Original Article

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The exopolysaccharide produced by Pantoea sp. BCCS 001 GH provides hepatoprotection in a rat model of bile duct obstruction

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Abstract

Liver injury is a severe clinical complication associated with various diseases or xenobiotics exposure. Hence, finding safe and clinically applicable hepatoprotective agents have great value. Several naturally-derived chemicals have gotten attention for their biological functions. Polysaccharides are bioactive and safe chemicals produced by a variety of microorganisms. Several exciting features, including radical scavenging and antioxidative properties, have been attributed to polysaccharides. Recently we found that the exopolysaccharide derived from Pentoea sp. BCCS 001 GH (Pentoan exopolysaccharide; PEPS) revealed significant antioxidant and radical scavenging properties in an in vitro model. Hence, the current study was designed to evaluate the in vivo hepatoprotective effects of PEPS. Bile duct ligated (BDL) rats received PEPS (0.05 and 0.1% w: v in drinking water), and serum biomarkers of liver injury, liver tissue histopathological alterations, and hepatic markers of oxidative stress were monitored. Severely elevated serum biomarkers of liver injury and histopathological changes, including inflammatory cell infiltration, necrosis, bile duct proliferation, and tissue fibrosis, were evident in BDL animals. Moreover, a significant amount of reactive oxygen species, increased level of lipid peroxidation, and defects in tissue antioxidant capacity were apparent in BDL rats. It was found that PEPS significantly improved liver function, blunted hepatic pathological changes, and counteracted oxidative stress in the liver tissue. The radical scavenging and antioxidant properties of PEPS seem to play a fundamental role in its hepatoprotective properties.

Keywords: Bile acid; Cholestasis; Fibrosis; Hepatoprotection; Oxidative stress.

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1. Introduction

Bile duct obstruction, cholestasis, is accompanied by the accumulation of several toxic chemicals in the liver. Bile acids and bilirubin are among the most cytotoxic chemicals affecting liver function (1, 2). Several mechanisms could be involved in the progression of liver impairment during cholestasis (1, 2). Severe oxidative stress is a well-known phenomenon in the liver of cholestatic animals (3, 4). Defects in tissue antioxidant capacity, reactive oxygen species formation, biomembrane lipids disruption, and cellular protein structure changes are attributed to cholestasis-induced oxidative stress in the liver (1, 4). Therefore, the

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administration of radical scavenging and antioxidant agents might be protective in this model.

Polysaccharides are polymeric compounds composed of a long chain of monosaccharides. Several organisms synthesize these compounds. Polysaccharides could be a part of an organism (e.g., attached to the cell surface) or released into the surrounding environment (5). A variety of microorganisms synthesize exopolysaccharides (EPSs) or extracellular polysaccharides. These compounds are located out of the organisms. Therefore, their separation and purification processes are more convenient than intracellular or structural polysaccharides. Hence, EPSs have found a plethora of applications in the food and pharmaceutical industries (6, 7). It has been well-documented that polysaccharides are bioactive compounds (8, 9). Different biological actions, including antitumor, antiaging, immunomodulatory, and anti-diabetic properties, have been attributed to polysaccharides (8-10). On the other hand, several studies mentioned the antioxidant properties of polysaccharides as their key mechanism of cytoprotection (6, 11). Interestingly some of these polysaccharides showed antioxidant and radical scavenging capacity similar to standard antioxidant molecules such as vitamin C (6).

Recently, we found that PEPS could significantly scavenge reactive oxygen species (ROS) in an in vitro environment. The current study evaluated the effects of PEPS supplementation in an in vivo model of liver injury associated with chronic oxidative stress. Changes in serum biomarkers of liver injury, hepatic tissue histopathological alterations, and oxidative stress markers in the liver were monitored to investigate the hepatoprotective effects of PEPS.

