Author’s response to reviews

Title: The Effect of Total Anthocyanin-base Standardized (Cornus mas L.) Fruit Extract on Liver Function, Tumor Necrosis Factor α, Malondealdehyde, and Adiponectin in Patients with Nonalcoholic Fatty Liver: A study protocol for a double-blind randomized clinical trial.

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Author’s response to reviews:

Dear Dr. Alison Coates

We appreciate your kind letter, where you informed us of your decision about the manuscript NUTJ-D-19-00006, entitled "The Effect of Total Anthocyanin-base Standardized (Cornus mas L.) Fruit Extract on Liver Function, Tumor Necrosis Factor α, Malondealdehyde, and Adiponectin in Patients with Nonalcoholic Fatty Liver: A study protocol for a double-blind randomized clinical trial". First of all, we would like to thank the reviewers and the associate editor for their great comments that improved the quality of our paper. The manuscript was revised according to the reviewers’ comments. The point-by-point responses to the reviewers’ comments are provided below. Revised texts are also highlighted in yellow.

Best regards,

Yours sincerely,
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Reviewer reports:

Reviewer #1: Review of Manuscript Number: NUTJ-D-19-00006
Title: The Effect of Total Anthocyanin-base Standardized (Cornus mas L.) Fruit Extract on Liver Function, Tumor Necrosis Factor α, Malondealdehyde, and Adiponectin in Patients with Non-alcoholic Fatty Liver: A study protocol for a double-blind randomized clinical trial submitted to: Nutrition Journal

The manuscript by Sangsefidi et al., describes the pre-intervention protocol for a human RCT intervention with Cornelian cherry fruit extract in patients with NAFLD. The study aims to conduct a 12 week parallel study to determine the effectiveness of the study treatment on a number of primary (identified in the introduction as liver function, TNF-alpha, MDA and adiponectin) and secondary endpoints of relevance to NAFLD. The subject area is of potential clinical interest and the interaction between flavonoids and NAFLD remains an understudied area in human RCTs. Hereafter, please find comments relating to the study design and information presented within the manuscript.

Main comments:
1. The clarity and consistency of which variables are primary endpoints could be improved. In the clinical trials directory (IRCT20180419039359N1), 8 well defined primary endpoints are identified, yet the manuscript is somewhat vague at times. In the abstract, and introduction, 'liver function' TNF-alpha, MDA and adiponectin are specifically mentioned, but it isn't until the 'primary outcomes' section where ALT, AST, CK-18M30, hepatic steatosis and liver fibrosis are mentioned. The actual markers of function (and injury? i.e. CK-18M30) should be explicitly identified in the abstract and introduction. Confirmation, in the introduction, that the study was powered on ALT, would also be of interest to the reader. As would some broad reference to the wide range of secondary outcomes assessed (i.e. parameters of insulin resistance and lipid / lipoprotein status).

Author: Thank you for your comments. We explicitly mentioned the primary outcomes related to liver function including ALT, AST, CK-18 M30, steatosis, and fibrosis of liver in the Abstract (Page: 2, Lines: 28-33) and Introduction (Pages: 4, 5; Lines: 92-98).

Abstract: The aim of this research will be to evaluate the effect of supplementation with total anthocyanin-base standardized cornelian cherry fruit extract on liver function (Serum levels of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), cytokeratin-18 fragment M30 (CK-18 M30), as well as steatosis and fibrosis of liver), tumor necrosis factor α (TNF-α), malondealdehyde (MDA), and adiponectin in patients with NAFLD.

Introduction: the present double-blind randomized clinical trial will be conducted to investigate the effect of supplementation with total anthocyanin-base standardized cornelian cherry fruit extract on the liver function (Serum levels of AST, ALT and CK-18 M30; steatosis and fibrosis of liver), TNF-α, MDA, and adiponectin in patients with NAFLD. Furthermore, serum concentrations of glucose, total cholesterol (TC), High density lipoprotein (HDL-C), low density lipoprotein (LDL-C), triglyceride (TG), insulin, and insulin resistance will be evaluated as the secondary outcomes.

