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Peer Review Report for "Rat Model to Investigate the Effect of Oleuropein on Cardiac Tissue in D-Galactose-Induced Aging"

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Review Timeline:

Submit Date:	9 Nov 2024
Revised Date:	1 Dec 2024
Accepted Date:	3 Dec 2024

Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Amir Jalali : Reviewer

22 Nov 2024

Major Comments:

- 1). Explain more about the relationship of markers to the results: Explain the importance of the markers: Explain why the markers under study were chosen and what role they play in answering your research question or hypotheses (lowering aging process). Direct connection of the markers to the results: Show specifically how these markers are related to the results of the paper. Use statistical data, empirical evidence, or reliable sources to support their connection.
- 2) Related research background: In the discussion of the paper, mention previous studies that have confirmed the importance or impact of similar markers. This can strengthen the scientific and practical value of your paper's results.
- 3) Strengthen the discussion: Evaluate the findings in the context of previous research: Place your findings in the context of similar research and show how your results are complementary, similar, or different from other studies.

- 4) Investigate mechanisms or processes: If possible, add to the discussion the underlying mechanisms or physiological and biological aging causes that explain the association of the markers with the results.
- 5) Practical or clinical impact of the results: Explain how the findings and markers under study can be applied in changing the aging process, such as in the diagnosis, treatment, or prediction of the main aging year of individuals.
- 6) Add clarity to the conclusion: In the conclusion section, briefly and precisely explain how your findings support the markers under study and what implications they have for future research or practical applications.
- 7) Use credible sources: Use credible scientific sources to support your claims about the importance of the markers in aging process. Systematic reviews, meta-analyses, or original research can be helpful. If there is a gap in the explanation of the importance of the markers in the introduction or research methods section, complete it as well.

These revisions will help strengthen the scientific arguments of the article and improve its clarity, increasing the likelihood of acceptance.

OPEN PEER REVIEW

Revision (1)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Reviewers' Comments:

Reviewer 1:

The manuscript "Rat model to investigate the effect of oleuropein on cardiac tissue in d-galactose-induced aging" was reviewed, and the following objections were raised:

1. It is better to check the grammar and written language again with a native editor or relevant software. The standard of English language in the articles should be suitable. For example: In the abstract section, it is better to revise the "This study pointed to the protective impact" to "This study aims to investigate..."

Response: Edited as following in the abstract and highlighted in red color;

This study aimed to investigate the protective

OR: The abbreviation "D-Gal" should be defined as "D-galactose" on first use for clarity.

Response: Edited as following in the abstract and highlighted in red color;

Background: d-galactose (D-Gal)

OR: "Wister" should be "Wistar"; "grown-up" is informal.

Response: Edited as following in the abstract and highlighted in red color;

Wister male adult rats

OR: "group 2" should be "second group".

Response: Edited as following in the abstract and highlighted in red color;

the **second** group

OR: "bunches" is informal.

Response: Edited as following in the abstract and highlighted in red color;

The rats in **groups** 3 to 5 were

OR: "organizations" should be "treatments".

Response: Edited as following in the abstract and highlighted in red color;

All **treatments** were

OR: "killed" is too informal; consider "euthanized".

Response: Edited as following in the abstract and highlighted in red color;

the rats were **euthanized** and serum

OR: "discoveries" is non-standard; also, the sentence is overly complex.

Response: Edited as following in the abstract and highlighted in red color;

The **findings showed** that D-Gal sufficiently **decreased** the GPx, CAT, and SOD, GSH levels plus **to** SIRT1 and PGC1 expression within the heart tissue (P<0.05).

OR: "essentially expanded" is not precise; "PC" is not defined.

Response: Edited as following in the abstract and highlighted in red color;

In methods of Abstract;

The levels of protein carbonyl (PC), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and expression of PGC1a and SIRT1 genes in heart tissue were determined. Heart tissue was maintained in 10% formalin for Hematoxylin-Eosin (H&E) and histological examination.

In results of Abstract;

The findings showed that D-Gal sufficiently decreased the GPx, CAT, and SOD, GSH levels plus to SIRT1 and PGC1 expression within the heart tissue ($P<0.05$). Also, D-Gal, significantly increased PC and MDA levels and serum cardiac markers (CK-MB, LDH, AST and cTnI) ($P<0.05$).

OR: "direction" is unclear; "reestablished" is awkward.

