Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Exogenous Intervention with Sodium Hydrosulfite Enhances the Quality of *Radix Scutellariae* by Modulating Antioxidases and Secondary Metabolites

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Submitted: 17-12-2017 Revised: 25-01-2018 Published: 23-01-2019

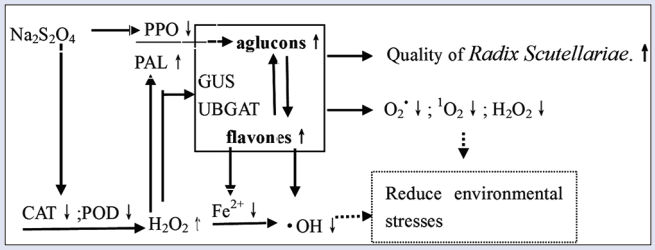
ABSTRACT

Introduction: The transformation of wild herbal medicine into cultivation results in decreased quality, becoming an issue of concern in China, even has a word, "traditional Chinese medical science would be destroyed by herbal medicine." Now, there is no way to acquire superior herb medicine. Reactive oxygen species (ROS) are generated under stress and represent key plant products that induce secondary metabolism. ROS may be important intermediates that link environmental stress to secondary metabolism. Sodium hydrosulfite ($Na_2S_2O_4$) is just one compound that produces $O_2^{\bullet-}$. **Materials and Methods:** The fresh roots of *Scutellaria baicalensis* were treated with 0.0004, 0.04, and 4 mmol/L $Na_2S_2O_4$, respectively. A stress model was established to elucidate the change of secondary metabolism, antioxidant enzyme system, and enzymes relating to flavonoids. **Results:** $Na_2S_2O_4$ regulated the activities of β-glucuronidase (GUS) and UDP-glucuronate baicalein 7-O-glucuronosyltransferase (UBGAT) and promoted the transformation of flavonoid glycosides into aglycones with greater antioxidant capacities. In fresh *S. baicalensis* roots, $Na_2S_2O_4$ decreased the activities of superoxide dismutase, catalase, and peroxidase (POD), which led to a substantial accumulation of hydrogen peroxide (H_2O_2). H_2O_2 increased the expression of phenylalanine ammonia lyase, GUS, and UBGAT genes and promoted the synthesis and transformation of flavonoids. Moreover, polyphenol oxidase regulated the flavonoid content to maintain the oxidation balance. Although the content of flavonoid glycosides was slightly reduced, the total flavonoid content remained high. Notably, the content of baicalein, an antioxidant compound, increased from 0.28% to 1.96%. **Conclusions:** ROS facilitated the dominant role of secondary metabolites and enhanced the quality of *Radix Scutellariae*.

Key words: Antioxidases, flavonoids, *Radix Scutellariae*, reactive oxygen species, sodium hydrosulfite

SUMMARY

- It elucidated a new mechanism of how *S. baicalensis* to match its surroundings
- A stress model was established with sodium hyposulphite for enhanced the Quality of *Radix Scutellariae*
- Contents of baicalein with the higher activities in adversity increased 15 times, it will bright prospects for clinic application and physiological research of plant.



Abbreviations used: APX: Ascorbate peroxidase, CAT: Catalase, GUS: β-glucuronidase, PAL: Phenylalanine ammonia lyase, POD: Peroxidase, PPO: Polyphenol oxidase, ROS: Reactive oxygen species, SOD: Superoxide dismutase, UBGAT: UDP-glucuronate baicalein 7-O-glucuronosyltransferase.

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DOI: 10.4103/pm.pm_598_17

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INTRODUCTION

Radix Scutellariae, the root of *Scutellaria baicalensis* Georgi., is a common medicinal plant that is widely utilized in traditional Chinese medicine. This plant is referred to as "Huang Qin" in Chinese, and it has remarkable antipyretic, anti-inflammation, and anticancer properties. The major compounds of this plant include baicalin, wogonoside, baicalein, wogonin, and other flavonoids. Figure 1 illustrates the biosynthetic pathways of these compounds. Previous studies have shown that aglycones also exhibit considerable antioxidant capacities by decreasing the reductive power of phenolic compounds.[1-3] Therefore, the activity of baicalein is significantly higher than the activities of baicalin, wogonin, and wogonoside.[2] Baicalein exhibits 2- to 5-fold higher antibacterial activity and 1- to 3-fold greater inhibitory effects on interleukin (IL)-1β converting enzyme than other flavonoids.[4-6]

Baicalein and wogonin are lipid-soluble and readily diffuse across membranes; thus, they exert strong pharmaceutical effects. A previous study showed that the bioavailability of baicalein is 7-fold higher than that of baicalin;[7] thus, the effects of baicalein has gained increasing attention. Transforming glycosides into aglycones through the cleavage of cellulose significantly increase antioxidant capacities and substantially inhibit cancer cell proliferation.[8,9] A recent report demonstrated that the best *Radix Scutellariae* is cultivated in Chengde city, China, and although it possesses a high content of baicalin, the

Cite this article as: Qi S, Huiru G, Jiahui W, Aihua Z, Xiangcai M. Exogenous intervention with sodium hydrosulfite enhances the quality of *Radix Scutellariae* by modulating antioxidases and secondary metabolites. Phcog Mag 2019;15:107-14.