2. Material and methods

2.1. Chemicals and reagents

2′,7′ Dichlorofluorescein diacetate (DCFH-DA), citric acid, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 4,2 Hydroxyethyl,1-piperazine ethane sulfonic acid (HEPES), dithiobis-2-nitrobenzoic acid, ethylenediaminetetraacetic acid (EDTA), reduced glutathione, malondialdehyde, meta-phosphoric acid, thiobarbituric acid, and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydroxymethyl aminomethane hydrochloride (Tris-HCl), potassium chloride, and n-butanol were purchased from Merck (Darmstadt, Germany). Kits for evaluating serum biomarkers of liver injury were purchased from Pars-Azmoon® Co. (Tehran, Iran).

2.2. Animals

Male Sprague Dawley rats (250-300 g; n = 24) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in polypropylene cages over wood-chip bedding in a standard environment (temperature of 23±1 ºC, a 12L: 12D photo schedule, and ≈40% relative humidity). Rats were allowed free access to a normal standard rodents' chow diet (RoyanFeed®, Isfahan, Iran) and tap water. Laboratory animal care and use were performed according to the guidelines approved by the ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1397.1051).

2.3. Bile duct obstruction surgery

Bile duct ligation (BDL) is an appropriate animal model to investigate the principal mechanisms, such as oxidative stress, in the pathogenesis of liver injury (12, 13). Moreover, the effects of therapeutic interventions on significant histopathological alterations (e.g., tissue fibrosis) could be readily monitored in the BDL model (14-16). For the BDL surgery, rats were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p), and a midline incision through the linea alba was made. The common bile duct was localized and doubly ligated (17-19). The sham operation involved laparotomy and bile duct manipulation without ligation.

2.4. Experimental setup

Rats were randomly allocated into four groups (n = 6/group). Animals were treated as follows: 1) Sham-operated (Vehicle-treated; tap water); 2) BDL; 3) BDL + PEPS (0.05% v: v in drinking water); 4) BDL + PEPS (0.1% v: v in drinking water). Water intake (milliliters) was measured daily, and tissue and serum samples were collected on day 14 after the BDL operation (20).

2.5. Serum biochemistry, tissue histopathology, and organ weight index

Blood samples were collected from the abdominal vena cava of the deeply anesthetized rats (thiopental, 90 mg/kg, i.p). Samples were transferred to gel-coated standard tubes (Vacutest® Kima; Italy), and serum was prepared by centrifugation (3000 g, 4 ºC, 20 min). A Mindray BS-200® autoanalyzer (Guangzhou, China) and commercial kits (Pars Azmoon®, Tehran, Iran) were used to evaluate serum biochemistry (21-23). For tissue histopathology evaluation, liver samples were fixed in buffered formalin solution (0.4% sodium phosphate monobasic, 0.64% sodium phosphate dibasic, and 10% formaldehyde in distilled water). Then, the paraffin-embedded tissue was prepared, and tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) (24). Previously reported scoring systems were used to monitor liver histopathological changes in the BDL animals (25). Liver fibrosis was assessed using Masson's trichrome staining and scored on the Ishak system to evaluate fibrotic tissue changes (26). A pathologist blindly analyzed samples. For evaluating organ weight indices, the liver and spleen were weighed, and the organ weight index was determined according to the formulae: Organ weight index = [Wet weight of organ (g)/ Bodyweight (g)]×100.

2.6. Reactive oxygen species (ROS) formation in the liver

Reactive oxygen species in the liver of BDL rats were estimated based on a previously described procedure using 2′, 7′ dichlorofluorescein diacetate (DCF) as a probe (27). Briefly, liver sample (500 mg) was homogenized in 5 mL of ice-cooled (4 ºC) Tris-HCl buffer (40 mM, pH=7.4). Then, samples of the resulted tissue homogenate (100 µL) were mixed with 900 µL of Tris-HCl buffer (40 mM, pH=7.4) and 10 µL of DCF (Final concentration of 10 µM). The mixture was incubated in the dark (10 min, 37ºC) (28-31). Finally, the fluorescence intensity was assessed using a FLUOstar Omega® plate reader (BMG Labtech®, Offenburg, Germany) at λ excit=485 nm and λ emiss=525 nm (27).

