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Extracts from *sojae semen germinatum* ameliorated carbon tetrachloride-induced liver injury in mice

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Abstract

Sojae semen germinatum (SSG) prepared from the germinating black soybean *Glycine max L. Merr.* is a traditional Chinese herbal medicine with heat-clearing, dampness-removing, and detoxifying properties. In the present study, we explored the effects and possible underlying mechanisms of petroleum ether extract (PEE), ethyl acetate extract (EAE), n-butanol extract (NBE) and aqueous fraction (AF) of SSG on the carbon tetrachloride (CCl_4)-induced liver injury in mice, an animal model representing pathological features of "dampness-heat" syndrome from the perspective of Traditional Chinese Medicine. Results showed that the EAE and NBE of SSG significantly protected mice against the CCl_4-induced increases of liver tissue index and serum transaminase levels, ameliorated the CCl_4-induced liver morphological changes. Compared with CCl_4 model controls, EAE and NBE of SSG decreased malondiadehyde (MDA) levels, enhanced glutathione (GSH) content and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in liver tissues, as well as reduced the number of apoptotic hepatocytes. Moreover, the CCl_4-induced increase of pro-inflammatory cytokine levels in livers was markedly suppressed by pre-treatment with EAE of SSG. Data suggested that SSG extracts showed protective effect on the CCl_4-induced liver injury *via* multiple pharmacological mechanisms including anti-oxidation, anti-inflammation and anti-apoptosis. EAE and NBE appeared to be superior to other fractions.

Keywords: *Sojae semen germinatum*; extracts; liver injury; carbon tetrachloride.

Practical Application: This study provided experimental data for medicinal and food uses of *Sojae semen germinatum* for the liver disorder patients with "dampness- heat" syndrome from perspective of Traditional Chinese Medicine.

1 Introduction

As a traditional Chinese herbal medicine prepared from the germinating seeds of black soybean *Glycine max L. Merr. (Leguminosae)*, *sojae semen germinatum* (SSG) has been used in Traditional Chinese Medicine (TCM) clinical practices for thousands of years based on its heat-clearing, dampness-removing, and detoxifying properties (Wang et al., 2020; He & Wang, 2013; Fan et al., 2016). Up to date, SSG has been officially included in the National Pharmacopoeia of People's Republic of China. According to the processing method described in the Chinese Pharmacopoeia, after the mature seeds of black soybeans (*Glycine max L. Merr.*) are germinated to the sprouts of 1 cm bud length, SSG is prepared through processing the dried bean sprouts with *Lophatherum gracile* and *Juncus effusus* (He & Wang, 2013). As a soy-derived medicinal material without toxicity, SSG has a neutral nature and a sweet flavor in TCM theory, and has a long history of both medicinal and food uses in Chinese perception and experience (Wang et al., 2020; He & Wang, 2013; Fan et al., 2016). However, the modern studies on pharmacological actions of SSG are still rare.

From the perspective of TCM, various hepatic disorders appear the typical symptoms and key pathophysiological characteristics of "dampness-heat" syndrome, one of the most common TCM syndromes (Dai et al., 2013; Cao et al., 2009). Among the current animal models of liver injury, the carbon tetrachloride (CCl_4)-induced hepatic damage has been demonstrated to properly represent the pathological features of "dampness-heat" syndrome in TCM, thus this model has been usually used in the experimental evaluation of potential therapeutic potentials of Chinese herbal medicines with heat-clearing, dampness-removing, and detoxifying properties (Xie et al., 2021; Cao et al., 2009; Xu et al., 2021). In the modern experimental research, the CCl_4-induced liver injury model has been well-accepted to share similar pathomechanisms, such as inflammatory damage, oxidative stress, and hepatocyte apoptosis, with chemical liver injury in humans (Li et al., 2018; Ahmad et al., 2023). Our previous studies showed that the extracts from SSG exerted the anti-inflammatory and anti-apoptotic activities in the *in vitro* and *in vivo* osteoarthritis models (Wang et al., 2020; Fan et al., 2016). Therefore, from the perspective of either TCM or modern scientific theory, it could be speculate that SSG and its active ingredients might have a protective effect on the CCl_4-induced liver injury. In the present study, we explored the effect of different fractions extracted from SSG on the murine liver injury progress induced by CCl_4 challenge and possible underlying mechanisms.

