Reviewer #2
We thank reviewer #2 for his/her helpful comments on our manuscript. Your comments helped us to improve the manuscript to a greater extent.

In this research article entitled “Identification of bioactive compounds in phenolic rich extract from marine red algae Gracilaria edulis (Gmelin) Silva to combat obesity and Type-2 diabetes” the authors investigated the antidiabetic and anti-obesity potential of several extracts/fractions of the marine red algae Gracilaria edulis. They observed that fractions of the algae extract showed different degrees of antioxidant, iron chelating, and radical scavenging activities. Moreover, the ethyl acetate fraction, the most promising one, showed potent α-amylase and α-glucosidase inhibitory activity. This is an interesting study that may contribute to the development of new oral therapies based on extracts of Gracilaria edulis. However, this reviewer has several concerns that will be pointed out below.

General comments – major concerns

1) Language and general editing: although I did not have major issues reading the manuscript, I guess it would benefit from careful editing. Thus, I suggest the authors get editing help from someone with full professional/editorial proficiency in English. –

Thank you. We already got the English editing done by MDPI professionals.

2) Description of the results should be improved: sometimes the description of the results is redundant (e.g. Page 4, lines 135-142), and the authors mention the same results twice in the same paragraph. This could be improved by rewriting the paragraph to give the information only once in a concise and comprehensive way.

We thank you for your comments as suggested corrections have been made in the paper with track changes.

3) Chosen references and wrong use of the literature: this is a very important topic, which will be further explored below:

- the chosen reference does not support data: I could not understand why/how the authors have chosen most of the cited studies in this manuscript, as almost all of them simply do not support the data they are supposed to. For instance:

a) Page 2, References 5 (line 44), 4 (line 47), 1 (line 49), 6 (line 52), 7 (line 61), among many others (throughout the manuscript), were used to support data but these studies/reviews did not discuss/mention such point/data. That is, they were wrongly placed in the present manuscript;

- References 5 (line 44)
  
  o As suggested we removed reference # 5 and cited a new referenc shown below that matches the comment.

Eleazu, C.; O. The concept of low glycemic index and glycemic load foods as panacea for type 2 diabetes mellitus. African Health Sciences 2016, 16 (2), 468-479.
• 4 (line 47): The high blood glucose level is linked with increased risk of hypertension, retinopathy, nephropathy, neuropathy and macro-vascular diseases.


  o As suggested following new reference has been cited.

• 1 (line 49), - Inhibition of carbohydrate digestive enzyme is one of the significant alternatives to management of obesity and chronic hyperglycaemia in diabetes patients [1].

Ref: Assaid, A.; Duarte, A. and Batista, P. M. Inhibition of digestive enzymes by medicinal plant aqueous extracts used to aid the treatment of obesity. Journal of Medicinal Plant research 2012, 6(47), 5826–5830.

  o Thank you for your comment but in the original paper, it mentioned that “Inhibitors of those enzymes, present in plants, offer a promising strategy to aid in the treatment of obesity, hyperglycemia associated with type 2 diabetes and hypertension, through the reduction of the starch breakdown and of the glucose absorption in the intestine (Kwon et al., 2006).” Therefore, we strongly think that this reference supports the data.

• 6 (line 52), - Alpha amylase and α-glucosidase enzymes are involved in the carbohydrate metabolism and they act synergistically to digest starch [6].


  o Thank you. As pointed out, this reference does not support the data even though it mentioned anti-obesity. Therefore, following new reference has been cited.


• 7 (line 61), - Inflammatory cells in adipose tissues secrete pro-inflammatory cytokines, which increase hepatic lipogenesis, generation of reactive oxygen species thus inducing oxidative stress 1

Furukawa, S. Increased oxidative stress in obesity and its impact on metabolic syndrome. The Journal of Clinical Investigation 2004, 114(12), 122-125

  o As suggested we added the following reference.

b) Page 2, lines 68-73; reference 10: “Gracillaria edulis (Gmelin) Silva is a red algae... against LPS-induced inflammatory responses” – First, Shanura et al. did not measure antidiabetic, antimicrobial, anti-coagulant, anti-inflammatory and anti-proliferative activities in extracts of G. edulis as suggested by the authors of the present manuscript. In addition, Shanura et al. did not measure anti-inflammatory activity of methanol extract and fractions of G. edulis against LPS-induced inflammatory responses. Therefore, this Ref. 10 should not be included here.
Here, mistakenly wrong reference is cited here. Same authors “Shanura Fernando et al,” has studied the anti-inflammatory activity of G.edulis against LPS induced inflammatory responses. I have added the new reference and link here.

