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ORIGINAL ARTICLE

Sagittaria sagittifolia Polysaccharide, a Chinese Herbal Extract, Protects against Isoniazid- and Rifampicin-induced Hepatotoxicity in *in-vitro* Model

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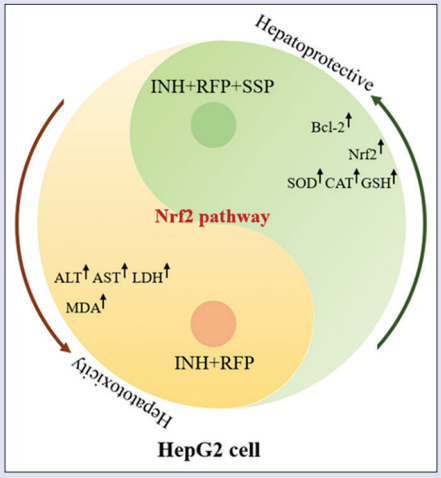
ABSTRACT

Background: Isoniazid (INH) and rifampicin (RFP) are first-line antituberculosis drugs; however, one of the most common adverse effects of their use is hepatotoxicity. *Sagittaria sagittifolia* polysaccharide (SSP) is the primary component of *S. sagittifolia*. Our previous research has confirmed the protective effect of SSP against INH and RFP-induced hepatic injury in *in vivo* model system. **Objectives:** In this study, we aimed to investigate the protective effect and the underlying mechanisms of SSP in cultured HepG2 cells after induction with INH and RFP. **Materials and Methods:** The study was designed as follows: normal (without any treatment), model (injury induced by INH + RFP), and SSP group (treated with SSP followed by INH + RFP). Cell viability, catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH), along with malondialdehyde (MDA), as well as alanine aminotransferase, aspartate transaminase, and lactate dehydrogenase, were assessed by corresponding kits. Real-time polymerase chain reaction and Western blot were used to evaluate the mRNA and protein expression of Bcl-2, Bax, Nrf2, and Keap1. **Results:** SSP had a potential protective effect as it increased the cell viability and reduced the intracellular levels of hepatic injury markers. SSP reduced the intracellular content of MDA and increased the activity of SOD, CAT, and GSH. It is noteworthy that the optimum protective effect was observed with a 0.5 mg/mL SSP at 24 h after treatment. In addition, the protection appears to be associated with its activation of nuclear factor E2-related factor 2 signaling pathway. **Conclusion:** SSP protected against INH + RFP-induced hepatotoxicity in HepG2 cells and this protection might via Nrf2 signaling pathway.

Key words: Hepatoprotective, HepG2 cell, isoniazid + rifampicin, Nrf2, *Sagittaria sagittifolia polysaccharides*

SUMMARY

- *Sagittaria sagittifolia* polysaccharide (SSP) protected against isoniazid + rifampicin-induced hepatotoxicity in HepG2 cells
- SSP's optimal concentration and treatment time were 0.5 mg/mL and 24 h, respectively
- SSP's protection might be via Nrf2 signaling pathway.



Abbreviations used: INH: Isoniazid; RFP: Rifampicin; SSP: *Sagittaria sagittifolia* polysaccharide; AST: Aspartate transaminase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; SOD: Superoxide dismutase; MDA: Malondialdehyde; CAT: Catalase; GSH: Glutathione; GCLC: Glutamate cysteine ligase catalytic; HO-1: Heme oxygenase 1.

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INTRODUCTION

Tuberculosis (TB) is an infectious respiratory disease and is considered to be a serious public health problem.[1] Isoniazid (INH) and rifampicin (RFP) are the most important first-line anti-TB drugs that are widely used in clinical setting.[2,3] However, these drugs are known to cause hepatotoxicity, which has been reported in about 5%–28% of the patients.[4-6] Although the possible reason for the hepatotoxicity has been attributed to the oxidative stress[7-9] and peroxidation of endogenous lipids,[10] the exact mechanism remains unclear.