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2 Materials and methods

2.1 Preparation of fractions extracted from SSG

Dried SSG was prepared according to the method recorded in the Pharmacopoeia of People's Republic of China (He & Wang, 2013). Then, as described in our previous study (Fan et al., 2016), the petroleum ether extract (PEE), ethyl acetate extract (EAE), and n-butanol extract (NBE) fractions of SSG were obtained through sequentially partitioning the crude ethanol extract with the corresponding solvent. The PEE, EAE, NBE, and the remaining aqueous fraction (AF) of SSG were evaporated to dryness.

2.2 Animals and groups

Kunming mice (male, 18-22 g) were obtained from Hubei Experimental Animal Research Center (Wuhan, China). After one week of acclimation, the animals were randomly divided into 7 groups of 10 mice in each group: (1) normal control group, (2) CCl_4 model control group, (3) CCl_4 + PEE of SSG group, (4) CCl_4 + EAE of SSG group, (5) CCl_4 + NBE of SSG group, (6) CCl_4 + AF of SSG group, and (7) CCl_4 + silymarin group. Mice in groups (3)-(6) were orally pre-treated with the corresponding extract fractions of SSG at the dose of 200 mg/kg for 7 consecutive days. As the positive control, silymarin (Tianjin Tasly Sants Pharmaceutical Co., Ltd, China) at dose of 100 mg/kg was orally administrated to mice in the group (7) for 7 days. At 2 h after the last administration of pre-treatment, all animals except those in the normal control group were intraperitoneally injected with the olive oil containing 0.5% CCl_4 (Jiangsu Qiangsheng Chemical Co., Ltd, China) at dose of 10 mL/kg. The normal control mice were administrated with the equal volume of vehicles (intraperitoneally injected with olive oil without CCl_4 + orally administrated with distilled water). At 24 h after CCl_4 challenge, all animals were weighed then sacrificed after collecting blood samples from their eyeballs. Liver samples were quickly collected and the liver masses were recorded. The liver tissue index of mice was expressed as the ratio of liver mass (g) to 100 g body weight. The experimental protocol was performed strictly in accordance with the procedures approved by the Animal Ethic Committee of the Wuhan University of Science and Technology. The flowchart of the experimental design of the study was shown as Figure 1.

2.3 Determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels

The blood samples collected from eyeballs were centrifuged to separate the serums. The levels of serum ALT and AST as liver