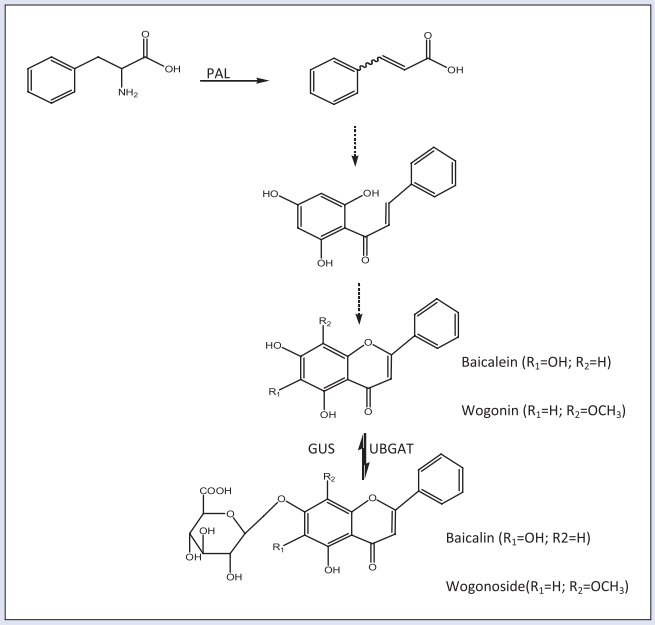

Figure 1: Synthesis and transformations of flavones in *Scutellaria baicalensis*

content of other flavonoids is not high.[10] Hence, baicalin may be an important index that influences herb quality. These findings indicate that baicalein may play an important role in antioxidative effects and quality evaluations.

Reactive oxygen species (ROS) are ubiquitous in plant cells. ROS could mediate stomatal closure, programmed cell death, gravitropism, and biotic and abiotic stress tolerance.[11] However, the global oxidation of ROS must be maintained at a stable level because extremely low ROS levels are also harmful to the growth, development, and physiological reactions of plants.[12] Increased levels of ROS may alter the configuration of adjacent molecules, decrease lipid bilayer stability, significantly damage single-stranded DNA, crosslink sulfur-containing proteins, and degrade polypeptides, thereby injuring plant cells and threatening their survival.[11] ROS levels in the plants are controlled via enzymatic components and nonenzymatic components. The enzymatic components include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and polyphenol oxidase (PPO). The nonenzymatic components include Vitamin C, glutathione, and polyphenols (such as flavonoids).[13] ROS generation rapidly increases under stress.[14,15] ROS function as secondary messengers and trigger various physiological responses in plants,[16,17] including synthesis of flavonoid compounds.[18-20] Flavonoids not only scavenge ROS molecules but also chelate the transition metal Fe^{2+} to avoid the Fenton's reaction, which transforms hydrogen peroxide (H_2O_2) into much more toxic $^{\cdot}OH$.[21] Therefore, ROS are closely associated with flavonoid compounds. The flavonoid content of *Radix Scutellariae* is as high as 10% in the dry root, and ROS can substantially improve the acclimatization of *S. baicalensis* by acting at the root.

Approximately 1200 years ago, the relationship between medicinal plant quality and different ecological environments were appreciated although the precise underlying mechanism was unclear. Determining the mechanisms underlying these interactions is one of the primary difficulties in herbal resource research. ROS are the initial products produced under environmental stress conditions and represent key plant products that induce secondary metabolism, and secondary metabolites are often medicinal ingredients. Therefore, ROS are likely a fundamental target for improving the quality of *Radix Scutellariae*. The high quality of *Radix Scutellariae* cultivated in Chengde city, Hebei, is related to the sufficient sun, dry climate, and low rainfall, which are essential factors that affect herb quality.[22-24] $O_2^{\cdot-}$ is the initial product generated in plants under ecological stress and is the essential reason for various physiological responses. Sodium hydrosulfite ($Na_2S_2O_4$) produces the same $O_2^{\cdot-}$ observed in stressed plants.[25] Therefore, $Na_2S_2O_4$ has the potential to induce metabolic processes that are observed under stress and closely resemble those observed in nature. The fresh roots of *S. baicalensis* are not only live organisms but also medicinal organs. Hence, we used fresh roots as research subjects to establish a biological model to elucidate the mechanisms of plant adaptations to the environment and clarify the relationship between the ecological environment and secondary metabolism to provide a scientific basis for improving the quality of *Radix Scutellariae*.

MATERIALS AND METHODS

Medicinal material collection and treatment

Fresh roots were isolated from 3-year-old *S. baicalensis* cultivated in the medicinal arboretum of Heilongjiang University of Chinese Medicine in October 2013. Large cylindrical roots were selected, washed, and stored at 20°C–25°C for 24 h to disperse any surface water. Each root was divided into four equal sections. One section was soaked in clean water for 30 s, and the other three sections were soaked in 0.0004, 0.04, or 4.0 mmol/L $Na_2S_2O_4$ in water for 30 s. Roots receiving the same treatment were grouped together and maintained at 20°C–25°C. The samples were collected from each group after 0, 3, 5, 7, and 9 days. The lifeless xylem, irrelevant to antioxidant system, was removed, and four samples of 0.5 g each were taken from each group and used to measure the activities of SOD, CAT, POD, and PPO. After removing the xylem, 0.1 g samples were used to analyze the expression of the phenylalanine ammonia lyase (PAL), UDP-glucuronate baicalein 7-O-glucuronosyltransferase (UBGAT), and β-glucuronidase (GUS) genes. The samples were stored at −80°C, and then, an additional 10 g of sample was dried at 55°C for 3 days and sonicated to measure the baicalin, baicalein, wogonoside, and wogonin contents. All tests were repeated three times.

Determination of hydrogen peroxide content

H_2O_2 was determined using a plant H_2O_2 ELISA kit that was purchased from Shanghai Yu Ping Biotechnology Limited Company.