In the past few decades, scientists have made tremendous progress in preventing hepatotoxicity during TB therapy, but there is still an urgent need to refine the novel drugs with a broad range of action.[11] With the gradual increase in research on Chinese medicines, more and more

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herbal products or extracts are being widely used in the treatment of hepatic disorders all over the world.[12,13]

Sagittaria sagittifolia, a perennial aquatic herbal plant of family *Alismataceae*, is grown in many areas in China; it is a medicinal and an edible plant.[14] In traditional Chinese medicine, *S. sagittifolia* is commonly used as a detoxifying agent and is used in the treatment of retention of the placenta, gonorrhea, sore, and hemoptysis.[15] A recent study demonstrated the antioxidative activity of *S. sagittifolia*.[16] Furthermore, it has been shown to play a potential protective role against cadmium- and CCl_4-induced hepatic injury.[17,18] In addition, our previous research has confirmed the protective effect of *S. sagittifolia* in INH + RFP-induced hepatotoxicity in both *in vitro* and *in vivo* model system.[19,20]

S. sagittifolia polysaccharide (SSP) is the primary component of *S. sagittifolia*. In our previous study,[21] we demonstrated the protective effect of SSP against the INH + RFP-induced hepatic injury using an *in vivo* model system. SSP markedly alleviated the hepatotoxicity. It not only downregulated the activities of aspartate transaminase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) in the serum but also upregulated the activities of antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT) in the liver. Probably, this protection may via Nrf2 signaling pathway.[21] In addition, our previous study[22] confirmed the role of SSP in protecting HepG2 cells by increasing the cell viability and reducing the levels of malondialdehyde (MDA), ALT, AST, and LDH. To extend our findings, as well as to verify our previous results, we explored the mechanism of action of SSP on INH + RFP-induced HepG2 cells. Therefore, in this study, we mainly focused on the time-dose-effect relationship of SSP on cell viability, hepatic injury markers, and antioxidant enzymes. Simultaneously, we aimed to find the optimal concentration and treatment time of SSP, as well as the effect of SSP on cell apoptosis and Nrf2 pathway *in vitro*.

MATERIALS AND METHODS

Materials and chemicals

SSP was isolated and purified from the tuber of *S. sagittifolia* obtained from Kunming City (Yunnan Province, China). *S. sagittifolia* was identified and authenticated by Dr. Xiuli Wang at Beijing University of Chinese Medicine, China, to be corms of *S. sagittifolia* L. in *Sagittaria* of *Alismataceae*. A voucher specimen (SS-201509-001) was deposited in the Department of Health Preservation and Rehabilitation for future reference. *S. sagittifolia* is an accepted name in the plant list (http://www.theplantlist.org; last accessed on December 25, 2017). INH, RFP, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM, high glucose), and culture supplements were purchased from Gibco-Invitrogen (Grand Island, NY, USA). AST, ALT, LDH, SOD, MDA, CAT, and glutathione (GSH) kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-Bax, anti-Bcl-2, anti-Nrf2, and anti-Keap1 were purchased from Abcam (Cambridge, MA, USA). Other chemicals were of analytical grade (Beijing HWRK Chemical Co., Ltd., Beijing, China).

Preparation of *Sagittaria sagittifolia* polysaccharide[21]

The tuber of *S. sagittifolia* were dried at 55°C and powdered. Next, 20 g of the powder was subjected to extraction thrice with 200 mL of boiling water. The polysaccharide in the filtrate was precipitated fractionally with alcohol. The protein in the product was removed as per the Sevag method and purified using diethylaminoethanol ion exchange cellulose (DEAE-52). The content of polysaccharide was 34.31% as determined by the phenol-sulfuric acid method.

Cell lines and cell culture conditions

In this study, we used HepG2 cells, the human hepatoma cell line, as this cell line shows similar functions as normal human hepatocyte.[23] In addition, compared with primary human hepatocytes, HepG2 cells are relatively easy to handle and are frequently used in research on biotransformation of xenobiotics.[24-27] In this study, HepG2 cells were obtained from the Cell Resource Center of Peking Union (Beijing, China). The cells were cultured in DMEM (high glucose) containing 10% FBS and 1% antibiotics (50 U/mL of penicillin and 50 mg/mL streptomycin) in a humidified incubator with 5% CO_2 at 37°C.