Response: Edited as following in the abstract and highlighted in red color;

Also, the protective effect of the OLE was confirmed by the histological examination.

OR: In Conclusion section: "Study" should be "The study"

Response: Edited as following in the abstract and highlighted in red color;

Conclusion: The study showed

OR: "exerts this protective effect through reducing" is slightly awkward.

And so on.

Response: Edited as following in the abstract and highlighted in red color;

The findings suggested that the protective effect of OLE was through the reduction of oxidative damage.

2. It is also advisable to indicate how the rats were acclimatized to their environment before the experiment commenced.

Response: Edited as following in the main text and highlighted in red color;

One week before the experiments, the rats were brought to the laboratory to adapt to the environment.

3. In the "Study Design" section: how were the dosages of oleuropein determined? What was

the reason for choosing eight weeks for this study? Please for each experimental group, describe the timing and frequency of procedures.

Response: Edited as following in the main text and highlighted in red color;

The reason for choosing this protocol was based on the previous studies conducted and confirmed, such as study conducted by khalili and et al that we referenced it in the main text/
Study Design.

One week before the experiments, the rats were brought to the laboratory to adapt to the environment. They were placed in 5 groups. Group 1 received normal saline for 8 weeks as a control. Also, the second one received D-Gal (100 mg/kg /IP) for the same period as a positive control group. Groups 3 to 5 as treatment groups were fed with a certain amount of ELO substance (doses of 20, 40, and 80 mg/kg, respectively,) orally, as well as they received D-Gal (100 mg/kg /IP) for 8 consecutive weeks as described by Khalili et al.¹⁶

Reference: Khalili HR, Adeli Behrooz HR, Rashidi Nooshabadi MR, Geravandi S, Mohammadi MJ, Foruozandeh H. Evaluation of potential anti-fibrotic effect of oleuropein on bleomycin-induced pulmonary fibrosis in rat. *Toxin Reviews.* 2020;39(2):188-96.

4. State whether randomization was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.

Response: Edited as following in the main text and highlighted in red color;

No specific method was used and we randomly assigned the rats to different groups, but no specific protocol was used for this assignment.

5. "Turkey's post hoc analysis" should read "Tukey's post hoc analysis".

Response: Edited as following in the main text and highlighted in red color;

Further analyses of the data were conducted using *Tukey's post hoc analysis*.

6. "means $\pm$ SEM" is mentioned in the method section, but "means $\pm$ S.E.M." is written in the result.

Response: Your right, *means $\pm$ SEM* is correct, we edited in results section, in caption of figures.

7. References should be in the correct format. For example: In a similar study Yang et al. showed that administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress decreased GSH and other enzymatic antioxidants such as SOD).³¹ Moreover, studies of Fu

et al. in 2018³², Yang et al. in 2016³¹, and study by Coban et al. in 2015³³ showed that subcutaneous administration of D-Gal for 8 weeks increased oxidative stress in different tissues. Bo-Htay et al., believed that D-Gal administration causes cardiac damage through upregulation of the expression of senescence markers. In addition, they suggested that increased oxidative stress, decreased antioxidant levels, and heightened apoptosis are some of the mechanisms involved in promoting aging induced by D-Gal. ³⁴

Response: Edited as following in the main text and highlighted in red color;

In a similar study, Yang et al. ³¹ showed that **the** administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress (decreased GSH and other enzymatic antioxidants such as SOD). **Also**, studies **conducted by** Fu et al. in 2018,³² Yang et al. in 2016,³¹ and Coban et al. in 2015³³ showed that subcutaneous administration of D-Gal for 8 weeks increased oxidative stress in different tissues. **Another study conducted by** Bo-Htay et al., **revealed** that D-Gal administration **could cause** cardiac damage through upregulation of the expression of senescence markers. In addition, they suggested that increased oxidative stress, decreased antioxidant levels, and heightened apoptosis are some of the mechanisms involved in promoting aging induced by D-Gal.³⁴

8. It is better to mention the limitations of this research to increase validity.

Response: Edited as following in the main text (end of Discussion section) and highlighted in red color;

The limitations of this study are; we should have used the rats that had naturally aged themselves to survey the effect of oleuropein, but these rats practically did not exist, and we used of D-Gal to induce aging. As well, regarding the complexity of the antioxidant-oxidative stress system, it seems that the lack of measurement of DNA damage indices and total antioxidant capacity is another limitation. Given the wide range of mechanisms for improving telomere function in heart tissue, it appears that the lack of measurement of key proteins in nuclear transcription such as NRFs and PPARs is a limitation.