2.7. Lipid peroxidation in the liver tissue

The thiobarbituric acid reactive substances (TBARS) were measured as an index of lipid peroxidation (27). For this purpose, 500 µL of tissue homogenate (10% w: v in KCl, 1.15% w: v) was added to 2 mL of TBARS assay reaction mixture (consisted of 0.375%, w: v thiobarbituric acid and 1% w: v metaphosphoric acid in double-distilled water, pH=2), vortexed (10 sec), and heated in a water bath (100 °C, 45 min). After the incubation period, samples were cooled, and 2 mL of n-butanol was added. Then, samples were mixed well (30 sec) and centrifuged (12000 g, 5 min) (27). Finally, the absorbance of developed color in the upper phase (n-butanol) was measured (λ = 532 nm; EPOCH® plate reader, BioTek®, USA) (27).

2.8. Hepatic glutathione content

For assessing hepatic GSH content, 5 mL of the liver homogenate (10% w: v in 40 mM Tris-HCl buffer, four °C) was added to 4 mL of deionized water (4 °C) and 1 mL of trichloroacetic acid (50%; w: v in double-distilled water). The mixture was vortexed and centrifuged (10,000 g, four °C, 15 minutes). Then, 2 mL of the supernatant was combined with 4mL of Tris-HCl buffer (40 mM, pH=8.9, 4ºC) and 100 µl of freshly-prepared DTNB solution (10 mM in methanol) (27). Finally, the absorbance was measured (λ=412 nm, EPOCH® plate reader, BioTek®, USA).

2.9. Ferric reducing antioxidant power (FRAP)

The FRAP assay was used to estimate the total antioxidant capacity of the liver tissue (27). For this purpose, the working FRAP reagent was freshly prepared by mixing ten volumes of acetate buffer (300 mM, pH=3.6), one volume of TPTZ (10 mM in 6N HCl), and one volume of ferric chloride (20 mM). Then, 50 µL of tissue homogenate (10% w: v in 40 mM Tris-HCl, pH=7.4, 4 ºC) and 150 µL of deionized water was added to 1.5 mL of the FRAP reagent (27). The mixture was incubated for five minutes (37 ºC, in the dark). Finally, the absorbance of the developed color was measured (λ=595 nm, EPOCH® plate reader, BioTek®, USA) (27).

Table 1. Serum biomarkers of bile duct injury in cholestatic animals.

Treatment	Serum ALP (U/l)	Serum Bilirubin (mg/dl)	Serum γGT (IU/l)
Sham-operated	1334±146	0.1±0.01	28±5.9
BDL	3741±173 ***	8.1±0.60 ***	242±34 ***
BDL + PEPS 0.05 %	3282±364 ns	7.8±0.54 ns	181±27 ns
BDL + PEPS 0.1%	3534±256 ns	6.5±0.60 ns	205±31 ns

Data are given as mean ± SD (n = 6). BDL: Bile duct ligation; PESP: Penthoan exopolysaccharide.
***Indicates significantly different from the control group (P < 0.001).
ns: not significant as compared with the BDL group.

2.10. Preparation and purification of PEPS

The microorganism (Pantoea sp. BCCS 001 GH) and culture conditions, as well as the preparation, purification, and physicochemical characterizations of PEPS used in the current study, are widely described in our previous investigations on this polysaccharide (32, 33). The purified PEPS was used in the present study.

2.11. Statistical analysis

Data are represented as mean±SD. Comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparison test as the post hoc. Liver tissue histopathological scores are shown as median and quartiles for five random pictures per group. The analysis of liver histopathological changes was performed by the Kruskal–Wallis followed by the Mann-Whitney U test. A P 0.05 was considered a statistically significant difference.

