Protective Effects of *Smilax glabra* Roxb on Carbon Tetrachloride Induced Rat Acute Liver Injury by Inhibiting the Activation of the Nucleotide-Binding Domain Leucine-Rich Repeat Containing Pyrin Domain containing 3 Inflammasome

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The experiment was conducted to investigate the protective effect of *Smilax glabra* Roxb. aqueous extract on carbon tetrachloride induced acute liver injury in rats by regulating the nucleotide binding leucine rich repeat receptors family pyrin domain containing 3 inflammasome. In this study, a rat acute liver injury model was established by a single intraperitoneal injection of 50 % carbon tetrachloride oil solution (1 ml/kg body weight). A total of 50 rats were randomly divided into control group, model group and 3 *Smilax glabra* Roxb. aqueous extract intervention groups (0.75, 1.5 and 3 g/kg body weight/d). *Smilax glabra* Roxb. aqueous extract or distilled water was orally administered by gavage once daily for seven consecutive days. Then the blood and liver samples were collected. The results showed that in the 3 intervention *Smilax glabra* Roxb. aqueous extract groups, the levels of serum aspartate aminotransferase and Alanine aminotransferase, the content of malondialdehyde in rat liver tissues significantly reduced and superoxide dismutase, catalase and glutathione increased significantly, the liver lobule structures were repaired and the liver cells were aligned in an orderly manner, the expressions of nucleotide binding leucine rich repeat receptors family pyrin domain containing 3 inflammasome, apoptosis associated speck like protein containing and caspase-1 proteins, Interleukin 1 beta messenger ribonucleic acid was decreased. *Smilax glabra* Roxb. aqueous extract exerted a protective effect against carbon tetrachloride induced acute liver injury by inhibiting the activation of nucleotide binding leucine rich repeat receptors family pyrin domain containing 3 inflammasome, which suggest a promising avenue for the exploration of *Smilax glabra* Roxb. aqueous extract in improving acute liver injury.

Key words: *Smilax glabra* Roxb, rat, acute liver injury

Acute liver injury (ALI) is a disease caused by drug poisoning, viral infection, immune reaction, or vascular disorders resulting in acute abnormal liver function and hepatocyte death. The clinical manifestation is acute hepatic dysfunction[1]. The pathogenesis of acute hepatic injury includes the activation of the innate immune, release of pro inflammatory cytokines and the alteration of signal transduction pathways related to hepatic inflammation, necrosis and apoptosis[2]. Its main features include the release of inflammatory cytokines, the overload of oxidative stress, an increase in the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatocyte apoptosis

and coagulation[3]. ALI can subsequently develop into acute liver failure, which is characterized by rapidly progressing hepatic encephalopathy and multiple organ failure with poor prognosis and high mortality rate[4].

The treatment of liver injury is based on different basic causes, there are no remarkable and effective medicine to cure it in clinic[5] and most of them are chemical

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synthetic drugs such as vitamins, nucleoside analogues, liver protecting and enzyme inhibiting drugs and α-interferon[6]. These drugs have some disadvantages such as significant side effects, adverse reactions and high prices, which greatly limited their clinical application[7]. Accordingly, it is an urgent need for rational drug use in clinical to develop safe and effective anti-liver injury drugs[8]. Considering the complex pathogenesis of ALI, natural herbal medication with anti-inflammatory, anti-oxidation and immunoregulatory function is usually recommended in China. Natural herbal medication routinely contains complex ingredients and plays a role in the multiple factors of the pathogenesis[9,10], as well as has the unique advantage in treating liver diseases due to its minor side effects. According to recent literature, Chinese Herbal medicine (CHM) has been one of the main hot points of hepatoprotective and widely used in clinical therapy[11]. Lots of single herbals or extracts, the active components of CHM and herbal formulas have been reported to exert protective effects on liver injury. In particular, Chinese patent medicine Fuzheng Huayu tablet has been proven to have good drug safety and patient tolerance by US Phase II clinical trials[12-14], which brought new opportunities and laid a good foundation for the development of CHM.

