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Extraction optimization of *Angelica sinensis* polysaccharides and its antioxidant activity in vivo

Fang Yu[a,*], Huali Li[b], Yu Meng[a], Di Yang[a]

[a] *Medical college, DaLian University, DaLian City, 116600, PR China*
[b] *Norman Bethune College of Medicine, Jilin University, PR China*

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ABSTRACT

Extraction of *Angelica sinensis* polysaccharides (ASP) was optimized by the utilization of response surface methodology, RSM. Through the analysis, extraction time and water/solid were found to be the most significant factors. Based on contour plots and variance analysis, optimum operational conditions for maximizing polysaccharides yield (5.6%) were found to be extraction time 130 min, water/solid 5, and extraction number 5. *A. sinensis* polysaccharides (150 and 300 mg/kg) were administered for 15 days. The hepatoprotective activity was assessed using various biochemical parameters. Serum aspartate aminotransferase (AST), alanine aminotransfere (ALT) and alkaline phosphatase (ALP) levels were significantly restored toward normalization by the extracts (150 and 300 mg/kg body weight). ASP (150 and 300 mg/kg body weight) significantly increased the levels of antioxidant enzymes. It can be concluded that ASP possesses significant protective effect against hepatotoxicity induced by carbon tetrachloride (CCl_4). This protective effect appears due to ASP antioxidant properties.

1. Introduction

Angelica sinensis (Danggui), sometimes referred to by its Chinese name as Dong Quai, is the root of *A. sinensis* (Oliv.) Diels, an herbaceous perennial plant belonging to the Umbellfrae Family (Hsieh, Lin, Lin, & Wu, 2000). *A. sinensis* has been known for a long time for its effects of cleansing blood and increasing circulation and utilized as a valuable remedy for anemia, menstrual irregularities, and constipation in traditional Chinese medicine (Ji et al., 1999; Liu et al., 2001; Ye et al., 2001; Yim et al., 2000). It is also used for treatment of anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases (Mei, Tao, & Cui, 1991; Pharmacopeia of the People's Republic of China, 2000; Tang & Eisenbrand, 1992). It is recorded that 70 formulae in China and 56 formulae in Japan contain Danggui (Huang & Song, 2001; Pharmacopeia of the People's Republic of China, 2000). Besides the common usage in Asia, Danggui is also used as a health food product for women's care in Europe and America. Therefore, the demand for Danggui is enormous throughout the world (Sarker & Nahar, 2004).

Liver is a vital organ where key metabolic events occur. Alcohol, drug use, and biological and chemical agents cause damage to the liver. If the damage incurred is not regenerated or repaired, then the normal structure of the liver is disturbed and liver functions begin to be hindered (Wolf, 1999). Many chemical agents such as CCl_4, acetaminophen, thioacetamide and polycyclic aromatic hydrocarbons cause liver damage in humans and animals (Siegers, Heger, Baretton, & Younes, 1988). CCl_4 is widely used to induce liver damage experimentally (Aleynik, Leo, Ma, Aleynik, & Lieber, 1997). Carbon tetrachloride (CCl_4), frequently used as a model hepatotoxic agent in test animals (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2007; Yang et al., 2008), is particularly damaging to humans, affecting the liver and others organs through the release of a harmful free radical—carbon trichloromethyl—generated by the phase I metabolizing enzyme cytochrome P450 2E1 (Wong, Chan, & Lee, 1998; Yu et al., 2002). Administration of CCl_4 to the liver results in cirrhosis, hepatocyte lesions, and necrosis (Song et al., 2007) through membrane lipid peroxidation (Lima et al., 2007) and inflammatory processes (Decker, 1990; Gonzalez-Périz et al., 2006). The inflammatory events cause neutrophil infiltration that releases free radicals, contributing to further inflammatory exacerbations during liver injury (Houle, Papez, Ferazzini, Hollenberg, & Vergnolle, 2005; Sener, Topaloglu, Sehirli, Ercan, & Gedik, 2007). The end products of lipid peroxidation, malondialdehyde (MDA), 4-hydroxinonenal, and other free radicals, can enter systemic circulation and affect other organs (Weber, Boll, & Stampfl, 2003).

We investigated the protective capacity of *A. sinensis* polysaccharides against CCl_4 induced liver oxidative injury in rats.

* Corresponding author. Tel.: +86 411 84412001; fax: +86 411 84412001.
E-mail address: fangyu1153@sina.com (F. Yu).