Therefore, as suggested following new reference has been cited to confirm the biological activities of G.edulis replacing # 10.


4) IC50 calculation: As I am not a specialist in pharmacology, I would like to understand how the authors can be sure of the IC50 values calculated if their dose-response curves do not reach a plateau showing the extracts/fractions maximum effect. They did not reach a concentration in which a further inhibition is not observed (we can clearly see that the maximum concentration tested did not inhibit 100%, regardless of the measurement). Please look at the correct way to calculate IC50. If necessary, the authors need to complete their experiments with lower or higher concentrations of the extracts/fractions.

We thank you for your comment. To calculate the IC50 values, we plotted a graph which fits the data with a straight line. Then, IC50 values are estimated using the linear equation (Y=mX+C). Therefore, we use the linear range of the graph (Y=50%) and it should not necessarily reach the plateau. The graph is given in the present manuscript for IC50 calculation and all graphs are fitted to the equation Y=mX+C and R2 is nearly equal to 1.

5) Effects in cellular models in vitro: The authors only show the inhibitory effects of G. edulis extracts/fractions in an isolated system using purified α-amylase and α-glucosidase. I would like to know whether they have tested this compound in a cellular, in vitro system. If not, I suggest the authors perform such experiments, as I believe it would increase the quality of the manuscript. Of note, this is only a suggestion, not a requirement for the acceptance of the manuscript.

We value comment, but since we are from a developing country (Sri Lanka), funds are extremely limited. This project is funded by a university grant and therefore, we are unable to complete the cellular models due to shortage of funding. In the future, we hope to evaluate the effects using cellular models.

6) Lack of appropriate experiments to determine the antidiabetic and anti-obesity effects of the G. edulis extracts/fractions: The authors state that their aim is “to appraise the antidiabetic and anti-obesity potential through inhibitory activities of carbohydrate digestive enzymes, glucose diffusion, and protein glycation.” However, I do not believe the authors managed to test their aim using the models described in the present manuscript.

- Antidiabetic effect

Herein, the authors measured carbohydrate digestive enzymes, glucose diffusion, and protein glycation. Although I agree that inhibition of α-amylase and α-glucosidase, as well as formation of AGE, are commonly used as parameters of antidiabetic properties in vitro, here the authors only measured the activity of these proteins in isolated systems (see point 5 above). In addition, I do not think that a
glucose diffusion assay determined using dialysis tubes can really reflect the complexity of the intestinal glucose transport into the bloodstream.

- **Anti-obesity effect**

Regarding the anti-obesity effects, the authors should have performed, at least, some experiments in differentiated adipocytes (e.g. 3T3-L1 cell line), showing that their extracts/fractions have antiadipogenic effects (e.g. inhibition of lipid accumulation).

- **In vivo studies**

Finally, I believe it is very difficult to discuss antidiabetic and anti-obesity effects without experimentation in vivo. I understand in vivo experiments are not precisely easy to address, but at least two simple experiments showing fasting glucose levels (experiment 1) and an oral glucose tolerance test (experiment 2) upon treatment with the adequate vehicles and *G. edulis* extracts/fractions need to be performed.

Here, we have used four main experiments to determine the anti-diabetic effect of *G. edulis*. Even though, in the present manuscript, inhibition of α-amylase and α-glucosidase enzyme is considered as experiments used to determine the anti-obesity effect, which also measures the anti-diabetic effects. Due to the lack of funds as a developing country, we have used these in-vitro models for determined anti-diabetic effect. Here, the effect of postprandial blood glucose level is measured from the inhibitory action of carbohydrate hydrolyzing enzymes and effect on diabetic complications are measured using the inhibitory action of formation of AGEs. Further, we have measured the extent of glucose diffusion by samples through dialysis membrane. Even though, the dialysis membrane does not reflect the complexity of the intestinal glucose diffusion, with the lack of grant, this is what we can perform to determine the effect on glucose diffusion.

In the future, we will try to complete an animal study and further to use 3T3-L1 cell line as you have mentioned to determine the anti-diabetic effect.

**Specific comments (divided by sections of the manuscript)**

**Title**

1) The correct name of the algae is *Gracilaria edulis*, with only one “l” in “Gracilaria”. -corrected

**Introduction**

1) Page 1, line 35: Please use an official reference to address this topic, such as Ref. 2. The authors can also consult the International Diabetes Federation atlas for more information (https://www.diabetesatlas.org/).