Determination of cell viability and biomarkers

Cells were plated in 96-well plates and cultured in a humidified incubator with 5% CO_2 at 37°C for 24 h. Next, HepG2 cells were divided into the following groups: Normal (no treatment), model (cells were treated with INH + RFP for 12, 24, or 48 h), and SSP (cells were separately treated with SSP for 0.125, 0.25, 0.5, 1, and 2 mg/mL; after 2-h SSP treatment, cells were treated with INH + RFP for 12, 24, or 48 h). Cell viability was measured by conducting MTT assay following the instructions.[28] The levels of AST, ALT, and LDH in the supernatants were determined using the corresponding kits.

Evaluation of malondialdehyde, superoxide dismutase, catalase, and glutathione levels

Intracellular activities of the antioxidant enzymes including CAT, SOD, and GSH, along with MDA, the marker of the extent of lipid peroxidation, were determined using the corresponding kits according to the manufacturer's instructions. Briefly, HepG2 cells were seeded in 6-well plates for 24 h and then divided into the following groups: normal (no treatment), model (cells were treated with INH + RFP), and SSP (cells were separately treated with INH + RFP + 0.125, 0.25, 0.5, 1, or 2 mg/mL SSP) for 24 h, after which the levels of aforementioned biomarkers were determined.

RNA extraction and real-time polymerase chain reaction

Total RNA was isolated using TRIzol reagent (invitrogen) and transcribed into cDNA. Quantitative real-time polymerase chain reaction (RT-PCR) was performed to quantify the mRNA levels of Bcl-2, Bax, Nrf2, and Keap1 with gene-specific primers in a 25 μL reaction system. Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control. The reactions were performed using an Applied Biosystems 2720 Fast RT-PCR System (Applied Biosystems, Foster City, CA, USA). The following program was set at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s.

Western blot analysis

After treatment, cells were harvested and total protein contents were measured using Bicinchoninic Acid Protein Assay Kit according to the supplier's protocol. The samples were subjected to 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore Corporation, Billerica, MA, USA). After incubating with primary antibodies against Bcl-2, Bax, Nrf2, Keap1, and actin at 4°C overnight, the membranes were then incubated with horse radish

peroxidase-labeled secondary antibodies at room temperature for 40 min. Blots were visualized by ECL Select Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and scanned by Bio-Rad ChemiDoc™ XRS System (Bio-Rad Laboratories, Inc., Hercules, USA).

Statistical analysis

The values were presented as mean ± standard deviation. One-way analysis of variance was performed for intergroup comparisons using SPSS software (version 21; IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Sagittaria sagittifolia polysaccharide decreased isoniazid- + rifampicin-induced hepatotoxicity

Investigating the markers of hepatic injury can help us to assess the hepatotoxicity induced by INH + RFP, as well as the effect of SSP. MTT assay indicated that the cell viability decreased significantly after induction with INH + RFP compared to normal control [Figure 1a]. However, it is clear that SSP can reverse the effect, as SSP promoted the cell viability [Figure 1a] and decreased the levels of AST [Figure 1b], ALT [Figure 1c], and LDH [Figure 1d] in a dose-dependent manner. The protective effect of SSP was more optimal when the treatment time was 24 h and the concentration was > 0.5 mg/mL. In addition, when compared to 0.5 mg/mL SSP, the aforementioned markers did not show any statistically significant difference at 1 mg/mL and 2 mg/mL concentration.

Sagittaria sagittifolia polysaccharide increased superoxide dismutase, catalase, and glutathione while decreased malondialdehyde

As shown in Figure 2a, the expression level of MDA was significantly decreased by SSP after 24 h treatment ($P < 0.05$ when compared to the model cells). However, as shown in Figure 2b-d, the expression levels of SOD, CAT, and GSH were significantly increased when the cells were treated with SSP ($P < 0.05$ when compared to the model cells). Our results showed that SSP at a concentration of 0.5 mg/mL plays a significant protective role. After combining the results presented in Figures 1 and 2, we chose 0.5 mg/mL as our optimal concentration, which is both effective and does not cause any potential side effects.

Sagittaria sagittifolia polysaccharide raised Bcl-2 expression while reduced Bax expression

Further experiments were conducted using 0.5 mg/mL SSP as the optimal concentration. As shown in Figure 3, the expression of Bcl-2 at both the mRNA and protein level was significantly upregulated by SSP, whereas the expression of Bax was downregulated ($P < 0.05$ when compared to the model cells).

