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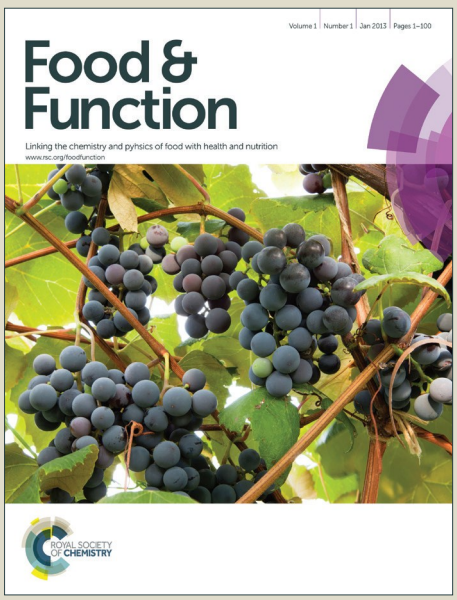

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Phenolic-rich lychee (*Litchi chinensis* Sonn.) pulp extracts offer hepatoprotection against restraint stress-induced liver injury in mice by modulating mitochondrial dysfunction

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Abstract: The pulp from lychee, a tropical to subtropical fruit, contains large quantities of phenolic compounds and exhibits antioxidant activities both *in vitro* and *in vivo*. In the present study, we investigated the mechanisms underlying the hepatoprotective effects of lychee pulp phenolics (LPPs) against restraint stress-induced liver injury in mice. After 18 h of restraint stress, increased levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were observed. High levels of thiobarbituric acid reactive substances (TBARS) were also found. Restraint stress causes liver damage, which was protected against by LPP pretreatment at a dosage of 200 mg/(kg · d) for 21 consecutive days. This treatment remarkably decreased serum ALT, AST and TBARS levels, elevated liver glutathione (GSH) content, and the activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT). Furthermore, respiratory chain complex and Na+-K+-ATPase activities were enhanced in liver mitochondria, while mitochondrial membrane potential levels and reactive oxygen species (ROS) production decreased. Thus, treatment with LPPs ameliorated restraint stress-induced liver mitochondrial dysfunction. These results suggest that LPPs protect the liver against restraint stress-induced damage by scavenging free radicals and modulating mitochondrial dysfunction. Thus, lychee pulp may be a functional biofactor to mitigate oxidative stress.

Keywords: Phenolics; Restraint stress; Liver injury; Mitochondrial dysfunction; Lychee; Hepatoprotective activity.

1. Introduction

Previous studies have demonstrated that long-term exposure to stress triggers numerous health problems and lifestyle diseases, including cardiovascular injury[1] and Alzheimer's disease[2]. Increased oxidative stress and diminished antioxidant protection are the primary contributors to the development of stress-induced diseases. Phenolics in fruits and vegetables have potent antioxidant properties, which may help combat oxidative stress and improve pro/antioxidant balance within the body[3].

Lychee, a tropical to subtropical fruit, has become increasingly popular throughout the world[4]. Recent work has suggested that lychee pulp, which is the most commonly consumed part of the fruit, contains large quantities of phenolic compounds. Several phenolics, including quercetin, kaempferol, trans-cinnamic acid, gallic acid, chlorogenic acid, (+)-catechin, caffeic acid, (-)-epicatechin and rutin, have been detected in lychee pulp extracts via high-performance liquid chromatography (HPLC) in tandem with mass spectrometry[5-7]. Our group previously isolated and purified major antioxidant compounds from lychee pulp. These included quercetin 3-*o*-rut-7-*o*-α-L-rha, rutin and (-)-epicatechin, which were subjected to cellular antioxidant activity and oxygen radical absorbance capacity assays. We also identified the structural formulae of these compounds using nuclear magnetic resonance and electrospray ionisation mass spectrometry[8]. Previous studies reported that fruit extracts rich in quercetin 3-rut-7-rha could reduce serum cholesterol and triglycerides in diabetic rats fed with high cholesterol diet[9]. Rutin exerts hepatoprotective effects[10] and antioxidant properties[11]. (-)-Epicatechin also provides cardiovascular

protection[12,13] as well as anti-inflammatory[14] and antioxidant effects.

Lychee pulp phenolic (LPP) compounds exhibit excellent antioxidant activities, including ferric reducing antioxidant power as well as 2,2-diphenyl-1-picrylhydrazyl and oxygen radical absorbance capacity, as demonstrated by cellular antioxidant activity assays[5, 7, 15]. However, whether *in vitro* methods can predict *in vivo* antioxidant activity is a matter of debate; as such, *in vivo* data are more robust. It has been reported that lychee pulp extracts can decrease alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels following CCl_4-induced liver injury[16]. The hepatoprotective effects of lychee pulp extracts on CCl_4-induced hepatotoxicity are believed to be related to lychee pulp's antioxidant properties.