Smilax glabra Roxb. (SGR), the dried rhizome of the liliaceous plant, is used as both medicine and food all the time in China. Since 1963, SGR has been listed in the Pharmacopoeia of the People's Republic of China. According to the theory of traditional Chinese medicine, SGR is sweet, light, mild and non-toxic and is associated with stomach and liver meridians. "Compendium of Materia Medica" of Li Shizhen in the Ming dynasty indicated that SGR can strengthen the spleen, stomach, bones and muscles, eliminate wind damp, ease joint movement, cure spasm and ostealgia, malignant sores and swollen welling abscess and is an antidote to mercury and silver poisoning. SGR has been extensively used worldwide for its marked pharmacological activities for treating syphilitic poisoned sores, limb hypertonicity, morbid leukorrhea, eczema pruritus, strangury due to heat, carbuncle toxin, headache, diarrhoea, hepatitis, cholecystitis, stranguria, tumours, gout, mercury poisoning and many other human ailments in clinical[15,16]. In the previous study, we found that SGR not only effectively cured gentamicin induced kidney injury in rats[17], but also showed a powerful protective effect on carbon tetrachloride

(CCl_4) induced ALI by tentative experiments. However, the protective mechanism has not been revealed yet. In this study, a pathological model of CCl_4 induced ALI in rats was established and *Smilax glabra* Roxb. aqueous extract (SGRAE) was used as a therapeutic drug. The histological changes of the liver were observed and the biochemical indexes of liver and the level of nucleotide binding leucine rich repeat receptors family pyrin domain containing 3 (NLRP3), apoptosis associated speck like protein containing (ASC) and caspase-1 protein in liver and messenger Ribonucleic acid (mRNA) expression levels of Interleukin 1 beta (IL-1β) were detected. Through inter group comparisons, we explored the protective effect of SGR on CCl_4 induced ALI in rats and its related molecular mechanisms, which will provide an experimental basis for the clinical use of SGR in the control of liver injury diseases.

MATERIALS AND METHODS

Reagents:

CCl_4 was purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). Cell lysis buffer for western blotting and immunoprecipitation (IP) was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). Phenylmethanesulfonyl fluoride (PMSF) was purchased from Sigma Aldrich Co., Ltd. (St. Louis, USA). EasySee Western Marker was purchased from TransGen Biotech Co., Ltd. (Beijing, China). Pro light horseradish peroxidase (HRP) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Bovine serum albumin or Fraction V (BSA-V) was purchased from Roche Co., Ltd. (Basilea, Switzerland). Ultrasensitive two step immunohistochemical detection reagent was purchased from ZSGB-Bio Co., Ltd. (Beijing, China). All antibodies were purchased from Cell Signaling Technology, Inc. (Lexington, KY, USA). Total RNA extraction reagent (RNAiso Plus) was purchased from Takara Bio Inc (Europe). HiScript Q Reverse Transcriptase SuperMix for quantitative polymerase chain reaction (qPCR) and AceQ qPCR Synergy Brands (SYBR) Green Master Mix purchased from Vazyme Biotech Co., Ltd (Nanjing, China). Primers were synthesized by Sangon Biotech Co., Ltd., Shanghai, China. All detection kits were purchased from Jiancheng Bioengineering Institute production, Nanjing, Jiangsu Province, China. Tris (hydroxymethyl) aminomethane (THAM) hydrochloride (Tris-HCl), Polysorbate 20 (Tween 20) and other reagents were purchased from Solarbio Life Sciences Co., Ltd. (Beijing, China).

Animals:

In total, 55 w old male Sprague Dawley (SD) rats (weight, 180-200 g) were obtained from Aier Matt Technology Co., Jiangsu Province, China (animal license: SCXK (Su), 2014-0007). The animals were maintained in standard housing facilities (temperature: 24±1º, humidity: 45±5 % and a 12 h light/dark cycle) and fed a standard laboratory diet, with *ad libitum* access to water. All animals were given a week to acclimatize before the experiment. All of the animals involved in this protocol were bred and managed in strict accordance with the Chinese regulations on the use and breeding of experimental animals and the guidelines developed by the Experimental Animal Research Institute of Anhui Agricultural University. This research was approved by the Animal Feeding and Use Committee (approval no. AHAU2018028) of Anhui Agricultural University.

Preparation and quality evaluation of SGRAE:

SGR was purchased from Hefei Lejia Herbal Pieces Co. Ltd. (Anhui, China) and authenticated/identified by Prof. Weijing Shi, which geographical origin was Guangxi Province of China and deposited at the herbarium of Hefei Lejia Herbal Pieces Co. Ltd. (HFLJHP) (Voucher No. 20181208), Hefei, Anhui, P. R. China. After super micro grinding, the powder of SGR was extracted twice using an SJC-II-10L Ultrasonic extraction apparatus (Wuxi Shangjia Biological Technology Co., Ltd., China) and aqueous extract of SGR (SGRAE) were obtained by filtering. Then, the filtrate was concentrated to desired concentrations by RE-52CS rotary evaporation (Shanghai Yarong Biochemical Instrument Factory, China) and stored at 4º after sterilization. For more details on the method and result of HPLC analysis of SGRAE, please see the reference[17].