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Table 1
Coded level combinations for a three-variable Box–Behnken design.

Run	Extraction time	Water/solid	Extraction number	Extraction percent (%)
1	−1.00 (90 min)	−1.00 (3)	0.00 (5)	3.81
2	1.00 (130 min)	−1.00	0.00	4.27
3	−1.00	1.00 (5)	0.00	4.31
4	1.00	1.00	0.00	5.83
5	−1.00	0.00 (4)	−1.00 (4)	4.07
6	1.00	0.00	−1.00	5.22
7	−1.00	0.00	1.00 (6)	4.1
8	1.00	0.00	1.00	5.32
9	0.00 (110 min)	−1.00	−1.00	4.04
10	0.00	1.00	−1.00	5.26
11	0.00	−1.00	1.00	4.03
12	0.00	1.00	1.00	5.28
13	0.00	0.00	0.00	5.05
14	0.00	0.00	0.00	5.04
15	0.00	0.00	0.00	5.09
16	0.00	0.00	0.00	5.06
17	0.00	0.00	0.00	5.07

2. Material and method

2.1. Preparation of the polysaccharides extract of Angelica sinensis

A. sinensis were collected in a local herb shop (Dalian city, China). Roots of *A. sinensis* were cut from the plant for extract production. The material weighting about 200 g was cleaned, extracted with 1000 ml of distilled water at boiling point, and concentrated to a final volume of 100 ml. Decoct was frozen and stored at −20 °C for subsequent lyophilization.

2.2. Optimization of Angelica sinensis polysaccharides

A number of factors such as extraction time, water/solid and extraction number subjected to extraction can significantly affect the polysaccharides extraction efficiency. Therefore, a standard RSM design (Box–Behnken) was used to identify the relationship between the response function (total amount of polysaccharides extracted) and the process variables (extraction time, water/solid and extraction number). Basing on our pre-test results, the experimental range of the selected process variables with their units and notation is given in Table 1. The response variable, extraction yield can be expressed as a function of the independent process variables according to the following response surface quadratic model:

$$R = \beta_0 = \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=1+1} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where β_0 is the constant coefficient, β_i, β_{ii}, β_{ij} are the coefficients for the linear, quadratic and interaction effect, x_i and x_j are the independent variables and ε is the error. A total of 17 experiments were performed in duplicate according to the Box–Behnken matrix in Table 2 and the average values were used in data analysis. The experimental data were analyzed by the software, Design Expert Version 7.1.6 (Stat-Ease, USA). The adequacy of the developed model and statistical significance of the regression coefficients were tested using the analysis of variance (ANOVA).

2.3. CCl_4-induced liver damage

SD rats weighing 160–180 g were housed in conventional cages with free access to water and rodent chow at 20–22 °C with a 12-h light–dark cycle. All procedures involving laboratory animal use were in accordance with the guidelines of the Instituted Animal Care and Use Committee of Our University for the care and the use of laboratory animals.

Animals were divided into four groups consisting of eight rats each. Animals of group I received 0.5%, w/v, carboxymethyl cellulose and treated as vehicle control. Group II to V received CCl_4 (20% CCl_4 in corn oil; 1 ml/kg body weight) intraperitoneally for every 72 h until the animal is inducted and this induction was confirmed by biochemical analysis. Group II received only carbon tetrachloride is considered as diseased group. Group III and IV received ASAE orally at the dose of 150 and 300 mg/kg body weight respectively. After 15 days of treatment, rats were anaesthetized and blood was collected from the retro-orbital sinus, and the serum was separated for assessment of different enzyme activities. The rats were then sacrificed by bleeding and the liver was carefully dissected, cleaned of extraneous tissue. Liver tissue samples were taken from the left liver lobe, and cut into two pieces. One piece was fixed in formalin for pathological examination. The other piece was utilized for the following biological analyses. Liver homogenates (10%, w/v) were obtained in 50 mM phosphate buffer (pH 7.0) and stored at −80 °C for analysis within 2 weeks.

Table 2
Regression coefficients of predicated second-order polynomial model for the response variable and analysis of the variance (ANOVA) for the fit of the experimental data to response surface model.

