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Research article

Novel antioxidant additive ENTAN molecule for animal production: Evaluation at the cellular level ☆

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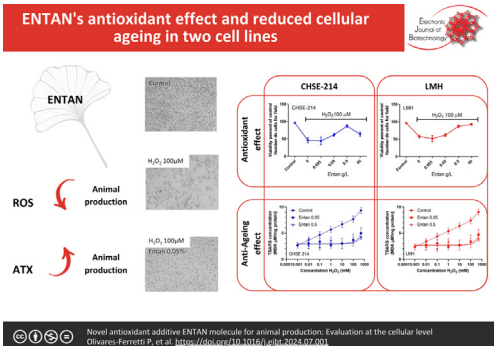
GRAPHICAL ABSTRACT



ENTAN's antioxidant effect and reduced cellular ageing in two cell lines.

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ABSTRACT

Background: Using cell lines to explore the function of organic compounds is fundamental in biotechnology. Evaluating new additives intended to improve animal production is challenging due to the complexity and uncertainty of in vivo testing. This study investigated the action of a compound with antioxidant properties using cells from terrestrial (LMH cells line) and aquatic vertebrates (CHSE-214).
Results: The results of our study provide reassuring evidence of the compound's safety for use in animal production. The compound demonstrated no adverse effects on cell viability, indicating its potential for safe application. Furthermore, the compound's antioxidant properties were evident, with a 100% recovery in both cell lines when exposed to hydrogen peroxide 0.1 mM. It also effectively reduced cellular ageing caused by metabolic processes, as measured by the TBARS formation in both cell lines, from 5 MDA μM/mg protein to 2.5 MDA μM/mg protein when used at 0.05 or 0.5 g/L. Notably, this action did not increase cell membrane oxidation, further supporting its safety profile.
Conclusions: These findings indicate that the compound has an antioxidant effect and can be used independently or in combination with metabolic stimulants in the diets of production animals. Applying this additive and its possible synergy with other compounds could help reduce oxidative stress and improve growth in animal production. The data generated in this study provide a solid basis for designing diets

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incorporating this additive to observe improvements in animal production based on activity observed at the cellular level.

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1. Introduction

Cellular models, as screening systems for innovative products, are widely used to evaluate novel molecules, vaccines, and cellular mechanisms [1,2]. Several models are available to study various cellular mechanisms, such as using murine cells to investigate ethanol's effects and studying Alzheimer's disease, among others [3]. The use of cell lines as an indicator of productive effectiveness in industry continues to be innovative. For example, studies carried out with salmonid cells have directly led to precision adjustments of additives in diets by indicating that including compounds such as nucleotides improves development under fasting conditions in production plants [4]. In another study, synergy was observed between two different molecules used as antioxidants in diets, leading to further optimisation of their dosages in salmon diets [5]. Without these advances, diet development would consume more resources and have a less significant impact on production. In addition, these models provide us with a detailed understanding of oxidation–reduction processes at the cell membrane level and of cellular responses. These processes lead to alterations in the membrane that result in ageing [6,7] and lead to the generation of biochemically quantifiable metabolites, such as thiobarbituric acid reactive substances (TBARSs), in membranes [6]. These molecules in the membrane can used as an ageing indicator preliminarily and can combine with cell viability to understand the effects of the oxidate stress in the cells [7,8].

Antioxidant agents help restore the balance of reactive oxygen species (ROS) [9,10]. These molecules are produced as part of the normal metabolism of cells and fulfil a physiological function by acting as signals of cellular catabolism [11]. However, under conditions of excess nutrients or rerouting to alternative biochemical pathways, more ROS are generated, overwhelming the protection mechanisms and giving rise to a toxic environment for both cells and organisms [12,13]. This situation occurs frequently in intensive farming; however, introducing additives or molecules to reduce the concentration of free radicals and improve the cellular response [14,15] has increased animal growth and health [16]. As in the previous report for our group, we used similar models to explore the cell effects of additives [17]. In this work, we can show evidence that solergy has an effect on the metabolic function of the hepatic cells, and improves the performance of the cells in stress conditions [17].

A growth stimulation model requires energy [18], but this stimulation also generates metabolic residues, including free radicals, which, in turn, cause oxidative stress. Molecules that promote growth are metabolically active and generate ROS [19,20]. The adverse effects of energy-rich diets have been described in fish, including resistance to lipid transport, inflammation, and pathologies such as cataracts [21,22].

The objective of this study was to demonstrate that the compound ENTAN acts as an additive with antioxidant properties. To this end, a series of experiments was carried out to evaluate its effectiveness in reducing the concentration of oxidants *in vitro* and its ability to protect cells in culture and use in the future for animal production diet.