Determination of enzyme activity

SOD activity (U) was measured using the nitroblue tetrazolium photoreduction method. The amount required for 50% inhibition of photoreduction was defined as one unit of enzyme activity.[26] CAT activity was measured using ultraviolet (UV) spectrophotometry, and one unit of enzyme activity was defined as a reduction of 0.1 at A_{240} per min caused by 1.0 g of sample at 25°C.[27] POD activity was determined using the guaiacol approach, and one unit of activity was defined as a change of 0.01 at A_{470} caused by 1.0 g of the sample within 1 min.[28] PPO activity was estimated according to the method of Ohkawa *et al.*[29]

A spectrophotometric approach was applied to measure the activity of PAL. A total of 1.0 g of fresh root with the xylem removed was ground in 10 ml of boric acid buffer (BAB) (0.1 mol/L, pH 8.8) in an ice bath. BAB contains 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA), 5% glycerol, and 5% polyvinylpyrrolidone. The stock solution was centrifuged at 10,000 rpm for 20 min at 4°C. Approximately 2 ml of BAB and 1 ml of 0.02 mol/L L-phenylalanine solution were added to each of three tubes. Subsequently, 1.0 ml of enzyme solution and 0.01 ml of H_2O were added to Tube 1; 1.0 ml of enzyme solution and 0.01 ml of 0.4 mol/L $Na_2S_2O_4$ were added to Tube 2; and 1.0 ml of H_2O was added to Tube 3 as a control. The reaction was performed in a water bath at a

constant temperature of 30°C. After 1 h, 0.2 ml of HCl (6 mol/L) was used to terminate the reaction. The obtained solutions were centrifuged at 13,000 rpm for 15 min. The absorbance at 290 nm was determined using UV spectrophotometry. One unit was defined as the amount of enzyme causing a change of 0.01 at 290 nm in 1 h.

High-performance liquid chromatography (HPLC) was used to analyze the effects of GUS and UBGAT. A total of 4.0 g of fresh roots was ground in 40 ml of phosphate buffer (0.2 mol/L, pH 6.8) containing 0.1% Vc, 0.02 mol/L catechol, 1.0 mmol/L EDTA, 1.0 mmol/L phenylmethylsulfonyl fluoride, and 0.6% polyvinylpyrrolidone. The resulting solution was centrifuged at 10,000 rpm for 20 min at 4°C. A total of 1.0 ml of the obtained supernatant was added to two tubes. Subsequently, 0.01 ml of H_2O, 0.01 ml of 0.02 mol/L H_2O_2, and 0.01 ml of 0.4 mol/L $Na_2S_2O_4$ were added into the three tubes, respectively. 1.0 ml aliquot of the supernatant and 0.01 ml of H_2O were added to a fourth tube as a control. The tubes were incubated in a water bath at 30°C for 1 and 3 h. The reaction was terminated by boiling at 100°C for 15 min. The samples were dried under nitrogen, resuspended in 12 ml of methanol solution, and centrifuged at 13,000 rpm for 15 min. The enzyme activity was determined based on the peak area of baicalin.

Analysis of phenylalanine ammonia lyase, UBGAT, and β-glucuronidase expression

Target RNAs were extracted using a plant polyphenol polysaccharide kit (ABigen Corporation, Beijing, China). Reverse transcription was performed using a HiFi-MMLV cDNA First-Strand Synthesis Kit (GeneCopoeia Inc., MD, USA). Real-time polymerase chain reaction (PCR) was performed to detect the gene expression. The target PAL fragment of *S. baicalensis* was 139 bp in length. The forward and reverse primers were 5'-TGACCTCGTGCCCCTG TCCTAC-3' and 5'-CAGCTCGAAGACCCTCCACTAACT-3', respectively. The 175-bp PCR fragment of the UBGAT gene was amplified using the primers: 5'-AGCCAA GGAAGCCATAGTCAACG-3' (forward) and 5'-CCCGAAACAAAGGAAGACGACA-3' (reverse). The 131-bp PCR fragment of the GUS gene was amplified using the primers: 5'-CAAATACTTTCATCAATGGTTTCTGGT-3' (forward) and 5'-AATGTA GTGCCG GTTTGGAGTA G-3' (reverse). The 138-bp actin fragment was amplified using the primers: 5'-TCGACTACGAGCAAGAGCTAGAAACA-3' (forward) and 5'-TCATTG ATGGCTGGAAGAGGACC-3' (reverse). The reaction system consisted of 10 μl of 2×Ultra SYBR Mixture (Kang Century Biotechnology Co. Ltd., Beijing, China), 0.4 μl (10 μM) of forward primer, 0.4 μl (10 μM) of reverse primer, 2 μl of template, and 6.8 μl of dH_2O. The real-time PCR program was initiated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. An ABI 7500 system (Applied Biosystems, CA, United States) was used for real-time PCR, and the $2^{-\Delta\Delta ct}$ method was used for data analysis.

Determination of baicalin, baicalein, wogonoside, and wogonin

The experimental samples were analyzed using a Waters ACQUITY HPLC. The trial samples were analyzed on a BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm). The mobile phases consisted of (A) acetonitrile with 0.1% formic acid and (B) H_2O with 0.1% formic acid. A gradient program was run at an initial concentration of 25% A for 5–15 min, 25% A→54% A between 15 and 22 min, maintained at 54% A from 22 to 23 min, 54% A→25% A for 23–25 min, and then maintained at 25% A. The flow rate was set at 1 ml/min, and the column temperature was 30°C. The detected wavelengths for baicalin, wogonoside, baicalein, and wogonin were 277, 279, 274, and 275 nm, respectively.

