Paul Tulkens
Academic Editor
PeerJ

Your reference	**Our reference**	**Date**
Manuscript No: **PeerJ-40525**		Oct. 15 2019

Dear Paul Tulkens,
Thank you for your letter dated Oct. 05, 2019 informing us that our manuscript needs to be revised. We have revised our manuscript according to the constructive comments and suggestions from you and other reviewers, and detailed answers to the comments are provided below.

Editor's Comments
1. As you can see, your paper was considered as interesting and valuable, but a number of comments and critiques need to be addressed. I'd like my-self to insist on the necessity to discuss the dosages used and to convince the reader that these dosages are meaningful if considering potential human therapy. One of the reviewers suggest hints in this connection but you may have other suggestions. In any case, we *must* be able to distinguish between what could be nice but somewhat gratuitous biological studies and studies that clearly show that the compounds discussed are amenable to use in humans. This includes dosages and potential toxicities.

Please, submit a detailed rebuttal where I can see clearly where and how you have taken all comments and suggestions into consideration. If you do not agree with some of these comments or suggestions, explain why. Your rebuttal will be an essential element for me to make a final decision on your paper. Please, note also that your revised version may enter a new round of review by the same or by different reviewers. I cannot, therefore, make any commitment about a final acceptance of your submission.

Reply: We definitely agree your concern on dosage and potential toxicity.
Actually, at the beginning of the study, we treated normal mice with normal diet and 50, 100 mg/kg of TQPE. During this pre-experiment, both 50 and 100 mg/kg dosages of TQPE had no significant effect on their body weight, appetite and hepatic histologic structure, as showed in Figure 1 and 2.

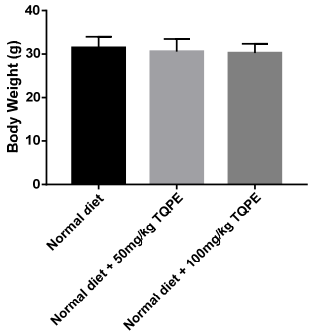
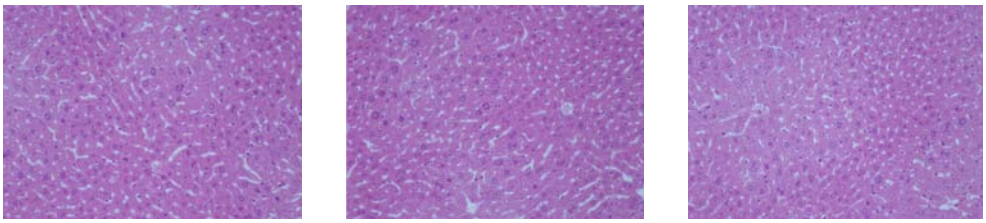


Figure 1. Body weight of normal mice treated with normal diet, 50 and 100mg/kg TQPE.



Nomal Nomal+50mg/kg TQPE Nomal+100mg/kg TQPE

Figure 2. Histological analysis of liver tissues (HE, 200× magnification)

In the formal study, we scaled down and used lower dosages of TQPE (15, 30 mg/kg). According to USA Food and Drug Administration's instruction, when comparing 30 mg/kg of TQPE to dosage used in human, it is approximately 175 mg/day for an adult. Some studies shown that polyphenol intake of an adult subject is about 50-1000mg/kg [1]. These polyphenols are from plant origin. The dose of TQPE (rich in polyphenols) is acceptable to patients for prescription made from plant extract. For the potential toxicity, we didn't perform the test in the present study.

[1] Gemma CB, Lina B. Effects of Polyphenol Intake on Metabolic Syndrome: Current Evidences from Human Trials[J]. Oxidative Medicine and Cellular Longevity, 2017, 2017:1-18.

This response is acceptable. However, in the choice of dose of dose please state the above reason in short.. something like, " Based on exploratory studies 50-100 mg/kg doses were found to be effective but stepped down doses were selected for this study to provide a good safety margin"

Reviewer: Prabhavathi Fernandes

Basic reporting

There are no concerns on basic reporting and language

Experimental design

As noted in the review, some points such as pharmacokinetics to determine the active component and dose justification is not reported

Validity of the findings

The data partially supports the findings but the deficiency is that we do not know which component is active. Also if the animals simply stopped eating the high fat food. There is an urgent need for adequate treatment for the growing NAFLD and hepatocellular carcinoma. This manuscript reveal a natural product extract that could be beneficial.

1. Simple editing:

All “et al. “ in the manuscript should be in italics.

Please search the entire manuscript and change.

Line 52. Replace restore with treat and prevent

Line 72. in the south of China, as well as in India and south east Asia.

Line 96. Five standard (lower case s)

Line 107: Delete The primary antibodies and replace simply with Antibodies to

Line 115: Change concentrated to concentrating

Line 112: Define TQPE as Trapa quadrispinosa pericarp extract. Also, the name of the plant must always be in italics.

Line 196. Please change dominated to predominant

Line 239. Define HOMA-IR. Homeostatic Model Assessment of Insulin Resistance.

Line 321. Please change to used in other clinical trials.

Line 332. .waste, that are usually discarded

Reply： Thank you for your careful review and valuable suggestion. We have checked throughout the manuscript and made correlative corrections in the revised version.