Sagittaria sagittifolia polysaccharide upregulated Nrf2 expression and downregulated Keap1 expression

Nrf2 mRNA expression level was determined by RT-PCR. As shown in Figure 4a, Nrf2 mRNA level was upregulated in INH + RFP-treated group (the model group) than that of the normal group ($P < 0.05$). Moreover, the level of SSP group was much higher than that of the model group ($P < 0.05$). Consistently, the protein level of Nrf2 increased in the cells treated with INH + RFP + SSP compared with cells treated with INH + RFP [Figure 4b and e] ($P < 0.05$).

Keap1 is a specific repressor of Nrf2. As shown in Figure 4c, compared to the INH + RFP-treated cells, mRNA expression of Keap1 in INH + RFP + SSP-treated cells showed a remarkable suppression. Consistently, the protein level of Keap1 decreased in the cells treated with INH + RFP + SSP compared with the cells treated with INH + RFP alone [Figure 4d and e] ($P < 0.05$).

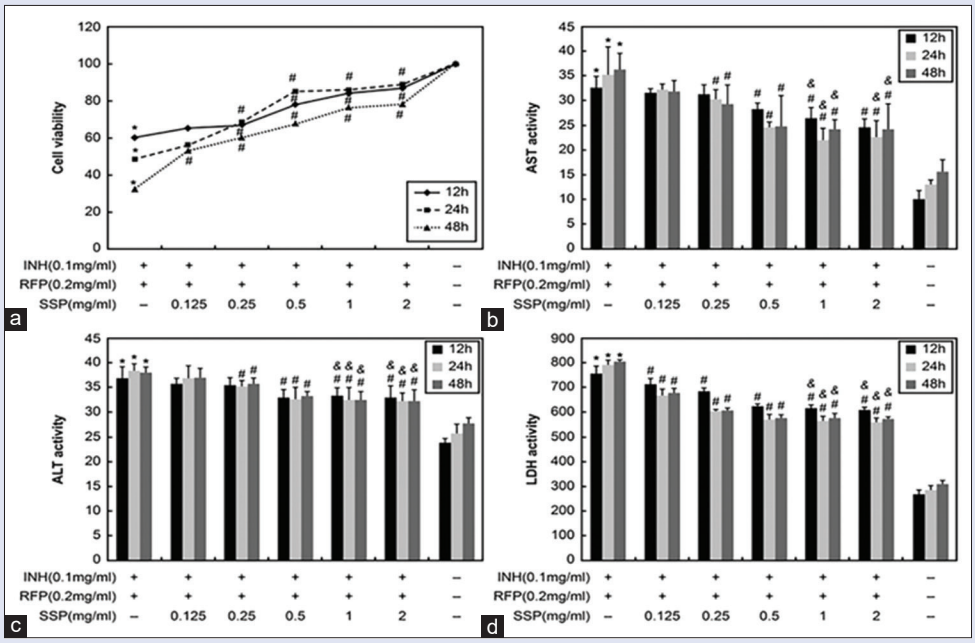

Figure 1: Effect of *Sagittaria sagittifolia* polysaccharide on Isoniazid + rifampicin-induced hepatotoxicity. (a) Effect of *Sagittaria sagittifolia* polysaccharide on cell viability. (b-d) Effect of *Sagittaria sagittifolia* polysaccharide on the activity of aspartate transaminase, alanine aminotransferase, and lactate dehydrogenase ($n = 6$). $^{*}P < 0.05$ compared to the normal group (no treatment), $^{\#}P < 0.05$ compared to the model group (isoniazid + rifampicin treated alone) and $P > 0.05$ compared to the *Sagittaria sagittifolia* polysaccharide 0.5 mg/ml group