9. *The references are too old and should be updated.*

Response: **We deleted the references before 2000 and replaced with new ones as followings;**

18. Srikanth M, Yadav A. Three cardiac biomarkers and their efficacy: A review. *Journal of Drug Delivery & Therapeutics.* **2022**;12.

19. Nouroozi RV, Noroozi MV, Ahmadizadeh M. Determination of protein concentration using Bradford microplate protein quantification assay. *Disease and Diagnosis*. 2015;4(1):11-7.
20. Cui X, Gong J, Han H, He L, Teng Y, Tetley T, et al. Relationship between free and total malondialdehyde, a well-established marker of oxidative stress, in various types of human biospecimens. *Journal of thoracic disease*. 2018;10(5):3088.
22. Alisik M, Neselioglu S, Erel O. A colorimetric method to measure oxidized, reduced and total glutathione levels in erythrocytes. *Journal of Laboratory Medicine*. 2019;43(5):269-77.

10. *I recommend reviewing all section titles to ensure they adhere to the same style, including font size, boldness, abbreviation, and capitalization.*

Response: We have reviewed all section titles to ensure they adhere to the same style, including font size, boldness, abbreviation, and capitalization. Please check again.

Reviewer

2:

Major

Comments:

1). explain more about the relationship of markers to the results: Explain the importance of the markers: Explain why the markers under study were chosen and what role they play in answering your research question or hypotheses (lowering aging process). Direct connection of the markers to the results: Show specifically how these markers are related to the results of the paper. Use statistical data, empirical evidence, or reliable sources to support their connection.

Response: Edited as following in the main text and highlighted in red color;

Increasing biomarkers such as MDA, PC, and decreasing antioxidant enzymes such as SOD, GPx, and CAT in some ways indicates an oxidative damage.³² Our findings showed that aging in d-galactose group was associated with elevation in both MDA and PC content and reduction of GSH, CAT, SOD and GPx activities. These findings indicated that intraperitoneal administration of D-Gal for 8 weeks increased oxidative stress in the heart tissue of rats. These findings are consistent with other studies. In a similar study, Yang et al.³³ showed that the administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress (decreased GSH and other enzymatic antioxidants such as SOD). Also, studies conducted by Fu et al. in 2018,³⁴ Yang et al. in 2016,³³ and Coban et al. in 2015³⁵ showed that subcutaneous administration of D-Gal for 8 weeks increased oxidative stress in different tissues. Another study conducted by Bo-Htay et al., revealed that D-Gal administration could cause cardiac damage through upregulation of the expression of senescence markers. In

addition, they suggested that increased oxidative stress, decreased antioxidant levels, and heightened apoptosis are some of the mechanisms involved in promoting aging induced by D-Gal.³⁶

Simultaneous management of OLE by gavage 8 weeks **primed** to remarkable alterations in tissue antioxidant position of heart and its cause's **increase** of GSH, CAT, SOD, and GPx activities and **reducing the** MDA, and PC in treated groups compared to the group **received** D-Gal alone. These findings **suggested** that the reduction in oxidative stress is likely due to increase of antioxidant defense mechanisms. These effects obtained from the present study are comparable **to another study conducted by Çoban et. al. in 2014.**³⁷ They evaluated the **positive** effect of olive leaf extract on oxidative stress in **vital** tissues of elderly rats. **The** findings **showed** that the consumption of this extract can decrease oxidative stress in **some organs such as** liver, heart, and brain of rats.³⁷

Some studies have shown that Sirt1 has a protective role against apoptosis, as well as it plays a role in the survival of cardiac myocytes and neurons under stress in vitro. Overexpression of Sirt1 (2.5-fold) to moderate (7.5-fold) in the hearts of transgenic mice reduces age-related effects, including cardiac hypertrophy, apoptosis, fibrosis, cardiac dysfunction, and expression of aging-related markers.^{38, 39} Our findings showed that D-Gal administration resulted in down-regulation of *sirt1* gene expression in cardiac tissue. OLE administration resulted in significant expression of *sirt1* gene in cardiac tissue **as shown by the current study.** Our findings **are accordant with** the findings of **a study conducted by** Ralph et al. They reported that a moderate increase in *sirt1* expression can provide a protection **for** heart **tissue** against oxidative stress, while also promoting the expression of antioxidant enzymes.⁴⁰