Questions that need to be answered:

2. Line 153. How do we know that the mice continue to eat the high fat diet after beginning treatment? It is possible that they lost their appetite and ate less. Was the amount of food eaten (or left in food tray) measured?

Reply： Unfortunately, the amount of food eaten was not measured in the present study. As we discussed above, both 50 and 100 mg/kg of TQPE did not affect the body weight of normal mice (Figure 1), so we judged that TQPE had no remarkable effect on their appetite. During daily observation, no obvious dietary loss was founded in the study.

This should be stated clearly. “ Although the amount of food eaten was not directly measured, observation by caretakers showed that the animals were feeding and the loss of weight was not simply related to decreased eating”

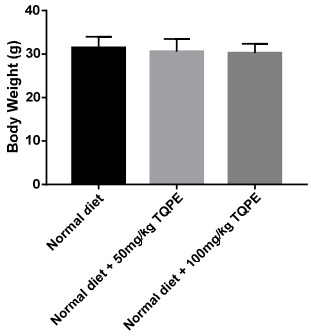


Figure 1. Body weight of normal mice treated with normal diet, 50 and 100mg/kg TQPE.

3. Line 153. How are these doses selected. Normally one selects does based on blood levels and scaled down from toxic limits

Reply： The therapeutic effect of 15, 30, 50 and 100 mg/kg TQPE treatment on HFD-induced NAFLD mice were performed in our pre-experimental study. TC and TG contents of NAFLD mice were regarded as important indexes in the study. We found that 30 mg/kg of TQPE had similar effect comparing with 50 and 100 mg/kg TQPE on TC and TG levels. So we chose 30 mg/kg as the highest dose in this study.

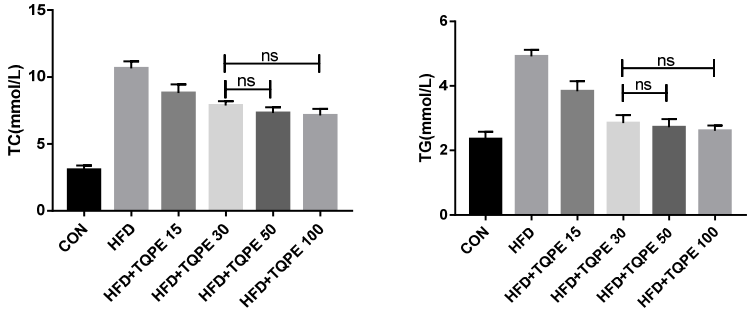


Figure 3. Effects of TQPE on TC and TG level in HFD-induced NAFLD mice.
Results are means ± SD (n = 6); ns = not significant

Text added by Reviewer above.

4. In the introduction the authors should note if the pericarp of TQ is used in China as natural medicine for liver disease. Is this used what led the authors to identify the active components?

Reply： Thank you for your valuable suggestion. The fruit of water caltrop is a kind of function food and folk medicine, which could be used to treat metabolic syndrome. One study reported that TQPE displayed hepatoprotective effect [1]. We also found that the pericarp extract of TQ had therapeutic effect on hyperglycemia and hyperlipidemia [2]. NAFLD is a hepatic manifestation of the metabolic syndrome, so in the present study we wanted to figure out whether TQPE had therapeutic effect on NAFLD as well.

We have mentioned hepatprotective activity of the pericarp of TQ in our revised version.

[1] Kim YS et al., 2014. Antioxidant activity and protective effects of *Trapa japonica* pericarp extracts against tert-butylhydroperoxide-induced oxidative damage in Chang cells. Food and Chemical Toxicology. 64:49-56.10.1016/j.fct.2013.11.018

[2] Lv H et al., 2019. *Trapa natans* pericarp extract ameliorates hyperglycemia and hyperlipidemia in type 2 diabetic mice. Revista Brasileira de Farmacognosia. 10.1016/j.bjp.2019.04.011

5. Also, there are other Trapa species (such as T. japonica and others. Is the activity specific to TQ?

Reply： According to previous reports, some other *Trapa* species such as *T. japonica* and *T. natans*, contain similar compounds as *T. quadrispinosa*. The extracts or compounds isolated from those species showed hypoglycemic or liver-protective effect. So we concluded that other *Trapa* species might have the similar activity.

6. Was there any plasma pharmacokinetic analysis on the TQPE conducted? Steady state plasma levels are essential. It will also show which of the components are actually absorbed. Of the several constituents of the TQPE it is possible that in the intestinal tract some of them may be converted and it is also likely that only one or more components are actually absorbed. What is the oral bioavailability of each component? This is important and needs to be determined prior to publishing this manuscript. The authors may be surprised that only one or more components are needed and also that there may be conversion in the gut.

Reply： Thank you for your valuable suggestion. So far, little attention has been paid to *T. quadrispinosa* pericarp extract (a kind of agricultural waste), and no related research of plasma pharmacokinetic analysis has been done. The purpose of the present study was to determine whether *Trapa quadrispinosa* pericarps extract (TQPE) could attenuate NAFLD induced by HFD in mice, and also to explore a possible mechanism of this action. We focused on investigation the therapeutic effect and molecular mechanisms of TQPE on NAFLD. Interestingly, as shown in Figure 3, compared with 50 and 100 mg/kg TQPE administration, TQPE at 30 mg/kg achieves the best treatment effect. This may be related to active ingredients achieving steady state plasma levels as the reviewer mentioned. This study found that polyphenolics (hydrolysable tannins) in TQPE are active compounds and have good effect on HFD-induced NAFLD, the actual absorbed components are still unknown. As you kindly suggested, we look forward to isolating pure compounds from TQPE, and conducting plasma pharmacokinetic analysis in order to figure out actually absorbed and active component of TQPE.