CC$_{l4}$ induced ALI rat model:

A total of 50 SD rats were randomly divided into 5 groups (10 rats in each group) after a week acclimatization: the control group, the model group and 3 SGRAE intervention groups (0.75, 1.5 and 3 g/kg body weight/d). Rats in 3 intervention groups were given different dosages of SGRAE and the rats in the model group and control group were given distilled water by intragastric administration for 7 d consecutively. 6 h after the last administration, rats in the intervention groups and model group were given 50 % CCl$_4$ (1 ml/kg) by intraperitoneal injection and rats in the control group were given normal saline and then they were observed in a 24 h period for gross behavioural change and mortality. No mortality was noted. 24 h after the injection, the rats were anesthetized with an intraperitoneal injection of 1.0 % pentobarbital sodium (40 mg/body weight) and then weighed and the blood samples were taken. The liver samples were collected after euthanasia by cervical dislocation. Considering the toxicity of CCl$_4$ when inhaled through respiratory tract or absorbed through skin, causing serious damage to heart, liver and kidney, we operated in a ventilated place and wore gloves, work clothes, masks and other protective facilities in the process of experimental operation and parameters were evaluated (loss of weight, possible changes in the social interactions.

Liver coefficient analysis:

The liver tissues of the rats were weighed to calculate the kidney coefficient (liver coefficient=kidney weight/body weight).

Serum analysis:

After fasting for 12 h, 3-5 ml blood was drawn from each rat's caudal vein. Samples were centrifuged at 2500 rpm for 5 min to separate the serum. Finally, serum levels of AST and ALT were detected using a Vet Test™ Automatic Biochemistry Analyzer (IDEXX Laboratories, Inc., USA).

Liver tissue biochemical analysis:

A portion of the liver was taken and made into a 10 % tissue homogenate using a tissue homogenizer. It was then centrifuged and the supernatant was collected. Levels of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and reduced glutathione (GSH) in the supernatant were detected by a spectrophotometer.

Histopathological examinations:

The liver tissues of rats were fixed in 4 % buffered formalin solution. The tissues were subjected to standard alcohol xylol processes and embedded in paraffin. The samples were then cut into 5 μm sections using a LS-2055+Paraffin Semiautomatic Machine (Shenyang Longshou Electronic Instrument Co., China) and stained with hematoxylin eosin (H&E). Liver sections were examined by light microscopy using a CX21FS1 Biological Microscope (OLYMPUS Co., Japan) and assessed at 20x magnification by randomly

selecting areas in the microscopic field at 10x in order to determine changes in degeneration and necrosis.

Western blot analysis:

Total proteins were extracted after cell lysis for Western and IP with PMSF. The bicinchoninic acid assay (BCA) was used for quantification of proteins. Equal amounts of protein were loaded onto a 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to electrophoresis. The proteins were transferred onto a nitrocellulose (NC) membrane and blocked with 5 % skim milk. Primary antibodies against anti-NLRP3, ASC, caspase-1 and α/β Tubulin were used. The membrane was incubated overnight at 4º and then washed with Tris buffered saline with 0.1 % Tween® 20 detergent (TBST), followed by incubation with peroxidase labeled secondary antibody. Protein visualization was achieved using enhanced chemiluminescence Western blot reagents and a Versa Doc™ 4000 MP multi spectral imaging system (Bio Red Co., USA).

Immunohistochemistry analysis: Before immunostaining, 5 μm thick liver tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol and washed in phosphate buffered saline (PBS). Tissue sections were blocked with BSA after antigen retrieval. Tissue sections were then incubated with primary antibodies against anti-NLRP3, ASC, caspase-1 followed by peroxidase labeled secondary antibody. After treatment with 3,3'-diaminobenzidine (DAB) condensed chromogen and staining with hematoxylin, tissue sections were examined under light microscopy.

