April 26, 2019

Dear Dr. Martirosyan:

We are thankful for the valuable comments, suggestions and corrections from the reviewers. We have addressed all the reviewers’ comments and revised the manuscript accordingly. All the revisions and inclusions are highlighted in red.

Response to Concerns:

**Reviewers Comments (1)**

1. Background could be improved by clarifying what was the aim of this investigation.

**Y: BACKGROUND**

**We changed descriptions as follows.**

**Page 2, lines 25**

~~, evidence of antitumor activity has not been obtained~~ without concrete evidence of active ingredients and anti-cancer mechanism.

**Page 3, line 3 to 7**

Thus, we purified the hydrophobic fraction of the moriche palm fruit to search for estrogenic compounds, and identified two hydroxypterocarpans with estrogenic activity. Pterocarpans belong to the flavonoids, and are structurally similar to isoflavonoids known as typical phytoestrogens. ~~Then w~~ We then investigated the properties of estrogenic effects of these hydroxypterocarpans.

1. Discussion section is very short, I recommend comparing with some other investigations in similar field. Also, you can get this way more citation in the discussion section also, since you have very limited citations/references for discussion portion

**Y: We added following description with references in Discussion**

Ptercarpans present in Legminosase plants [soybean leaves (27) and Japanese clover (23)] and Fabaceae plants [Babch (28) and *Sophora tonkinensis* (29)]. It’s structure is similar to that of isoflavons, and coumestans, which are well known phytoestrogens. However, as there have not been any studies reporting the existence of pterocarpans in fruits of Arecaceae plants rich in oil substances, such as palms, our finding is the first report to find estrogenic compounds in Arecaceae plants. On the other hand, several pterocarpans have been reported to exhibit estrogenic activities. Glyceollin I which accumulates in stressed soy bean stimulates both ER and ER (30). Medicarpin isolated from legumes strongly activates osteoblasts by stimulating ER and suppresses bone loss in rats (31). Thus we speculated that hydroxypterocarpans isolated from moriche palm might exhibit estrogenic activities.

Recently, several phytoestrogens in *Pueraria candollei var. mirifica* [32] which has potent estrogenic activity, have been reported to show adverse effects in Japanese female including atypical genital bleeding. These estrogenic compounds are miroestrol and isomiroesterol, which exhibit strong estrogenic activities similar to estradiol [33-35]. With this background of phytoestrogens in mind, mild phytoestrogens having estrogenic activity milder than soy isoflavons are favorable. LF and 8-HHP, which have been found to be mild phytoestrogens, will satisfy the demand of functional foods applicable for female symptoms caused by estrogen deficiency.

1. Conclusion part also very short, it can be expanded, such as “The activity of lespeflorin is 5.5-fold lower than that of daidzein and 16.2-fold lower than that of genistein.

**Y: We added following description in Conclusion**

8-HHP was a partial agonist bound to ER. This report is a first to have found estrogenic compounds in the oil fraction of palms.

**Reviewers Comments (2)**

**ABSTRACT:**

1. On the one hand, authors acclaim to isolate two hydroxypterocarpans from studied plant in background. On the other hand, they explain about evaluation of estrogenic activity and related receptor, cell and gene in the materials chapter. Apparently, there isn’t a suitable continuity between the content of these two parts and authors must consider it.

**Y: We separated the description as follows.**

**Background:** Phytoestrogens in edible plants, including soybean isoflavones and pomegranates, have been used for alleviation of premenopausal/postmenopausal syndromes and osteoporosis. *Mauritia flexuosa* (moriche palm) is grown in Peru and Brazil for its edible fruit that is said to contain phytoestrogens, but the relevant estrogenic compounds have not been identified. We investigated the constituents of moriche palm fruit extract and evaluated their estrogenic ability. ~~isolated two hydroxypterocarpans: lespeflorin G~~~~8~~ ~~(LF) and 8-hydroxyhomopterocarpan (8-HHP).~~

**Materials and methods:** Ethyl acetate fraction of dried moriche palm was purified by column chromatography and HPLC chromatography. To evaluate estrogenic activity, we performed (1) simulation of binding to the human estrogen receptor (ER), (2) investigation of the proliferative effect on MCF-7 cells, and (3) the estrogen-chemically activated luciferase gene expression (E-CALUX) assay.

