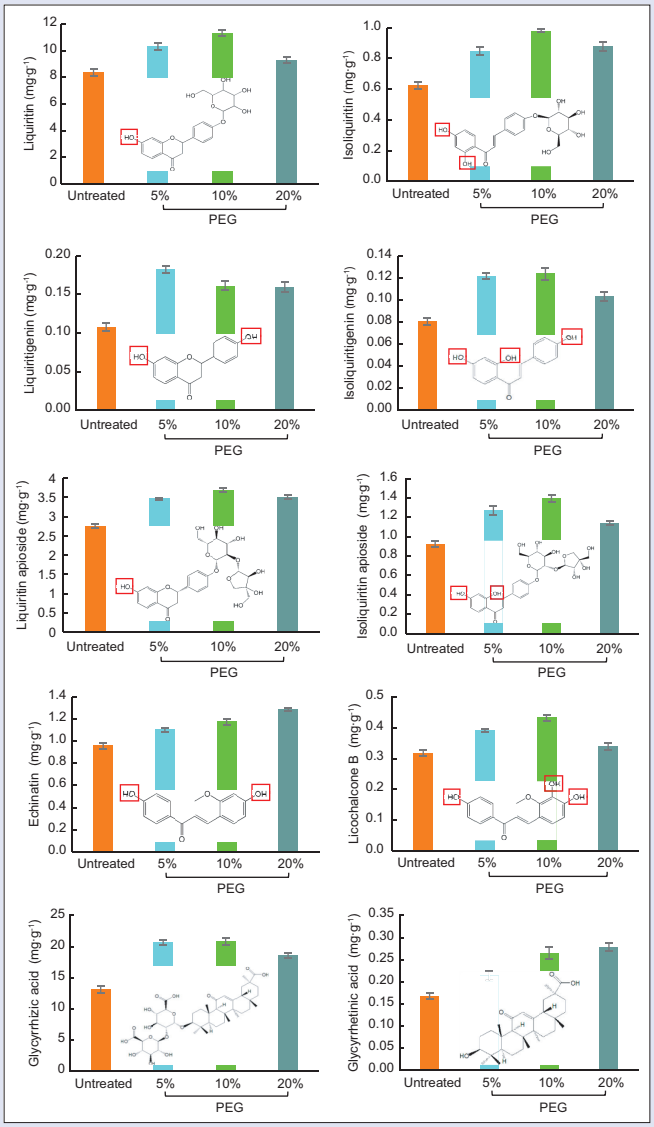

Figure 5: Effects of drought stress on secondary metabolites

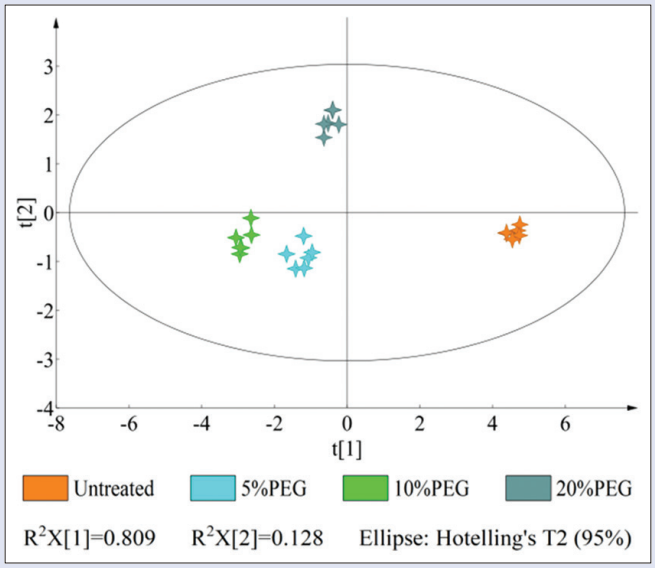

Figure 6: OPLS-DA of secondary metabolites

Detoxication

Licorice has detoxication on a variety of toxic phytomedicine. Aconite is a common phytomedicine with analgesic, anti-inflammatory, and cardiotonic properties.[48] However, the diester alkaloids, aconitine, neoconitine, and hypaconitine, contained in it have cardiotoxicity,[49,50] which is too strong to be used alone unless licorice is present,[51,52] so aconite is a good material to study licorice's detoxication. Myocardial zymogram (CK, LDH, and cTn-T) is an important index to judge a damaged myocardium.[53,54] Compared with the control, CK, LDH, and cTn-T in the model greatly increased [Figure 7], designating that aconite can cause serious damage to the heart. Compared with the model, CK, LDH, and cTn-T in each group with licorice were significantly decreased. Compared with the untreated, 10% PEG is the most outstanding and decreased CK, LDH, and cTn-T by 9.29%, 6.30%, and 14.61%, indicating that the detoxication of the drought-stress licorice on the cardiotoxicity caused by aconite greatly enhanced.