Furthermore, we provided more evidences regarding the effects of anthocyanins on liver function (Serum levels of ALT, AST, CK-18 M30; steatosis and fibrosis of liver), oxidative stress markers such as MDA, inflammatory markers such as TNF-α, lipid profile, glycemic control, insulin resistance, and adiponectin in the Introduction (Pages: 3, 4; Lines: 66-77).

Some human studies reported useful effects of anthocyanins on the levels of liver enzymes such as ALT
and AST (33, 34), oxidative stress markers such as MDA (35), inflammatory markers such as TNF-α (36, 37), lipid profile (35, 36, 38-40), glycemic control (38, 39), insulin resistance (40, 41) and adiponectin (41). However, very few clinical trials evaluated the effect of anthocyanins (37, 42, 43) on NAFLD. For example, supplementation with purified anthocyanins for 12 weeks was associated with a significant decrease in the level of Alanine Aminotransferase (ALT) and cytokeratin-18 fragment M30 (CK-18 M30). However, it improved the fibrosis scores of NAFLD patients (42). In addition, another research reported that intake of Hibiscus Sabdariffa extract, rich in anthocyanins, for 12 weeks improved liver steatosis in patients with fatty liver (43). A study found that consumption of bayberry juice, as a source of anthocyanins, for 4 weeks decreased the levels of TNF-α and CK-18 M30 among NAFLD patients (37).

2. Whilst the introduction mentions many associations between liver function, NAFLD and anthocyanin (in vitro / in vivo studies), there is no explicit rationale presented why ALT, AST, TNF-alpha, MDA (which isn't mentioned at all), adiponectin, CK-18 M30 are primary endpoints. Presumably, this is due to being a combination of markers of liver health, injury and fibrosis, inflammation, glucose and fatty acid regulation and oxidative stress - but the mechanism and rationale why these are selected in a NAFLD population, over insulin resistance, as an example of a secondary marker, is currently not clear enough.

Author: Thank you for your suggestion. We explained the relationship of some factors such as insulin resistance, oxidative stress, adiponectin, and inflammatory mediators with pathogenesis of NAFLD (Pages: 3; Lines: 53-59). We also provided more evidences regarding the effect of anthocyanins on our outcomes (primary and secondary) (Pages: 3, 4; Lines: 66-77). Finally, we explicitly described our rationale to select the primary and secondary outcomes in the Introduction (Pages: 4, 5; Lines: 77-98). NAFLD may be associated with factors such as insulin resistance (10, 11), oxidative stress, and adipokines such as adiponectin, cytokines, and other inflammatory mediators (10, 12). Insulin resistance increases lipolysis in adipose tissue, releases free fatty acids to liver, and causes inflammation in liver (10, 11). Moreover, mitochondrial dysfunction, oxidative stress, and increased inflammatory responses are related to damaged liver (10, 12). The low level of serum adiponectin was also observed in NAFLD patients, which was related to the rate of steatosis, fibrosis, and severity of NAFLD (13). Moreover, some human studies reported useful effects of anthocyanins on the levels of liver enzymes such as ALT and AST (33, 34), oxidative stress markers such as MDA (35), inflammatory markers such as TNF-α (36, 37), lipid profile (35, 36, 38-40), glycemic control (38, 39), insulin resistance (40, 41) and adiponectin (41). However, very few clinical trials evaluated the effect of anthocyanins (37, 42, 43) on NAFLD. For example, supplementation with purified anthocyanins for 12 weeks was associated with a significant decrease in the level of Alanine Aminotransferase (ALT) and cytokeratin-18 fragment M30 (CK-18 M30). However, it improved the fibrosis scores of NAFLD patients (42). In addition, another research reported that intake of Hibiscus Sabdariffa extract, rich in anthocyanins, for 12 weeks improved liver steatosis in patients with fatty liver (43). A study found that consumption of bayberry juice, as a source of anthocyanins, for 4 weeks decreased the levels of TNF-α and CK-18 M30 among NAFLD patients (37). Nevertheless, the earlier studies had various limitations: lack of precise examination of steatosis and fibrosis of liver by an accurate none-invasive method such as transient elastography (Fibroscan) (37, 42, 43), low study power (42), lack of assessing other important factors such as adipokines, adiponectin, as well as inflammatory and oxidative stress markers (37, 42, 43). Recent evidences demonstrated that results of transient elastography, as a non-invasive method, had appropriate consistency with results of biopsy, as an invasive but gold standard method for assessing NAFLD. Therefore, transient elastography can replace biopsy as a more accurate and non-invasive method in evaluating NAFLD (44-46). Cornus mas L. (cornelian cherry) is a fruit rich in anthocyanins (47). Although its protective effects on liver was reported in several animal studies (48-51), no clinical trial has ever investigated the effect of cornelian cherry fruit extract on NAFLD.
Considering the high prevalence of NAFLD as well as the limited number and limitations of clinical trials over the effect of anthocyanins on NAFLD, further clinical trials are required in this area. The future trials should conduct comprehensive investigations on important NAFLD variables and examine the impacts of anthocyanins on NAFLD. Therefore, the present double-blind randomized clinical trial will be conducted to investigate the effect of supplementation with total anthocyanin-base standardized cornelian cherry fruit extract on the liver function (Serum levels of AST, ALT and CK-18 M30; steatosis and fibrosis of liver), TNF-α, MDA, and adiponectin in patients with NAFLD. Furthermore, serum concentrations of glucose, total cholesterol (TC), High density lipoprotein (HDL-C), low density lipoprotein (LDL-C), triglyceride (TG), insulin, and insulin resistance will be evaluated as the secondary outcomes.