A total of 0.25 g of root powder (d <0.1 mm) was placed in a 25-ml volumetric flask, and 70% methanol was added to extract the total compounds under ultrasonic conditions for 30 min. The supernatant was filtered through a 0.22-μm microporous filter for ultra-performance liquid chromatography analysis.

Statistical analysis

All the experimental data were analyzed using Excel (Microsoft Corp) and expressed as the means ± standard mean of the error. Two-way ANOVA followed by *t*-test was done for statistical analysis, and the results were considered significant when $P < 0.05$.

RESULTS

Sodium hydrosulfite caused hydrogen peroxide fluctuations

Although the H_2O_2 level in the control group showed limited changes, the H_2O_2 levels in all $Na_2S_2O_4$ groups increased at day 1, decreased at day 3, and then increased gradually to levels close to that at day 1. The 0.0004 mmol/L and 0.04 mmol/L $Na_2S_2O_4$ groups returned to normality, whereas the 4 mmol/L $Na_2S_2O_4$ did not [Figure 2]. Large fluctuations were observed before day 3.

Sodium hydrosulfite induces differential changes in superoxide dismutase, catalase, POD, and polyphenol oxidase activity

$Na_2S_2O_4$ reduced the activity of SOD in the fresh roots of *S. baicalensis*. The reduction of CAT and POD activities began on day 3 and continued until day 9. CAT showed a rapid decrease, and its activity was reduced by 80%–85% after 3–5 days. $Na_2S_2O_4$ reduced PPO activity before the 3rd day, and this effect was significantly different after 5 days. This change was closely associated with the applied doses of $Na_2S_2O_4$. $Na_2S_2O_4$ concentrations of 0.0004 and 0.04 mmol/L increased PPO activity up to 60%–80%, whereas 4.0 mmol/L $Na_2S_2O_4$ generated a 60% reduction in enzyme activity [Figure 3]. The rapid change in SOD, CAT, and POD activities was synchronous and preceded the PPO activity.

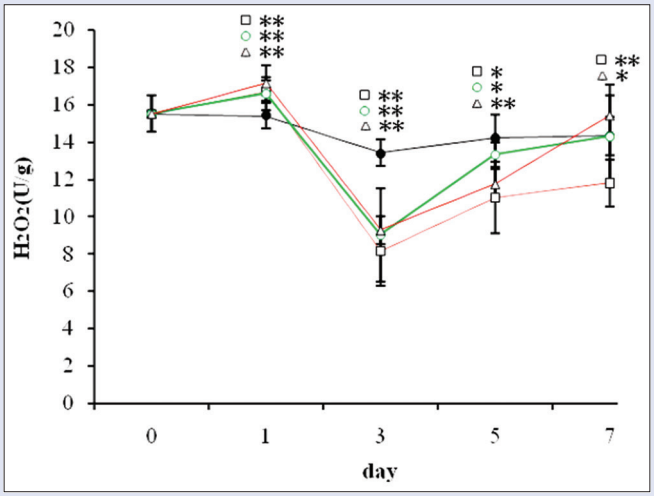

Figure 2: H_2O_2 levels in fresh roots treated with $Na_2S_2O_4$. The control group hardly changed but all $Na_2S_2O_4$ increased at day 1, dropped dramatically at day 3 and soon increased gradually to recover ground that was lost. $P < 0.05$ vs. day 0 was marked with "", $P < 0.01$ vs. day 0 with ""

Sodium hydrosulfite significantly modulated phenylalanine ammonia lyase, UBGAT, and β-glucuronidase expression

For enzymes associated with flavonoids, $Na_2S_2O_4$ did not increase PAL activity. The increased aglucons content was attributed to UBGAT and GUS. Compared with the inactivated enzyme group, the control, H_2O_2, and $Na_2S_2O_4$ groups had significantly lower baicalin content. The lower concentration of baicalin suggested that additional glycoside was converted into aglucon. Compared with the control, both the H_2O_2 and $Na_2S_2O_4$ groups decreased baicalin content within 1 h. However, H_2O_2 had an obvious effect after 1 h, whereas $Na_2S_2O_4$ had a dramatic effect only after 3 h [Figure 4]. Thus, $Na_2S_2O_4$ acted more slowly than H_2O_2.

The expression of genes encoding flavonoid-related enzymes was detected, and 3 days posttreatment, $Na_2S_2O_4$ was observed to slightly enhance PAL gene expression and significantly increase UBGAT expression in the 0.0004 mmol/L and 0.04 mmol/L treatments. At a concentration of 4.0 mmol/L, $Na_2S_2O_4$ augmented the expression levels of PAL and UBGAT. In addition, GUS expression was enhanced only at a dosage of 0.04 mmol/L [Figure 5].

Sodium hydrosulfite exerts an obvious influence on the biosynthesis of baicalin, baicalein, wogonoside, and wogonin

The most dramatic changes occurred 3 days after treatment. The $Na_2S_2O_4$ concentrations of 0.0004 and 0.04 mmol/L increased the flavonoid glycoside content to a certain extent. The baicalin content increased from 6.28% to 6.84% (from 1.50 to 1.53 mmol/g), and the wogonoside content increased rapidly from 3.35% to 4.12% (from 0.63 to 0.90 mmol/g). In contrast, the 4.0 mmol/L $Na_2S_2O_4$ treatment decreased the glycoside content and significantly increased the aglycone content. The baicalin and wogonoside contents decreased from 6.28% to 5.21% (from 1.50 to 1.24 mmol/g) and 3.35% to 2.83% (from 0.63 to 0.61 mmol/g), respectively. However, the baicalein and wogonin contents increased from 0.28% to 1.96% (from 0.08 to 0.72 mmol/g) and from 0.14% to 1.24% (from 0.04 to 0.22 mmol/g), respectively, which represented greater than 7-fold increases in baicalein and wogonin. The increased amount of aglucons, such as baicalein and wogonin, were well above the homologous glycoside contents. The change in baicalein and wogonin content was consistent with the different concentrations of $Na_2S_2O_4$. Overall, the different concentrations of $Na_2S_2O_4$ increased the total flavonoid content over varying periods. After 3 days, the content increased from 10.05% to 10.36% (from 2.25 to 2.31 mmol/g) at a $Na_2S_2O_4$ dose of 0.0004 mmol/L, to 11.46% (2.57 mmol/g) at a dose of 0.04 mmol/L, and to 11.95% (2.72 mmol/g) at a dose of 4 mmol/L [Table 1 and Figure 6].