Glycyrrhizin acid and glycyrrhetinic acid of triterpenoids are considered to be the main substances to relieve the toxicity of aconite. Glycyrrhizin acid can reduce the contents of free alkaloids by promoting the hydrolysis and lipid exchange of diester alkaloids.[55] Glycyrrhetinic acid has the structural characteristics of adrenocortical steroid hormone, can enhance the anti-stress ability,[56] and can also maintain the balance of calcium ions in cardiomyocytes through reducing the expression of calmodulin in the excitation–contraction coupling system of cardiomyocytes so as to alleviate the cardiotoxicity induced by aconitine.[57] In addition, the acid–base neutralization reaction of glycyrrhizin acid (or glycyrrhetinic acid) with diester alkaloids also can reduce cardiotoxicity.[58] Figure 5 and Figure 6 showed that the glycyrrhizin acid and glycyrrhetinic acid were heavily raised under drought stress, especially with 10% PEG (59.1% and 58.3%), which was consistent with the detoxification trend [Figure 7].

Anti-oxidation

As a natural antioxidant, licorice has been widely used in many fields, for instance, medicine, food, chemical industry, animal husbandry, etc.[59,60] The membrane lipid peroxidation induced by radicals is an important factor leading to a variety of diseases, and the pharmacological activities of licorice such as bacteriostasis, anti-inflammatory, anti-virus, and anti-depression are closely related to its anti-oxidation.[61] DPPH· and OH· are the main radicals in organisms, and the clearance rates of the two can directly reflect the anti-oxidation.[62,63] Figure 8 shows that the clearance rates of DPPH· and OH· of the drought-stress licorice heavily increased, especially, 10% PEG raised by 7.5% and 13.1%, showing that moderate drought stress notably enhanced the anti-oxidation of the drought-stress licorice.

Flavonoids take 2-phenylchromone as the basic nucleus and contain one or more -OH; the hydrogen atom in the -OH can combine with radical to form a resonance-stable semiquinone structure, terminate the radical chain reaction, and exert anti-oxidation.[64,65] The number and positions of -OH have vital effects on the anti-oxidation, especially located at C-5, C-7, and C-4′.[66,67] Figure 5 showed that flavonoids in the drought-stress licorice greatly were raised, particularly, isoliquiritigenin (-OH located at C-5, C-7, and C-4′), licochalcone B (-OH located at C-7, C-3′, and C-4′), liquiritigenin (-OH located at C-7 and C-4′), and echinatin (-OH located at C-7 and C-4′). The above four components of 5% PEG increased by 51.7%, 23.0%, 69.7%, and 15.2%, those of 10% PEG by 54.1%, 35.9%, 49.9%, and 22.8%, those of 20% PEG by 28.7%, 6.6%, 48.2%, and 34.2%, separately. On the whole, 10% PEG showed the greatest increase of aglycones.

