Author’s response to reviews

Title: The potential of antioxidant-rich Maoberry (Antidesma bunius) extract on fat metabolism in liver tissues of rats fed a high-fat diet

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Version: 2 Date: 13 Aug 2019

Author’s response to reviews:

6 August 2019

Dear Editor-in-Chief

Mohd Fadzelly Bin Abu Bakar, Ph.D.

BMC Complementary and Alternative Medicine

Thank you for your evaluation letter. Please find enclosed our manuscript no. BCAM-D-19-00712R1 entitled “The potential of antioxidant-rich Maoberry (Antidesma bunius) extract on fat metabolism in liver tissues of rats fed a high-fat diet” which we would like to be considered for publication as a Full length Article in BMC Complementary and Alternative. The grammatical structure of this study was proved by Enago™ – Language Editing Services.

According to reviewer’s comments, we corrected and answered with all the comments raised by the reviewers. We would like to thank you for allowing us to resubmit a revised manuscript. We also would like to take this opportunity to express our sincere thanks to the reviewers and editor for the valuable comments.
We have answered and revised the manuscript according to your suggestion as follows:

Editor Comments:

1. Kindly improve the abstract and discussion - what group of phytochemical(s) contribute to the fat metabolism in liver tissues of rats fed a high-fat diet.

Author’s Response:

   - As suggested by editors, we have improved the abstract and discussion according to editor and reviewers’s comments. The groups of phytochemicals contribute to the fat metabolism in liver tissues of rats fed a high-fat diet were added in discussion.

(See abstract: Page2).

(See discussion: Page17; lines 9-20).

2. Biological or antioxidative activity study of extracts requires detailed extract characterization. Chromatographic profiling (e.g. HPLC profile with at least the major peaks identified) should be carried out.

Author’s Response:

   - Our current study analyzes cyanidin and peonidin of extracts by HPLC as well as total polyphenol and flavonoid contents by chemical spectacular reactions. Previously, we reported the nutritive values and antioxidant activity of Maoberry extract following (Udomkasemsab et al 2019):

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>Fibre (g/100 ml)</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Moisture (g/100 ml)</td>
<td>30.49 ± 0.17</td>
</tr>
<tr>
<td>Fat (g/100 ml)</td>
<td>0.00</td>
</tr>
<tr>
<td>Ash (g/100 ml)</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Antioxidant activity</td>
<td></td>
</tr>
<tr>
<td>FRAP (mmol TE/100 ml)</td>
<td>1.51 ± 0.01</td>
</tr>
<tr>
<td>ORAC (mmol TE/100 ml)</td>
<td>15.28 ± 0.90</td>
</tr>
</tbody>
</table>
We have notice the editor’s consideration. Thus, we improve discussion part by adding the sentence following “The purple-black fruits are often rich in anthocyanins and phenolic compounds, which are known as potent antioxidants. Our results showed the significant amount of cyanidin and peonidin in Maoberry extract. This is consistent with the study of berry fruits that reported the highest amount of cyanidin and peonidin among all anthocyanins (Zheng 2003). Previously, we have reported that Maoberry extract are sources of many essential nutrients and high in antioxidants (Udomkasemsab et al 2019). Other bioactive compounds in Maoberry fruit (Antidesma bunius (L.) Spreng have been reported such as ascorbic acid, gallic acid, (-)-epicatechin, (+)-catechin, and cyanidin-3-O-glucoside (Sripakdee et al. 2015 and Butkhup et al, 2011). Synergistic interactions of those active ingredients available in Maoberry extract have the opportunity to improve the fat metabolism in the liver tissues. In addition to improving fat metabolism, Maoberry extract from our previous studies also showed a reduction of oxidative stress and inflammation in heart tissues (Udomkasemsab et al 2019). Therefore, this can confirm the ability of strong antioxidant activity that is possessed by Maoberry extract”

References


(See discussion: Page 17; lines 9-20).

