

Original Research

Polysaccharide derived from pomelo seed coat ameliorates APAP-induced liver injury in hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀)

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ABSTRACT

Ever-increasingly Citrus wastes have been generating during the industrial processing, which is troublesome to dispose them for the considerations of economic feasibility and environmental protection. Meanwhile, liver disease, which causes liver damage, is the one of the major threats for the further development of aquaculture, especially in marine fish. The present study explored the hepatoprotective effect of PSCP, a polysaccharide extracted from the pomelo seed coat, in the primary hepatocytes of hybrid grouper (*Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀). PSCP displayed considerably scavenging effect to the free radicals and strong inhibitive effect to the erythrocyte hemolysis. In vitro, we added PSCP (0, 100, 200 and 400 µg/mL) to the primary hepatocytes before incubation with Acetaminophen (APAP) (12 mM). The CKK-8 experiment displayed the administration of PSCP (100 and 200 µg/mL) substantially mitigated the reduction of hepatocyte viability inflicted by APAP. Moreover, we found that PSCP effectively alleviated APAP-induced oxidative stress, as shown by the significantly reduced level of reactive oxygen species and the elevated enzymatic activities of superoxide dismutase, catalase, and glutathione peroxidase, which indicated its anti-liver injury effect ($P < 0.05$). The more direct signs associating with the hepatoprotection of PSCP are reflected in the results of hematoxylin and eosin staining, as evident by the morphological recovery following the addition of PSCP. In brief, these findings showed that the therapeutic potential of PSCP on APAP-induced fish liver injury, which not only provided a new prospect in treating the liver impairment in aquatic animals, but to also improve the utilization of pomelo fruitlets.

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1. INTRODUCTION

The increasing incidence of diseases in fish has been along with the rapid expansion of aquaculture and becomes a major obstacle for the further development of aquaculture [1]. In intensive aquacultural system, a large quantity of antibiotics and chemicals have been used for the prevention and control of fish diseases, which in turn increases the expose of xenobiotics to fish [2]. The liver in fish is susceptible to xenobiotic-induced injury for its critical role in xenobiotic metabolism, its portal location in the circulation of blood, and its anatomic and physiologic structures [3]. Numerous chemical compounds have showed the potential to liver injury [4, 5]. Presently, a fish liver injury called “liver and gall syndrome” in China, manifested by the color change and enlargement (up to twofold or more of original size) in liver, along with the characteristics of emaciation, poor growth performance and feeding efficiency, has caused a considerable loss to aquaculture

in China [6]. However, until now there is no effective approach to treat the “liver and gall syndrome” and the use of Herbal medicines has garnered more and more attention to treat the disease.

Recently, Treatments using plant polysaccharides for disease prevention and medical application have drawn considerable attention owing to their myriad pharmacological properties and few side effect [7]. In the case of liver disease in fish, many studies have reported that polysaccharides derived from plant were efficacious in inhibiting reactive oxidative stress, excessive fat ingestion or environmental factor-induced liver pathologies [8–10].

Acetaminophen (APAP) which has been widely used over 50 years as an analgesic and antipyretic agent [11]. Although APAP is generally considered to be safe at the appropriate doses, acute liver failure induced by APAP overdose is the most common cause in USA and United States and many other countries worldwide [12]. When APAP is overdose, sulfation and

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glucuronidation, the major metabolic pathway of APAP, readily saturate and most APAP undergoes the oxidation under the help of hepatic cytochrome P450 2E1 to a toxic intermediate, N-acetyl-p-benzoquinone imine (NAPQI) which is efficiently detoxified by glutathione (GSH). [13]. However, when approximate 70%-80% GSH is depleted for detoxication, NAPQI binds to hepatocyte, resulting cellular injury. This is the irreversible process to cause oxidative insult and hepatocellular necrosis [14]. Moreover, given the antioxidant capacity of GSH, the reduced GSH also contributes to the accumulation of intracellular reactive oxygen and nitrogen species (ROS/RNS), which also resulted oxidative stress and further pathogenic factor, such as inflammation and apoptosis [15]. APAP is extensively used as a well-established chemical model to investigate the mechanism of oxidative stress and screen the hepatoprotective and antioxidative agents in different cells and species [11–13].