Since the bioavailability data is not available, at least a sentence should be added that "in future studies the active entity in the extract that is absorbed and found in the plasma of the rats will be measured"

7. Line 245. Was there a dose response between the 15 and 30 mg/kg doses tested

in each of the parameters?

Reply： Thank you for your careful review. According to your valuable suggestions, we have checked our original data, and found that there were statistical differences among the levels of TG, LDL, MDA, SOD and the protein expression of AMPK, SREBP, ACC and Akt between the 15 and 30 mg/kg doses of TQPE.

8. It would be helpful to have a Figure of the proposed NAFLD signaling pathways. I note the pathway as the last Figure. That Figure also needs clarification. There are several up and down arrows. Which of these are related to the TQPE treatment. It is not clear to me.

Reply： Thank you for your valuable suggestion. To avoid confusion, we deleted up and down arrows to make the figure clear to be understood. We have corrected these in the revised manuscript, and the figure in the Results of the revised manuscript has been changed as well.

Reviewer: Carla Carvalho

Basic reporting

The authors showed reduction of the diet induced NAFLD associated to obesity in ICR mice due to administration of a specific herb extract (TQPE), from the pericarp of an aquatic plant popular in south of China, which fruit is used as folk medicine and functional food. They also suggested that AMPK/SREBP/ACC intracellular pathway in the liver is involved in the detected effects.

The authors performed:
1- HPLC from the TQPE
2- liver histology by HE from the DIO with NAFLD mice;
3- body and liver mass;
4- AST, ALT, TG, TC, HDLc, LDLc, SOD, and MDA in blood samples;
5- WB in liver samples of AMPK, SREBP, and ACC.

The language of the manuscript is clear. The references provide sufficient background to its proposal.
The figures and tables are well done and convincing.
The raw data is shared. However, it should be interesting for readers to know which animal group corresponds to each band in the supplied PVDF membranes.
The hypothesis is clear and the data presented is adequate to confirm it.

Experimental design

The subject of the manuscript is within the scope of the journal.
The hypothesis is clearly defined and the aims of the study are adequately indicated within the research field.
The ethical approval for animal usage is described in the manuscript, however, I think it should include the following information in the Methods section and or in the legend figures and tables:

1- the total number of animals used in each parameter analyzed;

Reply： Thank you for your valuable suggestion. The legend of figures and tables in the Results of the revised manuscript have been changed accordingly.

2- the entire period of HFD intake;

Reply： Thank you for your valuable suggestion. In the present study, HFD intake period is 12 weeks, while the administration of TQPE (15, 30 mg/kg/day) is 8 weeks. We have mentioned HFD intake information in the Methods section of our revised manuscript.
CON: normal diet 12 week.
HFD: high fat diet 12 week.
HFD+TQPE 15 mg/kg: high fat diet 12 week including 8 week TQPE 15 mg/kg administration.
HFD+TQPE 30 mg/kg: high fat diet 12 week including 8 week TQPE 30 mg/kg administration.

3- the via of administration of the TQPE;

Reply： Thank you for careful review. The mice were injected with TQPE via intragastric administration.

4- if the animals were euthanized after any period of fast or in fed condition.

Reply： Thank you for careful review. In the end of experimental period, all the mice were fasted, then anesthetized and sacrificed by isoflurane gas.

Validity of the findings

The data presented is convincing. The statistical analysis performed is adequate, however there are 3 minor points that I would like to hear from the authors:

5- Is there any effect of the TQPE in control chow animals, such as reduced insulinemia or body weight?

Reply： Thank you for your valuable suggestion. As shown in Figure 1, the very high dose of TQPE (50 and 100 mg/kg) did not affect the body weight of control mice treated with normal diet. During daily observation these high doses of TQPE had no significant effect on their appetite.

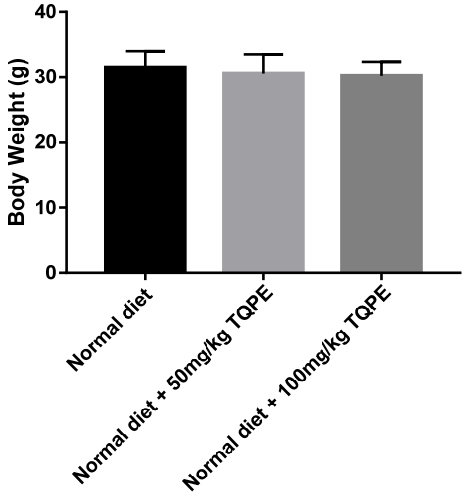


Figure 1. Body weight of normal mice treated with normal diet, 50 and 100mg/kg TQPE.

6- Is there any impact of the TQPE treatment on food and or water intake?

Reply： Thank you for your valuable suggestion. During daily observation high doses of TQPE (50, 100mg/kg) had no significant effect on the appetite of mice, and the body weight didn't change remarkably as shown in Figure 1.

7- Are the elevated AST and ALT blood levels associated to any other hepatic injury histological marker?