2. Methodology

2.1. ENTAN molecule

The molecule of ENTAN was provided by Igusol to our lab to test the different effects, and the ENTAN is described as a Natural product based on grape extracts, rich in a mixture of various polyphenols, including a high concentration of flavonols, anthocyanins and proanthocyanidins, making a natural plant bases antioxidants activity. The molecules were used in the concentration previously described for formulated animal production diets, diluted in the cultured media at concentrations of g/L described in curve concentration form 0.01 g/L to 1 g/L, at some experiments at 0.05 g/L or 0.5 g/L relative to a used in diet to animals.

2.2. Cell culture and cytotoxicity analysis

This study used two cell lines: CHSE-214 cells derived from *Oncorhynchus tshawytscha* embryos and LMH cells derived from *Gallus gallus* hepatocytes. Cell culture media and handling procedures were adapted from those described by Olivares et al. [4]. They will be cultured in DMEM/F12 + GlutaMAx (Gibco), supplemented with 10% foetal bovine serum (Gibco) and antibiotic–antimycotic solution (Corning). The cell cultures were subjected to controlled exposure to the additive as a function of time and concentration. A modified approach based on previous research was used to evaluate any toxic effects and establish safety thresholds for the additive [4].

2.3. Pharmacological tests to evaluate antioxidant power

Two strategies were implemented to evaluate the additive's antioxidant power, one was based on similar pharmacological approaches previously used with compounds such as silymarin [5,23]. In the first strategy, hydrogen peroxide (H_2O_2) was applied directly to cell cultures to induce controlled oxidative stress for 24 h. An H_2O_2 concentration curve (0.1 μM to 1000 μM) was developed and compared to a mean concentration of the compound, which had previously been validated in the safety test. In the second strategy, a constant concentration of H_2O_2 was maintained (100 μM), and the concentration of the compound was increased. In this way, it was possible to determine the concentration at which the additive effectively protected cells against oxidative damage.

2.4. Assessment of cellular ageing

The analysis of cellular ageing was carried out using the Abcam TBAR kit. Two hundred microliters of thiobarbituric acid (TBA) reagent were added to each standard and sample. The absorbance was measured at 535 nm (in a range of 525 to 545 nm); the intensity of the colour was proportional to the concentration of oxidised lipids. This measurement indicated the degree of cellular oxidation. The same cytotoxicity curves from the other stages of the study were used to correlate mortality with cellular ageing. This is an approach for measured cell ageing *in vitro* [24].

2.5. Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were carried out using Student's *t* test or analysis of variance (ANOVA). A probability value (p) less than 0.05 was considered a significant difference.

3. Results

3.1. Evaluation of toxicity and recommended doses

A safety curve was generated to verify the activity and recommended dose for the use of the additive. CHSE-214 cells from trout embryos and LMH cells from rooster hepatocytes were exposed to increasing concentrations of ENTAN for 24 h. Subsequently, a cell count was performed to determine the number of cells per area, and cell viability was calculated. As seen in Fig. 1A, the LD50 was 10 g/L, which indicates that the recommended doses were below the lethality threshold. Doses significantly higher than 50 g/L resulted in a decrease in viability, suggesting that the compound possesses biological activity (Fig. 1A). We investigated whether ENTAN affected cell ageing by measuring the change in TBARS levels in CHSE-214 and LMH cells exposed to increasing concentrations of the compound (Fig. 1B). Under these conditions, an increase was observed only at very high doses, an effect that may be related to the increase in cell mortality observed at these concentrations in both cell models.

3.2. Antioxidant effect

Because a baseline effect of ENTAN was identified, we evaluated whether the additive protected at the cellular level. For this, cells were exposed to a toxic concentration of H_2O_2, and increasing concentrations of ENTAN were added. The curves in Fig. 2A and Fig. 2B indicate that the viability of cells improved when the cells were incubated with ENTAN, indicating that the additive protected against damage caused by oxidation in trout (Fig. 2A) and rooster (Fig. 2B) cells. Recognising that H_2O_2 is an agent that induces cell death and ageing, we evaluated whether ENTAN reduces these effects to provide information on its mechanism of action. Fig. 2C and Fig. 2D show the change in TBARS concentration in the cell membranes of both models and how ENTAN protects trout cells (Fig. 2C) and rooster hepatocytes (Fig. 2D).