Citrus fruits, mainly cultivated in tropical and sub-tropical regions, are the most important fruit crop around the world, with a yearly production of over 120 million tons (FAO, 2016) [16]. Among these, a citrus variety commonly recognized as “pomelo” or “shaddock” has been growing in popularity in Southeast Asia and other places of the world. More and more pomelo wastes, such as peel, pulp, and seed residue, are generated with the ever-increasingly industrialized production of pomelo [17]. These wastes are no easy to dispose and may not be economically feasible to store after removing the remanent moisture or just simply burning and landfilling, especially the latter which could contribute the environmental pollution, aggravating the greenhouse effect. Hence, a requirement, therefore, exists for the design of strategies to effectively reutilize these waste products, thus, creating new added value and minimizing their derived negative environmental impact as far as possible. Several bioactive effect of the extractions of pomelo, like antimicrobial [18], anti-obesity [19] and antioxidant activities [20] have been discussed, which suggested they could be applied as an herbal medicine for some disease. Given the abovementioned context of the fish liver injury and the widely reported therapeutic potential of the plant polysaccharide, we assumed that the polysaccharide extraction from the pomelo waste may be used to be an effectual medicine for the liver injury.

The present study is primary aimed at extracting the polysaccharide from pomelo seed coat (PCSP) and evaluating its antioxidant capacity. Then also performing some biological analyses involving primary hepatocytes of hybrid grouper (*Epinephelus fuscoguttatus* ♂ × *Epinephelus lanceolatus* ♀) subjected to a liver injury inducer, APAP to evaluate the hepatoprotective effects of PCSP.

2. MATERIALS AND METHODS

2.1. Materials

Fresh pomelos were obtained from Meizhou Orchard in Guangdong Province, China. APAP was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). CCK-8 assay kits were purchased from the Biyuntian Institute of Biotechnology (Shanghai, China). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) were obtained from the Beyotime Institute of Biotech (Nantong, China). All other reagents and chemicals used in this study were

of analytical grade.

Hybrid grouper in the experiment were purchased from Daya Bay Breeding Test Center in Huizhou City, Guangdong Province (weighing ~25 g with an average body length of 10 cm).

2.2. The extraction of polysaccharide from Pomelo seed coat

The seeds taken from pomelo were extracted with boiled distilled water at a ratio of 1:30 (w/v). Then filtering using double gauze was conducted after the solution become cooling and thick. The resultant filtrate was precipitated with ethanol at 4 °C overnight and then lyophilized to obtain the PCSP for the further study.

2.3. The Composition analysis of PCSP

The content of galacturonic acid was determined directly by carbazole and sulphuric acid spectrophotometric method [21]. The detection of total polysaccharide was administrated according to the previous method [22]. The emulsifying activity index (EAI) and the emulsion stability index (ESI) were measured as previous described [23]. Turbidity measurements were made at room temperature using viscometer [24]. The protein content was determined by the Coomassie brilliant 88 blue G-250 method, bovine serum albumin serving as a standard (Bradford, 1976) [24]. The degree of esterification (DE) was detected using titration method [25]. The value of pH was determined using the method reported by Hao et al [26].

2.4. Antioxidant activity assays

Sample solutions of polysaccharides with various concentrations of 0, 2, 4, 6, 8 mg/mL were prepared. 1mL sample solution was taken for experiments. The total antioxidant capacity and scavenging capacity of DPPH free radicals and superoxide anion were conducted according to the previous literature [7, 8].