Reply： Thank you for your valuable suggestion. Hepatic cells swelled and occurred vacuolar, and induced damage of hepatic lobule structures. These changes were

associated with elevating of AST and ALT levels in the blood.

Comments for the author

The manuscript is very easy to read and the figures are well presented.

The inclusion of the translational information regarding the equivalent dose for human is positive, as well as the economical aspect of identifying the potencial beneficial usage of the pericarp since it is an agriculture wast.

However, I would like to see included in the manuscript some information regarding the above mentioned 7 points.

Reply： Thank you for your positive comments. We have revised our manuscript according to the constructive comments and suggestions, and made correlative corrections in the revised version. Thank you for your valuable suggestion.

The manuscript has been revised as above suggested. Finally, thank you for your patience in assisting us to arrive at an improved manuscript with a high standard of ***Peer J***.

Thank you for your attention.

Best regards,
Yours sincerely

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Polyphenol-rich *Trapa quadrispinosa* pericarp extract ameliorates high-fat diet induced non-alcoholic fatty liver disease by regulating lipid metabolism and insulin resistance in mice

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Abstract

In China, *Trapa quadrispinosa* (also called water caltrop) has long been used as a function food and folk medicine to treat diabetes mellitus for years. In the present study, the extract of *T. quadrispinosa* pericarp (TQPE) which mainly contains hydrolysable tannins was prepared to investigate the potential therapeutic action in non-alcoholic fatty liver disease (NAFLD) mice induced by high fat-diet (HFD). After the administration of TQPE (15, 30 mg/kg/day) for 8 weeks, the increased weight of body and liver were significantly suppressed. TQPE also ameliorated liver lipid deposition and reduced lipids parameters of blood in mice. Moreover, TQPE attenuated oxidative stress and showed a hepatoprotective effect in mice. TQPE was also found to decrease the value of homeostatic model assessment for insulin resistance. In addition, TQPE administration increased the phosphorylation of AMP-activated protein kinase (AMPK) and Acetyl-CoA carboxylase (ACC) and inhibited sterol regulatory element-binding protein (SREBP) in the liver tissue. Meanwhile, TQPE elevated insulin receptor substrate-1 (IRs-1) and protein kinase B (Akt) phosphorylation. These results reflected that, as a nature product, TQPE is a potential agent for

suppressing the process of NAFLD via regulation of the AMPK/SREBP/ACC and IRs-1/Akt pathways.

Introduction

As a common chronic liver disease, Non-alcoholic fatty liver disease (NAFLD) is defined by pathological accumulation of lipid in the liver without excess alcohol consumption (Golabi *et al*. 2017). Being a hepatic manifestation of metabolic syndrome, it is similar to those chronic metabolic disorders, such as obesity, insulin resistance, type 2 diabetes mellitus (T2DM), inflammation and cardiovascular disease (Bagherniya *et al*. 2018). NAFLD increases the risk of progressive liver injury, which appears as a continuum disease progression, from simple steatosis to liver failure and hepatocellular carcinoma (Suolang *et al*. 2019). NAFLD has emerged as a worldwide serious public health burden, epidemiology of NAFLD have highlighted surprisingly high prevalence in many countries (the estimated prevalence is 25-30% in adults) (Moore 2019; Ratziu 2018). Therefore, there is a great demand for exploring effective therapeutic agents to treat and prevent NAFLD.

The recent evidence indicated that fat accumulation and insulin resistance (IR) are intensely associated with the development and progression of NAFLD (Araujo *et al*. 2018; Fan *et al*. 2018; Jian *et al*. 2018). As a highly evolutionarily conserved sensor of cellular energy status, AMP-activated protein kinase (AMPK) plays a critical role in regulating hepatic lipid metabolism including lipolysis, glucose transport and gluconeogenesis (Brown & Goldstein 1997). Sterol regulatory element-binding protein (SREBP), a key transcription factor in regulating liver lipid synthesis, is the downstream of AMPK (Li *et al*. 2011). Acetyl-CoA carboxylase (ACC), a member of lipogenic factor, is the downstream target of SREBP. AMPK activation phosphorylates and inhibits ACC in adipose and hepatic tissues thus downregulate fatty acid synthesis (Bijland *et al*. 2013; Zhang *et al*. 2018). In NAFLD model of many studies, it was observed that the inhibition of phosphorylation of AMPK led to lipid accumulation by increasing SREBP and inhibiting ACC phosphorylation (Chen *et al*. 2019; Li *et al*. 2018b; Park *et al*. 2019; Zhou *et al*. 2017). Besides, IR is also strongly associated with hepatic lipid accumulation in NAFLD. Insulin signaling transduction is dependent on insulin receptor substrate-1 (IRs-1), and phosphorylation of IRs-1 give rise to insulin pathway activation (Fu *et al*. 2018; Saez-Lara *et al*. 2016). Moreover, for insulin signaling cascade conduction, Protein kinase B (Akt) is another essential factor. Impairment of Akt activity has been demonstrated under NAFLD condition, thus activated Akt (increased phosphorylation) could ameliorate hepatic steatosis and improve IR in NAFLD model (Fan *et al*. 2018; Jung *et al*. 2018). Therefore, targeting regulation of AMPK and insulin signaling pathway might be a new and useful therapeutic approach to drop lipid accumulation and insulin resistance in NAFLD.