3. Kindly include related previous study to support and strengthen your discussion.
Author’s Response:

- As suggested by editors, we have improved discussion according to editor and reviewers’s comments.

(See discussion: Page 14;lines 9-18, Page 16; lines 8-13).

: Page17;lines 9-20).

Reviewer’s Comments:

Reviewer reports:

Zulkhairi bin Amom (Reviewer 1): In the abstract and methodology sections, it was not clear whether high fat diet was given only for 4 weeks prior to the maoberry treatment and stop during the intervention, or it subsequently continued throughout the 12 weeks treatment. This information is essential because the findings will be very much affected by the fat/cholesterol stress in the body. On the other hand, the discussion section dictates high fat diet was given for 16 weeks (line 6, p15). Suggestion: the author is advise to clarify this in both abstract and methodology sections for better comprehension.

Author’s Response:

- We are thankful to the reviewer for the encouraging comments to improve our manuscript. In our study, high fat diet was given for 4 weeks to induce obesity and subsequently continued a treatment throughout more other 12 weeks with maoberry or statin. Total time for high fat feeding was 16 weeks.

Therefore, we modify the sentence in abstract from “The rats were fed with high fat diet for 4 weeks before being given the Maoberry extract or statin by gavage method for 12 weeks” to “The rats were fed with high fat diet for 4 weeks to induce obesity and subsequently continued more for 12 weeks with treatments of Maoberry extracts or STAT”

(See abstract: Page2;lines 11-13).

We also modify the sentence in methodology section from “all rats were received high fat diet (5.34 kcal/g of diet: carbohydrate 22%, fat 59%, and protein 19% base on nutrition distribution) for 4 weeks of induction phase. At week 6, rats were randomly divided into 5 groups, 12 rats of each group.” to “all rats were received high fat diet (5.34 kcal/g of diet: carbohydrate 22%, fat 59%, and protein 19% base on nutrition distribution) for 4 weeks to induce obesity and subsequently continued more for 12 weeks with treatments of Maoberry extracts or statin. During treatment, rats were randomly divided five groups with 12 rats in each group.”
Reviewer’s Comments:

Rabeta Mohd Salleh (Reviewer 2):

1. Explain why technique, which do not use animals, have been rejected as unsuitable because couldn't find the in vitro study done before.

Author’s Response:

-We would like to thank to the reviewers for the valuable comments. We have notice the editor’s consideration. Thus, we improve discussion part by adding the sentence following: “This current study focus on the liver tissue of rats which are induced to accumulate triglyceride by feeding high fat diet. Nonalcoholic fatty liver disease (NAFLD) is the accumulation of fat or triglyceride in the liver, in the absence of heavy alcohol use [Pavlides et al, 2015]. Pathogenic genesis to develop the accumulation of fat in the liver has found to link with many risk factors such as obesity, metabolic syndrome, excess calories, lipotoxicity, and oxidative stress [Pavlides et al, 2015]. There is a need to imitate the pathogenic and histological features of triglyceride accumulation be similar to human. It is very complex mechanisms in humans. This is very difficult to obtained in vitro study. Thus, rodents have mostly been used as experimental models of NAFLD to resemble the pathogenic and histological features of NAFLD (Lieber et al, 2004; Kucera & Cervinkov; 2014).

(See discussion: Page14; lines 9-17).

References:


2. Background: Sentence no 1, 2, 3 and 4, 6 and 7 please put references.
Author’s Response:

-We revised the background of the first paragraph. The references in this paragraph are all cited following: “The global prevalence of obesity in adults has dramatically increased over a period of time by almost three times from 3.2% in 1975 to 13% in 2016. (WHO). Thailand is one of the highest prevalence of obesity in Asia. In 2009, the prevalence of obesity was approximately 35%, increasing more than 2.5 times compared to 1991 (Aekplakorn, et al. 2014), which 66.5% of the same population group has abnormal blood cholesterol (Aekplakorn, 2014). Obesity and dyslipidemia are major risk factors associated with non-alcoholic fatty liver disease (NAFLD). Fat accumulation in the liver more than 5% without significant alcohol consumption was represented of NAFLD (Bedogni G et 2005). It is the most common liver disease worldwide, although there has not been a report of actual fatty liver levels due to different clinical and histological forms (Araujo, 2018). NAFLD causes increasing oxidative stress, tissue inflammation, and hepatocytes malfunction. Simple steatosis can progress to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and ultimately hepatocellular carcinoma [1]. Consumption of a high fat diet causes increasing body weight and enlargement of internal organs, including the liver, as well as dyslipidemia and obesity in rats [7-9] Feeding high fat diet to rats were able to attribute characteristics hypercholesterolemia which is relevance to human biology [8-12]. A high fat diet was therefore an option in this study

(See background: Page3;lines 10- Page4;lines2)

References


3. In background try to find the prevalence of obesity and dyslipidemia in Thailand and in the world.

Author’s Response:

- We added the prevalence of obesity and dyslipidemia in Thailand and in the world in background of the first paragraph following: “The global prevalence of obesity in adults has dramatically increased over a period of time by almost three times from 3.2% in 1975 to 13% in 2016. (WHO). Thailand is one of the highest prevalence of obesity in Asia. In 2009, the prevalence of obesity was approximately 35%, increasing more than 2.5 times compared to 1991 (Aekplakorn, et al. 2014), which 66.5% of the same population group has abnormal blood cholesterol (Aekplakorn, 2014).

(See background: Page3; lines 10-14).

4. The problem statement of this study was too weak.

Author’s Response:

- According to your suggestion, we revised the problem statement as mentioned in reviewer's Comment number 2.

(See background: Page3; lines 10-Page4; lines2).

5. Cooled extracts were centrifuged at 4 2,000 rpm for 30 minutes (HIMAC centrifuge, CR5BB2, HITACHI, Tokyo, Japan). What was the temperature for cooled extracts?

Author’s Response:

- To avoid confusion to reader, we adjust the whole sentence following “Then, the extracts were heated with a boiling water bath (Memmert, Duesseldorf, Germany) for 1 hour before cooling immediately in an ice bath. The solution layer was separated by centrifuging at 4 oC, 2,000 rpm for 30 min”

(See Method: Page5; lines 14-16).

6. Thiobarbituric acid reactive substances assay in serum and liver tissue: full name of the first word MDA.
Author’s Response:

- According to your suggestion, the word of “Malondialdehyde” is added before MDA (See Method: Page9;lines 11).

7. Why refer Baba SA, Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume? In determination of TFC. Their sample was root. And what was the modification you did?

Author’s Response:

- Once we started to determine total flavonoid contents, we referred the method from Baba SA and Malik SA who modified the method that analyzed the samples of propolis (Mihai et al. 2010). There are several research using the similar methods to analyze total flavonoid contents in various berry fruits (Li et al., 2013 & Souza et al 2014.).

   For total flavonoid contents, we modify the methods of Baba SA and Malik SA by adapting the volume of chemical reaction applicable to a 96 well microplate. The absorbance to detect total flavonoid contents were further applied from 510 nm to 410 nm. 410 nm was mentioned in method. Another information of “using a microplate reader” is added (See Method: Page7;lines 1).

References


8. Why refer Baba SA, Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume? In determination of Total polyphenol contents. Their sample was root. And what was the modification you did?
Author’s Response:

- As refer to comment number 7, once we started to determine total polyphenol, we referred the method from Baba SA and Malik SA who modified that analyzed the samples of fruit, vegetables (Kaur C & Kapoor HC, 2002). There are several researchers using the similar methods to analyze total phenolic contents in various berry fruits (Diaconeasa et al., 2015 & Álvarez et al 2016 & Li et al., 2013 & Souza et al 2014).

For total polyphenol contents, we modify the methods of Baba SA and Malik SA by adapting the volume of chemical reaction applicable to a 96 well microplate.