PGC-1 α is highly expressed in tissues with high energy demands, such as the heart, skeletal muscle, and liver. It is activated by various stimuli, including exercise, cold exposure, and fasting, and can induce the expression of genes involved in energy metabolism. PGC-1 α has been shown to play a role in the pathogenesis of metabolic disorders such as obesity, type 2 diabetes, and cardiovascular disease. Therefore, it has become an attractive target for the development of therapeutic interventions.⁴²⁻⁴⁴

The result of present study indicated that administration of D-Gal **caused** down regulation of PGC-1 α in heart tissue. This reduction was significant compared to the control (healthy) group. Co-administration of OLE with D-Gal for 8 weeks by gavage increased PGC-1 α gene expression, **showing a reduction** in the effect of d-galactose. **Of course, it should be noted this effect was more prominent and higher at a dose of 80 mg/kg OLE.**

Histopathological results of heart tissue also confirmed the findings of antioxidant tests, and gene expression, and showed that D-Gal consumption led to severe damage to heart tissue, and OLE administration reduced the severity of heart tissue damage. In histopathological studies of heart tissue, which were performed using conventional H&E staining, signs of D-Gal toxicity in the form of hemorrhage, myocardial irregularity, myofibrillar loss, and inflammatory cell infiltration were clearly evident in the heart tissue. Histopathological results of heart tissue in the present study are in line with studies conducted by He et al. in 2009, and Wang et al. in 2012.^{45, 46}

2) Related research background: In the discussion of the paper, mention previous studies that have confirmed the importance or impact of similar markers. This can strengthen the scientific and practical value of your paper's results.

Response: Edited as following in the main text and highlighted in red color;

Increasing biomarkers such as MDA, PC, and decreasing antioxidant enzymes such as SOD, GPx, and CAT in some ways indicates an oxidative damage.³² Our findings showed that aging in d-galactose group was associated with elevation in both MDA and PC content and reduction of GSH, CAT, SOD and GPx activities. These findings indicated that intraperitoneal administration of D-Gal for 8 weeks increased oxidative stress in the heart tissue of rats. These findings are consistent with other studies. In a similar study, Yang et al.³³ showed that the administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress (decreased GSH and other enzymatic antioxidants such as SOD). Also, studies conducted by Fu et al. in 2018,³⁴ Yang et al. in 2016,³³ and Coban et al. in 2015³⁵ showed that subcutaneous administration of D-Gal for 8 weeks increased oxidative stress in different tissues. Another study conducted by Bo-Htay et al., revealed that D-Gal administration could cause cardiac damage through upregulation of the expression of senescence markers. In addition, they suggested that increased oxidative stress, decreased antioxidant levels, and heightened apoptosis are some of the mechanisms involved in promoting aging induced by D-Gal.³⁶

Simultaneous management of OLE by gavage 8 weeks primed to remarkable alterations in tissue antioxidant position of heart and its cause's increase of GSH, CAT, SOD, and GPx activities and reducing the MDA, and PC in treated groups compared to the group received D-Gal alone. These findings suggested that the reduction in oxidative stress is likely due to increase of antioxidant defense mechanisms. These effects obtained from the present study

are comparable to another study conducted by Çoban et. al. in 2014.³⁷ They evaluated the positive effect of olive leaf extract on oxidative stress in vital tissues of elderly rats. The findings showed that the consumption of this extract can decrease oxidative stress in some organs such as liver, heart, and brain of rats.³⁷

Some studies have shown that Sirt1 has a protective role against apoptosis, as well as it plays a role in the survival of cardiac myocytes and neurons under stress in vitro. Overexpression of Sirt1 (2.5-fold) to moderate (7.5-fold) in the hearts of transgenic mice reduces age-related effects, including cardiac hypertrophy, apoptosis, fibrosis, cardiac dysfunction, and expression of aging-related markers.^{38, 39} Our findings showed that D-Gal administration resulted in down-regulation of *sirt1* gene expression in cardiac tissue. OLE administration resulted in significant expression of *sirt1* gene in cardiac tissue as shown by the current study. Our findings are accordant with the findings of a study conducted by Ralph et al. They reported that a moderate increase in *sirt1* expression can provide a protection for heart tissue against oxidative stress, while also promoting the expression of antioxidant enzymes.⁴⁰

3) Strengthen the discussion: Evaluate the findings in the context of previous research: Place your findings in the context of similar research and show how your results are complementary, similar, or different from other studies.