Oxidative damage causes mitochondrial dysfunction and thus has a critical role in the development of human diseases[17, 18]. Restraint stress can induce serious liver injury manifested as increased serum ALT and malondialdehyde (MDA) levels[19] and mitochondrial dysfunction in liver[20, 21]. Mitochondria are important for energy production and play pivotal roles in basic cellular processes, such as pyruvate oxidation, free radical generation and fatty acid metabolism[17, 22]. Mitochondrial membrane potential (MMP) and ATP synthase (ATPase) activity are key parameters used in the assessment of cellular energy metabolism. ATPase dysfunction has been associated with increased oxidative stress[18, 23]. LPP compounds exhibit good antioxidant activities and hepatoprotective effect on chemical-induced liver injury. However, the possible protection of LPP against the restraint stress-induced liver injury in mice and the mechanisms underlying the hepatoprotective effects of LPPs

remain unknown.

This report extends previous work regarding the structures, potential hepatoprotective effects and antioxidant activities of LPPs in mice subjected to restraint-induced stress. The mechanisms underlying LPP activity were further determined by evaluating mitochondrial function, which appeared to be improved. The findings of the present study provide evidence to promote the use of lychee pulp as a functional biofactor to mitigate oxidative stress.

2. Materials and methods

2.1. Plant Material: Lychee (cv. *Feizixiao*), which is one of the main cultivars in South China, was purchased at commercial maturity from a local fruit market in Guangzhou, China. Uniformly mature fresh fruits were selected and washed with tap water. The pericarp and seed were then manually removed. The fresh lychee pulp was weighed and immersed in chilled acetone/water (80:20, v/v).

2.2. Chemicals: Rutin and (-)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetic acid and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Deionised water was prepared using a Milli-Q water purification system (Billerica, MA, USA). ALT, AST, thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), xanthine oxidase (XOD) and Coomassie brilliant blue kits were all obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.3. Preparation and analysis of LPPs

LPPs were prepared as previously described[8]. In brief, samples (10 kg) were extracted twice with 20 L of chilled acetone/water (80:20, v/v). The supernatants were combined and concentrated at 45 °C. The concentrated supernatant (1 L) was then fractionated on a HPD826 resin (Cangzhou Bonchem Co., Ltd., Cangzhou, China) column (ø 10 cm, length 150 cm) to remove most of the non-phenolic compounds. Elution was performed using 10 L of deionised water and 95% aqueous ethanol (v/v). The organic phase fraction was collected, rotary evaporated and freeze-dried to produce LPP powder, which was then analysed using HPLC.

The phenolic composition and contents of the extract were determined using the HPLC-DAD method, which has been described previously[15]. Briefly, the extract was filtered before being applied to an Agilent Zorbox SB-C_{18} column (250 × 4.6 mm, 5 μm, Palo Alto, CA, USA) and eluted at a flow rate of 1.0 mL/min using a binary gradient that consisted of solution A (water/acetic acid 996:4 v/v) and solution B (acetonitrile) as the mobile phase. Elution was monitored based on spectrophotometric absorption at 280 nm. The gradient elution programme was as follows: 0-40 min, solution A 95-75%; 40-45 min, solution B 75-65%; and 45-50 min, solution B 65-50%, followed by a 5 min equilibration period with 95% solution A. Peak identities were confirmed based on retention times determined for standard compounds. The total phenolic content was determined according to a previously described method[24], the moisture content was evaluated based on the methodology of Varith *et al.*[25], total sugar was measured spectrophotometrically according to the

126 colorimetric method[26] and protein was assayed using a modified Kieldahl method[27].

127 The three major phenolics identified in the lychee pulp were quercetin
128 3-*o*-rutinoside-7-*o*-a-L-rhamnosidase, rutin and (-)-epicatechin. These components
129 constituted 230.03±15.14, 37.10±3.11 and 25.11±1.43 mg/g of the LPP freeze-dried
130 powder, respectively. The total phenolic content accounted for up to 53.40±2.37% of
131 the total weight of the LPP freeze-dried powder; other components included moisture
132 (10.00±1.63%), total sugar (15.01±0.42%) and protein (3.51±0.14%) as well as
133 unknowns.

134 **2.4. Animals and experimental design**

135 The experiment was approved by the Ethics Committee on Animals Experiment of
136 Guangdong Academy of Agricultural Sciences. The animal care and treatment
137 protocols complied with the national guidelines for the care and use of laboratory
138 animals. Seven-week-old pathogen-free male Kunming (KM) mice were purchased
139 from the Center of Laboratory Animal Science Research of Southern Medical
140 University (Guangzhou, China) and acclimated for 1 week before the experiment. All
141 animals were housed in a specific pathogen-free and environmentally controlled room
142 under controlled temperature (23±2 °C) and humidity (60±5%) conditions with a 12-h
143 light/dark cycle. The mice were fed a standard laboratory diet and provided with tap
144 water *ad libitum* in accordance with the national standards outlined in "Laboratory
145 Animal Requirements of Environment and Housing Facilities" (GB 14925-2010).