DISCUSSION

Sodium hydrosulfite caused hydrogen peroxide fluctuations and decreased antioxidant enzymes

$Na_2S_2O_4$ can generate $O_2^{\bullet-}$,[25] and $O_2^{\bullet-}$ in fresh roots can be converted into H_2O_2 by SOD; therefore, H_2O_2 can be used as an indicator to estimate ROS levels. At day 1, the increased H_2O_2 content was caused by SOD. At day 3, the H_2O_2 contents dramatically decreased and then gradually increased [Figure 2], indicating that *S. baicalensis* possesses strong antioxidant activity, which occurs via the mediation of antioxidant enzymes, such as CAT and POD, or secondary metabolites.

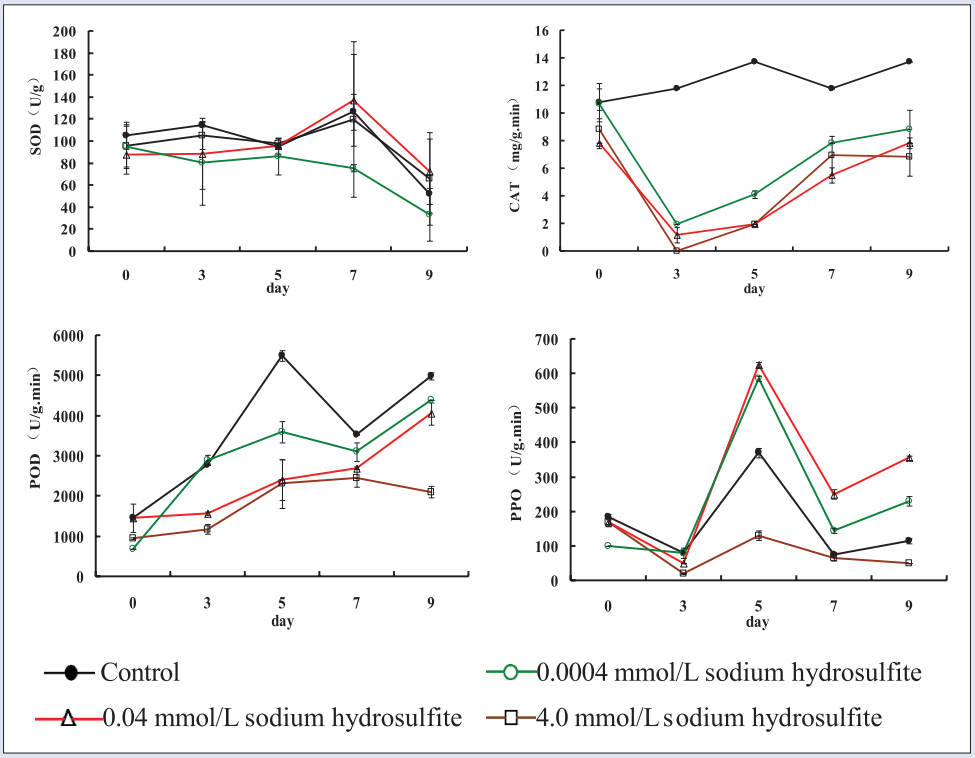

Figure 3: Effect of sodium hydrosulfite on the antioxidative enzyme system. Sodium hydrosulfite was bound to leading to increased reactive oxygen species. With reactive oxygen species, the superoxide dismutase, catalase, and POD activities suffered, indicating that under stress situation, antioxidative enzyme system fails to quench directly excessive reactive oxygen species in the plant cell. The activities of polyphenol oxidase were derived up at 0.0004 and 0.04 mmol/L sodium hydrosulfite, down at the 4.0 mmol/L, showing that flavones may take important role in keeping reactive oxygen species constant under heavy stress situation. The performance was repeated three times