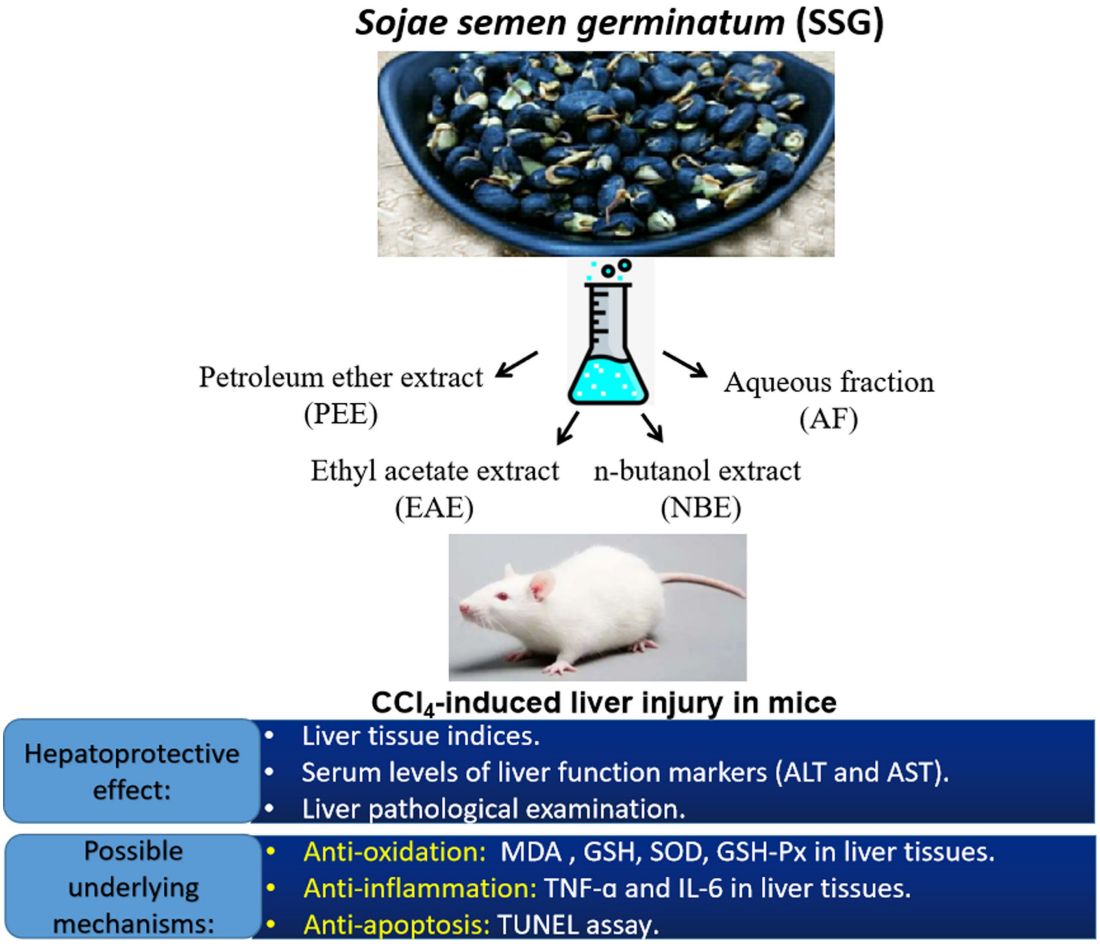

Figure 1. The flowchart of the experimental design of the study.

function markers were measured by enzymatic colorimetric methods according to kit protocols (Nanjing Jiancheng Bio-Engineering Co., Ltd., China).

2.4 Pathological examination of liver tissues

After being fixed in 10% neutral buffered formaldehyde for 24 h, the liver tissues were embedded in paraffin, cut into 4-6 μm-thick sections then stained with hematoxylin and eosin (H&E) for pathological examination of liver injury.

2.5 Measurement of malondiadehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in liver tissues

The liver tissues were homogenized on ice. Then, the levels of MDA and GSH as well as the activities of SOD and GSH-Px as oxidative stress parameters in the liver homogenates were measured according to the corresponding manufacturer's instructions (Nanjing Jiancheng Bio-Engineering Co., Ltd., China). The protein contents in homogenates were detected with the bicinchoninic acid assay kit (Nanjing Jiancheng Bio-Engineering Co., Ltd., China).

2.6 Determination of the tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in liver tissues

After homogenizing the collected liver tissues, the levels of TNF-α and IL-6 in liver homogenates were detected using ELISA kits according to kit protocols (R&D Systems, USA).

2.7 TUNEL assay

The 10% neutral buffered formaldehyde-fixed and paraffin-embedded liver tissues were cut into 5-μm sections. After the sections were deparaffinized and rehydrated, the apoptotic hepatocytes in the liver sections were detected using the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay according to the manufacturer's instruction (Roche, USA).

2.8 Statistical analysis

Results were shown as the mean ± SD. One-way analysis of variance (ANOVA) with post hoc Tukey test was employed for statistical analysis in SPSS 25.0 software. P value above 0.05 was statistically significant.