146 The mice were randomly divided into five groups of 10 animals each. These groups

were designated as normal control, model (restraint stress), LPP-L, LPP-M, and LPP-H treatment groups. LPP was dissolved in distilled water and mice were orally administered 50, 100 and 200 mg/kg body weight LPP in the afternoon per day for 3 weeks. The animals of normal control and model groups were given distilled water instead. The body weight and food intake were recorded twice a week. Thirty minutes after the final oral gavage, all animals except those in the normal control group were physically restrained in 50-mL polypropylene tubes with holes for 18 h before being sacrificed for serum and liver collection.

Serum was collected by centrifuging the blood samples at 3000 × g for 10 min at 4 °C and stored at -20 °C for later biochemical analysis. Liver samples were immediately excised, washed with chilled normal saline, blotted dry and weighed. The liver was cut into 2 portions, and one of which was used for mitochondria isolation. The other portion was stored at -80 °C for later biochemical determination.

2.5. Measurement of ALT and AST activities in serum

Serum levels of ALT and AST were measured using an automatic biochemical analyser (7600 Series, Hitachi, Tokyo, Japan) and commercial kits. Enzyme activities are expressed in units per litre (U/L).

2.6. Measurement of SOD, T-AOC, GSH, GPx, CAT and XOD activities in serum and liver

The frozen liver samples were homogenized in chilled normal saline in an ice bath to obtain a 10% (w/v) liver homogenate. The supernatant was collected by centrifuging

the homogenate at 4000g for 10 min at 4 ℃. Then the homogenate supernatant was aliquoted and stored at -80 ℃ for biochemical analysis. The Bradford method[28] was used to determine protein concentrations of liver homogenate with bovine serum albumin as a standard. SOD, T-AOC, GSH, GPx, CAT and XOD activities were quantified using a commercial kit according to the manufacturer's protocol. SOD activity was determined according to the xanthine and xanthine oxidase method. Briefly, 20 mL of sample and 20 mL of enzyme working solution were mixed thoroughly with 200 mL of WST-1 working solution (from the kit) in each well of a 96-well microplate. The plate was incubated at 37 °C for 20 min, and its absorbance at 450 nm was measured using an Infinite M200 PRO plate reader (Tecan Austria GmbH, Groig, Austria). The total antioxidant capacity (T-AOC) of the liver was assessed using a colorimetric method. Briefly, samples prepared as above and a working solution were added to test tubes containing phenanthroline substances and then incubated at 37 °C for 30 min. Fe^{3+} was reduced to Fe^{2+}, which then formed complexes with phenanthroline that could be measured at 520 nm. GPx activity was measured using a spectrophotometric assay that involved calculating the catalysis rate of the oxidation of GSH to GSSG. GSH content was determined using a DTNB-GSH reductase-recycling assay. CAT levels in the liver were determined based upon the decomposition of H_2O_2, which can be measured based on absorbance at 415 nm. XOD catalysed the oxidation of hypoxanthine to xanthine to produce superoxide anion radicals, which eventually resulted in a fuchsia adduct measurable at 530 nm using an Infinite M200 PRO plate reader.

2.7. Measurement of TBARS content in serum and liver

MDA is a product of lipid peroxidation and is therefore an indicator of this process. MDA reacts with thiobarbituric acid (TBA) to generate pink MDA-TBA adducts with measurable absorbance at 532 nm. MDA contents in serum and liver were determined by performing a TBARS assay using a commercially available kit according to the supplier's instructions and expressed as nmol MDA equivalents per mL or per mg of protein.

2.8 Isolation of liver mitochondria

The mitochondria were isolated from liver tissue by using a commercial tissue mitochondria isolation kit (Beyotime Institute of Biotechnology, Guangzhou, China) according to the manufacturer's instructions. Briefly, 100 mg fresh mice liver tissues were cut into small pieces and homogenized with Dounce tissue grinder in 1 ml isolation reagent (supplied by the commercial kit) in an ice bath. The homogenate was centrifuged at 600g for 5 min at 4 °C. Then, the supernatant was centrifuged again at 11,000 g for 10 min at 4 °C to obtain mitochondrial pellets. Mitochondrial pellets were then resuspended in 40 μL ice-cold preserving solution (supplied by the commercial kit). Three hundred milligrams of liver tissue of each animal was used to isolate mitochondria by repeating the above procedure 3 times and combining the suspension together. In order to get enough mitochondria for later analysis, mitochondrial suspensions from two mice in the same group were mixed together. The activity of mitochondria were determined by Janus green B staining and observed

with a microscope. Freshly isolated mitochondria were used to measure ROS generation and membrane potential, and the remaining mitochondrial suspension was aliquoted and stored at -80 ℃ for enzyme activity determination. Protein concentration in mitochondrial suspension was determined with Bradford method[28].