Response: Edited as following in the main text and highlighted in red color;

Increasing biomarkers such as MDA, PC, and decreasing antioxidant enzymes such as SOD, GPx, and CAT in some ways indicates an oxidative damage.³² Our findings showed that aging in d-galactose group was associated with elevation in both MDA and PC content and reduction of GSH, CAT, SOD and GPx activities. These findings indicated that intraperitoneal administration of D-Gal for 8 weeks increased oxidative stress in the heart tissue of rats. These findings are consistent with other studies. In a similar study, Yang et al.³³ showed that the administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress (decreased GSH and other enzymatic antioxidants such as SOD). Also, studies conducted by Fu et al. in 2018,³⁴ Yang et al. in 2016,³³ and Coban et al. in 2015³⁵ showed that subcutaneous administration of D-Gal for 8 weeks increased oxidative stress in different tissues. Another study conducted by Bo-Htay et al., revealed that D-Gal administration could cause cardiac damage through upregulation of the expression of senescence markers. In addition, they suggested that increased oxidative stress, decreased antioxidant levels, and

heightened apoptosis are some of the mechanisms involved in promoting aging induced by D-Gal.³⁶

Simultaneous management of OLE by gavage 8 weeks **primed** to remarkable alterations in tissue antioxidant position of heart and its cause's **increase** of GSH, CAT, SOD, and GPx activities and **reducing the** MDA, and PC in treated groups compared to the group **received** D-Gal alone. These findings **suggested** that the reduction in oxidative stress is likely due to increase of antioxidant defense mechanisms. These effects obtained from the present study are comparable **to another study conducted by Çoban et. al. in 2014.**³⁷ They evaluated the **positive** effect of olive leaf extract on oxidative stress in **vital** tissues of elderly rats. **The** findings **showed** that the consumption of this extract can decrease oxidative stress in **some organs such as** liver, heart, and brain of rats.³⁷

Some studies have shown that Sirt1 has a protective role against apoptosis, as well as it plays a role in the survival of cardiac myocytes and neurons under stress in vitro. Overexpression of Sirt1 (2.5-fold) to moderate (7.5-fold) in the hearts of transgenic mice reduces age-related effects, including cardiac hypertrophy, apoptosis, fibrosis, cardiac dysfunction, and expression of aging-related markers.^{38, 39} Our findings showed that D-Gal administration resulted in down-regulation of *sirt1* gene expression in cardiac tissue. OLE administration resulted in significant expression of *sirt1* gene in cardiac tissue **as shown by the current study.** Our findings **are accordant with** the findings of **a study conducted by** Ralph et al. They reported that a moderate increase in *sirt1* expression can provide a protection **for heart tissue** against oxidative stress, while also promoting the expression of antioxidant enzymes.⁴⁰

4) Investigate mechanisms or processes: If possible, add to the discussion the underlying mechanisms or physiological and biological aging causes that explain the association of the markers with the results.

Response: Edited as following in the main text and highlighted in red color;

Current studies prepared insight into the disease-protective mechanism of OLE and elucidated numerous of its impacts via oxidative stress modulation and antioxidant action.³⁷ OLE presented a capability to destroy the fabrication of reactive oxygen and nitrogen species (ROS and NOS, respectively) in vitro biochemical tests, in addition to in human cells, where it prohibited excessive ROS generation. It was furthermore revealed to reduce oxidative stress (OS) by the inflection of the ERK/Nrf2 pathway-mediated signaling, with downstream values for a diversity of cellular procedures.³⁸

Increasing biomarkers such as MDA, PC, and decreasing antioxidant enzymes such as SOD, GPx, and CAT in some ways indicates an oxidative damage.³⁹ Our findings showed that aging in d-galactose group was associated with elevation in both MDA and PC content and reduction of GSH, CAT, SOD and GPx activities. These findings indicated that intraperitoneal administration of D-Gal for 8 weeks increased oxidative stress in the heart tissue of rats. These findings are consistent with other studies. In a similar study, Yang et al.⁴⁰ showed that the administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress (decreased GSH and other enzymatic antioxidants such as SOD).