**Results:** Two hydroxypterocarpans were isolated including lespeflorin G8 (LF) and 8-hydroxyhomopterocarpan (8-HHP). The binding affinity of LF to ER was higher than that of 8-HHP, with inhibition constants of 81.9 nM and 1.99 M, respectively. However, LF and 8-HHP exhibited a similar proliferative effect on MCF-7 cells at 10 M. The E-CALUX assay showed that LF is a full ER agonist and 8-HHP is a partial agonist.

1. Authors should briefly say about estrogenic compounds from fruit of Mauritia flexuosa in the materials part, otherwise the necessary unity and totality would be damaged in this part.

**Y: We added following description in Materials and Methods in ABSTRACT**

Ethyl acetate fraction of dried moriche palm was purified by column chromatography and HPLC chromatography.

**Keywords:**

- It’s better to cite “estrogenic activity” instead of “estrogen”.

**Y: We have amended as you mentioned.**

BACGROUND:

1- Apparently, from all 24 references, 22 ones have been allocated to background and only one to discussion. It seems that the reference distribution is a little unusual.

**Y: We added 9 references with description in Discussion.**

1. hydroxypterocarpan is a kind of flavonoid with antioxidant property. So, authors should make bold this important or applicable character and explain about that. In this manner, they can increase the worth of their article.

**Y: We added description at the end of BACGROUND as follows.**

Pterocarpans belong to the flavonoids, and are structurally similar to isoflavonoids known as typical phytoestrogens.

3- Usually, referring would be related to the fact, idea, explanation, document or some items such as these. Emphasis on single words, especially without any specific elucidation, like references 2 – 3 – 4 – 5 – 6 – 9 – 10 – 11 and 12, couldn’t be comprehensive and informative.

**Y: Sorry, but I can’t agree with your manner. These references report specific estrogenic activities of distinct plant resources or applicable diseases for isoflavons. This way is effective in a view of reader’s friendly.**

MATERIALS AND METHODS:

*Isolation of estrogenic compounds form Peruvian moriche palm fruit:*

1- If it’s possible, authors should add the related atmosphere pressure and pH to the related condition, before extraction of residue.

**Y: I’m sorry but to do so is unusual regarding our experiences to submit journals for naturally occurring bioactive compounds.**

2- Citation of chemical measures due to laboratory materials and solutions, such as kg, L or g is good. But, concentrations of lab solutions or liquids are necessary and must be presented as mg/ml, g/L, etc. So, authors should consider this basic lab rule.

**Y: We must consult with editorial office about your suggestion, because there are no indication about units in the Journal submitting rules and micro g/mL or cells/mL are commonly used in a field of biological experiences.**

*Cell culture:*

- It’s necessary to use one reference in this chapter. If there isn’t any reference, authors must mention the related bases for exhibited quantities.

**Y: We added a reference [25] in MCF-7 experiment.**

25. Matsuda H, Shimoda H, Morikawa T, Yoshikawa M: Phytoestrogens from the roots of *Polygonum cuspidatum* (Polygonaceae): Structure-requirement of ydroxyanthraquinones for estrogenic activity. *Bioorg. Med. Chem. Lett*., **11**, 1839—42 (2001).

*Simulation of receptor binding:*

1- Estrogen receptor is a kind of protein that can be purchased from Sigma Company (E 1528-1VL). So, if it’s possible, authors can try to assay the changes of second and third structures of estrogen receptor α by CD (Cirular Dichroism) method before and after effect of lespeflorin G8. Besides simulation of receptor binding, CD can be impressive to clear the alterations of mentioned receptor and increases the value of present study.

**Y: Sorry but we can’t do that now.**

2- Authors must exhibit the related calculations due to Lamarckian Genetic Algorithm. This can clarify the readers mind and enhances the paper worth.

**Y: We cited reference of the algorithm as reference [24].**

*MCF-7 proliferation assay:*

- Such as some other chapters, this one needs the reference, unless authors present the respective principles.

 **Y: We added a reference (25) in MCF-7 experiment.**

*E-CALUX assay:*

- It’s important to know the general parameters of incubation including: temperature, pH, CO2, O2, with or without shaker, atmosphere pressure and humidity.

**Y: We added culture condition (at 37°C under a 5% CO2 atmosphere).**

*Statistics:*

- If authors used SPSS software for statistic assay, they must mention the related version.