2.9. Measurement of ROS generation in liver mitochondria

ROS were assayed using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)[29]. DCFH is oxidised in the presence of ROS into highly fluorescent 2',7'-dichloro fluorescein (DCF). Briefly, 2 μL of DCFH-DA was added to 50 μL of mitochondrial suspension and incubated in the dark at 37 °C for 15 min. The resulting fluorescence was measured using an Infinite M200 PRO plate reader with an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

2.10. Measurement of liver mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potentials ($\Delta\Psi m$) were assayed using the fluorescent probe Rhodamine 123. Mitochondrial suspension samples (50 μL) prepared as described above were mixed with membrane potential reaction buffer (150 μL) and 1 μL Rhodamine 123 (1 mmol L^{-1}) and accessed via flow cytometry at an excitation wavelength of 503 nm and an emission wavelength of 527 nm.

2.11. Measurement of liver mitochondrial complexes

The activities of mitochondrial complex I (NADH coenzyme Q10 oxidoreductase) and complex II (succinate coenzyme Q10 oxidoreductase) were measured spectrophotometrically according to reference[30]. Complex I activity was measured by

recording decreases in absorbance caused by the oxidation of NADH at 340 nm for 3 min. Complex II activity was measured by monitoring the reduction of 2,6-dichlorophenolindophenol at 600 nm for 3 min.

2.12. Measurement of liver mitochondrial ATPase

ATPase activity was determined at 37 °C by measuring the initial rate of inorganic phosphate release following ATP hydrolysis according to a previously described method[31]. Na^+-K^+-ATPase activity was calculated by determining the difference between total ATPase activity (Na^+-K^+-Mg^{2+}-ATPase) and Mg^{2+} ATPase activity via a colorimetric assay. One unit of ATPase activity is defined as the amount of enzyme required to produce 1 μmol Pi mg protein^{-1} h^{-1} via ATP hydrolysis.

2.13. Statistical analysis

All data are presented as the mean±standard deviation (SD). Biochemical indicators of serum and liver were analysed in 10 animals from each group. ROS levels, mitochondrial membrane potential, mitochondrial complexes activities and ATPases activity were determined 5 replicates in each group due to the combination of mitochondrial suspensions from two mice. One-way analysis of variance (ANOVA) was used to assess inter-group differences. SPSS 13.0 was used for statistical analysis. In cases of statistically significant differences, Dunnett's post hoc test was employed for multiple pairwise comparisons. Differences at $p < 0.05$ were considered statistically significant.

3. Results

253 **General conditions of mice**

254 No animal died during the whole experimental period. The body weights and food
255 intake of each group animals were equivalent (data not shown). Abnormal signs
256 related with toxicity were not observed in LPP treated animals including rough hair
257 coats, diarrhea, ataxia, hunched posture, and hypoactivity.

258 **Effects of LPP on ALT and AST levels in serum of restraint-stressed mice**

259 The mean serum ALT and AST levels in the normal control mice were 97.53±67.87
260 U/L and 55.73±18.93 U/L, respectively. These values significantly increased to
261 292.60 U/L and 320.47 U/L, corresponding to 1.98- and 4.75-fold increases over
262 baseline, respectively, after the mice were subjected to restraint-induced stress for 18
263 h (Fig. 1). All evaluated LPP doses decreased serum ALT and AST levels to varying
264 extents in restraint-stressed mice. The highest dose of LPP significantly decreased
265 serum ALT and AST levels ($p < 0.05$).

266 **Effects of LPP on biochemical indicators in serum and liver of restraint-stressed**
267 **mice**

268 Compared with the normal control group, serum SOD activity in the restraint-stressed
269 mice dramatically decreased ($p < 0.05$), as indicated in Table 1. Conversely, in the
270 restraint-stressed mice, TBARS levels were markedly higher than in the normal
271 control group ($p < 0.05$). LPP treatment increased SOD activity of restraint-stressed
272 mice and the middle and high doses of LPP significantly increased serum SOD
273 activity ($p < 0.05$). In contrast, TBARS content decreased in all treatment groups; in

the high-dose group, TBARS content was reduced to the level of the normal control group ($p > 0.05$).

The phenolics in lychee pulp affected biochemical indicators in the livers of the restraint-stressed mice. Compared with the normal control group, the restraint-stressed mice had lower GSH content ($p < 0.05$) and T-AOC capacity and higher TBARS levels ($p < 0.05$), as indicated in Table 1. Meanwhile, SOD, GPx and CAT activities decreased, while XOD activity increased in the restraint-stressed group. LPP administration reduced TBARS content from 17.41 to 8.04 nmol/mg protein, which was not significantly different from the value measured for the normal group ($p > 0.05$). XOD content also decreased. Meanwhile, GPx activity increased in a dose-dependent manner in the restraint-stressed mice treated with lychee pulp extract. The high-dose group exhibited significantly higher GPx activity than the model group ($p < 0.05$) and did not significantly differ from the normal group ($p > 0.05$). Similar results were found for SOD and CAT. GSH content and T-AOC capacity were accordingly elevated as SOD, GPx and CAT increased. Both the high-dose and middle-dose groups exhibited increased GSH content and T-AOC capacity compared with the restraint-stressed group ($p < 0.05$).