Nowadays, pharmacological studies have significantly expanded to screen natural products for exploration of novel pharmaceutical agents. Many studies revealed that medicinal plant extracts, herb formulas have remarkable therapeutic effect on NAFLD (Bagherniya *et al*. 2018; Chen *et al*. 2017; Li *et al*. 2018a; Suolang *et al*. 2019). *Trapa quadrispinosa*, also called water

chestnut or water caltrop, is a floating-leaf aquatic plant, which is commonly cultivated in the south of China, as well as in India and south east Asia. The fruit of water caltrop, is a function food and folk medicine, which could be used to treat metabolic syndrome such as diabetes mellitus (DM). But the pericarps of *T. quadrispinosa* were usually discarded in large quantities after the seeds had been harvested. Interestingly, recent researches have demonstrated that the pericarps of water caltrop also displayed multiple biological activities, including hypoglycemic (Huang *et al*. 2016), anti-tumor (Lin *et al*. 2013), anti-inflammatory (Kim *et al*. 2015), anti-oxidant effects and hepatprotective activity (Kim *et al*. 2014). To our knowledge, the therapeutic effect of *Trapa quadrispinosa* pericarps extract (TQPE) in high-fat diet (HFD) induced NAFLD, remains unknown.

The purpose of the present study was designed to determine whether *Trapa quadrispinosa* pericarps extract (TQPE) could attenuate NAFLD induced by HFD in mice, also explore a possible mechanism of this action.

Materials & Methods

Chemicals and Reagents

HPLC grade methanol used for the mobile phase in HPLC-DAD/QTOF analysis was obtained from Tedia Co. Inc. (Fairfield, OH, USA). Formic acid in HPLC grade was taken from Acros Organics (Geel, Belgium). Pure water was produced from a Milli-Q system (Millipore, Bedford, MA, USA). Five standard compounds isolated from *T. quadrispinosa* were prepared and identified according to our previous study (Lv *et al*. 2019). Gallic acid was obtained from National Institutes for Food and Drug Control of China (Beijing, China). The other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). A kit from KeyGen Biotechnology (Nanjing, China) was used for the protein extraction and BCA protein assay. Besides, the kits for detecting blood glucose, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), total cholesterol (TC), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), superoxide dismutase (SOD) and malonaldehyde (MDA) were all obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Determination of insulin by enzyme-linked immunosorbent assay (ELISA) commercially kits were purchased from Beyotime Institute of Biotechnology (Haimen, China).

The antibodies of AMPKα, p-AMPKα (Thr172), ACC, p-ACC, IRs-, p-IRs-1 (Tyr895), Akt, p-Akt (Ser473) for western blotting (WB) were purchased from Cell Signaling Technology (Danvers, MA, USA). SREBP was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-rabbit/anti-mouse IgG and HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, USA).

Preparation and characterization of *Trapa quadrispinosa* pericarp extract (TQPE)

The air-dried *T. quadrispinosa* (collected in Shandong Zaozhuang of China) pericarp powder (10.0 kg) was extracted twice with fifty litres 80% (v/v) ethanol by soaking at room temperature for 14 days. After concentrating in vacuum at 50 °C, the extract was suspended in distilled water and then partitioned by petroleum ether, ethyl acetate and normal-butanol successively. The ethyl acetate

Deleted: type of

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extract was first concentrated and then dissolved in water, and later applied to a column packed with macroreticular resin XAD16 (The Dow Chemical Company). The column was orderly eluted with a gradient of H_2O, 10%, 40%, 60% and 95% ethanol solution. The 40% ethanol elution was collected, concentrated and dried and finally the TQPE (175g) was obtained.

According to previous reported method, the total phenolic content of TQPE was measured by the Folin-Ciocalteu phenol reagent (Singleton & Rossi 1965). Briefly, 1.0 mg of TQPE extract was solved in 1.0 ml of methanol was used as test solution. After incubation with Folin-Ciocalteu phenol reagent, the test solution was added 20% sodium carbonate to develop a color and its absorbance was measured at 755 nm. The final value was expressed as gallic acid (standard) equivalent.

5.0 mg/ml of TQPE (dissolved in methanol) was injected in an Agilent 6530 accurate-mass quadrupole time-of-flight system (Agilent Technologies, CA, USA) for the HPLC-QTOF/MS analysis. Separation was conducted by an Agilent ZORBAX SB-C18 column (1.8 μm, 4.6 × 100 mm; Waldbronn, Germany). The mobile phase was composed of Methanol (A) and 0.1% formic acid (B) under gradient conditions (0-50 min, 10-50%A; 50-65 min, 50-100% A) at 1 ml/min. The QTOF-MS equipped with an electrospray ion source operated in the negative ion mode at 4 kV capillary voltage, 10 L/min drying gas and 350 °C gas temperature. Mass range of scanning was 100-1000 m/z. MassHunter Qualitative Analysis software (Agilent Technologies, USA) was used to perform measurement and analysis.

Furthermore, HPLC-DAD was performed by Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA) equipped with a diode array-UV detector at 210 nm. Nucleosil C18 column (4.6 mm × 150 mm, 3 μm, ThermoFisher Scientific, USA) was used for separation. The mobile phase was composed of Acetonitrile (A) and 0.05% trifluoroacetic acid (B) under gradient conditions as followed: 0-5 min, 5%A; 5-15 min, 5-10% A; 15-55min, 10-25% A; 55-65min, 25-100% A, the flow rate of HPLC was 1 ml/min.