2.5. The assay of APAP-induced hemolysis

The intracellular antioxidant capacity of PCSP was assessed by the suppression of erythrocyte hemolysis as previous described with slight modifications [27]. In brief, sample blood was collected from healthy male volunteers with average age of 22. Erythrocytes, separated from plasma by centrifugation at 1200 g for 10 min at 4 °C. were rinsed with PBS×3 (pH 7.4). An aliquot of 0.2 mL erythrocyte suspension was collected and mixed with 0.2 mL PBS (variable A) or PCSP (variable B) at different concentrations of 0, 0.625, 1.25, 2.5, 5, 10 mg/mL, respectively. After being incubated at 37 °C for 20 min and simultaneously gentle shanking, the suspension was treated with 0.4 mL of 200 mM APAP and continued the above incubation for another 2 h. The resultant solutions were finally diluted with 8 mL of PBS, centrifuged at 1200 × g for 10 min at 4 °C and investigated at the absorbance of 540 nm. The percentage of hemolysis inhibition was calculated with the following equation: % hemolysis inhibition $(1 - A/B) \times 100\%$.

2.6. The hepatoprotection of PSCP to hybrid grouper

2.6.1. Primary hepatocyte isolation and culture

The isolation steps for obtaining the grouper's primary hepatocytes was conducted following our previous experiment [28] with some modifications. Briefly, the liver was rapidly excised from disinfected juvenile hybrid grouper and rinsed several times in ice-cold PBS containing antibiotic (400 IU/mL penicillin and 400 IU/mL streptomycin). Subsequently, the excised liver was divided into small pieces ($\sim 1\text{mm}^3$) and digested with 2x the volume of 0.25% trypsin (1:20 w/v; Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Thereafter the trypsinized product was washed 3x with PBS and filtered through a 100-mesh sieve. The cell pellets were then collected after two different centrifugation steps (100 g for 2 min, followed by 50 g for 5 min), washed 3x with PBS and then re-suspended in MEM culture medium (Gibco, Thermo Fisher, Suzhou, China) supplemented with 15% fetal bovine serum and 100 IU/mL antibiotic (as above) at 25 °C in a 5% CO₂ incubator. Upon reaching confluency, cells were digested by 0.25% (w/v) trypsin-EDTA, and suspended at a density of 1×10^6 cells/mL. The viability of hepatocytes was measured using the trypan blue exclusion method. Only when the viability of Cells greater than 92% were allowed for the subsequent experiments.

2.6.2. Primary hepatocytes culture and treatment

The liver injury model was established in the hepatocytes challenged with APAP (12 mM). In this study, five groups were set up as follows: control (hepatocytes neither treated with PSCP nor exposed to APAP); model group (hepatocytes treated with APAP alone for 24 h) and pretreatment with PSCP groups (the cells were pre-incubated with 100, 200 and 400 $\mu\text{g/mL}$ of PSCP for 24 h, then subjected to APAP for 24 h again). After the administration of each treatment, the processed hepatocytes were centrifuged at 200 $\times$ g for 10 min, and their supernatants were collected for next biochemistry assay.

2.7. Hepatocyte viability assay

Viabilities of hepatocytes were assayed using a Cell Counting Kit 8 following by the manufacturer's instructions. Approximately 5×10^4 cells were incubated in triplicate in 96-well plates maintained for 24 h at 25 °C under 5% CO₂ and treated with indicated reagents (APAP or PSCP) prior to the addition of CCK-8 solution for 2–4 h. Subsequently, viable hepatocytes were determined by investigating the absorbance at 450 nm using a microplate reader (Thermo, MA, USA). The viability rate was calculation as:

$$R (\%) = \frac{\text{Experiment well A450}}{\text{Control well A450}}$$

2.8. Antioxidation status analysis

The supernatants that were obtained as mentioned above at section "Primary hepatocytes culture and treatment" were used to measure the activities of antioxidant enzymes, including glutathione

peroxidase (GSH-Px), catalase (CAT), and superoxide

dismutase (SOD). These measurements were conducted using different corresponding ELISA Kit according to the manufacturer's instructions.