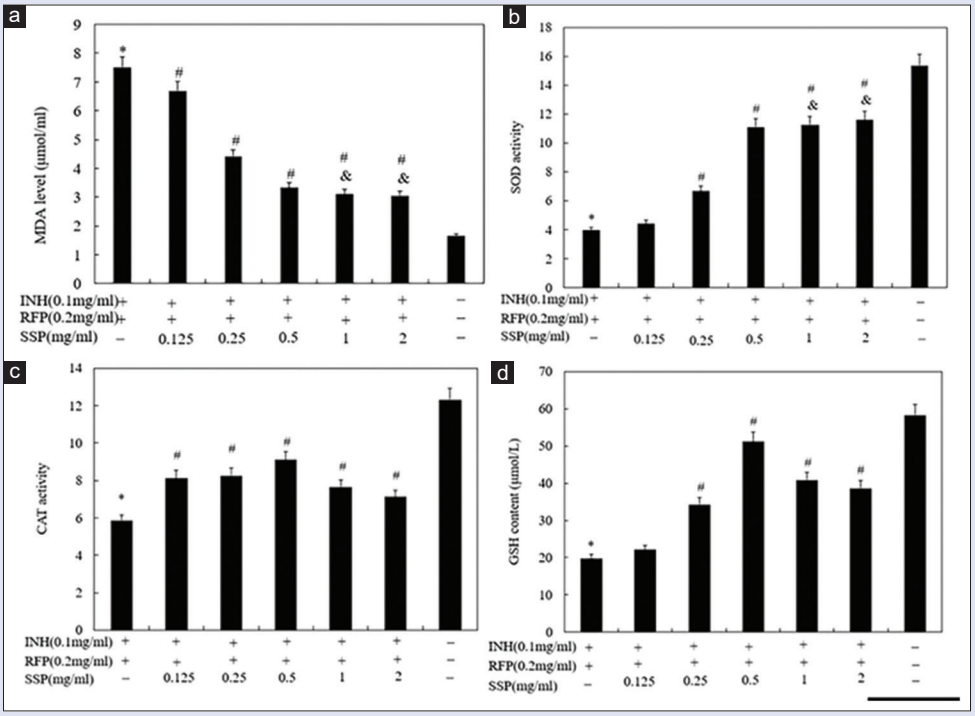

Figure 2: Effect of *Sagittaria sagittifolia* polysaccharide on levels of (a) malondialdehyde, (b) superoxide dismutase, (c) catalase, and (d) glutathione. **P* < 0.05 compared to the normal cells, #*P* < 0.05 compared to the model cells and *P* > 0.05 compared to the *Sagittaria sagittifolia* polysaccharide 0.5 mg/ml group