Animals

The male ICR mice were obtained from Shanghai Sino-British SIPPR/BK Lab Animal Co., Ltd. (China) and they were maintained in controlled condition as our previous study described (Jian *et al*. 2018).

Experimental protocol

At first stage, mice were provided either a normal chow diet (10% of calories) or a high fat diet (60% of calories) for 4 weeks, which were obtained from Shanghai Lab-oratory Animal Co. Ltd. (Shanghai, China). Then, the high fat diet-fed mice were randomly divided into 3 groups: one control group and two test groups, the test groups were administered with low dose of TQPE (15 mg/kg) and high dose of TQPE (30 mg/kg) per day. Normal control groups (CON) and high fat diet-fed mice control (HFD) group were treated with solution of 0.5% CMC-Na. All mice in each group were administrated relevant reagents for another 8 weeks. In the end of experimental period, all the mice were fasted, then anesthetized and sacrificed by isoflurane gas. Thereafter, the serum was separated from blood and the liver was removed and weighted. All the samples were stored at -80°C condition before use.

Biochemical parameters analysis

According to the commercial kits instructions, serum TC, HDL, LDL, TG, SOD, MDA, ALT and AST concentrations were determined in a microplate reader (Molecular Device, CA, USA). Serum insulin level was determined by using ELISA commercially kits. The HOMA-IR (Homeostatic model assessment for insulin resistance) value which was calculated as fasting blood glucose (mmol/L) × fasting serum insulin (mIU/L)/22.5, was represented insulin sensitivity.

Liver pathological examination

Liver tissues were fixed overnight at 4 °C in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were prepared for hematoxylin & eosin (H&E) stain. The histological images were observed and photographed under an optical microscope (Carl Zeiss, Germany).

Western blot analysis of liver tissue

The total protein from the liver tissues was extracted using a commercial kit. Proteins from the liver supernatant were quantified by a BCA kit. 25 μg of total proteins was separated on 8-10% SDS-PAGE gel then transferred onto a PVDF membrane from Millipore (MA, USA). The membrane was blocked for 2 h in 5% nonfat milk powder solution at room temperature. Then the membranes were incubated with primary antibody at 4°C overnight. At the second day, the membranes were incubated with secondary antibody at room temperature for 1-1.5 h. The Membrane-bound antibodies complexes were noticed by ECL detection (Santa Cruz Biotechnology, CA, USA). Tanon 5200 image system (Tanon, Shanghai, China) was used to examine the signal. β-actin was employed to demonstrate the standard proteins quantity. ImageJ software (NIH) was applied to quantify the density of western blotting bands

Statistical analysis

All results were expressed as mean ± SE from 10 mice. All statistical analyses were done through the GraphPad Prism Software(Version 7.02, CA, USA). One-way ANOVA with a post-hoc test was used for the statistical significance evaluation. $P < 0.05$ was indicated statistical significance.

Results

Analysis of chemical constituents and content of TQPE by HPLC-QTOF/MS and HPLC-DAD

According to Folin-Ciocalteu phenol test, the most abundant compound in the TQPE was polyphenolics (hydrolysable tannins), the content of which was accounting for 91.7 ± 2.1% (gallic acid equivalents). To furtherly understand the potential bioactive components in TQPE, a HPLC-QTOF/MS assay was carried out. The base peak chromatogram of TQPE was illustrated in Figure 1A. Eight phenolic compounds (R_t = 9.45, 23.07, 24.15, 24.76, 30.72, 35.58, 36.52 and 47.65 min) were tentatively identified according to previous studies (Hatano *et al*. 1990; Huang *et al*. 2016). Their structures were shown in Figure 1B. The predominated polyphenols in TPQE was gallic acid and its derivatives, which included hydrolysable gallotannins and gallates. We also used HPLC-DAD method to calculate the content of five polyphenols in TPQE, which was showed in Table 1. Gallic acid (Compound 1), 1,2,3-tri-*O*-galloyl-*β*-D-glucopyranose (Compound 2), 1,2,3-tetra-*O*-galloyl-4,6-*O*-hexahydroxydiphenoyl-*β*-D- glucopyranoside (Compound 6), 1,2,3,6-tetra-*O*-

galloyl-*β*-D-glucopyranose (Compound 7), 1,2,3,4,6-penta-*O*-hexahydroxydiphenoyl-*β*-D-glucopyranoside (Compound 8) were accounted for 696.56 ± 4.61mg/g.

Effect of TQPE on the body and liver weight of HFD mice

As showed in Figure 2A and Figure 2B, compared with the normal group, body weight in HFD group and their liver to body weight ratio rose obviously ($P < 0.001$). Administration of TQPE (15 and 30 mg/kg) significantly inhibited HFD induced growth in body weight and liver to body weight ratios ($P < 0.01$). Treatment with TQPE reversed the continuing weight gain in both body and liver.

Histopathological examination

Normal control group livers showed glossy and resilient appearance. In contrast, as showed in Figure 2C, the NAFLD mice livers were enlarged with yellow necrosis foci appearing. After the livers were treated with TQPE, the liver appearance improved in a dose-dependent manner (Figure 2C).