3. Results

Serum biomarkers of bile duct injury were evaluated to ensure that cholestasis was induced by the BDL operation (Table 1). In this context, severe elevation in serum γGT, bilirubin, and ALP levels indicate bile duct obstruction in the BDL group (Table 1). On the other hand, no significant changes in serum biomarkers f bile duct injury were detected when BDL animals were supplemented with PEPS (0.05 and 0.1%) (Table 1).

Signs of hepatomegaly and splenomegaly were evident in the BDL group (Figure 1). It was found that PEPS, at both doses of 0.05 and 0.1%, significantly improved the liver and spleen weight index in BDL rats (Figure 1). The effect of PEPS on the liver and spleen weight index was not dose-dependent in the current model (Figure 1).

Serum biomarkers of liver injury were significantly raised in the BDL group (Figure 2). On the other hand, both doses of PEPS (0.05 and 0.1%) significantly decreased markers of liver injury in the serum of BDL animals (Figure 2). The effect of PEPS on serum biomarkers of liver injury was not dose-dependent in the current study (Figure 2).

Oxidative stress biomarkers, including ROS formation, lipid peroxidation, and depleted liver tissue GSH, were evident in BDL rats (Figure 3). Moreover, liver tissue antioxidant capacity was significantly impaired in the BDL group compared to control animals (Figure 3). It was found that PEPS (0.05 and 0.1% w: v) significantly mitigated oxidative stress markers in BDL animals (Figure 3). The effect of PEPS on liver tissue biomarkers of oxidative stress was also not dose-dependent in the current investigation (Figure 3).

Liver histopathological alterations in the BDL rats included bile duct proliferation, necro-

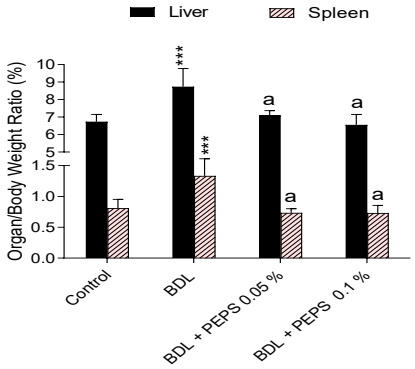

Figure 1. Organ weight index in bile duct ligated (BDL) animals (14 days after BDL operation). PEPS: Pentoan exopolysaccharide. Data are given as mean ± SD (n = 6). *** Indicates significantly different from the control group (P < 0.001). a Indicates significantly different as compared with the BDL group (P < 0.01).

Table 1. Scores of liver histopathological alterations in bile duct ligated (BDL) rats.

Treatment	Bile duct proliferation	Necrosis	Inflammation	Fibrosis
Sham-operated	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
BDL	3 (1, 2) #	1 (1, 1) #	3 (2, 2) #	3 (1, 2) #
BDL + PEPS 0.05 %	3 (1, 2) ns	0 (0, 0) a	2 (1, 1) a	1 (1, 1) a
BDL + PEPS 0.1%	3 (1, 2) ns	0 (0, 0) a	1 (0, 1) a	1 (0, 1) a

0 = absent; 1 = mild; 2 = moderate; and 3 = severe histopathological changes. PEPS: Pentoan exopolysaccharide.
Data are shown as median and quartiles for five random pictures per group.
# Indicates significantly different from the sham-operated group ($P < 0.05$).
a Indicates significantly different compared with the BDL group ($P < 0.05$).
ns: not significant as compared with the BDL group.