As suggested we completed the reference.  
**American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diab Care 2013, 36, 67-74.**

2) Page 2, line 77: “(…) samples were collected from different regions of the world (…)” – I do not fully agree with this statement. Based on the three studies presented so far in the manuscript, one collected samples from Sri Lanka (Shanura et al. 2017), while the other two (Koneri et al. 2017 and Patra et al. 2013) collected samples in the Mandapam region, India. As this region is geographically very near to Kalpitiya, Sri Lanka – where the authors of the present manuscript collected their samples –, I think it is an overstatement to say that other studies collected their samples from *different regions of the world*.

Thank you for your comment. As suggested we removed that part from the present manuscript.
Results

3) Page 3, lines 97-102: “The total flavonoid content… obtained in the chloroform fraction.” – This can be summarized in fewer words. It is very confused in the present form.

Thank you for your comment as suggest we changed it as follows:

The highest total flavonoid content was observed in ethyl acetate fraction (1461.49±75.22 µg QE/g) whereas the lowest was reported in chloroform fraction (289.39±9.55 QE/g).

4) Page 3, lines 111-112: “Free radical scavenging activity… scavenging activities.” – It is repetitive. The same has been stated in lines 107-108.

As suggested we removed the sentence.

5) Page 3, line 113: The value for IC50 presented in the text (i.e. 3.19±0.02 mg/mL) do not correspond to what is presented in Table 2 (i.e. 3.17±0.04 mg/mL). Please change it accordingly.

As suggested we changed the sentence accordingly.

6) Page 4, lines 135-142: “The ethyl acetate fraction… fraction (Table 3).” – This can be summarized in fewer words. It is very redundant in the present form.

Thank you. We reduced the number of words.

7) Page 5, lines 148-150: “The α-glucosidase activity… presented in Table 3.” – It is not the α-glucosidase activity of the crude methanol extract and the fractions, but the inhibitory activity of such extract and fractions that are being shown. This sentence should be rewritten.

We rewrote the sentence.

8) Page 5, lines 164-166: “The inhibition… more or less similar (Table A1).” – This “more or less similar” is compared to what? This needs clarification.

Changed it as follows:

The inhibition of glucose diffusion by hexane (52.01±0.96 µg/mL) and aqueous (52.69±1.31 µg/mL) fractions were more or less similar compared to the glucose concentration in the external solution.

9) Page 6, lines 183-185: “The crude methanol extract… inhibitory activities.” – This is not true. Only the chloroform fraction showed a different degree of inhibition, whereas all other fractions showed similar inhibitory activity.

As suggested we changed the sentence.

10) Page 8, Ref. 13: I could not find the compound “1H-Indole-2-carboxylic acid,6-(4-ethoxyphenyl)-224 3-methyl-4-oxo-4,5,6,7-tetrahydro-isopropyl ester” in the cited reference. Is there any other publication to support this statement, i.e. that this compound presents antidiabetic activities? Please cite other studies to support this sentence.
Since, this compound is an indazole derivatives, we assume that it contains anti-diabetic activities based on the information present in the cited reference. The following reference is also supported to the anti-diabetic activity of indole derivatives by insulin sensitizing and glucose lowering effect.


Discussion

11) Page 8, lines 244-247: “Oxidative stress is linked... production of adipocytokines.” – This is much more complex than simply activation of NADPH oxidase and decreased adipocytokine production. I would like to suggest the inclusion of a Review article as a reference for this subject, as it would be more appropriate to cover this wide subject.

Thank you for your fruitful comment as suggested a new reference has been cited.


12) Page 8, lines 244-247: “Therefore, the supplementation of antioxidants can reduce the risk of being obese.” – Abdali D and colleagues (Med Princ Pract. 2015;24(3):201-15) state that “the literature does not suggest antioxidant supplementation as a cure-all for obesity or for type 2 diabetes”, which contrasts with the statement made by the authors of the present manuscript. Therefore, the authors must include studies supporting the statement made in the manuscript.

This is not a statement obtained from the reference. As this is a discussion, I have discussed the way to prevent oxidative stress here. Removed that sentence.

13) Page 8, line 262: “(...) ethyl acetate fraction (0.43 and 0.809 mmol TE/g and).” – It seems that a piece of information is missing in this part of the sentence between parenthesis.

As suggested changed the sentence.

14) Page 8, line 264: “(...) the highest ferrous iron chelating activity (FICA) (...”) – Why did the authors change from “FICC” to “FICA”? This should be clarified and/or corrected if necessary.

As suggested we corrected this.