3. The evidence to suggest that the primary markers are modifiable over 12-weeks should be referenced in the introduction (if this data exists).

Author: Thank you for your attention. We presented the evidences that showed our primary outcomes were modifiable over 12-weeks (Pages: 3, 4; Lines: 71-77).

Supplementation with purified anthocyanins for 12 weeks was associated with a significant decrease in the level of Alanine Aminotransferase (ALT) and cytokeratin-18 fragment M30 (CK-18 M30). However, it improved the fibrosis scores of NAFLD patients (42). In addition, another research reported that intake of Hibiscus Sabdariffa extract, rich in anthocyanins, for 12 weeks improved liver steatosis in patients with fatty liver (43). A study found that consumption of bayberry juice, as a source of anthocyanins, for 4 weeks decreased the levels of TNF-α and CK-18 M30 among NAFLD patients (37).

4. Can the authors use a more accessible descriptor for the active treatment arm? There is a repetitive use of the description 'total anthocyanin-base standardized cornelian cherry fruit extract' which breaks the flow of the text in multiple places. It is correct to describe in longhand in the descriptor section of the interventions, but once described, perhaps paraphrase to Cornelian cherry extract, or anthocyanin extract, or cherry-anthocyanin extract (or similar).

Author: Thank you for your comment. We used the description 'total anthocyanin-base standardized cornelian cherry fruit extract' for our intervention at the first time in the paper. Then, we changed it to Cornelian cherry extract throughout the manuscript.

5. A more detailed description of the likely intervention end-products is required (if this can be estimated). For cherry-anthocyanin extract, are other flavonoid / phenolic compounds expected to be in the extract? or is this a purified anthocyanin? (if so, it would be good to make that explicit). How will the intervention material be presented and consumed; i.e. an encapsulated powder? Liquid? Syrup? How many capsules etc. per day? Regarding the placebo, the description is that it will be 'similar' - in what way? Macro/micronutrient composition? What comparative material is being used as a placebo filler?