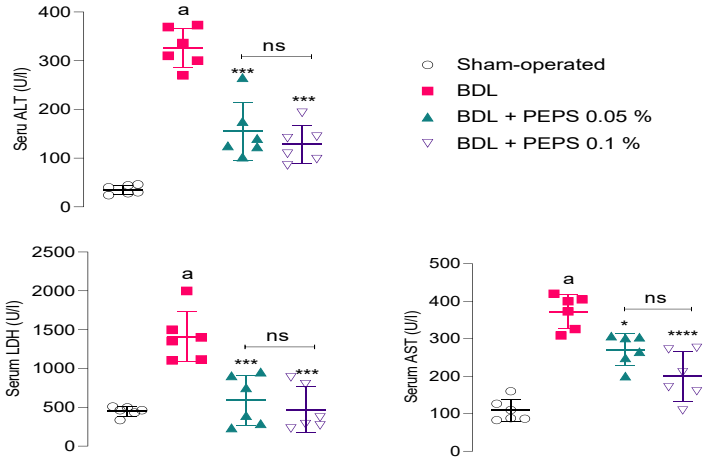

Figure 2. Serum biochemical measurements in bile duct ligated (BDL) cholestatic rats. PEPS: Pentoan exopolysaccharide. Data are given as mean ± SD (n = 6). a Indicates significantly different as compared with the sham-operated group ($P < 0.05$). Asterisks indicate significantly different from the BDL group (* $P < 0.05$ and *** $P < 0.001$).

sis, and inflammatory cell infiltration (Figure 4 and Table 2). Moreover, the Trichrome stain revealed significant collagen deposition in the liver of cholestatic animals (Figure 5 and Table 2). It was found that both doses of PEPS significantly ameliorated tissue fibrosis in cholestatic rats (Figure 5 and Table 2).

Notably, no significant difference in daily water intake was detected between control and PEPS-treated animals (Data not shown).

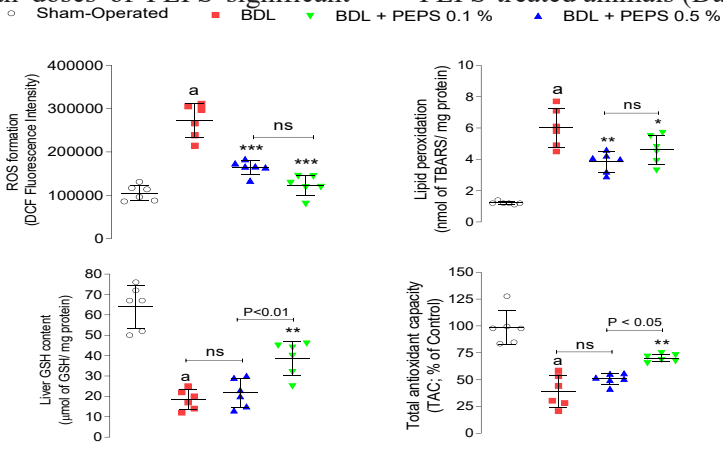

Figure 3. Liver tissue markers of oxidative stress in bile duct ligated (BDL) rats. PEPS: Pentoan exopolysaccharide. Data are given as mean±SD (n=6). a Indicates significantly different as compared with the sham-operated group ($P<0.05$). Asterisks indicate significantly different as compared with the BDL group (*$P<0.05$, **$P<0.01$ and ***$P<0.001$).