Thus, we add the information of “using a microplate reader (Tecan Sunrise, Männedorf, Switzerland).

(See Method: Page6;lines 11).

References

Kaur C., Kapoor HC. Anti-oxidant activity and total phenolic content of some Asian vegetablesInt. J. Food Sci. Technol. 2002;372002153161


Author’s Response:

- At the time of Kongkachuichai determine anthocyanidins in the sample of vegetables, she adapted the methods that examined anthocyanidins in Bilberry extract which is a kind of berry (Zhang et al. 2004). We directly employed the method from Kongkachuichai et al. 2015 to analyse Anthocyadins (cyanidin and peonidin) contents. Thus, we cited Kongkachuichai et al 2015.

References


10. Statistics analysis: Please use SEM

Author’s Response:

- SEM are applied through method and result parts

(See Method: Page11;lines 9).

(See Results: Page13;lines 7-10, Figures 1-3, Table 2).


Author’s Response:

- Our sample size had calculated and clarified at the process of animal ethics application. We employed PS Power and sample size calculation program version 3.1.2 [http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize] to calculate the sample size using the equation

\[ n_i = 2 \left( \frac{Z_{1-\alpha/2} + Z_{1-\beta}}{\sigma^2 / \delta^2} \right) \]

Which \( n_i \) = Sample size,

\( \sigma \) = Standard deviation of cholesterol (parameter of cholesterol is used for sample size calculation)

\( \delta \) = Difference between two group of means of cholesterol

The sample size was calculated from variants as shown in following Table.
The variants for sample size calculation in PS program

Power, Difference between 2 groups of mean (δ), Type I error Standard deviation (σ), The ratio of control to experiment subjects (m), Sample size (N)

<table>
<thead>
<tr>
<th>Study</th>
<th>Power</th>
<th>δ</th>
<th>σ</th>
<th>m</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yang et al., 2010</td>
<td>0.95</td>
<td>1.07</td>
<td>0.05</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Jurgoński et al., 2013</td>
<td>0.95</td>
<td>9.1</td>
<td>0.05</td>
<td>6.3</td>
<td>1</td>
</tr>
<tr>
<td>Takahashi et al., 2014</td>
<td>0.95</td>
<td>0.85</td>
<td>0.05</td>
<td>0.54</td>
<td>1</td>
</tr>
</tbody>
</table>

The number of sample size was calculated from related researches that employ high fat models. From the sample size calculation program, the less sample sizes (N) for rat per group are equivalent to 12. This is the less number of sample size that statically enough to see the difference of the cholesterol between normal control and high fat groups.

To clarify this issue, we adjust the sentence of “The number of sample size is appropriate for significance difference analysis with precision and accuracy” to “The number of sample size of 12 was calculated from related researches that employ high fat models (Yang et al, 2010; Jurgoński et al., 2013; Takahashi et al; 2014).

(See Method: Page7;lines 20-21).

References


12. Why this happened? The improvement of fat metabolism in liver tissues of rats fed a high-fat diet were observed by in Maoberry extracts treatment group but after 12 weeks of experimental study, no significant difference was observed between three different dosages of Maoberry extract feeding groups and HF. Please explain.
Author’s Response:

- Compared to liver tissues in HF groups, the significantly different improvements of fat metabolism in MH groups were observed in the markers of (1) liver triglyceride levels, (2) fat accumulation in liver pathology, and (3) gene expression of key enzymes of lipid production (GPAR-1 & ACC) anti-inflammation (TNF-alpha & IL-6). We revised the conclusion following:

“After 12 weeks of experimental study, compared to liver tissues in HF groups, the significantly different improvements of fat metabolism in MH groups were observed in the markers of liver triglyceride levels, fat accumulation in liver pathology, and gene expression of key enzymes of lipid production (GPAR-1 & ACC) anti-inflammation (TNF-alpha & IL-6). The underline mechanism that might link to fat metabolism might be through the process of antioxidant activity and bioactive ingredients in Maoberry extract accompanied with down-regulated the gene expression of key enzymes of lipid production and anti-inflammation. The exact molecular mechanism on fat metabolisms are still uncertain and complicated. Further carefully designed studies are need to access and clarify in human.”