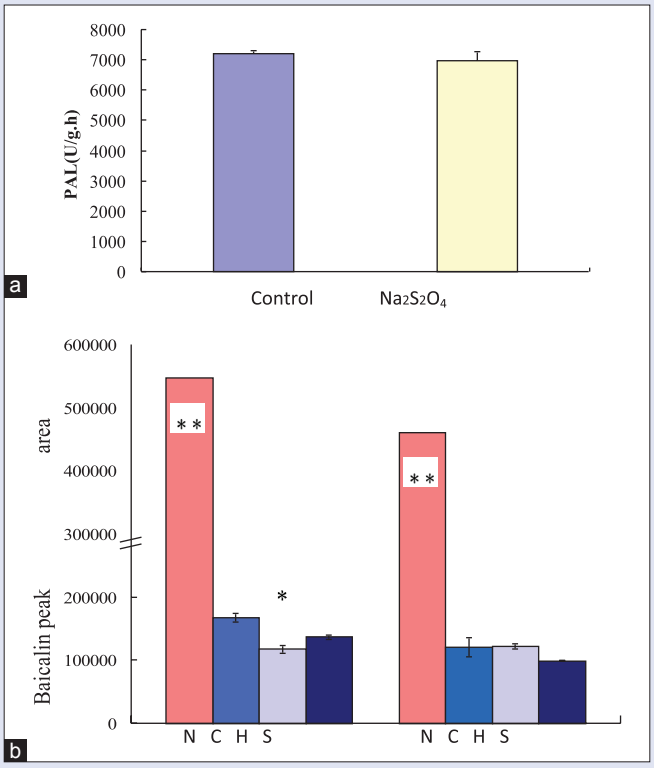

Figure 4: (a) Effect of sodium hydrosulfite on the phenylalanine ammonia lyase activity. sodium hydrosulfite show no signs of increasing the activity of phenylalanine ammonia lyase. (b) Integrate effect of hydrogen peroxide on UBGAT and β-glucuronidase activity. "N," "C," "H," and "S" stands for denatured enzyme, control with normal enzyme, treated with hydrogen peroxide, and treated with sodium hydrosulfite, respectively. Compared with denatured enzymes, the baicalin contents in the other groups decreased remarkable. Compared with control group, the baicalin contents in hydrogen peroxide group decreased remarkable at the 1 h, but the sodium hydrosulfite group decreased to some extent at the 3rd h, indicating sodium hydrosulfite acts through hydrogen peroxide. $P < 0.05$ marked by "*," $P < 0.01$ by "**" Control 0.0004 mmol/L sodium hydrosulfite 0.04 mmol/L sodium hydrosulfite 4.0 mmol/L sodium hydrosulfite

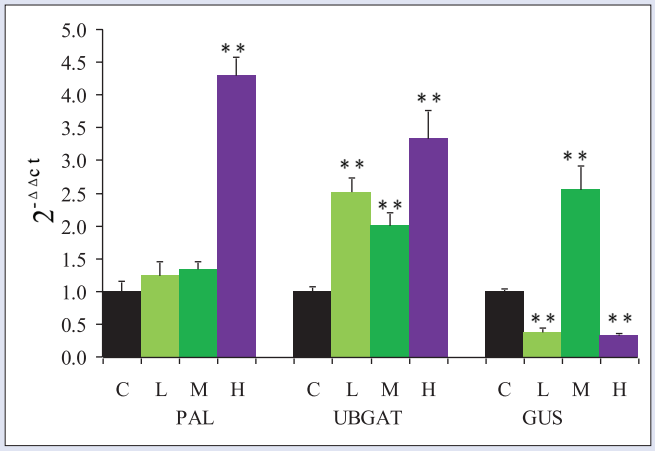

Figure 5: Effect of sodium hydrosulfite on the phenylalanine ammonia lyase, UBGAT, and β-glucuronidase gene expression. "C," "L," "M," and "L" stands for control, 0.0004, 0.04, and 4.0 mmol/L sodium hydrosulfite, respectively. Ruling the $2^{-\Delta\Delta ct}$ of the control of every gene as 1.0. Compared with the control, the 0.0004 and 0.04 mmol/L sodium hydrosulfite made the phenylalanine ammonia lyase increase slightly, UBGAT heavily, during the 3–5 day. The 4.0 mmol/L sodium hydrosulfite promoted the phenylalanine ammonia lyase and UBGAT heavily. At the same time, the GUS had overexpressed only at the 0.04 mmol/L. $P < 0.05$ marked by "*," $P < 0.01$ by "**" Control 0.0004mmol/L sodium hydrosulfite 0.04mmol/L sodium hydrosulfite 4.0 mmol/L sodium hydrosulfite

The activities of SOD, CAT, and POD are closely associated with ROS levels. $Na_2S_2O_4$ reduces SOD, CAT, and POD activity. CAT activity was reduced by 80%–85% within three days. Here, a consequence of the 4 mmol/L $Na_2S_2O_4$ with the highest content was production of the far more ROS, resulting in decreased the activities of CAT, POD, and PPO more seriously. APX activity may also be significantly decreased under drought stress conditions.[30] CAT, POD, and APX all catalyze the decomposition of H_2O_2 into H_2O and oxygen and decreases in the activities of these enzymes cause H_2O_2 to accumulate. In addition, $Na_2S_2O_4$ rapidly increases H_2O_2 contents. Among all ROS molecules, H_2O_2 has the lowest activity and is the least harmful to plants,[27] and it can persist for extended periods and is the only ROS molecule that can diffuse over long distances in cells.[31,32] $O_2^{\bullet-}$ is dismutated to H_2O_2 either nonenzymatically or by SOD activity and then converted to H_2O, suggesting that H_2O_2 may play an important role in regulating physiological functions.

Sodium hydrosulfite promotes the transformation of glycosides into aglycones

UBGAT transforms the redundant and initially synthesized flavonoid aglycones into glycosides, and GUS converts baicalin into aglycones under stress. The flavonoid changes represent the comprehensive effects of both UBGAT and GUS. The detection of only one of these enzymes does not indicate the balance between flavonoid glycosides and aglycones. In the present study, we used the baicalin content as an index to investigate the effects of H_2O_2 and $Na_2S_2O_4$ on UBGAT and GUS. The changes in baicalin content reflect how $Na_2S_2O_4$ affects physiological metabolism and adaptation. The inactivated enzyme group showed normal baicalin content in cells, which was likely associated with the cell chamber effect. Moreover, the inactivated enzyme group had higher baicalin content than the control, H_2O_2, and $Na_2S_2O_4$ groups, which might reflect the effect of enzymes. ROS can alter enzyme structures,[33] thereby influencing enzyme activity. One hour after adding ROS to the reaction system, the $Na_2S_2O_4$ group showed decreased baicalin content although the content of this compound was still higher than that in the H_2O_2 group. After 3 h, the $Na_2S_2O_4$ group had the lowest content [Figure 4], suggesting that $Na_2S_2O_4$ generates $O_2^{\bullet-}$, which is then converted into H_2O_2 by SOD. The structural modification of enzymes enhances GUS activity, decreases UBGAT activity, or both. These changes promote the transformation of flavonoid glycosides into aglycones and improve the capacity of scavenging ROS. Scutellaria glycosides and aglycones act as a buffer pair in solution to maintain the global oxidation of ROS balance. This balance does not involve any other processes and can rapidly react to environmental changes.