Real time quantitative polymerase chain reaction (RT-qPCR) analysis: Liver tissues were stored at -80º. Liver samples were ground with liquid nitrogen and total liver RNA was extracted with RNAiso Plus and then reverse transcribed to Complementary DNA (cDNA) according to the specifications. Samples were then amplified using qPCR according to the instructions of the AceQ qPCR SYBR Green Master Mix. The PCR conditions

TABLE 1: OLIGONUCLEOTIDE SEQUENCE OF QUANTITATIVE RT-Q PCR

Gene	Sequence (5'-3')	Product length
β-actin	F: CCCATCTATGAGGGTTACGC	150 bp
	R: TTTAATTGTCACGCACGATTTC	
IL-1β	F: GCCTGACAGACCCCAAAAGA	177 bp
	R: CTCCACGGGCAAGACATAGG	

were as follows: 95º for 10 min, 40 cycles of 95º for 15 s and 60º for 1 min and 95º for 15 s. Oligonucleotide sequences of PCR are listed in Table 1.

Statistical analysis:

Data were analyzed by conducting analysis of variance (ANOVA) and Dunnett's test using SPSS (v 19.0; IBM, USA). Results of all groups are shown as mean±standard deviation (SD). Association between two groups was determined by Dunnett's multiple comparison tests. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Organ coefficient, also known as visceral body ratio, is the ratio of the weight of an organ to its body weight. In normal condition, the ratio of organs to body weight was relatively constant. After being poisoned, the weight of damaged organs can be changed, so the organ coefficient will also change. Organ coefficient increased, indicating organ congestion, edema or hypertrophy, etc. In this study, rat liver coefficient in the model groups was significantly higher when compared with the control group ($p < 0.05$), the coefficient in the 3 SGRAE intervention groups was significantly lower than that in the model group ($p < 0.05$) and there was no significant difference between the high dose SGRAE group and the control group ($p > 0.05$), as shown in Table 2.

Serum aminotransferases ALT and AST are the most effective indicators for early liver injury[18] and 1 % liver cell necrosis will double the ALT level[19]. When hepatocytes are damaged, ALT and AST are released in large amounts into the blood and serum ALT and AST levels are increased. In our current study, the levels of serum ALT and AST in the model group were significantly increased, indicating that CCl_4 caused severe liver damage in rats. Compared with the model group, serum AST and ALT levels of the 3 SGRAE intervention groups were significantly lowered ($p < 0.05$) and no significant difference was showed ($p > 0.05$) between the high dose SGRAE group and the control group, as shown in Table 3. This indicated that SGR can alleviate CCl_4 induced ALI to a certain extent, consistent with the histopathological results.

SOD and GSH are the important antioxidant enzymes in the body, which can effectively eliminate free radicals and inhibit free radical induced lipid peroxidation[20]. MDA is the final metabolite of lipid peroxidation and its level reflects the degree of oxidative stress caused by

toxophores[21]. Our study team found that SGRAE can protect gentamicin induced renal injury by regulating the mechanism of apoptosis induced by oxidative stress activated caspase-3 protein[17]. In the current research, compared to the model group, SOD, CAT and GSH of the 3 SGRAE intervention groups increased significantly ($p < 0.05$) and MDA decreased significantly ($p < 0.05$) in liver tissues. None of the oxidative stress indexes of liver in the high dose SGRAE group showed significant difference when compared with the control group ($p > 0.05$), as shown in Table 4, which is consistent with previous findings[19,22]. The results suggested that SGR ameliorated CCl_4 induced liver injury by increasing SOD and GSH levels and reducing MDA and the hepatoprotective effects of SGR may be related to antioxidative stress.

Pathological changes in liver tissue were observed by H&E staining. In the control group, the structure of rat hepatic lobules was complete and the liver cells arranged regularly, as shown in fig. 1A. Obvious liver structure of rats in model group, hepatic lobule structure was not clear, liver cells arranged in disorder and showed intrahepatic cholestasis, as shown in fig. 1B. These findings are consistent with the previous findings[20]. In the 3 SGRAE intervention groups, the normal liver lobule structures began to recover and the liver cells were aligned in an orderly manner. Cell morphology in the high dose SGRAE group was similar to the control group as shown in fig. 1C, 1D and 1E.