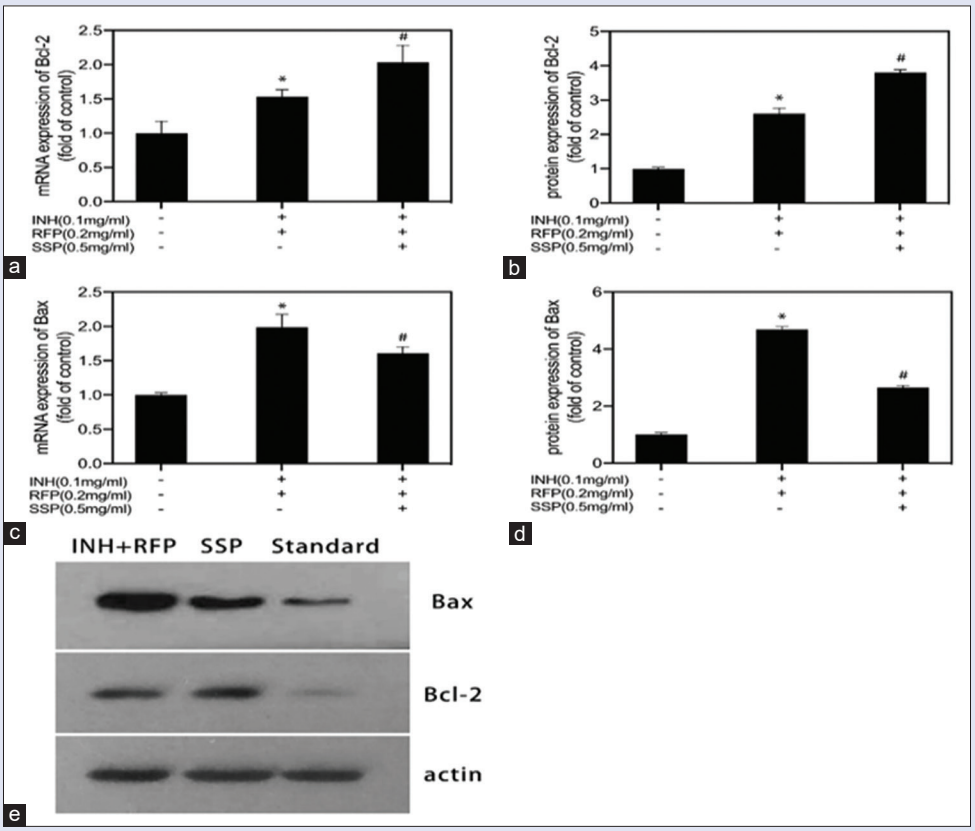

Figure 3: Effect of *Sagittaria sagittifolia* polysaccharide on levels of Bcl-2 (a, b and e) and Bax (c, d and e) (n = 3) **P* < 0.05 compared to the normal cells, #*P* < 0.05 compared to the model cells

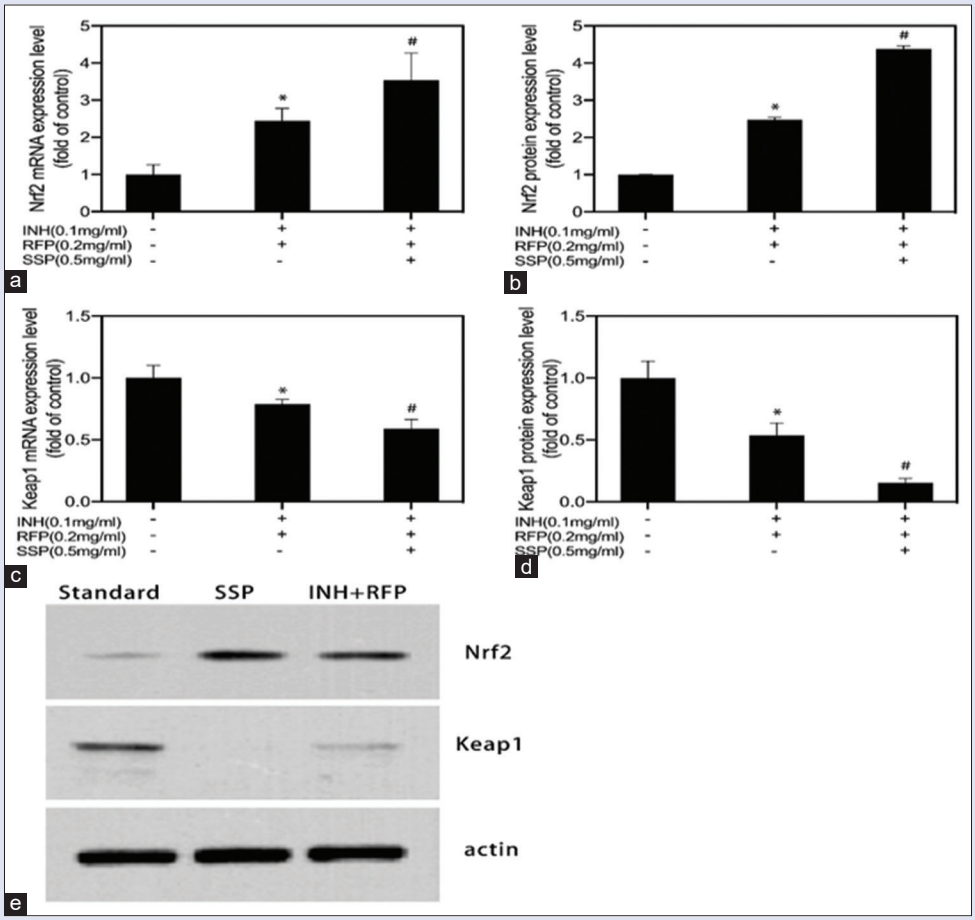

Figure 4: Effect of *Sagittaria sagittifolia* polysaccharide on the expression of Nrf2 and Keap1. (a and c) mRNA expression levels of Nrf2 and Keap1. (b, d and e) protein expression levels of Nrf2 and Keap1. ($n = 3$) $^{*}P < 0.05$ compared to the normal cells, $^{\#}P < 0.05$ compared to the isoniazid + rifampicin-treated cells

DISCUSSION AND CONCLUSION

Till date, INH and RFP are the first-line drugs in treating TB although severe hepatotoxicity could be induced.[10] Endogenous lipid peroxides are the major contributors of cytotoxicity of INH and RFP, whereas the activation of Nrf2-mediated endogenous antioxidant systems may reduce the damage of reactive metabolites.[11] Our results proved that SSP exerts hepatoprotective effect by mediating the antioxidant and anti-apoptotic activity, and this effect might be achieved via Nrf2 signaling pathway.