Figure 2D displayed liver histology photo sections. In the control group, liver tissues showed normal liver lobular structure without fatty accumulation or inflammatory. In HFD treated mice, liver sections revealed significantly higher damage of hepatic lobule structures compared with normal mice, while lipid droplets were also spotted in liver cells. As displayed in Figure 2D, after treating with TQPE in 15 and 30 mg/kg, hepatocyte swelling as well as quantities and volumes of lipid droplet were all alleviated. Morphology of liver lobular structure almost regained normal status, especially in the mice treated with 30 mg/kg TQPE.

TQPE ameliorates Lipid Parameters in NAFLD mice

Relevant lipids parameters were detected to evaluate the effects of TQPE on lipid metabolism in NAFLD mice. TC, TG and LDL content were markedly elevated ($P < 0.001$; Figure 3A, B and D) along with a decrease in HDL level (Figure 3C; $P < 0.05$) in NAFLD mice compared to the normal group. In Figure 3A-C, the data showed that administration of TQPE (15 mg/kg) significantly reduced TC, TG and elevated HDL compared with the HFD group ($P < 0.01$). Furthermore, after treatment with a high dose of TQPE at 30 mg/kg, besides above effects, it dramatically decreased LDL but increased HDL level (Figure 3A-D; $P < 0.001$).

TQPE changes the oxidative stress balance and reduces liver injury in NAFLD mice

As demonstrated in Figure 4A and B, HFD group mice showed less adequate SOD and surplus level of MDA in the serum compared to the control group. This phenomenon implying an increased oxidative stress and a decrease of antioxidant capacity in HFD-induced NAFLD mice. Treatment with TQPE ameliorated this symptom by significantly raising SOD and reducing MDA level ($P < 0.001$, Figure 4A and B). In Figure 4C and D, compared with normal mice, the contents of ALT and AST were seen elevating in the NAFLD mice. Administration of TQPE (15 and 30 mg/kg) significantly reduced ALT and AST levels indicating the recovery from liver damage induced by HFD-induced NAFLD mice ($P < 0.001$).

Effects of TQPE on serum insulin and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

Insulin concentration in serum and HOMA-IR value were calculated to evaluate the effect of TQPE on insulin signal.

In HFD mice, both the serum insulin concentration and HOMA-IR value were increased significantly, which indicating that there was an insulin resistance in NAFLD mice ($P < 0.001$, Figure 5). Both the value of serum insulin concentration and HOMA-IR were significantly decreased after the treatment of TQPE (15 and 30 mg/kg) in comparison with the HFD mice ($P < 0.001$, Figure 5A and 5B). The data indicated that TQPE suppressed HFD-induced IR in NAFLD mice.

TQPE changes pathways participated in lipid metabolism and insulin resistance

For further investigate the mechanism of TQPE preventing NAFLD, we detected the change of proteins expression including AMPK, SREBP, ACC, IRs-1 and Akt in the liver tissue, which were involved in lipid metabolism and insulin resistance. In Figure 6A-C, the phosphorylation of AMPK and ACC were decreased along with an increase in SREBP in HFD group in comparison with control group ($P < 0.001$). Meanwhile, compared with control group, in HFD mice liver, it showed a drop of IRs-1 and Akt phosphorylation ($P < 0.001$; Figure 6D and E). Treatment with TQPE at a high dose of 30 mg/kg markedly recovered the phosphorylation level of AMPK, ACC and inhibited the expression of SREBP in HFD-induced NAFLD mice ($P < 0.001$; Figure 6A-C). Additionally, TQPE (15 and 30 mg/kg) administration repaired prohibition of IRs-1 and Akt phosphorylation in NAFLD mice ($P < 0.01$; Figure 6D and E).

Discussion

The current study investigated the therapeutic effect of *Trapa quadrispinosa* pericarp extract (TQPE), on lipid accumulation and insulin resistance in HFD-induced NAFLD, and worked out the possible mechanism. We concluded that TQPE attenuated HFD-induced lipid accumulation in the liver, probably was mediated by the AMPK activation and SREBP-mediated lipogenesis inhibition. TQPE also improved IR in NAFLD possibly through upregulating the levels of p-IRs-1 and p-Akt protein (Figure 7).

It is widely accepted and confirmed that hepatic steatosis is the earliest stage of NAFLD, which is manifested as accumulation of lipid in the liver. In nutrient oversupply states, ectopic lipid accumulation is closely related to hyperglycemia and hyperlipidemia. AMPK is a crucial key and important metabolic sensor and regulator of lipid and glucose metabolism in diverse tissues and cells (Cheng *et al*. 2016; Day *et al*. 2017). Activated AMPK promotes energy production, meanwhile, and represses ATP-consuming processes, to maintain energy status balance (Gonzales *et al*. 1992; Ruderman & Prentki 2004). Both SREBP and ACC are two downstream effectors of AMPK. For instance, AMPK activation inhibits fatty acid synthesis via a reduction in the transcriptional activation of SREBP, which is a key transcription factor during *de novo* lipogenesis in the regulation of lipogenic genes including *acc1* in the liver (Ahmed & Byrne 2007; Zhou *et al*. 2017). AMPK activation also could switch off fatty acid synthesis through ACC phosphorylation in adipose and hepatic tissues (Bijland *et al*. 2013; Zhang *et al*. 2018). As described, AMPK activation might reduce hepatic lipid accumulation via lipogenesis inhibition, indicating that

AMPK is a potential therapeutic target for the treatment of hepatic steatosis in NAFLD (Zhou *et al*. 2017). Recent studies showed that many compounds are able to activate AMPK in animal and cellular models to improve lipid metabolism in NAFLD, by inhibiting SREBP and increasing phosphorylates of ACC (Chen *et al*. 2019; Kang *et al*. 2019; Park *et al*. 2019). In our study, we explored the lipid contents comprising TC, TG and LDL, the concentration of which were significantly lowered after TQPE treatment, while the HDL level was enhanced in HFD-induced NAFLD ($P < 0.01$; Figure 3). We also found that TQPE at dose of 30 mg/kg/day could obviously activate the phosphorylation of AMPK and ACC, while inhibit SREBP expression ($P < 0.001$; Figure 7A-C). These results demonstrated that AMPK axis is associated with the protective effects of TQPE in lipid metabolism under NAFLD status.