5) Practical or clinical impact of the results: Explain how the findings and markers under study can be applied in changing the aging process, such as in the diagnosis, treatment, or prediction of the main aging year of individuals.

Response: Edited as following in the main text and highlighted in red color;

The effect of oleuropein has been shown to prevent heart aging. So now, if we include olives (the source of oleuropein) in the diet of humans, will certainly benefit from its effects.

6) Add clarity to the conclusion: In the conclusion section, briefly and precisely explain how your findings support the markers under study and what implications they have for future research or practical applications.

Response: Edited as following in the main text and highlighted in red color;

The expression of genes involved in the aging process increased. Oxidative stress factors were practically suppressed, such as MDA. In fact, the oxidative phase decreased and the antioxidant phase increased. PGC1 increased. This means that this gene actually functions to increase the number of heart mitochondria, so this means, improving heart function. So, based on the results obtained in this study, we can examine the effect of this substance on other factors affecting aging.

7) Use credible sources: Use credible scientific sources to support your claims about the importance of the markers in aging process. Systematic reviews, meta-analyses, or original research can be helpful. If there is a gap in the explanation of the importance of the markers in the introduction or research methods section, complete it as well. These revisions will help strengthen the scientific arguments of the article and improve its clarity, increasing the likelihood of acceptance.

Response: We used of this references for explanation of the importance of the markers in the introduction;

6. Salisbury D, Bronas U. Reactive oxygen and nitrogen species: impact on endothelial dysfunction. *Nursing research*. 2015;64(1):53-66.
7. Kalantari H, Forouzanmehr H, Khodayar MJ, Siahpoosh A, Saki N, Kheradmand P. Antioxidant and hepatoprotective effects of Capparis spinosa L. fractions and Quercetin on tert-butyl hydroperoxide-induced acute liver damage in mice. *Journal of traditional and complementary medicine*. 2018;8(1):120-7.
8. Junqueira VB, Barros SB, Chan SS, Rodrigues L, Giavarotti L, Abud RL, et al. Aging and oxidative stress. *Molecular aspects of medicine*. 2004;25(1-2):5-16.
9. Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, et al. Oxidative stress, aging, and diseases. *Clinical interventions in aging*. 2018;13(8):757-67.
10. Sergiev P, Dontsova O, Berezkin G. Theories of aging: an ever-evolving field. *Acta Naturae (англоязычная версия)*. 2015;7(1 (24)).
11. Zhalehjo N, Panjehpour M. Biochemical and Genetic Theories of Aging. *Iranian Journal of Ageing*. 2014;9(3):232-43.
12. Atabati H, Yazdanpanah E, Mortazavi H, Bajestani SG, Raoofi A, Esmaeili S-A, et al. Immunoregulatory effects of tolerogenic probiotics in multiple sclerosis. *Reviews on New Drug Targets in Age-Related Disorders: Part II*. 2021:87-105.

Reviewer 3:
 Thanks for the effort of the authors,

++Introduction

1. To establish a deeper context, expand on the specific pathways of oxidative stress and aging (e.g., the role of SIRT1/PGC1 in mitochondrial function).

Response: Edited as following in the main text and highlighted in red color;

Sirtuins are class III proteins of a family of histone deacetylase enzymes that catalyze hydrolysis of histone acetylated end, deform chromatin, produce heterochromatin, and thus inhibit transcription of mitochondrial pathway-dependent cell death genes such as P53. Among the sirtuins, sirtuin1 (SIRT1) in the heart tissue by mechanism, Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1 α) and inhibition of Nuclear factor kappa B (NF- κ B) increase mitochondrial biogenesis and resulting in NAD modulating and increases cellular respiration. As a result, oxidative stress decreases and telomere length increases.¹¹⁻¹³ Sirtuins (SIRT) are NAD⁺-dependent deacetylases that are very preserved between yeast and humans. SIRT1 is the greatest largely studied member of this family [16]. SIRT1 plays a positive role in autophagy, and longevity and can activate AMPK/mTOR signaling in diverse pathologies.¹⁴

Peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1- α) is a key transcriptional factor that is broadly complicated in mitochondrial quality control of different physiological actions, plus cardiac roles and metabolism of skeletal muscle. Generally, PTCs with

containing PGC1- α satisfy their metabolic energy demands.¹⁵ Reports showed the association between PGC1- α and mitochondrial quality control on dysfunctional mitochondria in PTCs through kidney aging.¹⁶

2. Similar studies on other natural compounds should be mentioned for comparative analysis to strengthen the rationale for choosing OLE.