Author: Thank you for your suggestions. We presented a more description of our extract (Page: 6; Lines: 128-130) and placebo (Page: 6; Lines: 133-135). We also explicitly described the administered amounts and forms of the extract and placebo in the Method (Page: 8, 9; Lines: 187-197). Since the prepared extract does not have purified anthocyanins, the extract's total flavonoid/phenolic content should be measured. Furthermore, the necessary microbiology measurements will be performed for the prepared extract. The placebo will be prepared using diluted water, caramel color, allura red color, and natural flavorings with a color, appearance, taste, and texture similar to the cornelian cherry extract, but without any anthocyanins.
The participants in the intervention group will receive the total anthocyanin base-standardized cornelian cherry fruit extract with 320 mg.d-1 of anthocyanin daily for 12 weeks (42). The total anthocyanin content will be measured before and after concentration and in distinct intervals during the trial. Then, the total daily amount of extract consumed by each participant will be adjusted based on the amount of total anthocyanin in the extract. Finally, the total daily amount of extract consumed by each participant will be determined based on the amount of total anthocyanin in the extract, which is equal to 320 mg.d-1. The control group will also receive the placebo, matched with the extract in terms of appearance, taste, color, and texture (but without any anthocyanins) for 12 weeks. The participants will be asked to keep the intervention and placebo, which are presented as liquid in the refrigerator to ensure food safety.

6. Regarding the balancing of the treatment groups during randomisation - the authors have identified that age and sex will be used. Considering the primary endpoints, can the authors justify why BMI (likely to be highly correlated with adiponectin) and a marker of NAFLD progression (i.e. fatty liver grade 1, 2, 3) are not being included to ensure the groups are similar?
Author: Thank you for your attention. We will consider BMI and the severity of fatty liver (fatty liver grade 1, 2, and 3) along with age and gender in randomization process (Page: 8; Lines: 183-184). The participants will be also stratified based on age, gender, BMI, and severity of fatty liver (fatty liver grade 1, 2, 3).

7. Can the authors please clarify the cut-off being used for treatment adherence? In various places (e.g. study population section, intervention section), the text seems to suggest that anyone consuming more than >20% will be deemed as compliant. I'm assuming this is incorrect, as a high quality RCT would be benchmarking treatment adherence at >80-85%. Perhaps the authors meant that non-compliance of greater than 20% would mean an exclusion (i.e. <80% intake). An RCT with only >20% adherence to treatment would be unpublishable.
Author: We appreciate your nice comment. Please forgive us that we were unable to properly express our meaning. We revised our sentences regarding the adherence of patients in the Method (Page: 7; Lines: 161-63; Page: 9; Lines: 210-211).
Data of the patients with any of the following conditions will not be analyzed at the end of study: patients who take less than 80 percent of the administered extract or placebo.
In the case that a patient in each group consumed less than 80 percent of the administered extract or placebo, his/her data will not be analyzed at the end of the study.

8. A lack of biological sampling to confirm compliance to intervention, through i.e. anthocyanin / phenolic metabolite analysis (either in urinary or serum analyses) is a limitation and may reduce the impact of the study outputs.
Author: Thank you for your comment. Nevertheless, anthocyanin / phenolic metabolite analysis (either in urinary or serum analyses) need financial supports and facilities and we have financial limitations. Thus, it is not possible to perform this analysis.

Hereafter are additional minor comments for specific sections:
Abstract
* Confirm that the extract group will receive it for 12 weeks (it currently only identified that for the placebo).
Author: Thank you, it was a good comment. Thus, we revised the manuscript according to your suggestion (Page: 9; Lines: 190-192).
The participants in the intervention group will receive the total anthocyanin base-standardized cornelian cherry fruit extract with 320 mg.d-1 of anthocyanin daily for 12 weeks (42).
The word 'the beneficial effects' should be removed and replaced with 'the effect' - the research question should be objective, without a suggestion of preconceived benefits.

Author: Thank you for your attention. It was corrected according to your comment (Page: 2; Lines: 25, 42; Page: 3; Line: 63; Page: 12; Line: 278-279).

