Reviewer #2

Comments and Suggestions for Authors

Dear Editor and Authors,

The manuscript 'Protective Effects of Velvet Antler Methanol Extracts on Hypoxia-Induced Damage in Caenorhabditis elegans through HIF-1 and ECH-8 Mediated Lipid Accumulation' by Ru Li 1#, Hongyuan Li 2#, Xiaohui Wang 2,3* and Yinghua Peng 1 is a research manuscript on velvet antler extract protective effect on C. elegans after low oxygen stress. The Authors concluded that velvet antler extract (MEs) enhance the survival of C. elegans under hypoxic conditions through the stabilization of hypoxia-inducible factor-1 (HIF-1) and the promotion of lipid accumulation, both of which are crucial for mitigating cellular damage. 30 references were collected on the topic. The Figures are of good quality.

Reply: We thank the reviewer very much for the positive comments and thank you for taking the time to review our manuscript and for your valuable comments.

1. How and when was the methanol extract of velvet antler added to C. elegans- it should be explained?

Reply: We appreciate the reviewer's suggestion. The methanol extracts of velvet antler (MEs) was added to the C. elegans culture at the L1 larval stage. Specifically, the extract was incorporated into the nematode growth medium (NGM) plates at a final concentration of 0.1, 0.4, and 1 mg/mL. The C. elegans were exposed to the MEs from the L1 stage until they reached young adulthood to ensure continuous exposure and to assess the effects throughout their development. This information has been added to the Materials and Methods section for clarity in the updated manuscript. The detail is as follows:

"*MEs was added to the C. elegans culture at the L1 larval stage. Specifically, the extract was incorporated into the nematode growth medium (NGM) plates at a final concentration of 0.1, 0.4, and 1 mg/mL. The C. elegans were exposed to the ME from the L1 stage until they reached young adulthood to ensure continuous exposure and to assess the effects throughout their development.*"

2. How was the MEs prepared? What was its origin? Was the methanol removed and how?

Reply: We appreciate the feedback from the reviewer. MEs were prepared and chemical composition analyses as described in the previous study (2020, Biomed Pharmacother, 121, 109668), specifically as follows: antler velvet from sika deer (*Cervus Nippon*) was provided by the Zuojia Sika Deer Farm (Jilin, China). It was sectioned at a thickness of 2-3 mm, and then lyophilized, homogenized, and stored at -80°C until extraction. Preparation of deer antler velvet water extracts (WEs): Antler velvet powder was mixed with ultrapure water at the proportion of 1:10, and ultrasonicated for 20 min in an ice-bath. The supernatant was obtained after centrifugation at 8000 g for 15 min and then frozen dry by lyophilizer. The yield of WEs was 19.4% (w/w) of the dried sample. Preparation of deer antler velvet methanol extracts (MEs): 7 g of antler velvet powder was mixed with 210 mL methanol and was refluxed at 80°C for 1 h. The supernatant

was obtained after centrifugation at 8000 g for 15 min. The methanol solvent was removed under vacuum with a rotary evaporator. The yield of MEs was 3.2% (w/w) of the dried sample.

3. What was the composition of MEs?

Reply: We appreciate the feedback from the reviewer. The identifications of chemical compounds in the extracts MEs was carried out by using nuclear magnetic resonance (NMR) on a Bruker AV-300 spectrometer (300 MHz 1H, 75 MHz 13C) in the previous study (2020, Biomed Pharmacother, 121, 109668), which primarily contain terpenoids, phenols, steroids, lipids, and glycosides.

4. Discussion

More discussion with literature is necessary. In all the discussion only 3 references are cited and only in one sentence about the velvet antler properties and usage in East Asian medicinal practices. Please discuss your results.

Reply: We are grateful for the constructive feedback from the reviewer. In the revised manuscript, we have included additional references to discuss our results in the context of existing research. Specifically, we have integrated studies that highlight the role of HIF-1 as a lipid-binding protein essential for lipid reserves under hypoxic conditions, as previously reported. Additionally, we discuss how HIF-1 modulates hypoxia-induced lipolysis in *C. elegans* by inhibiting PKA and ATGL proteins, underscoring its central role in nematode lipid metabolism during oxygen deprivation. The detail is as follows:

(1) "*In prior research, HIF-1 has been identified as a lipid-binding protein whose functionality critically relies on lipid reserves under hypoxic conditions (Cell Rep. 2014, 9, 349-365.). Furthermore, HIF-1 modulates hypoxia-induced lipolysis in C. elegans by inhibiting PKA and ATGL proteins, emphasizing its central role in nematode lipid metabolism during oxygen deprivation (Mol Cell Biol. 2019, 39, e00390-18). In our study, RNAi-mediated suppression of HIF-1 during hypoxia markedly decreased lipid content, accompanied by a corresponding reduction in in vivo ATP metabolism.*"

(2) "*Lipid accumulation appears to be a critical adaptive response to hypoxia facilitated by MEs. This process appears to be regulated through sophisticated modulation of lipid storage, particularly via the action of the enzyme ECH-8. Lipid metabolism significantly influences the body's nutritional balance during processes such as fasting and hypoxia. In C. elegans, lipid storage metabolism critically impacts survival (Front Cell Dev Biol. 2023, 11, 1104725; Nat Commun. 2020, 11, 4627). Disruption of the lipid oxidation pathway was observed to affect lipid droplet formation, which is crucial for lipid storage and membrane fluidity.*"