Our results demonstrated a significant increase in the levels of ALT, AST, and LDH after treating the cells with INH + RFP. However, this effect was suppressed by SSP at different concentrations, which indicates that SSP plays a hepatoprotective role. Moreover, SSP increased cell viability, which also confirmed its hepatoprotective effect. In addition, our results on the time-dose-effect relationship of SSP suggested that the optimum treating time was 24 h. As increased ALT, AST, and LDH as well as decreased cell viability are conventional indicators of hepatocellular necrosis,[29] our results indicated that SSP might have a beneficial effect in INH + RFP-induced hepatocyte necrosis.

As mentioned before, lipid peroxidation and free radicals are the primary causes of drug hepatotoxicity. In this study, our results confirmed the detrimental effect of INH and RFP. After treating with INH and RFP, the increased MDA and decreased SOD, GSH, and CAT activity indicated a disturbance in the homeostasis in cellular antioxidant system, which would eventually lead to irreversible damage including necrosis or apoptosis. SSP is effective in maintaining the homeostasis. After treating with SSP, the level of antioxidants was significantly increased and the level of MDA significantly decreased. SSP showed a significant protective effect at a concentration of 0.5 mg/mL. These results strongly confirmed SSP's protective effect in HepG2.

Both Bcl-2 and Bax belong to the Bcl-2 family. Bcl-2 and Bax are antiapoptotic and pro-antiapoptotic proteins.[30] In this study, we found that the Bcl-2 expression significantly reduced, whereas the Bax expression increased after INH + RFP treatment. SSP could reverse this adverse effect. After treating with SSP, the Bcl-2 level exhibited a significant increase and the Bax level showed an obvious decrease, indicating an antiapoptotic effect of SSP on INH + RFP-treated HepG2.

To clarify the mechanisms underlying the antioxidative and antiapoptotic activity of SSP, we investigated the Nrf2 signaling pathway. Under physiological conditions, Nrf2 is located in the cytoplasm and its activity is suppressed by the binding of Keap1.[31] When oxidative stress occurs, the cysteine residue of Keap1 is modified, and thus, Nrf2 could disassociate and translocate into the nucleus where it could induce the transcription of cytoprotective genes[32] that code for antioxidant enzymes (SOD and CAT), antioxidants (GSH), and Phase II detoxification enzymes, among which heme oxygenase-1 (HO-1) and glutamate-Lcysteine ligase catalytic (GCLC) subunit are the most important.[33,34] As a protector of various stimuli, HO-1 is proved to have hepatoprotective effect against apoptosis, while GCLC shows

the ability to lessen cellular damage.[35] In addition, Nrf2 signaling pathway is also responsible for anti-apoptotic effect, as some researchers have confirmed that activating Nrf2 signaling pathway can increase the expression of Bcl-2 and decrease the expression of Bax.[36] In this study, we obtained similar results. SSP increased Nrf2 and suppressed Keap1 gene and protein expression. Considering the higher activity of SOD and CAT, increased GSH and decreased MDA, as well as increased Bcl-2 and decreased Bax expression levels, all the aforementioned findings indicated that Nrf2 signaling pathway was upregulated. This might be the main factor in SSP-mediated antioxidative and antiapoptotic activity in HepG2. When we combine the results of this study with those of our previous study, the role of SSP in INH + RFP-induced hepatotoxicity at least partially via Nrf2 signaling pathway is unequivocal.

In summary, this study showed that SSP protects against INH + RFP-induced hepatotoxicity in HepG2 cells and this protection might be via Nrf2 signaling pathway. Our results suggested a new strategy to prevent or alleviate INH + RFP-induced hepatotoxicity, thus providing a potential alternative to clinical treatment in future. Further studies should validate the role of Nrf2 signaling pathway by studying the effects of SSP on hepatic injury in mice lacking Nrf2.

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Conflicts of interest

There are no conflicts of interest.

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