Introduction
* Give greater detail of how this study will address the limitations identified; e.g. rather than just saying that steatosis and fibrosis were previously identified using imprecise methods - give details of the validated and precise methods that are being used in this study.

Author: You kind comments are appreciated. We explained the limitations of earlier studies in more details (Page: 4; Lines: 77-85).

The earlier studies had various limitations: lack of precise examination of steatosis and fibrosis of liver by an accurate none-invasive method such as transient elastography (Fibroscan) (37, 42, 43), low study power (42), lack of assessing other important factors such as adipokines, adiponectin, as well as inflammatory and oxidative stress markers (37, 42, 43). Recent evidences demonstrated that results of transient elastography, as a non-invasive method, had appropriate consistency with results of biopsy, as an invasive but gold standard method for assessing NAFLD. Therefore, transient elastography can replace biopsy as a more accurate and none-invasive method in evaluating NAFLD (44-46).

Methods
* Mention the word 'parallel' in the study design description.

Author: Thanks for your attention. It was revised according to your suggestion (Page: 5; Line: 102).

* What do you mean by 'authenticity'? that they were genuinely cherries? Or that their anthocyanin content will be confirmed?

Author: Your attention is appreciated. We mean that authentication of the fruits will be determined by specifying the voucher number (SSU0029) in the Department of Pharmacognosy, School of Pharmacy, Yazd Shahid Sadoughi University of Medical Sciences (Page: 5; Lines: 115-117).

The fruits' authenticity will be determined by specifying their voucher number (SSU0029) in the Department of Pharmacognosy, School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd.

Preparation of placebo
* First sentence, delete everything before 'A placebo of similar extract..' The current start to the sentence isn't about the placebo.

Author: Thank you for your suggestion. It was corrected according to your comment (Page: 6; Lines: 133-135).

Study population
* Will a nationally / internationally recognised validated method be used to define NAFLD by the clinicians? if so, please refer to this, to provide objectivity that all clinicians would judge the same patients are having NAFLD.

Author: Thank you for your comment. No confirmed accurate method has ever been recognized to diagnose NAFLD except liver biopsy. However, diagnosis of NAFLD will be according to the guidelines of American Gastroenterological Association and American Association for the Study of Liver Diseases in the present study (Page: 7; Lines: 144-152).

In the current research, nonalcoholic steatohepatitis (NASH) will be defined based on the guidelines of
American Gastroenterological Association and American Association for the Study of Liver Diseases (54). According to this guideline, NASH is defined as the presence of hepatic steatosis proved by ultrasonography or inflammation with hepatocyte injury (ballooning) with or without fibrosis (54). Moreover, NAFLD will be diagnosed by ultrasonography based on following criteria: an increase in hepatic echogenicity via renal echogenicity as a reference, presence of enhancement and lack of differentiation of periportal, and bile duct walls reinforcement due to great hyperechogenicity of the parenchyma (55).

* Regarding consumption of medications that affect liver function - presumably, this includes over the counter medications such as painkillers? Can this be included in the description.
Author: Thank you for your attention. In the study method, consumption of any medicine or supplement that affects liver function was previously listed as the exclusion criteria, which can also include consumption of over the counter medications (such as painkillers) (Page: 7; Lines: 156-157).

* Are drugs being monitored during the study too - currently, it is written that the exclusion only counts for one month before the study.
Author: We appreciate your comment. We will also ask the patients not to consume the medicines listed in exclusion criteria during the study. These explanations were presented in the method section (Page: 7; Lines: 160-161). Moreover, we previously mentioned that patients who need a special treatment due to a specific medical reason during the study will be excluded from research (Page: 7; Lines: 161-164). We will also ask the patients not to consume the medicines in exclusion criteria list during the study.
Moreover, data of the patients with any of the following conditions will not be analyzed at the end of study: patients who take less than 80 percent of the administered extract or placebo as well as participants who are under a special treatment due to a specific medical reason during the study.