3 Results and discussion

3.1 Effect of extracts from SSG on CCl_4-induced liver injury in mice

The hepatoprotective effects of different fractions extracted from SSG on CCl4-induced liver injury in mice were assessed using liver tissue indices, serum levels of liver function markers (ALT and AST), and the liver pathological examination. As shown in Table 1, no significant difference in body weight was found between any group (P>0.05). The CCl_4 challenge markedly increased the liver tissue indices of mice ($P < 0.05$). However, pre-treatment with silymarin, EAE and NBE of SSG significantly protected mice against the CCl_4-induced increase of liver tissue index ($P < 0.05$). Similarly, mice pre-treated with silymarin, EAE and NBE of SSG showed significantly lower serum transaminases ALT and AST levels than CCl_4 model controls ($P < 0.05$; $P < 0.01$). As shown in Figure 2, obvious hepatocyte edema, hemorrhage, inflammatory infiltration and destruction of hepatic architecture were found in CCl_4-challenged livers. These pathological changes of liver damage were less severe in CCl_4 + silymarin, EAE or NBE of SSG group than CCl_4 model group. These data collectively suggested the hepato-protective effects of EAE and NBE from SSG on the CCl_4-induced liver injury.

Previous studies have demonstrated the the liver protective effects of soy diet (Khan, 2012), soybean oil (Gao et al., 2019) and soy-derived active components, such as kaempferol galactosides separated from Japan Jindai unripe soybean leaves (Zang et al., 2018), an arabinogalactan from black soybean (Sun et al., 2018), and isoflavones-enriched soy protein (Sarhan et al., 2012). Interestingly, evidences showed that the germinated soybeans contain more functional substances

Table 1. Effect of extracts from SSG on liver tissue indices, and serum transaminases ALT and AST levels of CCl4-challenged mice (Mean ± SD, n = 10).

Group	Body weight (g)	Liver tissue index (g/100 g body weight)	AST(U/L)	ALT(U/L)
Normal control	27.1 ± 1.7	5.07 ± 0.57	9.8 ± 2.1	17.7 ± 6.8
CCl_4 model control	23.2 ± 3.2	5.80 ± 0.37*	131.2 ± 41.7**	122.4 ± 19.5**
CCl_4 + PEE of SSG	25.0 ± 3.2	5.55 ± 0.58	114.3 ± 47.2	109.8 ± 27.1
CCl_4 + EAE of SSG	26.6 ± 3.7	5.44 ± 0.30$^{\#}$	83.9 ± 28.5$^{\#\#}$	78.5 ± 21.6$^{\#\#}$
CCl_4 + NBE of SSG	25.4 ± 1.5	5.43 ± 0.35$^{\#}$	89.2 ± 26.4$^{\#\#}$	79.3 ± 22.7$^{\#}$
CCl_4 + AF of SSG	25.9 ± 1.6	5.80 ± 0.45	126.0 ± 45.8	117.9 ± 25.3
CCl_4 + silymarin	26.3 ± 3.4	5.32 ± 0.31$^{\#}$	56.1 ± 18.6$^{\#\#}$	68.8 ± 25.6$^{\#\#}$

*P < 0.05, **P < 0.01 *vs.* normal control group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 *vs.* CCl_4 model control group.