Response: Edited as following in the Introduction section and highlighted in red color;

In addition, it is believed that natural remedy, especially plant-based medicines, are safer and more sustainable than synthetic drugs, leading to an increasing trend towards their use in medicine.¹⁹ Polyphenols are a type of chemical that naturally occur in plants, fruits, and vegetables. Among these phenolic compounds, those found in olive fruit and leaves are particularly noteworthy due to their significant pharmacological properties. These compounds have been shown to possess a range of health benefits and are considered to be one of the most important factors contributing to the therapeutic potential of olive products.²⁰ Over the years, numerous studies investigated the various physiological properties of Oleuropein (OLE). OLE is a polyphenolic component with great antioxidant effect equivalent to a hydrosoluble analog of α -tocopherol or vitamin E. Many studies revealed that oleuropein has many biological effect in both animals and human such as antioxidant, antidiabetic, anticancer, anti-inflammatory, hypolipidemic and hypoglycemic.²¹ In-vitro studies have demonstrated the ability of OLE to scavenge free radicals, protect against oxidative stress, and reduce inflammation.²² Oleuropein cause enhanced cardiac dysfunction subsequent ischemic reperfusion damage that obvious by reduced LVEDP, and augmented max and min dp/dt, LVDP. These cardioprotective properties of oleuropein can be ascribed to its antioxidant features.²³ Also, considering that aging is increasing dramatically in the world and it is predicted that in the coming decades, there will be more elderly people who suffer from debilitating diseases, so this survey was conducted to consider the protective impact of OLE on D-Gal -induced aging in the heart tissue.

3. Clearly define terms such as "programmed aging theory" and "free radical theory" with references to avoid ambiguity for unfamiliar readers.

Response: Edited as following in the main text included the proper references for this section and highlighted in red color;

The programmed aging theory is the concept that aging is genetically programmed to occur with time, and this route of weakening eventually leads to death⁴. The free radical theory

suggests that as the body gets older, its capacity to counteract free radicals decreases and aging is caused by accumulated damage to cells and tissues induced by free radicals.⁵

4. Goldsmith TC. Evolution of aging theories: Why modern programmed aging concepts are transforming medical research. *Biochemistry (Moscow)*. 2016;81(12):1406-12.

5. Patil RB, Vora SR, Pillai MM. Antioxidant effect of plant extracts on phospholipids levels in oxidatively stressed male reproductive organs in mice. *Iranian Journal of Reproductive Medicine*. 2009;7(1).

4. Justify why D-Gal was chosen as an aging model over other available models (e.g., natural aging, other chemical inducers).

Response: Edited as following in the main text and highlighted in red color;

Considering the proven effect of this substance on the aging process and the existence of many studies in this field, and its availability and ease of use, this substance was used in the present study. Furthermore, the use of natural compounds required a long period of time, which we were unable to use due to time constraints.

++Method and materials

5. Provide a rationale for choosing male Wistar rats and clarify if the results can be generalized to both genders.

Response: Edited as following in the main text and highlighted in red color;

Male and female Wistar rats could be used for this study, and there was no specific advantage, and the results obtained are generalizable to both groups.

6. Elaborate on why specific doses (20, 40, 80 mg/kg) of OLE were chosen for the study. Include references for dose selection.

Response: Edited as following in the main text and highlighted in red color;

The reason for choosing this protocol was based on the previous studies conducted and confirmed, such as study conducted by Khalili and et al that we referenced it in the main text/*Study Design*.

One week before the experiments, the rats were brought to the laboratory to adapt to the environment. They were placed in 5 groups. Group 1 received normal saline for 8 weeks as a control. Also, the second one received D-Gal (100 mg/kg /IP) for the same period as a

positive control group. Groups 3 to 5 as treatment groups were fed with a certain amount of ELO substance (doses of 20, 40, and 80 mg/kg, respectively,) orally, as well as they received D-Gal (100 mg/kg /IP) for 8 consecutive weeks as described by Khalili et al.¹⁶

Reference: Khalili HR, Adeli Behrooz HR, Rashidi Nooshabadi MR, Geravandi S, Mohammadi MJ, Forouzanmehr H. Evaluation of potential anti-fibrotic effect of oleuropein on bleomycin-induced pulmonary fibrosis in rat. *Toxin Reviews*. 2020;39(2):188-96.