Ethical considerations and trial registration
* Informed consent is taken after protocol review, and presumably before any screening. Figure 1 is incorrect in this respect, and the 'consent forms' text should be moved to before 'screening for eligible participants'
Author: Thank you for your attention. It was corrected according to your comment (Figure 1).

Randomization:
* The text currently says 'using a random method.' - this should say 'using a method of randomisation' as the method isn't random. Can the authors add the reference for the Random Allocation Software?
Author: Thank you for your comment. It was revised according to your comment and the related reference was added for Allocation Software (Page: 8; Lines: 184,185).

Intervention:
* The text says 'The control group will also receive a placebo with the same dose for 12 weeks' - again, I think this is an error and could conceivably say, 'The control group will also receive a placebo, matched for the same weight, appearance, taste and colour (but without any anthocyanin) for 12 weeks'
Author: Thank you for your attention. It was revised according to your comment (Page: 9; Lines: 196-198).

The control group will also receive the placebo, matched with the extract in terms of appearance, taste, color, and texture (but without any anthocyanins) for 12 weeks.

Data collection:
* Is a 3d food diary sufficient? Food intake records are notoriously poor indicators of habitual intake (but relatively accurate of monitored 3d intake) and perhaps another measure (FFQ?) might give a more rounded assessment of typical intakes?
Author: Thank you for your comment. Our main objective is to compare the means of energy and nutrients' intake (macro/micronutrients) in participants before and after the intervention. Then, if necessary, we will adjust it as a covariate in the analysis. Food record is a more accurate method for assessing mean intake of nutrients than FFQ. FFQ is better for evaluating habitual intakes and ranking of participants (Nutritional Epidemiology Book, Professor Walter Willett; chapter 4, 5). Patients will be also randomly assigned into intervention and control groups; of course, they will be matched in terms of factors such as dietary intakes. Furthermore, the intervention group will be compared with control group. Meanwhile, several RCT assessed the effect of supplementation with anthocyanins such as Zhang et al, 2015; Li et al, 2015 and Yang et al, 2017. They applied food record for evaluation of diet. Therefore, application of food record for assessing diet seems to be appropriate in the current study.

* Is there any monitoring of change in weight / adiposity? (which would conceivably affect adiponectin, and almost every other cardiometabolic endpoint).

Author: Your consideration is appreciated. We explained the assessment of anthropometric parameters (e.g., weight, height, Body Mass Index (BMI), waist circumference) in the method section previously. Furthermore, hip circumference will be measured before and after intervention (Pages: 10; Lines: 225-227; 229-232).

Meanwhile, anthropometric parameters (e.g., weight, height, Body Mass Index (BMI), waist and hip circumference) will be measured with minimal clothing and without shoes at the baseline and the end of study.

BMI will be calculated after dividing the body weight (kg) by the square of height (m). Waist circumference will be also measured at the midway between the lowest ribs and the iliac crest. Moreover, hip circumference will be measured over the largest part of the buttocks.

* Is Waist and hip measurement possible in this population? This may provide relevant measure of central adiposity for a NAFLD population.

Author: We explained assessing anthropometric parameters (e.g., weight, height, Body Mass Index (BMI), waist circumference) in the method section previously. Furthermore, hip circumference will be measured before and after intervention (Pages: 10; Lines: 225-227; 229-232).

Meanwhile, anthropometric parameters (including weight, height, Body Mass Index (BMI), waist and hip circumference) will be measured with minimal clothing and without shoes at the baseline and the end of study.

BMI will be calculated after dividing the body weight (kg) by the square of height (m). Waist circumference will be also measured at the midway between the lowest ribs and the iliac crest. Moreover, hip circumference will be measured over the largest part of the buttocks.

Primary outcomes

* The elastography and fibroscan method should be described somewhere *(methods?), including a reference to the equipment used and the validated protocol used.

Author: Thank you for your recommendation. Measurement of steatosis and fibrosis liver using transient elastography (Fibroscan) was explained in the method section (Page: 11; Lines: 244-246).