Source	Sum of squares	*df*	Mean square	*F* Value	*p*-Value Prob > *F*	
Model	5.91	9	0.66	106.19	<0.0001	Significant
A–A	2.37	1	2.37	382.34	<0.0001	
B–B	2.57	1	2.57	414.64	<0.0001	
C–C	2.450E−003	1	2.450E−003	0.40	0.5491	
AB	0.28	1	0.28	45.41	0.0003	
AC	1.225E−003	1	1.225E−003	0.20	0.6698	
BC	2.250E−004	1	2.250E−004	0.036	0.8542	
A2	0.24	1	0.24	39.53	0.0004	
B2	0.30	1	0.30	48.16	0.0002	
C2	0.087	1	0.087	14.02	0.0072	
Residual	0.043	7	6.186E−003			
Lack of fit	0.042	3	0.014	37.68	0.0022	Significant
Pure error	1.480E−003	4	3.700E−004			
Cor total	5.96	16				
Std. dev.	0.079		*R*-Squared		0.9927	
Mean	4.76		Adj *R*-squared		0.9834	
C.V. %	1.65		Pred *R*-squared		0.8873	
Press	0.67		Adeq precision		36.801	

Table 3
Effect of ASP on serum AST, ALT, ALP, TP, ALB and A/G.

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	TP (mg/L)	ALB (mg/L)	A/G
Control	29.46 ± 1.62	89.36 ± 9.03	92.63 ± 8.08	77.82 ± 8.39	43.23 ± 5.03	1.22 ± 0.14
CCl_4	71.61 ± 6.41[b]	175.72 ± 16.39[b]	213.87 ± 22.84[b]	69.21 ± 7.29[a]	33.15 ± 2.84[a]	0.83 ± 0.09[b]
CCl_4 + ASP (150 mg/kg)	57.93 ± 3.59[d]	153.93 ± 16.72[d]	173.96 ± 18.92[d]	75.16 ± 8.37[c]	39.04 ± 4.41[c]	0.95 ± 0.07[c]
CCl_4 + ASP (300 mg/kg)	36.85 ± 2.22[d]	121.23 ± 11.71[d]	131.11 ± 14.09[d]	76.94 ± 6.61[c]	42.17 ± 4.89[d]	1.06 ± 0.08[d]

[a] $p < 0.05$, compared with control group.
[b] $p < 0.01$, compared with control group.
[c] $p < 0.05$, compared with CCl_4 group.
[d] $p < 0.01$, compared with CCl_4 group.

2.4. Biochemical assay

Serum was collected as mentioned above. ALT, ALP, TP ALB and AST activities were then determined under the manufacturer's instruction (Biovision US).

Serum NO and NOS levels were determined using the enzyme immunoassay (EIA) kit.

The tissues malonaldehyde (MDA) concentration was determined using the method described by Jain, McVie, Duett, and Herbst (1989), based on TBA reactivity. Briefly, 0.2 ml supernatant obtained from tissues, 0.8 ml phosphate buffer (pH 7.4), 0.025 ml BHT and 0.5 ml 30% TCA were added to the tubes and mixed. After 2 h incubation at −20 °C, the mixture was centrifuged (4000 × g) for 15 min. After this, 1 ml supernatant was taken and added to each tube, and then 0.075 ml of 0.1 M EDTA and 0.25 ml of 1% TBA were added. These tubes with Teflon-lined screw caps were incubated at 90 °C in a water bath for 15 min and cooled to room temperature. The optical density was measured at 532 nm for tissues MDA concentration (Novaspec II Pharmacia-Biotech, Biochrom Ltd., UK).

The tissues glutathione (GSH) concentration was measured using the method described by Beutler, Dubon, and Kelly (1963). Briefly, 0.2 ml fresh supernatant was added to 1.8 ml distilled water. Three milliliters of the precipitating solution (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml distilled water) was mixed with haemolysate. The mixture was allowed to stand for approximately 5 min and then filtered (Whatman N 42). Two milliliters of filtrate was taken and added into another tube, and then 8 ml of the phosphate solution (0.3 M disodium phosphate) and 1 ml DTNB were added. A blank was prepared with 8 ml of phosphate solution, 2 ml diluted precipitating solution (three parts to two parts distilled water), and 1 ml DTNB reagent. A standard solution of the glutathione was prepared (40 mg/100 ml). The optical density was measured at 412 nm in the spectrophotometer.