Sodium hydrosulfite inhibits the antioxidant enzyme system by enhancing flavonoid biosynthesis via the accumulation of hydrogen peroxide

A messenger substance rapidly responds to environmental changes.[34] H_2O_2 is a primary messenger among ROS that dramatically alters metabolism over a short period. This molecule can even induce the expression of more than 30 proteins within 30 min.[35] PAL is an enzyme that catalyzes the synthesis of flavonoids. The concentration of

Table 1: Effect of different sodium hydrosulfite on the flavones (mmol/g)

$Na_2S_2O_4$	Day	Baicalin	Wogonoside	Baicalein	Wogonin	Total flavones
Control	0	1.50	0.63	0.08	0.04	2.25
	3	1.41	0.73	0.10	0.05	2.29
	5	1.59	0.80	0.05	0.03	2.47
	7	1.53	0.75	0.07	0.04	2.39
	9	1.59	0.80	0.06	0.04	2.49
0.0004 mmol/L	0	1.50	0.63	0.08	0.04	2.25
	3	1.44	0.73	0.10	0.04	2.31
	5	1.73	0.91	0.10	0.04	2.78
	7	1.69	0.87	0.10	0.04	2.70
	9	1.63	0.80	0.13	0.05	2.61
0.04 mmol/L	0	1.50	0.63	0.08	0.04	2.25
	3	1.53	0.90	0.09	0.05	2.57
	5	1.61	0.85	0.10	0.05	2.61
	7	1.50	0.81	0.10	0.04	2.45
	9	1.52	0.78	0.10	0.05	2.45
4.0 mmol/L	0	1.50	0.63	0.08	0.04	2.25
	3	1.17	0.61	0.72	0.22	2.72
	5	1.44	0.73	0.46	0.15	2.78
	7	1.26	0.68	0.68	0.2	2.82
	9	1.24	0.62	0.84	0.23	2.93

$Na_2S_2O_4$: Sodium hydrosulfite

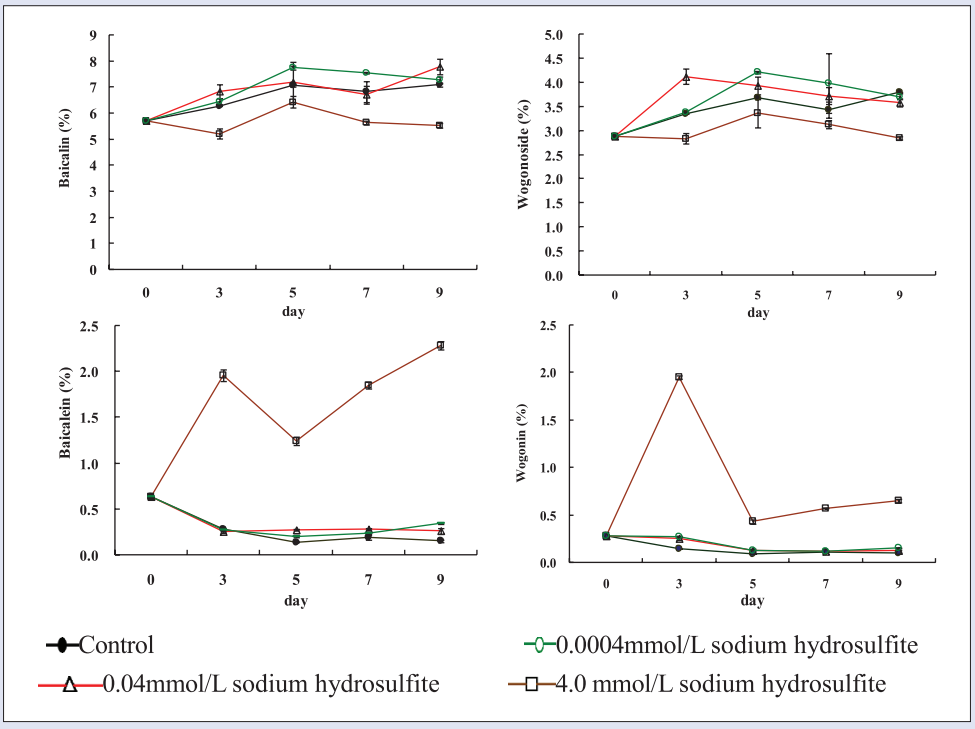

Figure 6: Effect of sodium hydrosulfite on the contents of four flavones. The contents of both baicalin and wogonoside in roots of *Scutellaria baicalensis* increased soon after treatment with 0.0004 and 0.04 mmol/L sodium hydrosulfite, but the contents of both baicalein and wogonin failed to decrease, indicating that under slightly stress, flavonoid glycosides was the basic and effective tool. At 4.0 mmol/L sodium hydrosulfite, the contents of both baicalin and wogonoside decreased, while the contents of both baicalein and wogonin increased insignificantly, showing that the aglucons had higher activity. Data were based on three times repeat