TABLE 2: LIVER COEFFICIENT OF RATS IN DIFFERENT GROUPS (N=10)

Group	Liver coefficient
Control group	0.307±0.035[a]
Model group	0.429±0.055[b]
Low dose SGRAE group	0.379±0.030[c]
Middle dose SGRAE group	0.371±0.027[c]
High dose SGRAE group	0.307±0.024[a]

Values are expressed as mean±SD for ten rats in each group. The same letter in the same line indicates no significant difference ($p > 0.05$) and different letters indicate significant difference ($p < 0.05$)

TABLE 3: SERUM LEVELS OF AST AND ALT IN RATS OF DIFFERENT GROUPS (N=10)

Group	AST (U/L)	ALT (U/L)
Control group	23.859±1.234[a]	26.738±1.044[a]
Model group	56.197±1.111[b]	77.659±2.297[b]
Low dose SGRAE group	39.093±1.922[c]	54.142±1.594[c]
Middle dose SGRAE group	34.718±1.461[d]	38.505±1.349[d]
High dose SGRAE group	24.791±1.257[a]	27.437±2.303[a]

Values are expressed as mean±SD for ten rats in each group. The same letter in the same line indicates no significant difference ($p > 0.05$) and different letters indicate significant difference ($p < 0.05$)

Studies in recent years have shown that the inflammasome plays a huge role in the onset and development processes of a variety of liver diseases[23-25]. The inflammasome is a poly protein complex present in the cytoplasm and is one of the important components of innate immunity. The inflammasome can recognize the molecular patterns of different pathogens and cause the release of various cytokines and inflammatory mediators through signal transduction and cell activation, ultimately triggering an inflammatory response[26]. Currently, various inflammasomes, including NLRP1, NLRP3, NLRC4, absent in melanoma-2 (AIM2) and retinoic acid inducible gene I (RIG-Ia), have been identified[26]. The most important of these is the NLRP3 inflammasome, which is formed mainly through the aggregation of NLRP3, ASC and caspase-1[27]. The NLRP3 inflammasome is expressed in a variety of cells in liver tissues and can be activated. Its activation requires the binding of the locally recurrent domain specific structure and the factor activated or released by the stressed cells, as well as the sequential opening of the protein structures to expose the pyrimidine (PYD) so that the ASC can bind. Through the PYD-PYD interaction, the inactive pro caspase-1 is recruited by ASC caspase recruitment domain (CARD) to enable self-cleavage by pro-caspase-1 to complete the self-activation process. The activated caspase-1 cleaves the inflammatory factor substrates (e.g., pro-IL-1β) to facilitate inflammatory responses[28-30]. In this study, compared with the control group, protein expression of NLRP3, ASC and Caspase-1 were increased in the model group; compared with the model group, three proteins were lowered in the 3 SGRAE intervention groups, as shown in fig. 2. Results of immunohistochemical staining showed no evidence of NLRP3, ASC and Caspase-1 in the control group, as shown in fig. 3A, 4A and 5A. In the model group, NLRP3, ASC and Caspase-1 was seen in liver cells, as shown in fig. 3B, 4B and 5B, while in the 3 SGRAE intervention groups, NLRP3, ASC and Caspase-1 decreased and was rarely seen in the high dose SGRAE group, as shown in fig. 3C-3E, 4C-4E, 5C-5E.

IL-1β is a pro inflammatory cytokine, which can widely participate in various pathological injuries such as tissue destruction and edema formation. As mentioned before, the aggregation of NLRP3, ASC and caspase-1 proteins forms the NLRP3 inflammasome and then induces maturation and release of IL-1β[27]. Studies have shown that SGR has anti-inflammatory effect[31]. SGRAE could inhibit Prostaglandin E_2 (PGE_2) synthesis

TABLE 4: LEVELS OF LIVER OXIDATIVE STRESS INDEXES IN RATS OF DIFFERENT GROUPS (N=10)

Group	SOD (U/mg Pro)	CAT (U/mg Pro)	MDA (nmol/mg Pro)	GSH (mg/g Pro)
Control group	141.894±16.947[a]	16.861±1.07 [a]	0.721±0.130[a]	20.993±1.170[a]
Model group	55.901±8.100[b]	6.772±0.973[b]	3.416±0.459[b]	7.718±1.318[b]
Low dose SGRAE group	88.676±10.949[c]	9.156±1.232[c]	2.243±0.330[c]	12.254±1.224[c]
Middle dose SGRAE group	127.321±10.395[d]	11.959±1.215[d]	1.322±0.333[d]	14.890±1.490[d]
High dose SGRAE group	161.292±10.426[a]	16.867±1.327[a]	0.796±0.111[a]	20.094±0.889[a]