3.3. Antioxidant power of ENTAN

ENTAN protected the two cell types studied from oxidative attack. However, it is unknown whether ENTAN blocks the effect of oxidation in production models. To address this issue, cell cultures were exposed to increasing concentrations of H_2O_2, and the mean lethal dose (LD50) of peroxide was determined, both in the presence and in the absence of two concentrations of ENTAN (Fig. 3). Fig. 3A shows the change in LD50 in trout embryonic cells, and in Fig. 3B, a similar effect is observed in LMH cells, with the effects in both cell types being concentration dependent. In Fig. 3C, curves for ENTAN and H_2O_2 were used to evaluate the mean value of the effect in salmonid cells. In addition, in the photomicrographs in Fig. 3D, changes in the density of the cultures can be observed in the experimental conditions evaluated. These data indicate that ENTAN can minimise the increase in the concentration of free radicals in cell models, thus protecting cells, and suggest that these results can be extended to the level of animal production.

3.4. TBARS values

The decrease in mortality observed due to oxidative stress in the cell lines suggests a cellular protection mechanism based on the antioxidant effect of ENTAN. To evaluate this trend, a TBARS concentration curve, because other cell studies used this model for ageing in cells [24,25], was constructed a TBARS curve, when cells were exposed to an increasing concentration of H_2O_2, both in the presence and absence of ENTAN at two different concentrations, as shown in Fig. 4A and Fig. 4B. Both concentrations reduced membrane oxidation, which resulted in a lower rate of cellular ageing and an increase in the viability of the cultures, even under conditions of metabolic stress. These findings indicate that ENTAN reduces oxidative stress following a dose–effect relationship, which resulted in less lipid oxidation in both cell models and improved the quality of the membranes, allowing for an increase in culture viability, even under conditions of metabolic stress, such as those found in animal production models.

4. Discussion

The data obtained suggest that ENTAN has antioxidant and protective properties at the cellular level, with possible applica-

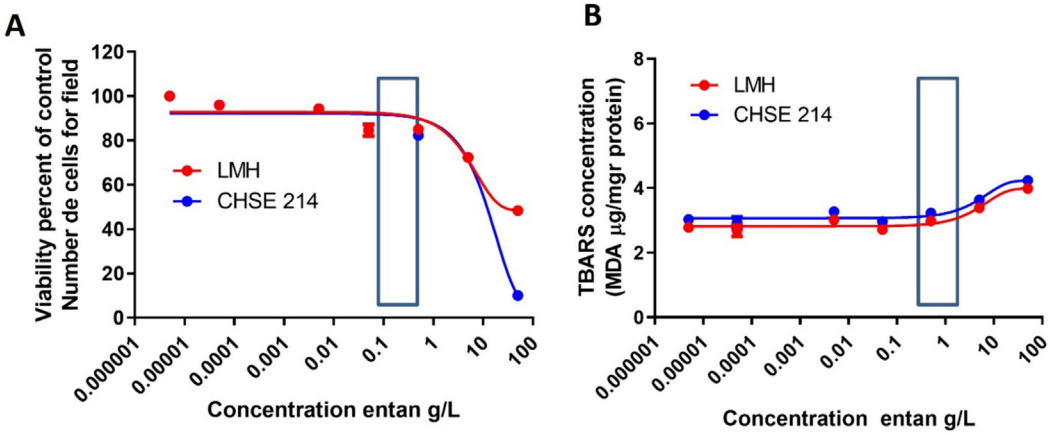

Fig. 1. Safety curve. Curves for CHS-E214 cells (trout embryo) and LMH cells (rooster hepatocytes). In (A), a curve for increasing concentrations of ENTAN from 0 to 50 g/L suggests an LD50 of 15 g/L, which would be 3 times higher than the recommended dose for ENTAN (0.5 g/L). In (B), a curve shows increasing ENTAN concentrations from 0 to 50 g/L and the concentration of TBARS in both cell lines. The curves represent 3 independent experiments, each with 3 replications, and the points represent the mean ± SEM.