Effects of LPPs on mitochondrial function in the livers of restraint-stressed mice

Nearly all the mitochondria became blue-green in colour in normal control mice as visualized under a light microscope with a green filter (Fig. 2). There were markedly fewer blue-green mitochondria in the restraint-stressed group versus the normal

295 control group. The number of blue-green mitochondria decreased in response to
296 restraint stress but was partially and dose-dependently restored following pretreatment
297 with lychee pulp extract.

298 LPPs altered ROS generation and membrane potential in liver mitochondria of
299 restraint-stressed mice. The mice subjected to restraint stress exhibited approximately
300 2-fold higher ROS production than the normal control group ($p < 0.05$) (Table 2).
301 However, pretreatment with LPP significantly attenuated the elevation in ROS level
302 in a dose-dependent manner. The high-dose group had an approximately 40% lower
303 ROS level than that in the restraint-stressed group ($p < 0.05$).

304 Restraint stress increased MMP levels, as indicated in Table 2. When the mice were
305 subjected to restraint stress, their MMP levels increased, which was indicated by their
306 high fluorescence intensities. Approximately 2-fold higher fluorescence intensity was
307 observed in the model group compared with the normal control group ($p < 0.05$).
308 After the addition of LPPs, the fluorescence decreased; in the high-dose group, this
309 value was approximately 26% lower than in the model group ($p<0.05$).

310 LPPs exerted positive effects on respiratory chain complex and ATPase activities in
311 liver mitochondria from restraint-stressed mice. The restraint-stress group did not
312 exhibit significant differences in respiratory chain complex I or Mg^{2+}-ATPase
313 activities compared to the normal group. Pretreatment with lychee pulp extract also
314 had little effect on these activities, as indicated in Table 3. However, restraint stress
315 significantly decreased respiratory chain complex total ATPase and Na^{+}-K^{+}-ATPase

316 activities ($p < 0.05$) by 23% and 50%, respectively, compared to normal mice.
317 Administration of LPP partially blocked the restraint stress-induced depletion of
318 respiratory complex and Na^+-K^+-ATPase activities. At 200 mg/(kg · d), lychee pulp
319 extract could reverse decreased activity of liver Na^+-K^+-ATPase to a level comparable
320 to the normal control group.

321 **4. Discussion and conclusions**

322 In recent years, numerous studies have demonstrated that lychee pulp contains large
323 quantities of phenolic compounds, which scavenge superoxide anion and hydroxyl
324 radicals both *in vitro* and *in vivo*[7, 8, 15, 16]. In accordance with our previous reports, the
325 major phenolic compounds that we identified in lychee pulp were quercetin
326 3-*o*-rutinoside-7-*o*-a-L-rhamnosidase, rutin and (-)-epicatechin [8]. Lv *et al*. determined
327 that quercetin rhamnosyl-rutinoside accounted for the majority of the total phenolic
328 content of a *Feizixiao* cultivar using HPLC–MS [32]. This result is in accordance with
329 our results and provides further support for our conclusions. Quercetin 3-rut-7-rha,
330 rutin and (-)-epicatechin together accounted for up to 53.40% of the total weight of
331 LPPs. All of these compounds also exhibited good antioxidant activity. Based on their
332 predominant contents and significantly higher antioxidant activity than other
333 phenolics in lychee pulp, quercetin 3-rut-7-rha, rutin and (-)-epicatechin would be the
334 major contributors to lychee pulp antioxidant activity in the present study. Mosaddegh
335 *et al*. found that *Paliurus spina-christi* fruit extracts rich in quercetin 3-rut-7-rha and
336 rutin could reduce the levels of serum cholesterol and triglycerides in rats[9]. Quercetin
337 3-rut-7-rha is a rutin derivative, with rhamnose substitution at 7-hydroxyl group. A

previous study showed that alkylation of the hydroxyl at position 7 enhanced free radical scavenging activity[33]. Therefore, it can be deduced that quercetin 3-rut-7-rha would exhibits good antioxidant activity *in vivo*. Rutin is used as a vasoprotectant[34] and exerts hepatoprotective effects[10] because of its excellent antioxidant activities[10, 11]. Indeed, its antioxidant properties are an important component of its biological activities. (-)-Epicatechin also provides cardiovascular protection[12, 13] as well as anti-inflammatory[14] and antioxidant effects. Additionally, (-)-epicatechin and its metabolites protect against oxidative stress via their direct antioxidant effects[35].