Evaluation will be conducted while patients are lying in a dorsal decubitus position with their right arm in maximum abduction (57).

Reviewer #2: The proposed study does have merit for those working in NAFLD.

It needs to undergo a spelling and grammatical review for errors.

Author: Thank you for your suggestion. Corrections were made throughout the manuscript.
A major consideration is to consider and include stability data of the extract prior to supplementation. There is no information included in the manuscript around food safety measures/food grade nature of the extract. Appropriate recognised measures included microbiology and sample stability data need to be considered and included.

Query how stable is the extract at room temperature, or do participants need to keep the concentrated extract under refrigeration to avoid issues with food borne pathogens and also to ensure anthocyanin is not degraded below dose 320mg/day.

Author: We appreciate your recommendations. We considered the necessary microbiology measurements and other essential measures to ensure about the food safety and adequacy of anthocyanin dose. These explanations are presented in the method section (Page: 6; Lines: 129-130; Page: 9; Lines: 192-200).

The necessary microbiology measurements will be performed for the prepared extract.

The total anthocyanin content will be measured before and after concentration and in distinct intervals during the trial. Then, the total daily amount of extract consumed by each participant will be adjusted based on the amount of total anthocyanin in the extract. Finally, the total daily amount of extract consumed by each participant will be determined based on the amount of total anthocyanin in the extract, which is equal to 320 mg.d-1. The control group will also receive the placebo, matched with the extract in terms of appearance, taste, color, and texture (but without any anthocyanins) for 12 weeks. The participants will be asked to keep the intervention and placebo, which are presented as liquid in the refrigerator to ensure food safety.

Whilst there is food diary to be undertaken, this also needs to be scrutinised for anthocyanin-rich foods in the diet. With the current data collection/protocol a baseline 3 day sample and 3 days at end of trial is not enough to determine anthocyanin content of the diet. If the diet of participants is naturally rich in anthocyanin or if there is significant variation between participants of within participant within the trial, this will confound results.

Without more accurate information regarding dietary intake, it creates weakness in the project design.

Author: Thank you for your comment. Our main objective is to compare the means of energy and nutrients’ intake (macro/micronutrients) in participants before and after the intervention. Then, if necessary, we will adjust it as a covariate in the analysis. Food record is a more accurate method for assessing mean intake of nutrients than FFQ. FFQ is better for evaluating habitual intakes and ranking of participants (Nutritional Epidemiology Book, Professor Walter Willett; chapter 4, 5). Patients will be also randomly assigned into intervention and control groups; of course, they will be matched in terms of factors such as dietary intakes. Furthermore, the intervention group will be compared with control group. Meanwhile, several RCT assessed the effect of supplementation with anthocyanins such as Zhang et al, 2015; Li et al, 2015 and Yang et al, 2017. They applied food record for evaluation of diet. Therefore, application of food record for assessing diet seems to be appropriate in the current study.

What form will the extract be given in? It is not clear from the protocol

Author: Thank you for your attention. The necessary explanation was added with regard to the administered forms of extract in the method section (Page: 9; Lines: 198-200).

The participants will be asked to keep the intervention and placebo, which are presented as liquid in the refrigerator to ensure food safety.

Reviewer #3: The manuscript by Zohreh Sadat Sangsefidi et al is a study protocol for a double-blind randomized clinical trial exploring the protective function of total anthocyanin-base standardized (Cornus mas L.) fruit extract in patients with non-alcohol fatty liver. I have several concerns about this
1. This manuscript is lack of innovation. This trial is highly similar to the article by Zhang et al. (Medicine (Baltimore). 2015 May;94(20):e758. doi: 10.1097/MD.0000000000000758.), except for the source of anthocyanins and several biomarkers.