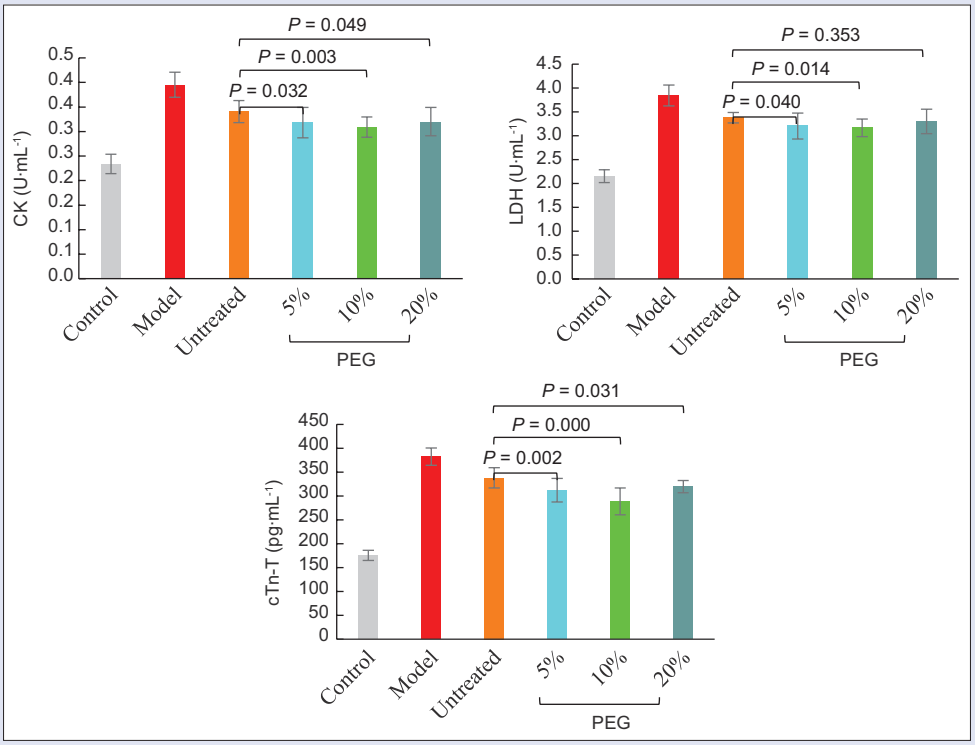

Figure 7: Effects of licorice with drought stress on CK, LDH, and cTn-T

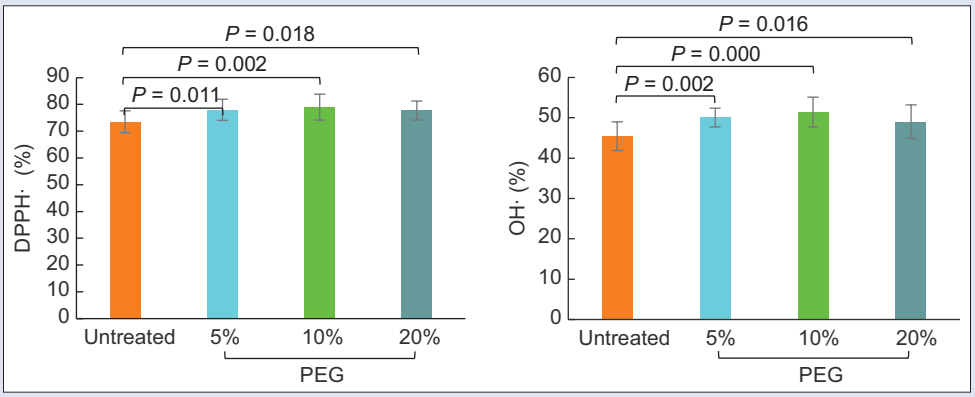

Figure 8: Effects of licorice with drought stress on DPPH• and OH•

CONCLUSION

Drought stress simulated with PEG could induce a massive accumulation of H_2O_2, which enhanced the gene expression and activities of HMGR, SQS, β-AS, and PAL; with this, the biosynthesis of secondary metabolites greatly boosted, and the detoxication and anti-oxidation of licorice also enhanced. Drought treatment could effectively solve the problems of the quality decline of cultivated licorice.

Acknowledgements

The authors wish to thank for providing financial support from the National Natural Science Foundation of China (81573523), National Survey of Traditional Chinese Medicine Resources (GZY-KJS-2018-004), and Scientific Research fund of Heilongjiang University of Chinese Medicine (2019MS27).

Ethics statement

Licorice, the root of *G. uralensis* Fisch., was collected in full compliance with the guidelines and regulations of Heilongjiang Province, China. Meanwhile, the collection was carried out in accordance with relevant legislation and with the permission of regulatory authorities.

All protocols involving animals were conducted in accordance with the guidelines and provisions of the Laboratory Animal Management Regulations of Heilongjiang Province, China. Moreover, the number and discomfort of animals were also minimized according to the guidelines for experiments with living animals in China.

Financial support and sponsorship

This work was supported by the National Natural Science Foundation of China (81573523), the National Survey of Traditional Chinese Medicine

Resources (GZY-KJS-2018-004), and the Scientific Research fund of Heilongjiang University of Chinese Medicine (2019MS27).

Conflicts of interest

There are no conflicts of interest.

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