2.9. Histological observation

Histological analyses were conducted following the method reported previously [28]. The cell culture was dried in the alcohol lamp and placed into a 6-well plate. Until cool, approximately 2×10^4 hepatocytes were pipetted onto the culture. 1.5mL MEM culture medium was filled in after the hepatocytes attached to the well and placed in an incubator at 25 °C and with 5% CO₂ overnight. APAP and/or PSCP was added, before that the hepatocytes were washed three times in 0.1 M PBS for 2 min each. 4% paraformaldehyde was used to fixed the sample for 20 min, and repeated the above washing operation for 4 times. Subsequently, the fixed samples were dehydrated several times with ascending graded ethanol, embedded in paraffin, and cuted into 5- μm thickness section. The section was then stained with hematoxylin and eosin (H&E) and investigated morphologically and photographed under a light microscope (MshOt MS60).

2.10. Intracellular ROS measurement

The content of ROS of various experimental groups were measured using a ROS

assay kit as described previously [29]. Briefly, the hepatocytes were dyed with 10 $\mu\text{mol/L}$ DFCH-DA (dichloro-dihydro-fluorescein diacetate) under dark environment at 25 °C for 20 min. After being diluted with 1% Triton X-100, the solution was washed with PBS for 3 times to remove the remanent DFCH-DA. The level of ROS was detected by an inverted fluorescence microscope (Nikon, Japan) at $\lambda_{\text{ex}} = 475 \text{ nm}$, $\lambda_{\text{em}} = 525 \text{ nm}$.

2.11. Statistical analysis

All results are presented as the mean $\pm$ standard deviation (SD). Comparisons among all groups were performed using a one-way analysis of variance (ANOVA). $P < 0.05$ was denoted as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of PSCP

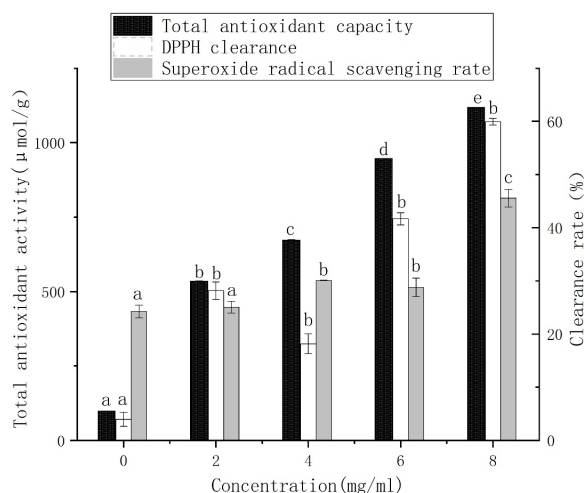
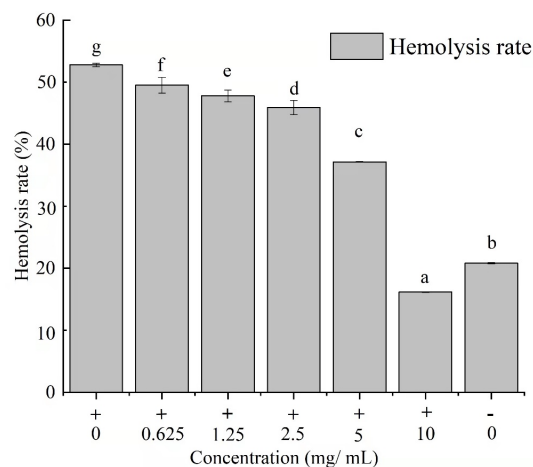
As exhibited on Table.1, the contents of total sugar, protein and galacturonic acid and the DE of PSCP were 54.5, 0.33, 30.24 and 90.7 %, successively. While its EAI and ESI were measured as 0.06 A and 19.35 min, respectively. The turbidity was 23479mPa.s. The pH of the sample under 25 °C was determined as 5.31.