Values are expressed as mean±SD for ten rats in each group. The same letter in the same line indicates no significant difference (p>0.05) and different letters indicate significant difference (p<0.05)
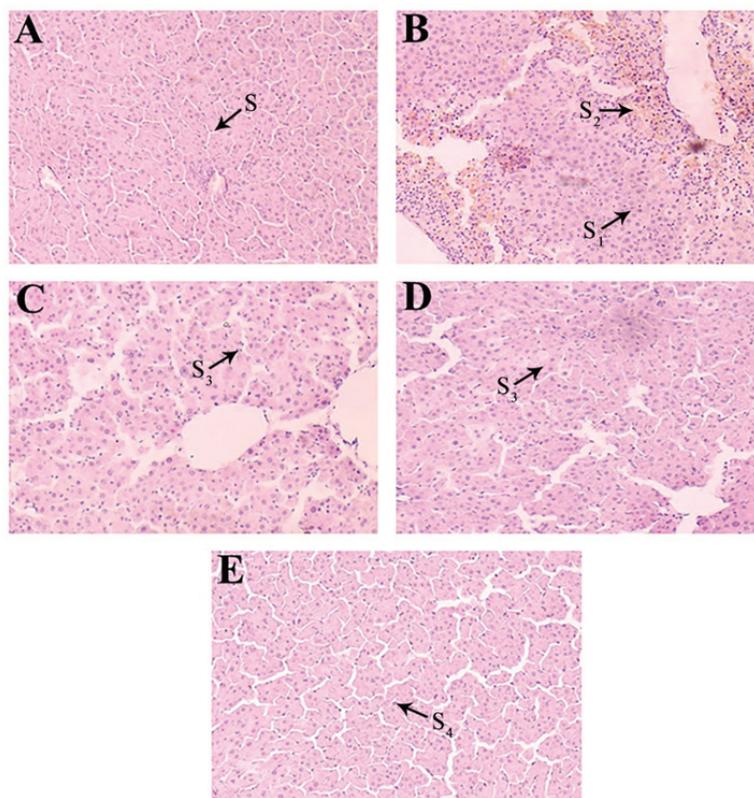
Fig. 1: Pathological changes in rat liver tissue
Pathological changes in rat liver tissue of different groups (H&E, magnification: 100x), (A) control group, (B) model group, (C) low dose SGRAE group, (D) middle dose SGRAE group, (E) high dose SGRAE group. Those labeled S is the neat and orderly hepatic cell cord, S_1 is the disorder or disappearing hepatic cord, S_2 is the intrahepatic cholestasis, S_3 is the recovering hepatic cord, S_4 is the near normality hepatic cord.
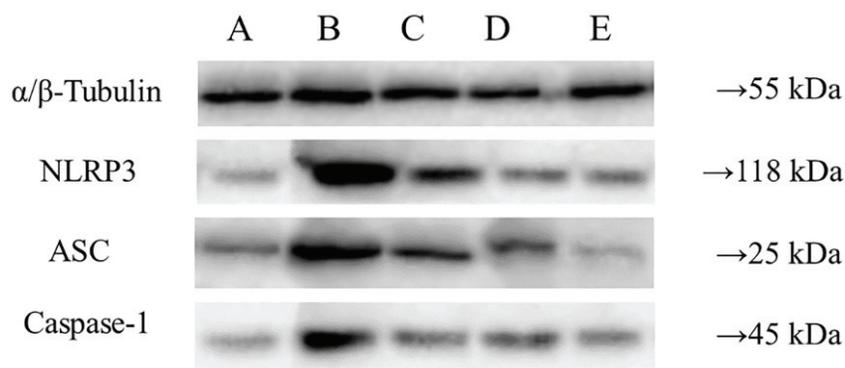
Fig. 2: Expression of α/β-Tubulin, NLRP3, ASC and Caspase-1 protein in rat liver tissue
Expression of α/β Tubulin, NLRP3, ASC and caspase-1 protein in rat liver tissue of different groups, (A) control group, (B) model group, (C) low dose SGRAE group, (D) middle dose SGRAE group, (E) high dose SGRAE group