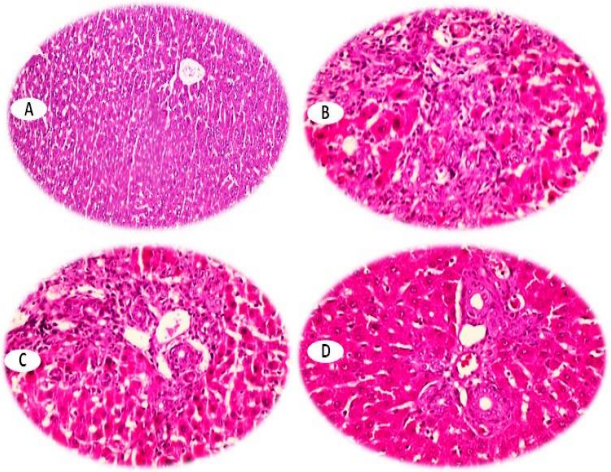

Figure 4. Liver tissue histopathological changes in bile duct ligated (BDL) rats. Hematoxylin and Eosin (H&E) stain revealed significant bile duct proliferation, inflammatory cells infiltration, and tissue necrosis in the BDL group. It was found that PEPS significantly decreased liver histopathological alterations (Table 2). A: Sham-operated; B: BDL rats; C and D: BDL rats treated with PEPS (0.05 and 0.1% respectively). PEPS: Pentoan exopolysaccharide.

4. Discussion

Cholestasis is a serious clinical complication that could lead to liver impairment (34). Hence, finding safe and clinically applicable agents could be of great value against this disorder. A plethora of evidence indicates the pivotal role of oxidative stress and its associated complications in the pathogenesis f cholestasis-induced liver injury(4); thus, several antioxidant molecules have been applied to mitigate oxidative stress in the liver during cholestasis (35). In the current study, we found an exopolysaccharide produced by Pantoea sp. BCCS 001 GH (Pentoan exopolysaccharide; PEPS) significantly improved liver function and counteracted hepatic oxidative stress in cholestatic rats. The effects of PEPS in mitigating oxidative stress seem to play a crucial role in its mechanism of hepatoprotection in cholestasis.

Various liver diseases have been identified in humans, leading to hepatic failure and patient death. Moreover, a wide range of xenobiotics could induce liver injury (36-38). Therefore, researchers always considered finding adequate and

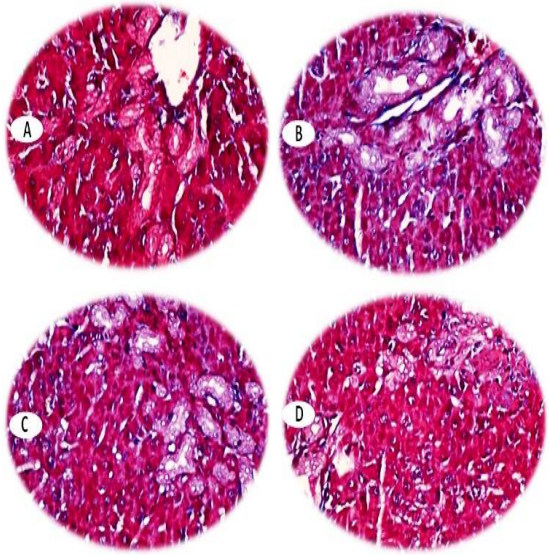

Figure 5. Trichrome stain revealed significant collagen deposition (blue area) in the liver of BDL rats (Table 2). It was found that PEPS significantly ameliorated tissue fibrosis in cholestatic animals (Table 2). A: Sham-operated; B: BDL rats; C and D: BDL rats treated with PEPS (0.05 and 0.1% respectively). PEPS: Pentoan exopolysaccharide.

safe hepatoprotective agents. On the other hand, it has been well documented that oxidative stress and its associated complications play a pivotal role in liver injury with different etiologies (39-41). BDL model is a reliable tool for investigating oxidative stress-induced liver injury and finding potential hepatoprotective agents (42-46). Cytotoxic bile acids are the primary culprits involved in the pathological changes of the liver in the BDL model (47). These compounds could affect several cellular targets, including proteins, lipids, and nucleic acid (48). Moreover, vital organelles such as mitochondria are also affected by bile acids (49). Oxidative stress is an essential mechanism of bile acid-induced liver injury during cholestasis (1, 3, 4, 50). The adverse effects of bile acids on hepatocytes' mitochondria seem to be a key mechanism for generating ROS and induction of oxidative stress (51). Therefore, several studies investigated the hepatoprotective properties of antioxidant molecules in the BDL model (14, 25, 42, 45, 46, 52, 53). Other molecules, such as manganese, are also involved in liver injury mechanisms during cholestasis (54, 55). Manganese is also able to induce significant oxidative stress in various organs (54, 56). Manganese could significantly facilitate mitochondria-mediated ROS formation and oxidative stress (54, 55).