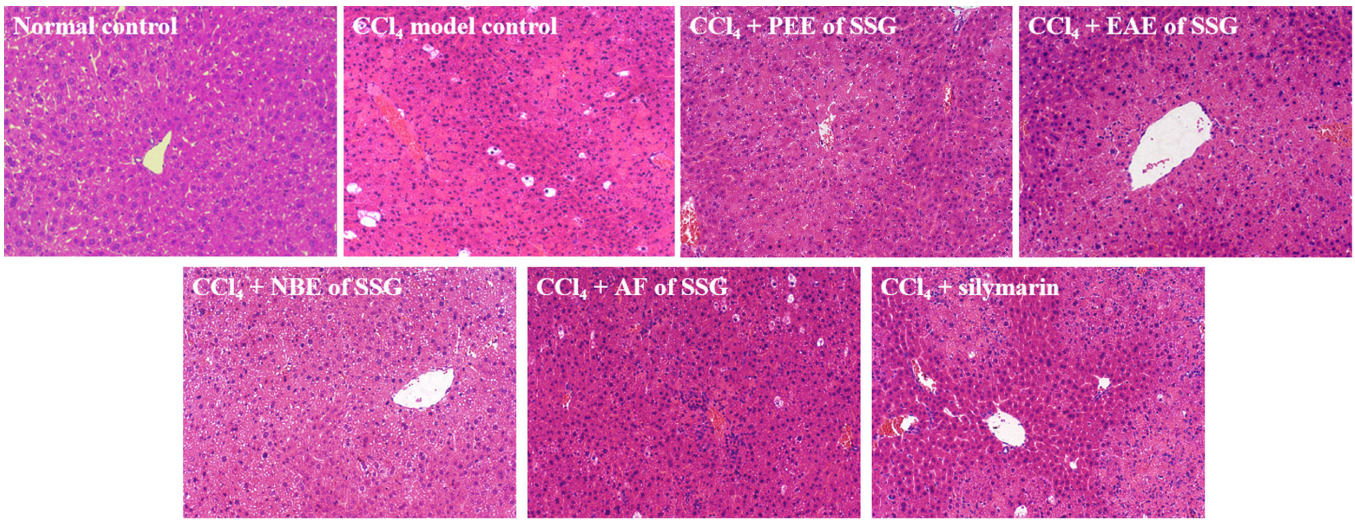

Figure 2. Effect of extracts from SSG on the CCl_4-induced pathological changes of liver tissues.

Table 2. Effect of extracts from SSG on MDA, GSH, SOD and GSH-Px levels in CCl_4-challenged livers (Mean ± SD, n = 10).

Group	MDA (nmol/mg prot)	GSH (nmol/mg prot)	SOD (U/mg prot)	GSH-Px (U/mg prot)
Normal control	2.33 ± 0.70	120.5 ± 32.5	149.7 ± 31.1	108.6 ± 15.4
CCl_4 model control	4.62 ± 0.85**	60.2 ± 15.4**	92.4 ± 25.0**	51.7 ± 12.8**
CCl_4 + PEE of SSG	4.28 ± 0.75	65.4 ± 13.6	99.5 ± 28.9	68.1 ± 20.7#
CCl_4 + EAE of SSG	3.55 ± 0.92##	80.8 ± 17.9##	119.5 ± 26.3#	75.7 ± 18.0##
CCl_4 + NBE of SSG	3.51 ± 0.88##	74.8 ± 20.9#	116.6 ± 24.1#	70.9 ± 17.5##
CCl_4 + AF of SSG	4.51 ± 0.72	63.7 ± 18.5	92.8 ± 29.2	57.3 ± 19.5
CCl_4 + silymarin	3.08 ± 0.81##	86.3 ± 11.0##	121.7 ± 22.4##	80.4 ± 13.3##

**P < 0.01 *vs.* normal control group. #P < 0.05, ##P < 0.01 *vs.* CCl_4 model control group.

than non-germinated soybeans, thus are more beneficial for human health (Huang et al., 2014; Borges-Martínez et al., 2022). Germination enhanced the antioxidant capacities as well as the contents of L-ascorbic acid, phenolics and isoflavone in soybean. For example, the contents of genistein, daidzein and total aglycone in germinated soybeans were found to be approximately 2-3 folds of those in the non-germinated soybeans (Huang et al., 2014). The ethanolic extract from germinated soybean embryos has been reported to inhibit hepatic inflammation and ameliorate the fatty liver injury (Kwon et al., 2020). As a kind of processed bean sprout, SSG is also a soy-derived traditional Chinese herbal medicine. The present study firstly showed that this soy-derived Chinese medicine could relieve the liver swelling, improve the liver function, and alleviate liver histopathology in the CCl_4-challenged mice, supporting the potential of SSG as well as its EAE and NBE as protective agents against liver disorders. From the unique perspective of TCM, SSG has the effects of clearing "heat", removing "dampness", and relieving "toxin", which could explain its effectiveness in the CCl_4-induced hepatic damage representing "dampness-heat" syndrome in TCM (Xie et al., 2021; Cao et al., 2009; Xu et al., 2021).