(See conclusion: Page19; lines 21-page 20; line 1).

13. Yang et al. (2010) reported that a freeze-dried powder of mulberry fruit consumption showed a significant decrease of liver triglyceride in hyperlipidemia rats but yours did not use freeze-drying.

Author’s Response:

- The use of freeze-dried sample should be used in our research. We are accepted that this is limitation of our study. The sentence of “However, freeze-dried Maoberry was not employed in our study. This could be one of possible limitations that may affect our study. Freeze-dried Maoberry might ameliorate adverse effects of lipid metabolism more more apparent. Freeze drying is used for food preservation. Previous study has shown that freeze dried blueberries stored for 3 months were able to maintain the levels of anthocyanins and antioxidant activity as fresh blueberries (Lohachoompol et al. 2004).” are added in discussion.

(See Discussion: Page16; lines 8-13).

References


Reviewer’s Comments:

Siti Fatimah Sabran, Ph.D. (Reviewer 3): This project conducted in vivo study which becomes the strength of the whole manuscript. However, there are three (3) major weaknesses for this manuscript.
1) Methods (for example method of extraction, antioxidant evaluation) were not appropriately described and impossible to be replicated if following the current written methods.

Author’s Response:

- The Methods of Maoberry extract preparation, anthocyanidins contents determination in Maoberry extract, total polyphenol and flavonoids contents was carefully revised and expanded.

- The Methods of Maoberry extract preparation are revised and expanded as “Maoberry fruits with black color without visible injuries were used for preparing of Maoberry extract. Briefly, Maoberry fruits were washed and homogenized using a blender (TEFA blenforce, TEFAL, Bangkok, Thailand). The 40 mesh filter was employed to eliminate seeds and marc. Total soluble solid contents of juice were measured approximately 18 % Brix using a hand-held refractometer (Master, Atago Co, Ltd, Tokyo, Japan) and concentrated to 60 % concentration (v/v) using rotary vacuum evaporator (BUCHI rotavapor R-200, BUCHI, Flawil, Switzerland). Extracts were packed and preserved in several airtight bottles at −20°C until used.”

The Methods of Anthocyanidins contents determination in Maoberry extract are revised and expanded as “Then, the extracts were heated in a boiling water bath (Memmert, Duesseldorf, Germany) for 1 hour before cooling immediately in an ice bath. The solution layer was separated by centrifuging at 4 °C, 2000 rpm for 30 min (HIMAC centrifuge, CR5BB2, HITACHI, Tokyo, Japan) and filtrated through a 0.45 μm membrane filter (Chrom Tech®, Milford, MA, USA) before injection to high performance liquid chromatography (HPLC) system.”

- The Methods of Total flavonoids contents are revised and expanded as “. The mixture was allow at room temperature for 5 minutes in the dark. Then, 60 µL of 1 M sodium hydroxide (NaOH; Merck Millipore, Darmstadt, Germany) and 72 µL distilled water were added.”

(See in Method: Page 5 ;lines 2-8

Page 5 ;lines 14-19

Page6 ;lines 20-23)

2) English (grammar, spellings, sentence structures) should be improved to avoid confusion to the readers.

Author’s Response:

- We are thankful to the reviewer for concerning, the grammatical structure of this article was proved by Enago™ – Language Editing Services. They help to edit our manuscript by native English editors and PhD holders.
3) Abstract was poorly written especially the results.

Author’s Response:

- Abstract was carefully revised and expanded in view of editor and previous reviewers. The importance findings of this study are added.

(See in Abstract: Page2).

We would like to express our gratitude to all the reviewers and editor for the encouraging comments to improve our manuscript.

We really hope that our manuscript will be acceptable for publication in BMC Complementary and Alternative Medicine.

Sincerely Yours,

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