0.0004 and 0.04 mmol/L $Na_2S_2O_4$ enhanced PAL gene expression and flavonoid glycoside content [Table 1 and Figure 4], and this increase in glycoside content was greater than that of aglycone after 3 days. This result might reflect the fact that a low concentration of baicalin is sufficient to eliminate excess ROS. Hence, excess glycosides are not necessary. After treatment with 4.0 mmol/L $Na_2S_2O_4$ for 3 days, the total flavonoid content was significantly augmented. The baicalein content increased from 0.08 to 0.72 mmol/g, and the wogonin content increased from 0.04 to 0.22 mmol/g, which represented an approximately 7-fold increase. This result might reflect the fact that the synthesis of flavonoid aglycones occurs earlier than the synthesis of flavonoid glycosides and the overexpression of PAL promotes aglycone accumulation [Figures 1 and 6]. The baicalin and wogonoside content was noticeably reduced, which indicates the conversion of large amounts of flavonoids

into quinones by excess ROS.[36] When highly expressed, UBGAT can convert excess baicalein and wogonin into low-activity baicalin and wogonoside [Figure 5]. However, the observed production of UBGAT was not sufficient to compensate for its consumption; consequently, the content of baicalin and wogonoside decreased from 1.50 to 1.17 mmol/g and from 0.63 to 0.61 mmol/g, respectively. Under ROS stress, reduced GUS expression promotes the accumulation of highly active aglycones although the synthesis of aglycones, such as baicalein, occurs earlier than the synthesis of glycosides. At this point, the aglycone content is already high, and the transformation of aglycones via GUS is unnecessary. Therefore, reducing GUS expression under stress is a favorable mechanism [Figure 5]. In normal cells, flavonoids are stored in the form of glycosides.[37] In response to ROS stress, flavonoid glycosides are rapidly converted into aglycones,[38] and this conversion may represent a stress-response strategy in *S. baicalensis*.

At 0.04 mmol/L, $Na_2S_2O_4$ clearly increased GUS activity on day 3, which is consistent with the change in flavonoids after 3–5 days [Figure 6]. However, the slightly increased PAL expression in response to 0.0004 and 0.04 mmol/L $Na_2S_2O_4$ and greatly enhanced GUS expression in response to 0.04 mmol/L $Na_2S_2O_4$ were not significantly positively correlated with baicalein, baicalin, wogonin, or wogonoside, which may be explained by multiple factors. Transcription, translation, and postmodification delay the increase of flavonoids, and flavonoid contents are continuously reduced by ROS. However, the most important reason is that the sudden artificial increase in ROS disrupts the natural metabolism of *S. baicalensis* and causes an unnatural status that is difficult to quickly restore. Overall, $Na_2S_2O_4$ enhances the biosynthesis of flavonoids, and the flavonoid content reached a peak at day 3. Although baicalin and wogonoside decreased from 6.28% to 5.21% and 3.35% to 2.83%, respectively, baicalein and wogonin increased from 0.28% to 1.96% and 0.14% to 1.24%, respectively. The increased flavonoid contents broke down the redundant ROS, which led to the lowest H_2O_2 levels [Figure 3].

Polyphenol oxidase maintains the biosynthesis of flavonoids

Many studies have shown that the induction of phenolic secondary metabolites reflects a plant response to environmental stress.[18,39] PPO activity was minimally altered after treatment with $Na_2S_2O_4$ for 3 days. A dramatic change occurred after 5 days, which was later than the changes in antioxidant enzymes and flavonoids. Different doses of $Na_2S_2O_4$ had varying effects on PPO activity. The antioxidant capacity of enzymes was replaced by flavonoids in the fresh roots treated with $Na_2S_2O_4$. The sudden addition of $Na_2S_2O_4$, even at low doses of 0.0004 or 0.04 mmol/L, induced excess flavonoids, and the PPO activity increased by 60%–80% to scavenge redundant flavonoids and prevent further reductions of ROS. The high dose of $Na_2S_2O_4$ (4.0 mmol/L) generated large amounts of ROS, and many flavonoids were required to maintain this balance. As a result, PPO activity was reduced by 60% [Figure 3].

CONCLUSION

ROS are produced in plants under stress. $Na_2S_2O_4$ can act as an unfavorable factor that generates $O_2^{\bullet-}$, which is subsequently converted into H_2O_2, $^{\bullet}OH$, and other ROS. Based on this theory, organisms treated with $Na_2S_2O_4$ can be used as models for studying environmental stress. These organisms can produce secondary metabolites that are the same as or similar to those generated under natural conditions. $Na_2S_2O_4$ has strong reducing abilities and transforms into nontoxic $NaHSO_4$; thus, $Na_2S_2O_4$ is widely applied as a tap water dechlorinating agent, a beer bottle disinfectant, a bleaching agent, and a food leavening agent. In recent years, the role of antioxidases under ecological stress has been emphasized, whereas the roles of nonenzymatic components have been undervalued. Reports on the dominant roles of flavonoids in response to ecologic stresses are rare. In the present study, we revealed that secondary metabolites play far greater role, while the antioxidase declines, in response to $Na_2S_2O_4$ stimuli. $Na_2S_2O_4$ significantly enhances the quality of *Radix Scutellariae*.

Acknowledgement

We wish to thank for providing financial support from the National Science Foundation (81573523) and Heilongjiang University of Chinese Medicine for innovative talents project.

Financial support and sponsorship

We wish to thank for providing financial support from the National Science Foundation (81573523).

Conflicts of interest

There are no conflicts of interest.

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