Table 1 Physicochemical index of PSCP

Physicochemical index	Value
Galacturonic acid	30.24 ± 0.70%
Total polysaccharide	54.5 ± 0.87%
Emulsifying activity index	0.06 ± 0.002A
Emulsion stability index	19.35 ± 0.83 min
Turbidity	23479 ± 170.7mPa·s
Protein content	0.33 ± 0.03 %
Degree of esterification	90.7 %
pH	5.31

3.2. Antioxidant activity of PSCP

DPPH, a very stable nitrogen-centered free radical, can be regarded as a reliable indicator to reflect whether existing a substance being capable to reducing peroxide free radicals and interrupting lipid peroxidation chain reaction [30]. Superoxide anion radicals, produced in the metabolic process of organism, can damage the macromolecules, including such as lipids, proteins, nucleic acids and polyunsaturated fatty acids and have a strong association with the aging and pathological changes of organisms. It is also the important indicator to show the substance's antioxidant capacity [31]. In addition, "total antioxidant capacity" is widely administrated to analyze the antioxidant effect of plant extract [32]. In the present study, as showed in Figure1, the scavenging rates to DPPH and total antioxidant capacity of PSCP significantly enhanced almost corresponding with the increase of concentration, which maximized when treating with 8 mg/ml PSCP ($P < 0.05$). While the scavenging superoxide anion radicals of PSCP also intensified in proportion to concentration, albeit with varying degree. Within 0 to 6 mg/ml, the increased trend was unobvious, even slightly declined in 6 mg/ml PSCP treated group, but reached the peak as well at the highest concentration. These finding suggested that PSCP have appreciably antioxidant ability and scavenging effect for the free radicals.

**Figure 1** Total antioxidant capacity, scavenging capacity to DPPH and superoxide radicals of PSCP**Figure 2** Protective effects of PSCP on APAP-induced erythrocyte hemolysis assay. Erythrocytes were preconditioned with various concentrations (0-10 mg/mL) of PSCP for 25 min prior to AAPH (12 mM) treatment for 2 h. "+" means APAP administration was done, while "-" wasn't.

Furthermore, the antioxidant activity of PSCP were studied using the APAP-induced erythrocyte hemolysis which is the well-established model to investigate the free radical-induced damage. As seen in Figure 2, in the control group (untreated cells), the erythrocyte hemolysis rate was 20.83%, while the rate in the group subjected to APAP alone was 52.79%. With the addition of PSCP, the erythrocyte hemolysis rate demonstrated the dose dependent trend. The suppressed degree of PSCP was progressive as the concentration increased, which, as mentioned above, maximized at 8 mg/ml (16.16% erythrocyte hemolysis), even lowering the figure in the control group. These results indicated that PSCP have the strong protective effect against erythrocyte hemolysis.

3.3. The effect of PSCP to the viability

To confirm that PSCP alleviated the APAP-induced cytotoxicity in the hepatocytes, we preconditioned cells with PSCP (100, 200, and 400 μg/mL) before exposing them to APAP. Viabilities on hepatocytes (Figure 3) was significantly reduced in the model group challenged with APAP relative to the control group ($P < 0.05$). Reversely, the PSCP-treated group demonstrated improved