It has been reported that LPPs can decrease serum ALT and AST activities in livers that have suffered CCl_4-induced damage[16]. Increased serum ALT and AST activities also serve as markers of liver damage in restraint-stressed mice. In the present study, we observed that LPPs pretreatment could attenuate restraint stress-induced liver damage in mice. This result arose from the antistress effects exerted by LPPs, as reflected by the recovery of ALT and AST activity in the serum. In addition, mice subjected to restraint stress for 18 h exhibited accelerated formation of ROS[19]. Imbalances between ROS scavenging and generation provoked by restraint stress can lead to excessive ROS levels. Harmful free radicals subsequently react with proteins and lipids, thereby resulting in oxidative damage. Lipid peroxidation was observed in restraint-stressed mice. Our study suggested that the content of TBARS, which are end products of lipid peroxidation, was elevated in both serum and liver. Previous studies have indicated that immobilisation stress induces increased TBARS levels[36]. Increases in ALT, AST and TBARS levels in the serum and livers of restraint-stressed

mice suggest that this stress induces damage to hepatic cell structure. However, the administration of lychee pulp extract significantly altered hepatic pathologic damage, as reflected by increased ALT, AST and TBARS levels.

There are two major intracellular antioxidant defence systems: low molecular weight antioxidants (such as GSH) and antioxidant enzymes (including GPx, SOD and CAT). GSH is an important intracellular antioxidant that utilizes a non-protein thiol to quench ROS[37]. GPx, a GSH-related enzyme, can degrade lipid hydroperoxides into their corresponding alcohols. SODs catalyse the breakdown of superoxide anions into oxygen and hydrogen peroxide, which can be further catalysed by GPx and CAT enzymes into water[38]. The mechanism driving XOD activity differs from those driving SOD, GPx and CAT activities. XOD overexpression can catalyse the oxidation of hypoxanthine to xanthine and generate unwanted free radicals[39]. In our present study, mice subjected to restraint stress exhibited decreases in GPx, CAT, and SOD activities and GSH content and increases in XOD activity and MDA levels in the liver. Similar changes in intracellular antioxidant defence system indicators have been reported by Li, et al. [40]. All of the above results are associated with antioxidant capacity; this is further supported by the T-AOC data corresponding to the livers of the stressed mice. However, treatment with lychee pulp extract significantly altered oxidative stress status and compensated for hepatocellular damage. Similar results have been previously observed in restraint-stressed mice pretreated with bilberry extracts or myelophil[36].

ROS are produced during cellular respiration. Mitochondria are the most important

382 cellular source of ROS [41]. Dysfunctional mitochondria produce excessive amounts of
383 ROS. Imbalances in ROS levels result in damage to cellular macromolecules, such as
384 membrane lipids[42]. Because of the decreased level of GSH and reduced activities of
385 SOD, GPx and CAT, increased ROS production was observed in liver mitochondria of
386 restraint-stressed mice in the present study. Mitochondria are specific targets of
387 oxidative stress, which results in impaired mitochondrial function[43]. MMP level and
388 ATPase activity are key parameters used to assess mitochondrial functioning under
389 physiological and pathological conditions[23, 44]. The observed 2-fold increase in MMP
390 levels in the liver mitochondria of restraint-stressed mice clearly indicates that
391 mitochondrial ROS generation is also activated by MMP, which was demonstrated via
392 cytofluorometric analysis of $\Delta\Psi m$. This conclusion is in accordance with the low
393 ATPase content measured in cells from patients with mitochondrial ATPase
394 deficiency[44]. ATPase dysfunction decreases mitochondrial synthesis of ATP. This
395 results in elevated mitochondrial ROS production, which is associated with increased
396 oxidative stress[44]. Our results demonstrated that ATPase, Na^+-K^+-ATPase and
397 mitochondria respiratory chain complex II activity decreased in restraint-stressed
398 mice as a consequence of mitochondrial respiratory chain dysfunction.
399 Na^+-K^+-ATPase acts as an energy-transducing ion pump and signal transducer[45].
400 When Na^+-K^+-ATPase function is impaired, the fluidity of the mitochondrial
401 membrane decreases. This change blocks electron transfer and therefore decreases
402 respiratory chain complex activity [46, 47]. Pretreatment with LPPs could attenuate MMP
403 and enhance respiratory chain complex and ATPase activities in mitochondria, thus

blocking ROS generation. These observations are in agreement with Bao *et al.*'s report that treatment with bilberry extract enhanced mitochondrial complex II activity, Na+-K+-ATPase activity and MMP ($\Delta\Psi m$) in restraint-stressed mice[20]. These findings indicate that LPPs exhibit potent protective effects against restraint stress-induced liver damage by scavenging free radicals and modulating mitochondrial dysfunction.

In summary, restraint stress-induced liver damage is primarily caused by oxidative stress. Pretreatment with LPPs provides hepatoprotection, which is associated with mitochondrial protection and antioxidant activities. Lychee pulp is therefore a potential candidate functional food.