The activity of superoxide dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 ml reaction mixture contained 100 mM Tris/HCl (pH 7.8), 75 mM NBT, 2 μM riboflavin, 6 mM EDTA, and 200 μL of supernatant. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% as described by Winterbourn, Hawkins, Brian, and Carrell (1975).

The activity of catalase (CAT) was measured according to the method of Aebi (1974). The reaction mixture (1 ml) that contained 0.78 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of liver supernatant, and 0.02 ml of H_2O_2 (0.5 M) was prepared. The reaction was started by adding H_2O_2 and decomposition was monitored by following the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated by using an extinction coefficient of 0.043 $mM^{-1}\,cm^{-1}$.

Glutathione peroxidase (GSH-Px) activity was analyzed by a spectrophotometric assay. A reaction mixture consisting of 1 ml of 0.4 M phosphate buffer (pH 7.0) containing 0.4 mM EDTA, 1 ml of 5 mM NaN_3, 1 ml of 4 mM GSH, and 0.2 ml of supernatant was preincubated at 37 °C for 5 min. Then 1 ml of 4 mM H_2O_2 was added and incubated at 37 °C for further 5 min. The excess amount of GSH was quantified by the DTNB method as described by Sharma and Gupta (2002). One unit of GSH-Px is defined as the amount of enzyme required to oxidize 1 nmol GSH/min.

2.5. Histological analysis

On day 21, when the animals were sacrificed, the livers tissues were removed and stored in 10% formalin after washing with normal saline. The tissues embedded in paraffin and sectioned with 5 μm thickness and then stained with hematoxylin eosin for microscopic assessment.

2.6. Statistical analysis

Values were expressed as mean + SEM. To evaluate differences between the studied groups, one way analysis of variance (ANOVA) with LSD post hoc test was used to compare the group means and $p < 0.05$ was considered statistically significant. SPSS, for Windows (Version 15.0) was used for statistical analysis.

3. Results and discussion

The experiment was conducted by using a three-level three-factor Box–Behnken design with four replicates at the central point. The coded and actual levels of the three variables in Table 1 were selected to maximize the extraction yield of total polysaccharides. The regression coefficients of both the parameters were high and lack of fit statistic was significant ($p < 0.05$). These results along with high F-values indicate that the models for extraction yield values were significant ($p < 0.0001$) and adequate.

The contour and three-dimensional plots presented in Fig. 1 were produced for each pair of factors, whereas the third factor was taken as a constant at its middle level. Results from Fig. 1 further confirm analysis of variance. During the polysaccharides extraction, the extraction time and water–solid ratio are important. Therefore, the best combinations of process variables for response functions are found. The process variables for best combination of response function are extraction time 130 min, extraction number 5, and water–solid ratio 5.

$$R_1 = +5.06 + 0.54 * A + 0.57 * B + 0.018 * C + 0.27 * A * B + 0.018 * A * C + 7.500E - 003 * B * C - 0.24 * A^2 - 0.27 * B^2 - 0.14 * C^2$$

The AST, ALT and ALP secretion reflected the specific function of liver cell. Serum ALT activity was the most frequently relied upon laboratory indicator of hepatotoxic effects (Amacher, 1998, 2002). As a general rule, ALT levels greater than 3 times the upper limits of normal had been identified as a marker for liver injure (Holt & Ju, 2006). ALP was primarily a marker of hepatobiliary effects and

Table 4
Effect of ASP on serum NO, NOS, MDA and GSH levels.

Group	NO (μmol/L)	NOS (U/L)	MDA	GSH
Control	17.38 ± 1.32	38.07 ± 1.32	3.95 ± 0.26	281.52 ± 19.84
CCl_4	10.69 ± 0.92[b]	30.14 ± 1.59[b]	6.71 ± 0.37[b]	173.24 ± 9.38[b]
CCl_4 + ASP (150 mg/kg)	13.57 ± 0.78[d]	35.18 ± 1.72[c]	5.59 ± 0.29[c]	225.16 ± 13.27[d]
CCl_4 + ASP (300 mg/kg)	14.82 ± 0.91[d]	36.92 ± 1.64[d]	4.28 ± 0.18[d]	269.05 ± 15.05[d]

[b] $p < 0.01$, compared with control group.
[c] $p < 0.05$, compared with CCl_4 group.
[d] $p < 0.01$, compared with CCl_4 group.