NLRP3
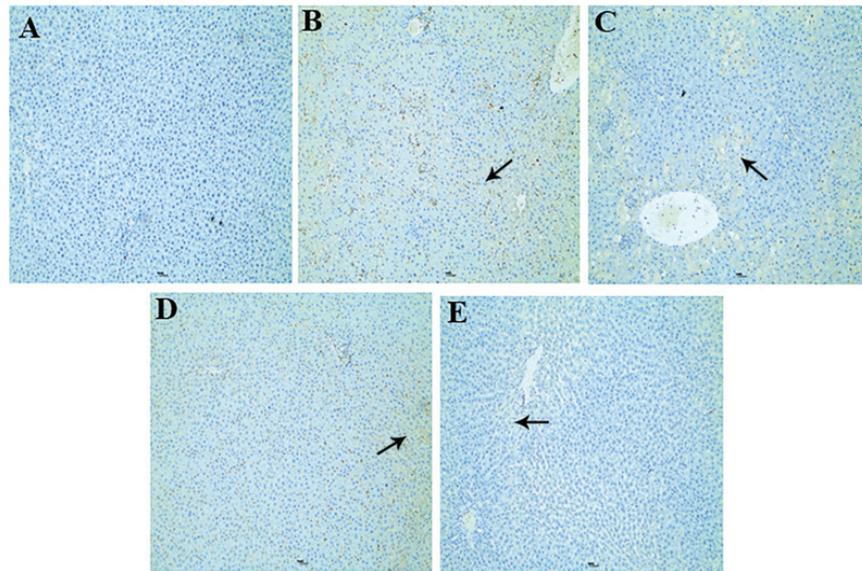
Fig. 3: Immunohistochemical assays on expressions of activated NLRP3 protein in rat liver tissue
Immunohistochemical assays on expressions of activated NLRP3 protein in rat liver tissue of different groups (magnification: 200x), (A) control group, (B) model group, (C) low dose group, (D) middle dose SGRAE group, (E) high dose SGRAE group

ASC
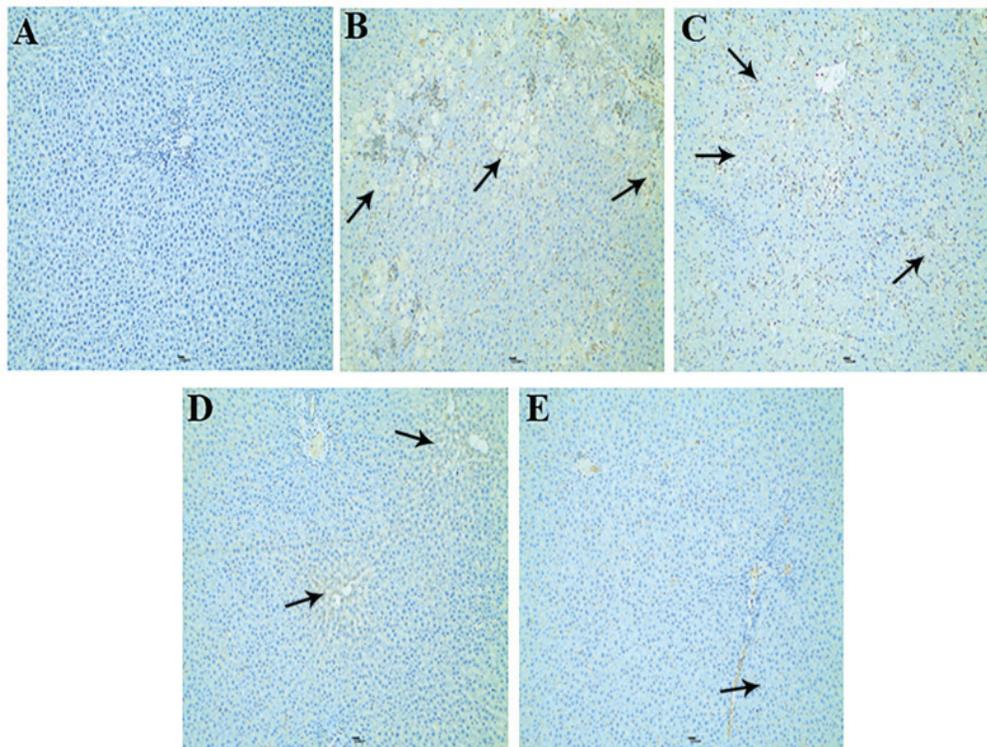
Fig. 4: Immunohistochemical assays on expressions of activated ASC in liver tissues of rats
Immunohistochemical assays on expressions of activated ASC in rat liver tissue of different groups (magnification: 200x), (A) control group, (B) model group, (C) low dose SGRAE group, (D) middle dose SGRAE group, (E) high dose SGRAE group