Author: Thank you for your attention. Very few clinical trials evaluated the effect of anthocyanins (37, 42, 43) on NAFLD. Nevertheless, the earlier studies had various limitations, including lack of precise examination of steatosis and fibrosis of liver by a fairly more accurate none-invasive method such as transient elastography (Fibroscan) (37, 42, 43), low study power (42), lack of assessment of other important factors including adipokines such as adiponectin, as well as inflammatory and oxidative stress markers (37, 42, 43). Recently, evidences demonstrated that transient elastography, as a non-invasive method had appropriate consistency with the results of biopsy, as a gold standard method for assessing NAFLD. Biopsy is an invasive method, while transient elastography is non-invasive. Therefore, this method, as a fairly more accurate none-invasive method can be used in evaluating NAFLD, in the case that biopsy is not possible (44-46).

In comparison with the study by Zhang et al. (2015), our research has more strengths. We will apply a combination of non-invasive markers including AST, ALT, CK-18 M30, steatosis and fibrosis of liver, inflammatory markers such as TNF-α, oxidative stress markers such as MDA, and adiponectin to assess NAFLD. Evidences indicated that using a set of non-invasive markers such as the mentioned ones could be an appropriate method to examine NAFLD when liver biopsy is not possible (Baršić et al, 2012; Fitzpatrick et al, 2014). However, Zhang et al. did not evaluate TNF-α; MDA and adiponectin in their survey. Transient elastography, as a fairly more accurate method will be used for assessment of hepatic steatosis and fibrosis in the present research; whereas, Zhang et al. applied a non-invasive scoring system as a formula for evaluating fibrosis. Hepatic steatosis was not also examined in the study of Zhang et al. Furthermore, the extract and placebo will be prepared by ourselves in the present research.

2. Transient elastography and ultrasonic techniques are used to diagnose fatty liver and liver fibrosis respectively, which are not gold standard for diagnosing fatty liver and liver fibrosis. Moreover, there is only one doctor to make the diagnosis and grade the severity of fatty liver.

Author: Thank you for your comments. Biopsy is a gold standard for evaluation of NAFLD. However, evidences demonstrated that transient elastography, as a non-invasive method had appropriate consistency with the results of biopsy as a gold standard method. Biopsy is an invasive method, while transient elastography is non-invasive. Therefore, this method as a fairly more accurate none-invasive method can be used in evaluating NAFLD in the case that biopsy is not possible (44-46). Although, diagnosis of NAFLD will be performed by a gastroenterologist, it will be based on a specific criteria (the guidelines of American Gastroenterological Association and American Association for the Study of Liver Diseases). These explanations were presented in the method section (Page: 7; Lines: 144-152).

In the current research, nonalcoholic steatohepatitis (NASH) will be defined based on the guidelines of American Gastroenterological Association and American Association for the Study of Liver Diseases (54). According to this guideline, NASH is defined as the presence of hepatic steatosis proved by ultrasonography or inflammation with hepatocyte injury (ballooning) with or without fibrosis (54). Moreover, NAFLD will be diagnosed by ultrasonography based on following criteria: an increase in hepatic echogenicity via renal echogenicity as a reference, presence of enhancement and lack of differentiation of periportal, and bile duct walls reinforcement due to great hyperechogenicity of the parenchyma (55).

3. When grouping, only age and gender matching were considered, while the severity of fatty liver and BMI were not taken into account.

Author: We appreciate your kind consideration. We will consider the participants' BMI and severity of
fatty liver (fatty liver grade 1, 2, and 3) along with age and gender in randomization process (Page: 8; Lines: 185-186).

The participants will be also stratified based on age, gender, BMI, and severity of fatty liver (fatty liver grade 1, 2, 3).

4. The article did not specify season for the experiment. Because anthocyanins can be obtained from a variety of foods, the amount of anthocyanin consumed by food may vary in different seasons. This is also a confounding factor that may affect the outcome.

Author: Thank you for your attention. However, the present study is a double-blind randomized parallel clinical trial. One control group was considered along with one intervention group in parallel design. Then, sampling, assessments, and interventions will be performed simultaneously for both groups and consequently the intervention group will be compared with the control group. Thus, time-trend effect or seasonal changes are unlikely.