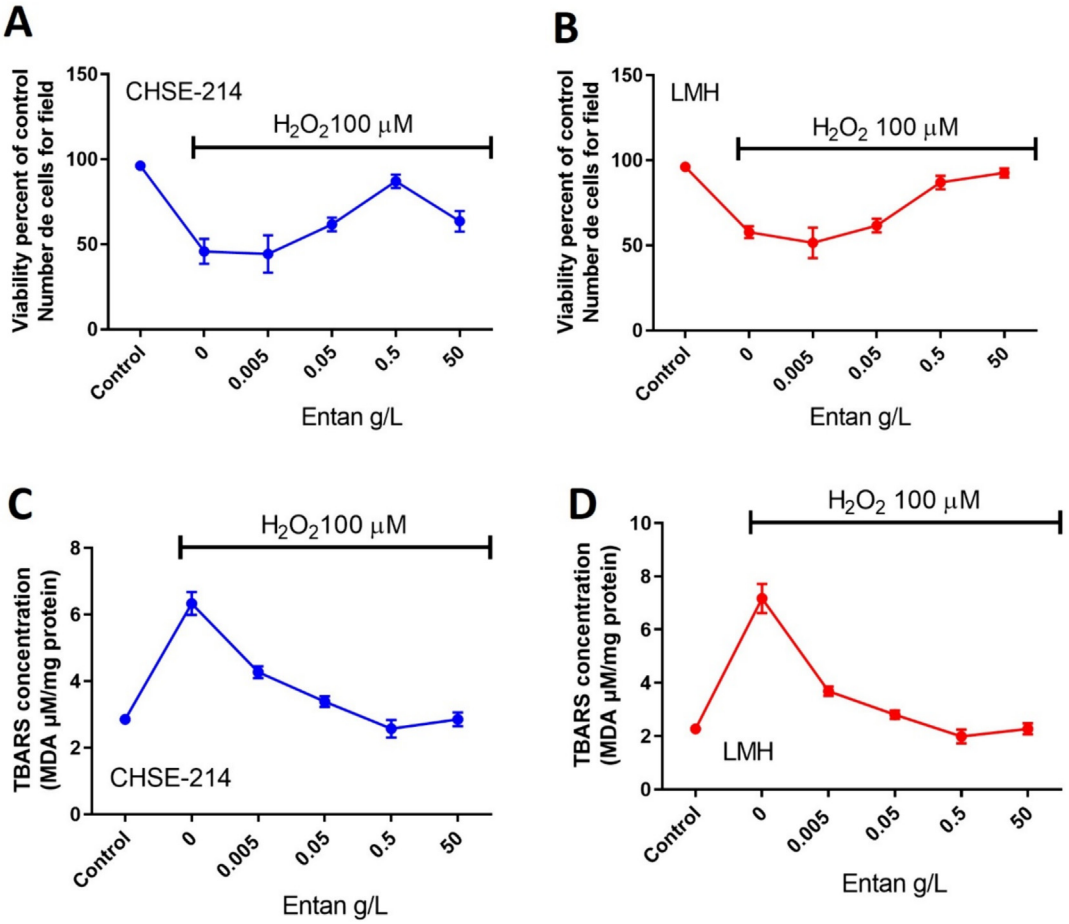

Fig. 2. Antioxidant effect. In (A), the number of CHSE-214 cells was quantified after 24 h of culture in 0.1 mM H_2O_2 in the presence of ENTAN from 0 to 50 g/L. (B), quantification of the number of LMH cells after 24 h of culture in 0.1 mM H_2O_2 in the presence of ENTAN from 0 to 50 g/L. (C) quantification of TBARS in CHSE-214 cells after 24 h of culture in 0.1 mM H_2O_2 in the presence of ENTAN 0 to 50 g/L. (D) quantification of TBARS in LMH cells after 24 h of culture in 0.1 mM H_2O_2 in the presence of ENTAN from 0 to 50 g/L. The graphs are representations of 3 independent experiments, with three replicates each. Each point represents the mean ± SEM.

tions in animal production [1]. In previous work, we used cell models to explore the metabolic y oxidant status; we used solely another compound for metabolic activation and presented [17], but in another report, the cell models are used to explore additives effect in salmon cells, for example [5] or test the toxic effect of invasive algae [25], the results from solergy are useful for diet design. Now, we explored other additives for use in the diet. The safety curves presented in Fig. 1 indicate that the recommended doses are safe for animals at the cellular level. This finding is significant because it indicates that ENTAN is a compound devoid of adverse effects at low doses and only shows biological activity at high concentrations, which supports its biologically active capacity.

ENTAN is effective in reducing the damage caused by oxidative stress at recommended doses. This is evidenced in Fig. 2, where a significant decrease in cell mortality induced by hydrogen peroxide is observed in the presence of ENTAN. These results suggest that the additive can reduce cell mortality by reducing cell oxidation. This observation is further reinforced in Fig. 3, showing that the concentration of TBARS in cell membranes, a marker of lipid oxidation, increases with increasing concentrations of hydrogen peroxide. However, this increase in the marker concentration was not observed in the presence of ENTAN. These findings allow us to conclude that ENTAN is an effective antioxidant that prevents cell oxidation in two cell lines, which translates into improving cell viability [16].