Impaired responsiveness to insulin evokes insulin resistance in NAFLD, hence, the improvement of insulin sensitivity is important for the treatment of NAFLD. Insulin receptor substrate-1 (IRs-1) is a core factor in insulin signaling transduction. Research showed that phosphorylation of IRs-1 at Ser accounts for IR, while phosphorylation of IRs-1 at Tyr is required for responses of insulin stimulates (Bhattacharyya *et al*. 2015; Xiao *et al*. 2018) . In metabolic disorders, such as NAFLD and T2DM, impaired IRs-1 level was observed, for instance, lipid accumulation in the liver was closely associated with increased serine phosphorylation of IRs-1 (Araujo *et al*. 2018; Dallak 2018; Dong *et al*. 2019). Restoration of IRs-1 might be a beneficial treatment for curing NAFLD (Yang *et al*. 2018; Zhou *et al*. 2018). Protein kinase B (Akt) as downstream of IRs-1, plays an essential role in insulin signaling cascade. In response to upstream signal of IRs-1 on Tyr, activated Akt increases hepatic glucose uptake, glycogen synthesis and decreases lipogenesis (Cignarelli *et al*. 2019; Geidl-Flueck & Gerber 2017; Guo 2014). Moreover, hepatic Akt activation enhances the insulin sensitivity (Cignarelli *et al*. 2019; Ke *et al*. 2015). Therefore, IRs-1/Akt is important for hepatic insulin signaling, regulates this pathway might be beneficial for treating NAFLD. In the current work, the level of insulin and HOMA-IR value increased significantly in HFD-induced NAFLD ($P < 0.001$; Figure 5). This mechanism supported by decreased hepatic phosphorylation of IRs-1 on Tyr ($P < 0.001$; Figure 6D). In parallel, there was also a significant decline in phosphorylation of Akt ($P < 0.001$; Figure 6E) in NAFLD mouse model. TQPE administration with different doses (15 and 30 mg/kg/day) reversed the high level of insulin and HOMA-IR value compared with NAFLD mice ($P < 0.001$; Figure 5). This effect might be associated with restore phosphorylation of IRs-1 and Akt ($P < 0.05$; Figure 6D, E) by TQPE to improve IR in NAFLD model.

In addition, ALT and AST are two hepatic enzymes, which are well-known key biochemical markers for detecting liver damage in NAFLD mode (Katsagoni *et al*. 2017). In our study, HFD treatment induced increasing of ALT and AST, while the administration of TQPE (15 and 30 mg/kg/day) could revise these parameters ($P < 0.001$; Figure 4C, D). In comparison with a dose of 2000 mg/kg of *T.* natans pericarp extract displaying antihyperglycemic effect in rats, we found that a lower dose of 30 mg/kg of TQPE, also exerted a therapeutic action on HFD-induced NAFLD in mice, while no significant abnormal changes were found in the present study (Yasuda *et al*. 2014). According to USA Food and Drug Administration's instruction (Food and Drug

Administration. 2005), the dose of 30 mg/kg TQPE used in human is approximately 200 mg/day, thus this dose is acceptable for patients compared to other clinical trials used (250-960 mg/day) (Chen *et al*. 2016; Qiu *et al*. 2013; Ried *et al*. 2013).

Conclusions

The present study demonstrated that *Trapa quadrispinosa* pericarp extract (TQPE) administration significantly improve the metabolic parameters including the insulin resistance in high-fat diet-induced NAFLD mice. The protective mechanisms of TQPE may be attributed to regulate AMPK/SREBP/ACC and IRs-1/Akt signal pathways, which are involved in lipid metabolism and IR, respectively. TQPE treatment provides a novel therapeutic strategy to prevent HFD-induced NAFLD, however, there were still some limitations in this study, more detailed mechanisms of TQPE require further analysis.

The pericarps of *T. quadrispinosa* is a kind of agricultural waste, that are usually discarded in large quantities after the seeds had been harvested. From economic aspect, the present study helps to discover polyphenol-rich extraction from this waste with medicinal value.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

Formal analysis, T.Y.J. and X.Q.D.; Investigation, T.Y.J., H.L., X.Q.D and Y.X.W.; Methodology, Y.Y.Z. and J.W.L.; Project administration, H.L. and J.C.; Resources, H.G.; Writing - original draft, T.Y.J. and H.L.; Writing – review & editing, J.C..

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All animal experiments for the study followed the Guide for the Care and Use of Laboratory Animals was approved by the Animal Ethics Committee of China Pharmaceutical University (certificate number: SYXK2016-0011, approval date: 27 January 2016 to 26 January 2021).

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