3.2 Effect of extracts from SSG on CCl_4-induced liver oxidative stress

Oxidative stress is the common pathophysiological basis involved in various liver injuries. In the CCl_4-challenged liver tissues, after the metabolism by hepatic cytochrome P450 enzyme, CCl_4 is converted to the trichloromethyl free radical (•CCl_3) and/or trichloromethyl peroxyl radical (•OOCCl3), sequentially induces liver oxidative stress damage (Li et al., 2018). Coincidentally, soybean products have been well demonstrated to be enriched with antioxidant compounds (Huang et al., 2014; Zang et al., 2018; Sun et al., 2018; Sarhan et al., 2012; Gao et al., 2019). In order to explore the possible mechanisms underlying the hepato-protective effect of SSG extracts, we evaluated the indices of liver oxidative stress, including the levels of a lipid peroxidation product MDA and a non-enzymatic antioxidant GSH, as well as the activities of antioxidant enzymes SOD and GSH-Px.

As shown in Table 2, the CCl_4 challenge induced an imbalance between oxidative stress and antioxidant defenses in liver, manifested as the enhanced MDA levels, the decreased GSH levels and the inhibited activities of antioxidant enzymes

SOD and GSH-Px ($P < 0.01$). In line with the above results showing hepato-protective effects of EAE and NBE from SSG, the oxidative imbalance induced by CCl_4 was significantly restored by pretreatment with silymarin, EAE or NBE of SSG ($P < 0.05$; $P < 0.01$), suggesting the EAE and NBE of SSG might possess anti-oxidative activity, which is at least partly responsible for their hepatoprotective effects.

3.3 Effect of extracts from SSG on the levels of pro-inflammatory cytokines in livers of CCl_4-challenged mice

Inflammatory damage is one of crucial events associated with CCl_4-induced liver injury (Li et al., 2018). As shown in Figure 3, significant increases in levels of pro-inflammatory cytokines TNF-α and IL-6 were observed in liver tissues collected from CCl_4 model control mice ($P < 0.01$). However, the CCl_4-induced elevations of TNF-α and IL-6 in livers were markedly suppressed in mice pre-treated with silymarin, PEE, EAE or NBE of SSG ($P < 0.05$; $P < 0.01$). In particular, compared to the CCl_4 model control group, the levels of TNF-α and IL-6 in the CCl_4 + EAE of SSG group was declined by 22.4 and 25.7%, respectively.

TNF-α has been considered as the earliest proinflammatory cytokine released mainly by damaged hepatic macrophages during inflammatory process of acute liver tissue injury. As a key lymphokine, IL-6 mediates chemotaxis of inflammatory cells, exacerbates the inflammatory cascade, finally leads to tissue damage (Xie et al., 2021). In line with the known anti-inflammatory properties of EAE of SSG (Wang et al., 2020; Fan et al., 2016) and other soybean bioactive compounds (Kusumah & Gonzalez de Mejia, 2022), the present study showed that, among different fractions extracted from SSG, the EAE appeared to exhibit the strongest inhibitory activity against the CCl_4-induced liver inflammation.

3.4 Effect of extracts from SSG on hepatocyte apoptosis in CCl_4-challenged mice

As a classic hepato-toxicant, CCl_4 could caused the cell apoptosis contributing to the morphological changes even death of hepatocytes, and the development of liver injury (Li et al., 2018). As shown in Figure 4, TUNEL assay in this study revealed that the CCl_4 challenge induced severe hepatocyte apoptosis in mice. Importantly, pre-treated with silymarin, EAE and NBE of SSG could decreased the CCl_4-enhanced number of TUNEL-positive apoptotic hepatocytes, suggesting EAE and NBE of SSG were effective in protecting the hepatocytes against the CCl_4-induced cellular damage.