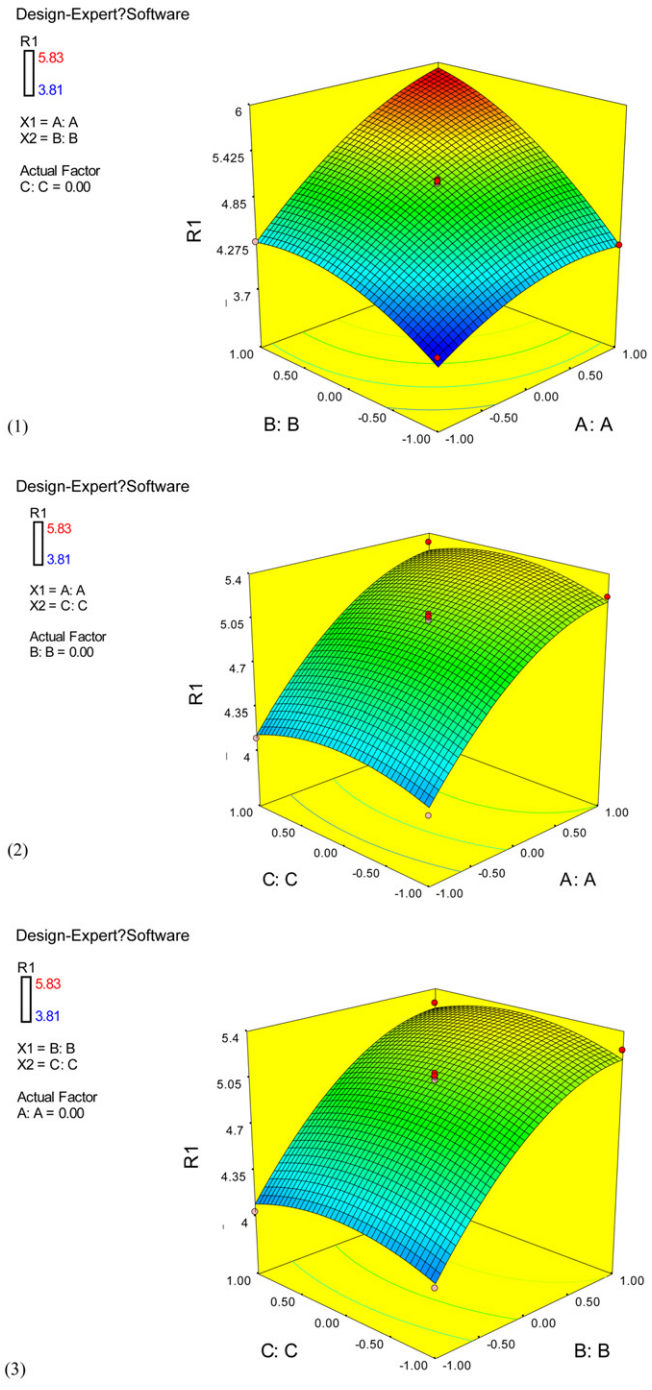

Fig. 1. Response surface showing the effect of extraction time, water/solid and extraction number on polysaccharides yield.

cholestasis (Ramaiah, 2007). In humans, increased alkaline phosphatase levels had been associated with drug induced cholestasis (Wright & Vandenberg, 2007).

The activities of AST, ALT, and ALP were significantly increased in plasma of rats treated with CCl_4 (Table 3), whereas the levels of TP, ALB and A/G were significantly decreased. This indicated that CCl_4 induced severe cell membrane damage and significant enzyme secretion. The changes of TC, PL and TG in this study also reflected damage to hepatocytes. Treatment of *A. sinensis* polysaccharides significantly decreased plasma AST, ALT, and ALP activities in a dose-dependent manner (CCl_4 + ASP groups). *A. sinensis* aqueous extract inhibited the AST, ALT, and ALP leakage simultaneously and improved liver function.

Nitric oxide (NO) is a highly reactive oxidant that is produced through the action of iNOS and plays an important role as a vasodilator, neurotransmitter and in the immunologic system as a defense against tumor cells, parasites and bacteria (Lee et al., 2007). iNOS is one of potential downstream targets of TNF-α for the of hepatocyte proliferation (Salkowski, Detore, McNally, van Rooijen, & Vogel, 1997). Indeed, hepatic expression of iNOS is critical for liver repair in various liver injury models (Yaylak et al., 2008; Zeini, Hortelano, Traves, Martin-Sanz, & Bosca, 2004). iNOS produces NO from L-arginine; NO production is associated with a shift from hepatocyte quiescence to proliferation during the priming phase of liver regeneration (Yaylak et al., 2008; Zeini et al., 2004).