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Table and figure captions

Table 1. Effects of LPPs on biochemical indicators in serum and liver tissues from restraint-stressed mice

Table 2. Effects of LPPs on ROS generation and membrane potential changes in liver mitochondria from restraint-stressed mice

Table 3. Effects of LPPs on respiratory chain complex and ATPase activities in liver mitochondria from restraint-stressed mice

Fig. 1. Effects of LPPs on ALT and AST activities in serum from restraint-stressed mice

Fig. 2. Effects of LPPs on liver mitochondrial function in restraint-stressed mice

Table 1. Effects of LPPs on biochemical indicators in serum and liver tissues from restraint-stressed mice

	Normal	Model	LPP-L	LPP-M	LPP-H
Serum					
SOD (U/mL)	230.31±5.98d	157.42±8.24a	163.56±8.33a	180.76±7.32b	203.77±8.51c
TBARs (nmol MDA equivalent/mL)	1.12±0.44a	3.04±0.78b	2.61±0.98ab	2.13±0.55ab	1.34±0.33a
Liver Tissue					
GSH (mg/g prot)	8.56±1.39c	3.06±0.67a	4.49±0.85ab	5.77±1.01b	6.34±1.43b
TBARs (nmol MDA equivalent/mg prot)	6.57±1.12a	17.41±1.61c	13.07±1.80b	9.65±2.11a	8.04±1.39a
GPx (U/mg prot)	43.76±3.23c	34.20±1.98a	36.77±2.69ab	39.60±2.31abc	40.53±1.87bc
SOD (U/mg prot)	125.31±7.30c	99.75±6.32a	101.32±6.31a	109.73±7.13ab	118.75±5.41bc
XOD (U/mg prot)	36.37±1.38a	48.70±1.90c	46.39±3.46bc	45.59±3.82bc	41.14±2.03ab
CAT (U/mg prot)	325.49±7.49c	300.61±5.79a	302.45±6.92a	307.06±9.13ab	318.78±5.55bc
T-AOC (U/mg prot)	0.58±0.08c	0.18±0.10a	0.24±0.07a	0.39±0.06b	0.43±0.07b

Normal: normal control group, administered distilled water by oral gavage for 3 consecutive weeks; Model: restraint stress group, administered distilled water by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30

min after the final oral gavage and lasting for 18 h before being sacrificed; LPP groups: administered 50 (LPP-L), 100 (LPP-M), or 200 (LPP-H) mg LPP per kg body weight per day by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed. Biochemical indicators were quantified using commercial kits. Values are reported as the mean ± SD (n = 10). Values within each column without a common letter are significantly different ($p < 0.05$).

Table 2. Effects of LPPs on ROS generation and membrane potential changes in liver mitochondria from restraint-stressed mice

	Normal	Model	LPP-L	LPP-M	LPP-H
ROS (RFU)	452.62±4.58 a	891.26±32.13e	791.96±13.63d	594.33±13.58 c	553.73±3.90b
Membrane potential (FI)	9.61±0.71 a	19.92±1.26 c	18.84±1.83c	15.93±2.36 b	13.98±1.29b

Normal control group administered distilled water by oral gavage for 3 consecutive weeks; restraint stress model group administered distilled water by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed; LPP groups administered 50 (LPP-L), 100 (LPP-M), or 200 (LPP-H) mg LPP per kg body weight per day by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed. ROS were assayed using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Mitochondrial membrane potentials were assayed using the fluorescent probe Rhodamine 123. Values are reported as the mean ± SD (n = 5). Values within each column without a common letter are significantly different ($p < 0.05$).

Table 3. Effects of LPPs on respiratory chain complex and ATPase activities in liver mitochondria from restraint-stressed mice

	Normal	Model	LPP-L	LPP-M	LPP-H
Complex I (nmol min^{-1} mg^{-1})	746.25±77.37 a	611.68±108.54 a	656.54±116.70 a	728.58±76.76 a	698.67±122.45 a
Complex II (μmol min^{-1} mg^{-1})	3.86±0.56 c	0.88±0.08 a	1.07±0.14 a	1.16±0.11 a	1.93±0.26b
Total ATPase (μmol Pi mg $prot^{-1}$ h^{-1})	56.70±8.33 a	43.32±6.07 b	46.03±5.86 a	49.14±6.93 a	51.74±6.97 a
Mg^{2+}-ATPase (μmol Pi mg $prot^{-1}$ h^{-1})	26.49±2.50 a	26.90±2.96 a	25. 94±1.80 a	23.21±3.31 a	20.27±2.11 a
Na^{+}-K^{+}-ATPase (μmol Pi mg $prot^{-1}$ h^{-1})	30.21±5.83 b	16.42±3.11 a	20.09±4.06 a	25.93±3.62 ab	31.47±4.86 b

Normal control group administered distilled water by oral gavage for 3 consecutive weeks; restraint stress model group administered distilled water by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed; LPP groups administered 50 (LPP-L), 100 (LPP-M), or 200 (LPP-H) mg LPP per kg body weight per day by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed. Mitochondrial complexes and ATPase activity were measured by spectrophotometric analysis. Values are reported as the mean ± SD (n = 5). Values within