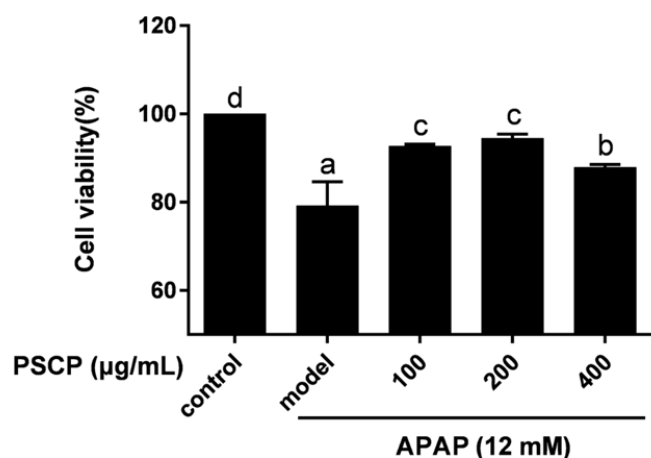


Figure 3 Effect of PSCP on Cell viability induced by APAP. Control: neither treated with PSCP nor subjected to APAP; Model: samples treated with APAP for 24 h alone. The hepatocyte viability was tested by CCK-8 assay according to the absorbance value. And the morphological results were investigated under a light microscope (original magnification 100 ×). Bars without same superscripts (a–f) differ significantly ($P < 0.05$).

hepatocyte viability. Particularly in the group treated with 100 and 200 µg/mL PSCP, the viabilities were significantly increased compared to the model group ($P < 0.05$). These results suggested that the treatment of PSCP mitigated the cytotoxicity induced by APAP in grouper hepatocytes.

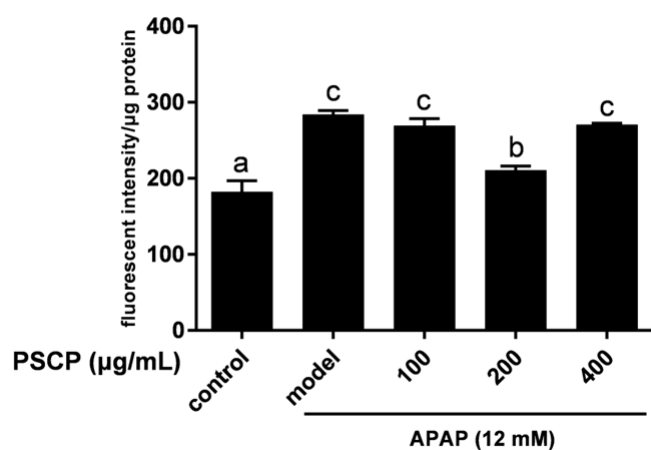


Figure 4 Effect of PSCP on the ROS content in the hepatocytes exposed to APAP. Model: samples treated with APAP for 24 h alone. Bars without same superscripts (a–f) differ significantly ($P < 0.05$).

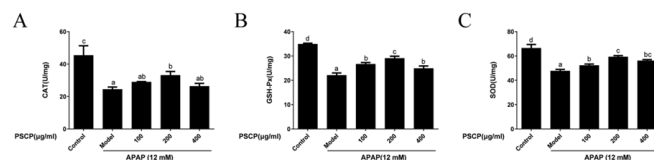


Figure 5 Effect of PSCP on the activities of the antioxidant enzymes in the hepatocytes exposed to APAP. Model: samples treated with APAP for 24 h alone. Bars without same superscripts (a–f) differ significantly ($P < 0.05$).