Caspase-1
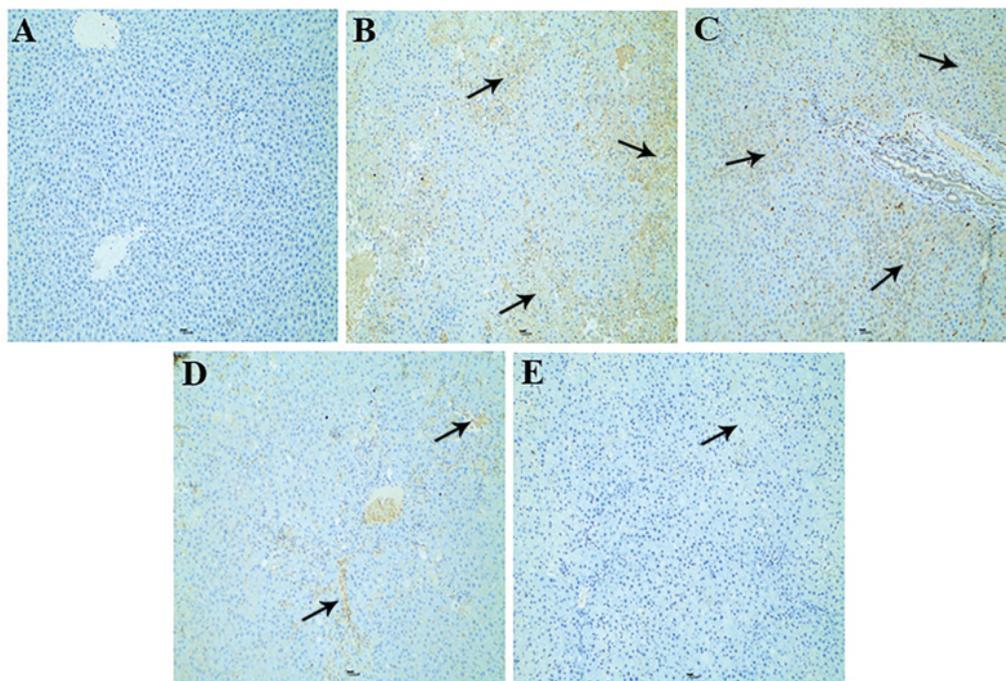
Fig. 5: Immunohistochemical assays on expressions of activated caspase-1 in liver tissues of rats
Immunohistochemical assays on expressions of activated caspase-1 in rat liver tissue of different groups (magnification: 200x), (A) control group, (B) model group, (C) low dose SGRAE group, (D) middle dose SGRAE group, (E) high dose SGRAE group
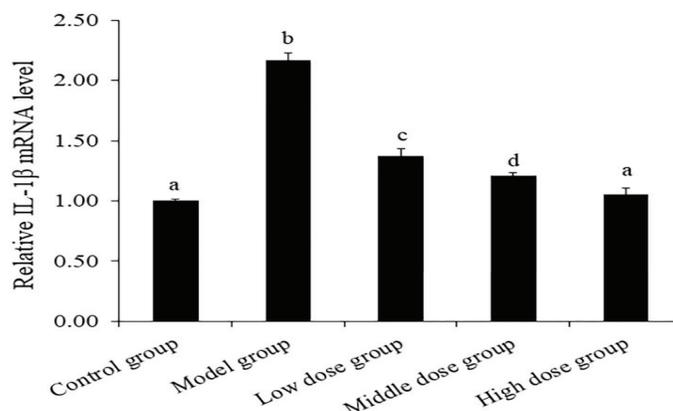
Fig. 6: IL-1β mRNA expression in rat liver tissue
IL-1β mRNA expression in rat liver tissue of different groups. Values are expressed as mean±SD for in each group. Not the same standard letter or letters mean significant difference (p>0.05) are not the same letters indicate significant difference (p<0.05), (A) control group, (B) model group, (C) low dose SGRAE group, (D) middle dose SGRAE group, (E) high dose SGRAE group

and release, which significantly down regulates the functional activity of macrophages, inhibited IL-1β, Tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) production[32]. In this study, the expression of IL-1β mRNA in the model group was significantly higher than the control group and 3 SGRAE intervention groups (p<0.05) and there was no significant difference between the high dose SGRAE group and the control group (p>0.05), as shown in Table 1. The lower expression of IL-1β mRNA may be one of reasons for SGR inhibiting the activation of NLRP3 inflammatory in CCl$_4$ induced ALI in rats (fig. 6).

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