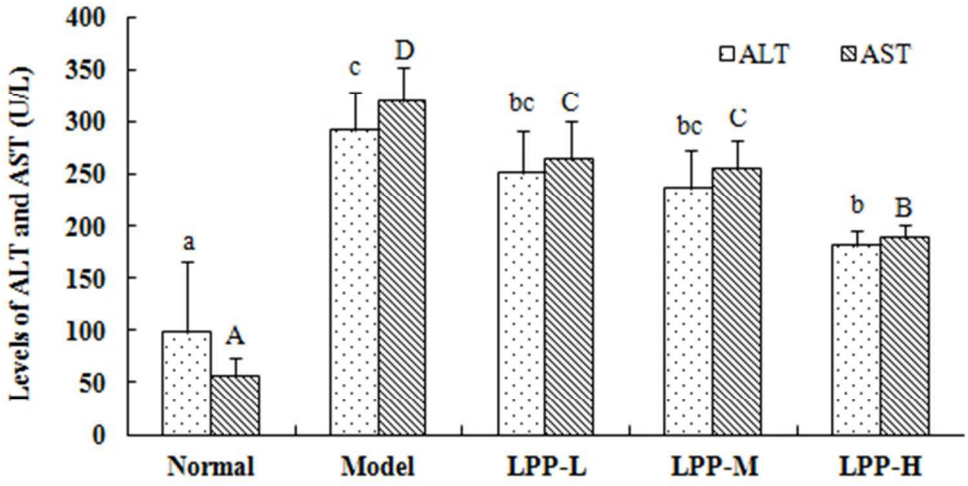
each column without a common letter are significantly different ($p < 0.05$).

Fig. 1. Effects of LPPs on ALT and AST activities in serum from restraint-stressed mice.

Normal control group administered distilled water by oral gavage for 3 consecutive weeks; restraint stress model group administered distilled water by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed; LPP groups administered 50 (LPP-L), 100 (LPP-M), or 200 (LPP-H) mg LPP per kg body weight per day by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed. Levels of ALT and AST were measured using an automatic biochemical analyser. Values are reported as the mean ± SD (n = 10); Bars labelled with different letters are significantly different ($p < 0.05$).

Fig. 2. Effects of LPPs on liver mitochondrial activity in restraint-stressed mice

Normal represents Normal control group, which were administered distilled water by oral gavage for 3 consecutive weeks; Model represents restraint stress model group, which were administered distilled water by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min later after the final oral gavage and lasting for 18 h before being sacrificed; LPP groups administered 50 (LPP-L), 100 (LPP-M), or 200 (LPP-H) mg LPP per kg body weight per day by oral gavage for 3 consecutive weeks and then subjected to restraint stress starting 30 min after the final oral gavage and lasting for 18 h before being sacrificed. The mitochondria were stained by Janus green B and observed under light microscope with a green filter.



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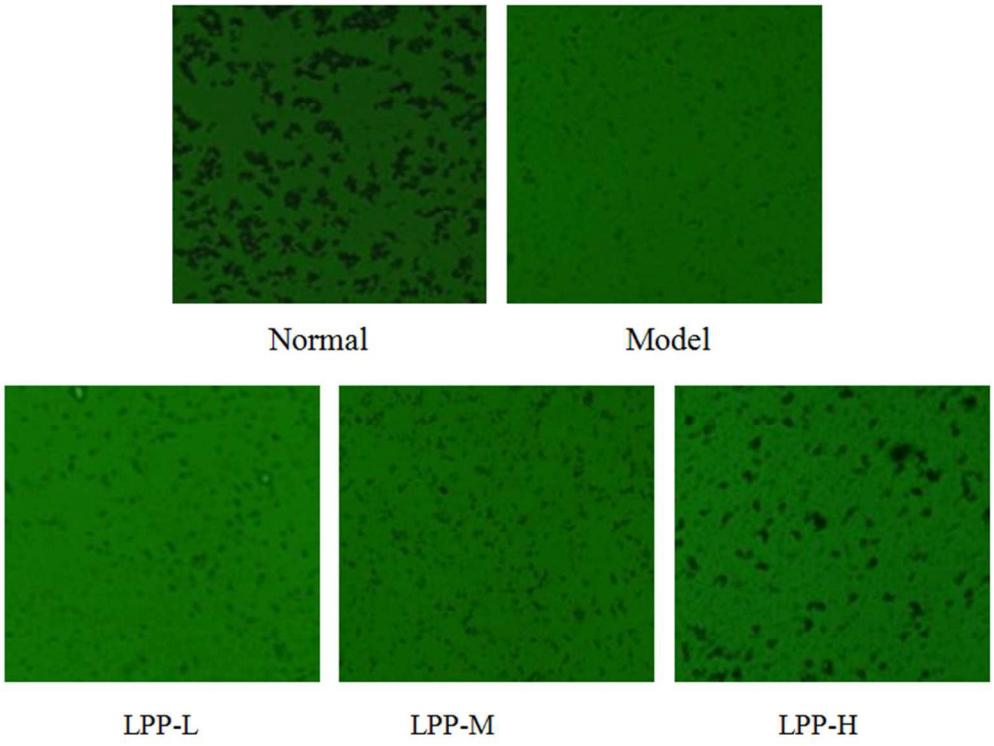

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