NO levels and NOS activities in the livers of control and tested groups are shown in Table 4. In CCl_4 group, a significant decrease of NO levels and NOS activities was observed in plasma (39% and 21%), as compared to those of control group. In CCl_4 + ASP group, *A. sinensis* aqueous extract treatment dose-dependently significantly enhanced NO levels and NOS activities in plasma as compared to those of CCl_4 group.

The factors and mechanisms involved in hepatic damage have been extensively studied, such as alcoholism, intoxication, and immunodeficiency. The chemical-induced hepatic damage, especially using the inducer of CCl_4, is one of the popular models to investigate the chemopreventive function of phytochemicals or nutraceuticals in the food-related research field. In addition, a great quantity of free radicals were formed that accompanied with the metabolism of CCl_4. Oxidative stress plays a crucial role in the development of CCl_4-induced hepatotoxicity (Sureshkumar & Mishra, 2006), and a connection between oxidative stress and lipid peroxidation has been reported (Kota, Krishna, & Polasa, 2008). Numerous studies noted that CCl_4 is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P450, generating a highly reactive carbon-centered trichloromethy radical, leading to initiating a chain of lipid peroxidation and thereby causing liver fibrosis (Aleynik et al., 1997; Ashok, Somayaji, & Bairy, 2001; Bahceioglu et al., 1990; Fang, Lai, & Lin, 2008; Halliwell & Gutteridge, 1998; Weber et al., 2003). Based on the free radical theory, it is one of the possible strategies to inhibit or mitigate the damage that induced by toxic radical. That is the reason we evaluated the

Table 5
Effect of ASP on liver SOD, CAT and GSH-Px.

Group	SOD (U/mg)	CAT (U/mg)	GSH-Px (U/mg)
Control	241.28 ± 19.83	51.28 ± 3.18	49.08 ± 2.17
CCl_4	128.19 ± 10.27[b]	21.67 ± 1.42[b]	20.14 ± 1.32[b]
CCl_4 + ASP (150 mg/kg)	189.27 ± 12.06[d]	33.29 ± 1.72[d]	32.49 ± 1.74[d]
CCl_4 + ASP (300 mg/kg)	231.05 ± 16.11[d]	46.03 ± 2.66[d]	43.81 ± 2.05[d]

[b] $p < 0.01$, compared with control group.
[d] $p < 0.01$, compared with CCl_4 group.

antioxidant and hepatoprotective efficacy of *A. sinensis* aqueous extract.

MDA and GSH levels in livers are presented in Table 4. MDA is a product of lipid peroxidation. As expected, a significant increase in MDA levels were observed in CCl_4 group compared to those of control group. *A. sinensis* polysaccharides treatment induced a significant decrease of MDA in livers compared to CCl_4 group.

CCl_4 not only initiates lipid peroxidation but also reduces tissue GSH-Px, CAT and SOD activities, and this depletion may result from oxidative modification of these proteins (Augustyniak, Wazkilwicz, & Skrzydlewaka, 2005). SOD, CAT and GSH-Px activities in livers are presented in Table 5. A significant decrease in SOD, CAT and GSH-Px activities in livers was observed in CCl_4 group compared to those of control group. Biochemical analyses showed a significant increase of the main enzymatic antioxidant defences (SOD, GSH-Px and CAT) in livers of CCl_4-treated rats (CCl_4 + ASP).

The light microscopy examination of the transverse section of control rat liver clearly illustrates complete hepatic lobules with well formed hepatocytes with distinct portal triads. Hepatic cells were arranged in cord like fashion, which are separated by sinusoids and central vein was seen clear. The liver sections of CCl_4 intoxicated rats showed massive fatty changes, necrosis, ballooning and degeneration in hepatic plates and loss of cellular boundaries. Treatment with the aqueous extract was effective in restoring the CCl_4 induced histopathological lesions when compared to CCl_4 per se, however highest dose was found to be more effective.

4. Conclusion

In summary, our results indicate that treatment with ASP after the establishment of CCl_4-induced hepatic oxidative damage significantly reduces and even reverses the oxidative injury in rats. This effect is related to an increased removal of lipid peroxidation and enhanced antioxidant enzymes activities.

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