7. Include the criteria for scoring histopathological changes to provide a more standardized evaluation.

Response: Edited as following in the main text and highlighted in red color;

According to the inflammatory cell infiltration, congested vessels and myofibrillar loss the heart tissue were scored as typical (-), mild (+), moderate (++), or severe (+++).²⁵

Kammon AM, Brar RS, Sodhi S, Banga HS, Singh J, Nagra NS. Chlorpyrifos chronic toxicity in broilers and effect of vitamin C. *Open Vet J*. 2011;1(1):21-7. Epub 2011 Feb 25. PMID: 26623275; PMCID: PMC4655760.

++Discussion:

8. Review the potential molecular mechanisms by which OLE enhances antioxidant enzyme activities, referencing recent literature.

Response: Edited as following in the main text and highlighted in red color;

Current studies prepared insight into the disease-protective mechanism of OLE and elucidated numerous of its impacts via oxidative stress modulation and antioxidant action.³⁷ OLE presented a capability to destroy the fabrication of reactive oxygen and nitrogen species (ROS and NOS, respectively) in vitro biochemical tests, in addition to in human cells, where it prohibited excessive ROS generation. It was furthermore revealed to reduce oxidative stress (OS) by the inflection of the ERK/Nrf2 pathway mediated signaling, with downstream values for a diversity of cellular procedures.³⁸

Increasing biomarkers such as MDA, PC, and decreasing antioxidant enzymes such as SOD, GPx, and CAT in some ways indicates an oxidative damage.³⁹ Our findings showed that aging in d-galactose group was associated with elevation in both MDA and PC content and reduction of GSH, CAT, SOD and GPx activities. These findings indicated that

intraperitoneal administration of D-Gal for 8 weeks increased oxidative stress in the heart tissue of rats. These findings are consistent with other studies. In a similar study, Yang et al.⁴⁰ showed that the administration of 150 mg/kg of D-Gal for 8 weeks increased oxidative stress (decreased GSH and other enzymatic antioxidants such as SOD).

9. Elaborate on the potential translational value of OLE in treating human cardiovascular diseases related to aging and oxidative stress.

Response: Edited as following in the discussion section and highlighted in red color;

In total, the results of the present study and other reports indicated the role of OLE in improving cardiac function and reducing oxidative stress. Therefore, this issue should be confirmed with further studies and its use in humans, which requires further investigation.

10. Clearly state the limitations of using a D-Gal-induced aging model and potential differences with natural aging.

Response: Edited as following in the main text and highlighted in red color;

The limitation of galactose is probably that because it is a sugar, in addition to its role as an inducer it can be consumed by cells, and the inducing effect is reduced. The difference between digalactose in inducing aging and the natural aging process is that natural aging requires time, but digalactose can lead to aging induction in a short period of time.

11. Suggest future studies, including exploring OLE's effect on other organs or its combination with other therapeutic agents for enhanced cardioprotection.

Response: Edited as following in the conclusion section and highlighted in red color;

Considering our study, the findings showed that oleuropein administration increased the levels of SOD, GPx enzymes, and PGC1- α , Sirt1 gene expression and decreased MDA and PC levels in heart tissue of rats, it is suggested that the effect of this substance on mitochondrial biogenesis and improving telomere and telomerase enzyme function be evaluated in future studies.

12. Highlight how the findings could influence the development of new pharmacological interventions targeting oxidative stress in aging.

Response: Edited as following in the main text and highlighted in red color;

In practical, the effect of oleuropein has been shown to prevent heart aging. So, if olives (the source of oleuropein) include in the diet of humans, will certainly benefit from its effects.

OPEN PEER REVIEW

Revision (1)

Amir Jalali : Reviewer

2 Dec 2024

Dear Editor in Chief,

All necessary changes were made correctly to enhance the transfer of information to others in the world. This article is of high value and will definitely have high citations. Considering the presence of oleuropein in olives, it is necessary to refer to Surah Noor of the Quran, which mentions figs and olives. It refers to Imam Hassan (AS) and Abu Abdullah Al-Hussein (AS), who are the lords of the youth of Paradise. So the Quranic emphasis on it has already been mentioned.