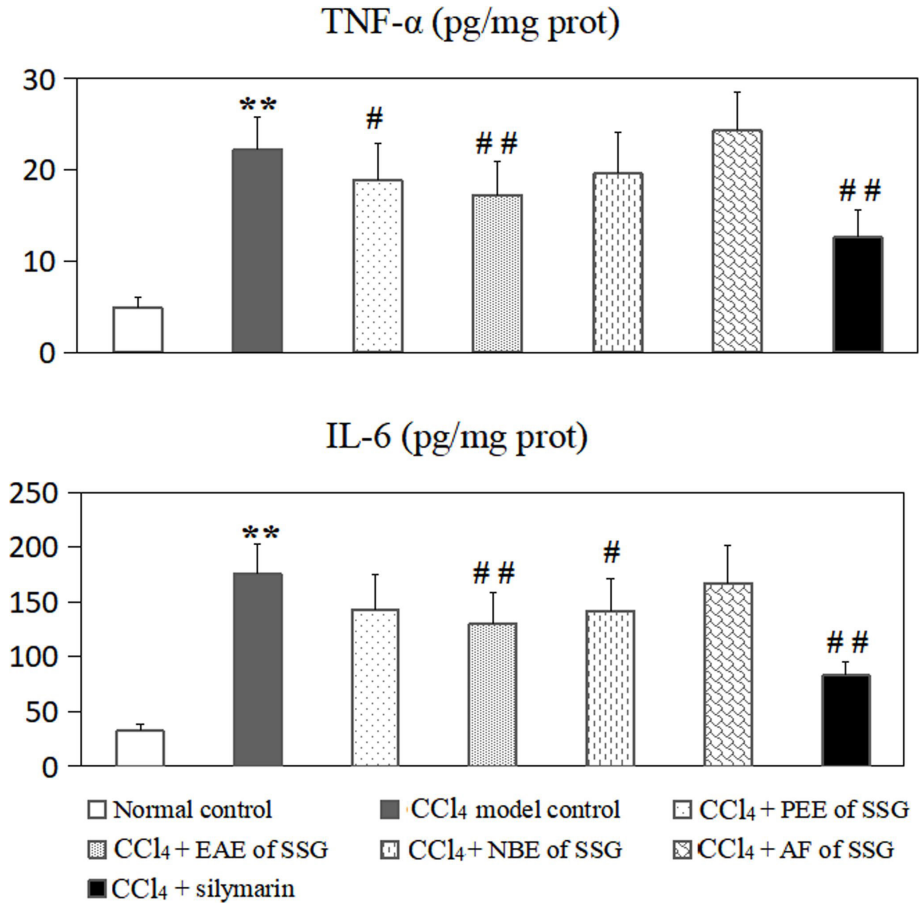

Figure 3. Effect of extracts from SSG on the levels of pro-inflammatory cytokines TNF-α and IL-6 in livers of CCl_4-challenged mice. n = 10. $^{**}P < 0.01$ *vs.* normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* CCl_4 model control group.