Natural sources-derived chemicals have been considered protective agents against many human diseases (57-60). Polysaccharides are naturally-derived bioactive chemicals with radical scavenging and antioxidant capability (11, 61-63). Many biological actions, including antitumor, antiaging, immunomodulatory, and anti-diabetic properties, have been attributed to polysaccharides (8-10, 64, 65). PEPS is a heteropolysaccharide composed of glucose and galactose. The structural and physicochemical characterization of PEPS has been determined in our previous study (32, 33). Our data in an in vitro model indicated that PEPS could efficiently inhibit hydroxyl (OH•) and superoxide (O2•-) radicals (32, 33). Previous studies mentioned that the antioxidant properties of polysaccharides depend on their monosaccharide composition, degree of substitution, and glycosidic bonds (66). Therefore, changes in the structure of polysaccharides could alter their bioactivity (66). For example, phosphorylation of these compounds or adding molecules such as selenium to polysaccharides could enhance their biological activities (e.g., antioxidant properties) (66). These modifications might also enhance PEPS activity and provide better biological activity for this polysaccharide. Further studies on PEPS derivatives are needed to understand their cytoprotective properties.

The antioxidant properties of naturally-derived polysaccharides have repeatedly been mentioned (11, 61-63). Interestingly, it has been found that the liver tissue antioxidant capacity is significantly enhanced after polysaccharide treatment (11, 61-63). For example, it has been found that the polysaccharide derived from Flammulina velutipes greatly improved catalase activity in various tissues, including the liver (67). Catalase is an important enzyme involved in the homeostasis of cellular ROS. The current investigation found that PEPS significantly enhanced liver antioxidant capacity, decreased hepatic ROS levels, and prevented biomembrane damage (lipid peroxidation) in the BDL liver injury model. On the other hand, tissue fibrosis is a complicated process that finally leads to organ failure (68). The BDL animal model is an excellent tool for investigating the antifibrotic properties of various agents (69, 70). It is well known that liver fibrosis is mechanistically connected to oxidative stress (68, 71). In this context, a wide range of antioxidant molecules has been applied to blunt hepatic fibrosis (71, 72). In the current study, PEPS significantly alleviated the progression of hepatic fibrosis in the BDL animals (Fig.5 and Table 2). The antifibrotic properties of PEPS make this molecule an excellent candidate for managing a wide range of liver diseases associated with tissue fibrosis (e.g., fatty liver or alcoholic liver disease). Although further studies are needed to clarify the precise antifibrotic mechanisms of PEPS, based on the current study's data, this molecule's antioxidant activity could be directly related to its antifibrotic properties in the liver.

The extraction of exopolysaccharides could be easily set up on industrial scales (32, 33). Therefore, these complex polymeric carbohydrates could be produced as a value-added bioma-

terial (73, 74). Exopolysaccharides considerably impact the industry as hydrocolloids, bio-sourced materials, and antioxidants (75). Previously, we found that Pantoea sp. BCCS 001 GH could produce high amounts of exopolysaccharides in fermentation broth (32, 33). Hence, an effective and cheap antioxidant molecule could be produced for biomedical and industrial applications.

5. Conclusion

Collectively, PEPS provided significant hepatoprotection in the current study. The effects of PEPS on oxidative stress and its associated complications seem to play a crucial role in its hepatoprotective properties. These data indicate that PEPS could be a valuable natural antioxidant for various liver diseases connected with oxidative stress. Further studies are warranted to enhance our understanding of the biological effects of PEPS and its applications in biomedical sciences and food industries.

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Conflict of Interest

None declared.

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