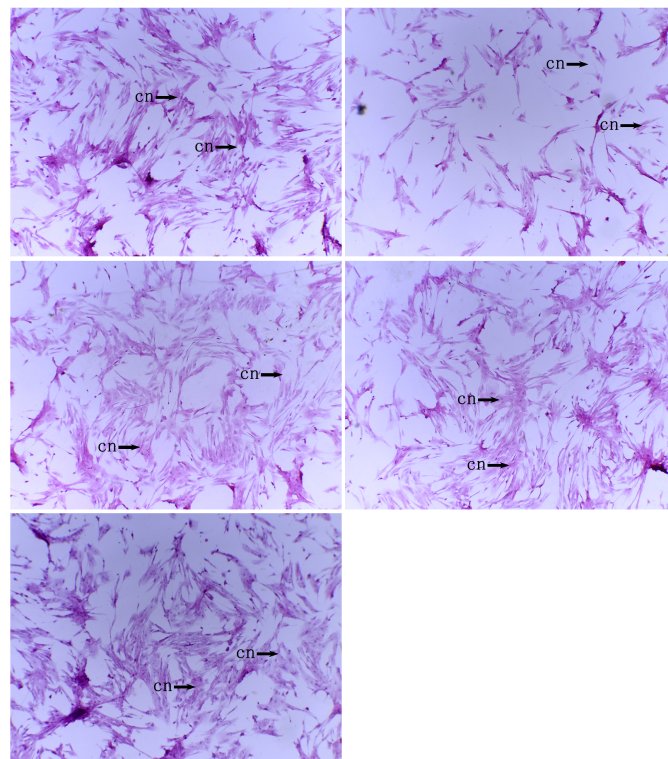


Figure 6 The performances of H&E staining. (A) Control; (B) Model; (C) APAP+ PSCP (100 µg/mL); (D) APAP+PSCP (200 µg/mL); (E) APAP+ PSCP (400 µg/mL); Control: neither treated with PSCP nor subjected to APAP; Model: samples treated with APAP for 24 h alone. cn: cell nucleus; va: vacuolation.

3.4. Effect of PSCP on antioxidant status

The occurrence of liver injury in fish could have various factors. While among them, oxidative stress is commonly recognized as a contributing cause. Even in some reports, oxidative stress is considered as the precursor to various liver diseases [33]. Therefore, mitigating the insult of oxidative stress could be the effectual strategy to prevent the development of liver failure. In this study, the performances of oxidative stress could be observed in model group (solely treated with APAP), as evident by the dramatically reductions in the ROS content (Figure 4) and the antioxidant enzymatic activities, namely CAT, GPx and SOD (Figure 5) relative to that in control group ($P < 0.05$). On the contrary, the changes above induced by APAP were reversed to different degree after the treatment of PSCP, in a manner corresponding well with

the changes in the viability detection. These results indicate the inhibitory effect of PSCP to oxidative stress in hepatocytes, which implied its therapeutic potential to APAP-induced liver injury.

3.5. Effect of PSCP on APAP-induced Morphological changes

The more direct sign of hepatoprotection of PSCP could be investigated in the morphological changes of the hepatocytes stained with H&E (Figure 6). In the recent study, the hepatocytes exposed to APAP alone displayed the characteristics of severe cellular necrosis, fuzzy cell contours, and nuclear deterioration appeared, while no abnormalities on the hepatocytes could be found in the control group, suggesting APAP have caused some damages to the hepatocytes. While in the group treated with PSCP, the signs of recovery were observed. Especially in the group treated with 200 $\mu\text{g/mL}$ PSCP, where the decline in necrotic areas and improved cellular morphologies were occurred. These morphological changes confirmed that the positive effect of PSCP to the liver injury induced by APAP.

4. CONCLUSION

In this study, we demonstration the treatment of PSCP ameliorated the APAP-induced insult in primary hepatocytes of hybrid grouper. Collectively, these results signified that PSCP may be employed as a potential regiment to mitigate the liver damage in fish, which provide a new prospective for reusing the Citrus waste. Further studies in vivo and fermentation study in vitro are needed to investigate and confirm its therapeutic potential to fish liver.

5. ABBREVIATIONS

Acetaminophen (APAP); catalase (CAT); DFCH-DA (dichlorodihydro-fluorescein diacetate); emulsifying activity index (EAI); emulsion stability index (ESI); glutathione (GSH); glutathione peroxidase (GPx); hematoxylin and eosin (H&E); N-acetyl-p-benzoquinone imine (NAPQI); PSCP (pomelo seed coat polysaccharide); reactive oxygen species (ROS); superoxide dismutase (SOD).

6. FUNDING

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7. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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