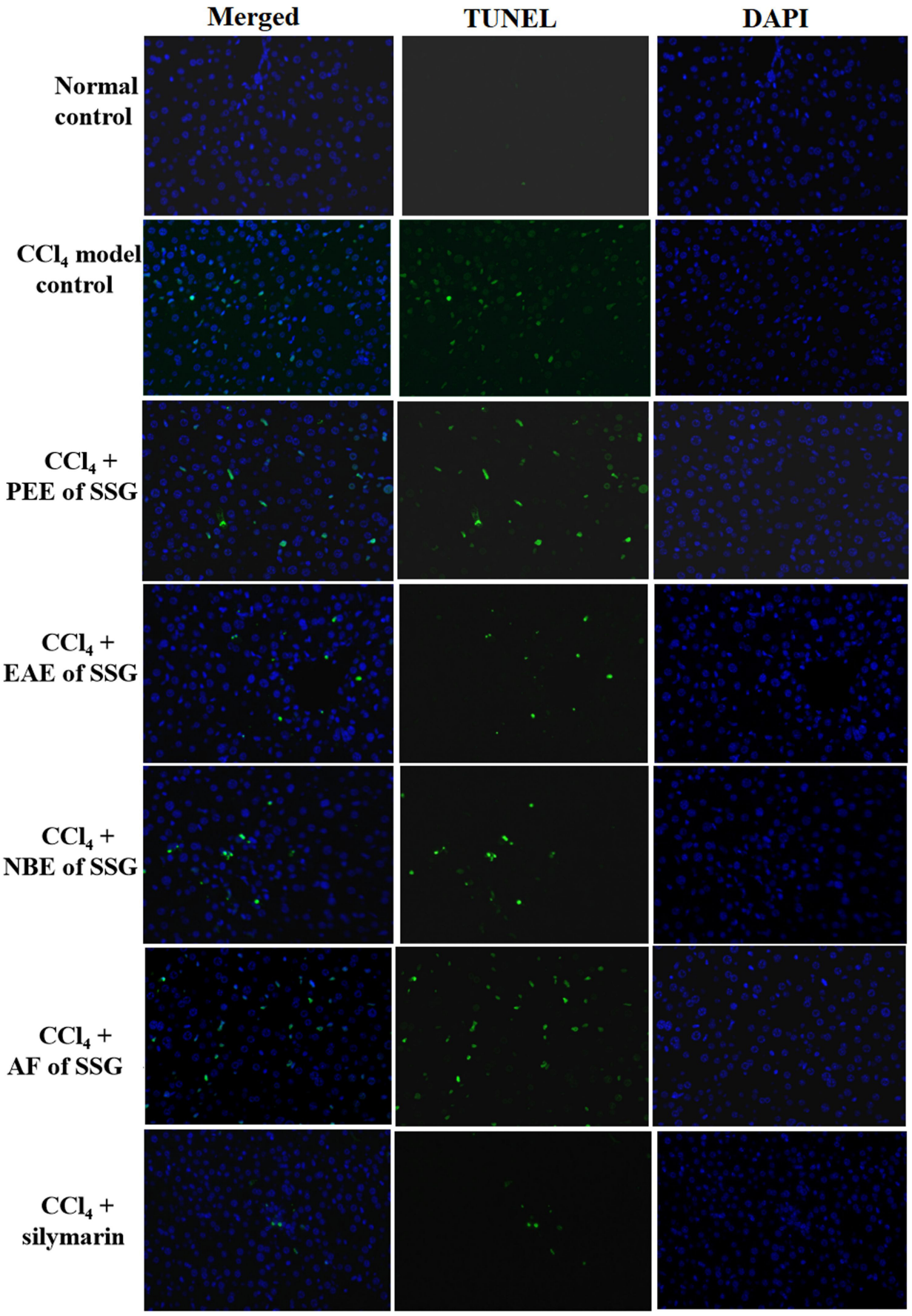

Figure 4. Effect of extracts from SSG on the number of TUNEL-positive apoptotic hepatocytes in CCl_4-challenged mice.

4 Conclusions

To the best of our knowledge, this is the first study providing experimental evidence supporting the medicinal and dietary uses of SSG, a germinated soybean-derived traditional Chinese herbal medicine with heat-clearing, dampness-removing, and detoxifying properties, for liver disorder patients with "dampness-heat" syndrome from perspective of TCM. In the present study, we demonstrated that extracts from SSG showed protective effect on the CCl_4-induced liver injury representing "dampness-heat" syndrome in TCM via multiple pharmacological mechanisms including anti-oxidation, anti-inflammation and anti-apoptosis. In general, EAE and NBE might mainly account for the hepato-protective effect of SSG extracts, and EAE appeared to be more active than the other extract fractions.

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