 **Y: We didn’t used SPSS.**

RESULTS:

*Hydroxypterocarpans in moriche palm fruit:*

- Authors say about orange spots in chromatography due to G8 (LF) and (8-HHP). So, it’s necessary to exhibit the picture of related gel.

**Y: We added a figure of TLC spots as new Fig. 2.**

*Simulation of binding to the human estrogen receptor (ER):*

1- Authors should emphasize that the important aim of molecular docking study is not only the investigation about binding between LF or 8-HHP and ER, but also the prediction of binding conformations between studied biomolecules. In this manner, readers can cognize the philosophy of simulation.

**Y: Yes, we totally agree with you and the recognition is expressed by current sentences.**

2- Authors try to present binding parameters. This is good, but apparently the content of table 1 is defective. So, they should add other parameters like: binding electrostatic energy, location of probable salt bridges and secondary structure style of protein receptor to the list of recent table.

**Y: We added electrostatic energy in Table 1. But we can’t calculate other parameters you mentioned because of lacking calculation ability of the software.**

*Estrogen-dependent cell proliferation:*

- How did authors achieve the number 25%? What was their main basis or standard to determine percent (%) as the unit of data in fig 3?

**Y: The value “25%” is the relative increase rate when compared to control group (without any sample). We added “compared to the control**”.

*E-CALUX assay:*

1- The unit of EC50 value has been cited as µM in the text, but as M in the table 2. This paradox must be eliminated.

**Y: We changed the value to µM.**

2- The presentation of data in this chapter is good, but the related explanation isn’t enough. Hence, at the end of second sentence, authors should cite that LF curve is nearer to 17β-estradiol curve than the 8-HHP one.

**Y: We added a following sentence at the point you mentioned.**

**The shape of inhibition curve of LF is more similar to 17-estradiol than that of 8-HHP.**

DISCUSSION:

1. This chapter is too brief and must be expanded.

**Y: We added following description with references in Discussion**

Ptercarpans present in Legminosase plants [soybean leaves (27) and Japanese clover (23)] and Fabaceae plants [Babch (28) and *Sophora tonkinensis* (29)]. It’s structure is similar to that of isoflavons, and coumestans, which are well known phytoestrogens. However, as there have not been any studies reporting the existence of pterocarpans in fruits of Arecaceae plants rich in oil substances, such as palms, our finding is the first report to find estrogenic compounds in Arecaceae plants. On the other hand, several pterocarpans have been reported to exhibit estrogenic activities. Glyceollin I which accumulates in stressed soy bean stimulates both ER and ER (30). Medicarpin isolated from legumes strongly activates osteoblasts by stimulating ER and suppresses bone loss in rats (31). Thus we speculated that hydroxypterocarpans isolated from moriche palm might exhibit estrogenic activities.

Recently, several phytoestrogens in *Pueraria candollei var. mirifica* [32] which has potent estrogenic activity, have been reported to show adverse effects in Japanese female including atypical genital bleeding. These estrogenic compounds are miroestrol and isomiroesterol, which exhibit strong estrogenic activities similar to estradiol [33-35]. With this background of phytoestrogens in mind, mild phytoestrogens having estrogenic activity milder than soy isoflavons are favorable. LF and 8-HHP, which have been found to be mild phytoestrogens, will satisfy the demand of functional foods applicable for female symptoms caused by estrogen deficiency.

2- Discussion is an opportunity for challenging not exhibiting. Authors only say about their data and methods, without any interaction between their study and other similar ones. Scientific comparison can be important for clarification of reader mind and increment of article value.

**Y: We added above sentences.**

CONCLUSION:

- This is one of the shortest conclusions that can be recorded. Authors could mention about medical or operational effect of LF such as antioxidant or lipid reducer. This article has been connected to medicine field and in addition to basic science (to be a full ERα agonist), must acclaim in the clinic area. Medicine includes both of basic science and clinical one.

**Y: We added following description in Conclusion**

8-HHP was a partial agonist bound to ER. This report is a first to have found estrogenic compounds in the oil fraction of palms.

ABBERVIATIONS:

- This chapter isn’t completed. Some abbreviations such as BGILuc4E2, RPMI-1640 or MCF-7 (GCRBO 134) wouldn’t be seen in the list.

**Y: These are not abbreviations but proper nouns.**

I hope that the reviewers’ comments have been properly addressed and I am looking forward the revision of my revised manuscript

Sincerely



Hiroshi Shimoda Ph. D.