15) Page 9, lines 272-273: “Inhibition of key metabolic carbohydrate digestive enzymes... medicinal plants.” – The authors must include studies supporting the statement made in the manuscript.

As suggested we cited a new reference.


16) Page 9, lines 283-284: “In the present study... previous study used water as a solvent.” – Which is this “previous study” mentioned by the authors?

As suggested we highlighted the previous study.
17) Page 9, lines 286-287: “Then the glucose is diffused through the intestinal wall and increases the postprandial blood glucose level.” – This mechanism is not so simple and relies on several enzymes (e.g. SGLT1 and Na+/K+-ATPase) and transporters (GLUT2) that will facilitate glucose absorption by intestinal cells and its transport into the bloodstream. The authors should be careful with this sort of statement to not give the wrong information to readers.

Thank you for pointing out this. We changed the sentence accordingly.

18) Page 9, lines 294-296: “Therefore, the finding of this assay... inhibiting glucose absorption.” – I disagree with this statement because, as explained above (point 17), glucose absorption by intestinal cells is not a simple mechanism depending only on diffusion through a membrane.

As suggested we changed accordingly.

19) Pages 9-10, lines 307-309: “Further, GC-MS analysis... 2,5-dimethylhexane-2,5-dihydroperoxide.” – There is not enough evidence in the literature to support this statement. Once again, the authors should be careful with this sort of statement.

We cited a new reference to support this.


Materials and Methods

20) Page 10, lines 314-315: The title of the topic is repeated.

Thank you for pointing out the mistake we removed the word.

21) Page 11, line 347: “(... pre-plate reading was taken at 415nm.” – What does “pre-plate reading” mean? This should be clarified, as it is used throughout the methods’ section.

We explained the meaning of “pre-plate reading”

22) Page 12, line 412: “The anti-amylase activity (...”) – I believe the authors mean “The a-amylase activity”

Thank you. We changed accordingly

23) Page 12, line 419: “Sample negative was carried out in an identical way without adding enzyme.” – This is not a “sample negative”, but an “enzyme negative” control:

As suggested we changed it as follows

“Another experiment was carried out in an identical way by replacing enzyme solution with acetate buffer to determine the absorbance produced by the sample itself.”

24) Page 14, line 462: “All the experiments were performed using four replicates.” – Are these four replicates from four different algae extractions or only experiments repeated four times with the same algae sample? In other words, did the authors test different algae extractions in their study?

It means the same experiment repeated four times to reduce errors.
25) Regarding Statistical analysis in general: the authors mentioned they performed One-way ANOVA as statistical test, but only showed statistics in Table 1 (or at least they included different letters among different fractions). The authors must include statistical analyses in all tables/figures and show comparisons among fractions.

Statistically analyzed and we mentioned this all tables and figures.

References

26) Formatting: the references’ format is not following the journal's guidelines and, then, must be formatted accordingly.

27) References mentioned in the text that do not discuss/support the statement they are supposed to: Refs 1, 5, 4, 6, 7, 9, 10, 13, 14, 17, 19, 21, 25, 26, and 27.

Some of the new references have been cited.

- 19-Support the data.
- 21- Support the data.

28) Reference 15: the name of the journal is wrong. The correct one is “Oxid Med Cell Longev.”. Please change it accordingly.

Thank you for pointing our this. We changed it accordingly.

29) Reference 18 has been previously cited as Ref. 7. Please change it accordingly.

Thank you. It was corrected

30) Reference 29 does not describe the technique the authors say it describes. Please change it accordingly.

The method has modified accordingly.

Figures and Tables

31) I did not understand why the authors separated in “Figures A” or “Figure 3”? Figures A1, A2 and A3 are very important to be considered as “Suppl. Material”. In addition, “Figure 1” and “Figure 2” are missing in the main manuscript.
We corrected this accordingly

Furthermore, figure legends must be improved and give a better description of the figure as well as statistical analysis where appropriate.

32) In Table 1, what does the letter a-e mean? It is not written in Table legend.

Thank you. We included this.

33) In Table 4, the authors must provide the literature supporting each one of the "reported biological activities" mentioned in the table

Cited new references

- Phthalic acid-6-ethyloct-3-y1 2-ethylhexyl.ester


- 1,2-dimethoxy-4 (1,3-dimethoxy-1-propenyl) benzene


- Benz(b)1,4-oxazepine-4 (5H)-thione, 2,3-dihydro-2,8-dimethyl


- 2-acetoxymethyl-3-(methoxycarbonyl)biphenylene