Taken together, the results from our experiments suggest that the additive ENTAN is of great relevance to improving animal production processes. In addition, this raises the possibility that it can act synergistically with other compounds present in the diets, as has been observed in previous studies exploring other additives [5]. The results of our study allow for an understanding of the action of this molecule in a short period and have provided information on its expected mechanism of action in diets. In addition, we have explained some of the mechanisms at the production level that have already been described for similar molecules [18,19]. The results of this study not only offer new perspectives for the animal production industry but also highlight the value of *in vitro* models in research. While it is important to recognise that *in vitro* models cannot address all the unknowns related to cellular processes within animal production, we should not underestimate their value. These models, such as those used in our research, can significantly reduce the need for more extensive and expensive *in vivo* testing. Our research represents an important step in evaluating the beneficial properties of ENTAN in the context of animal production. The compound ENTAN, identified as an effective antioxidant agent, has significant potential to improve the health and growth of animals in intensive production environments. As demonstrated in this study, its ability to reduce oxidative damage at the cellular level suggests that it could contribute to the optimisation of animal diets, mitigating the adverse effects of metabolic stress and promoting healthier growth. In addition, the demonstrated safety at

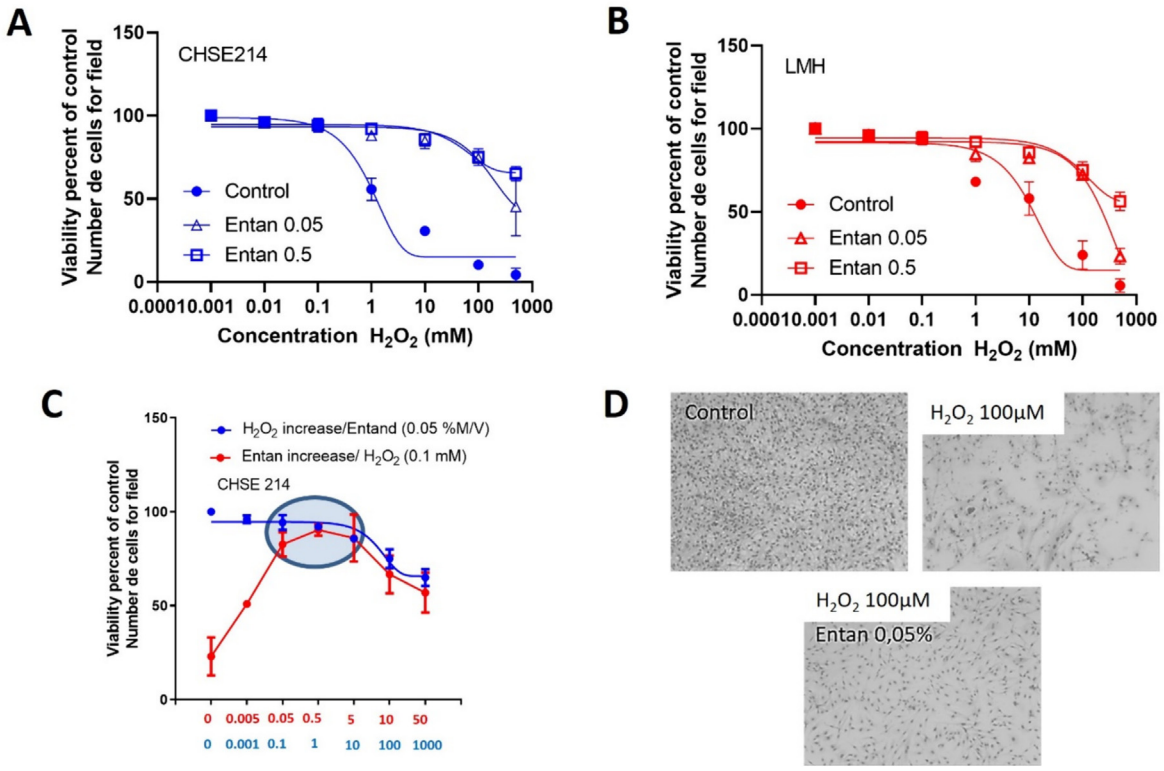

Fig. 3. Curve of concentration values. Curves for CHSE-214 cells (trout embryo) and LMH cells (rooster hepatocytes). In (A), a curve for increasing concentrations of hydrogen peroxide for both cell lines in the presence or absence of ENTAN at concentrations of 0.05 and 0.5 g/L. (B) quantification of the number of LMH cells after 24 h of culture in H_2O_2 at increasing concentrations in the presence of ENTAN at 0.05 and 0.5 g/L. In (C), curves for ENTAN against peroxide and peroxide against ENTAN. In (D), the image illustrates ENTAN and antioxidant data in CHSE2-214 cells. The curves represent 3 independent experiments, each with 3 replicates, and the points represent the mean ± SEM.

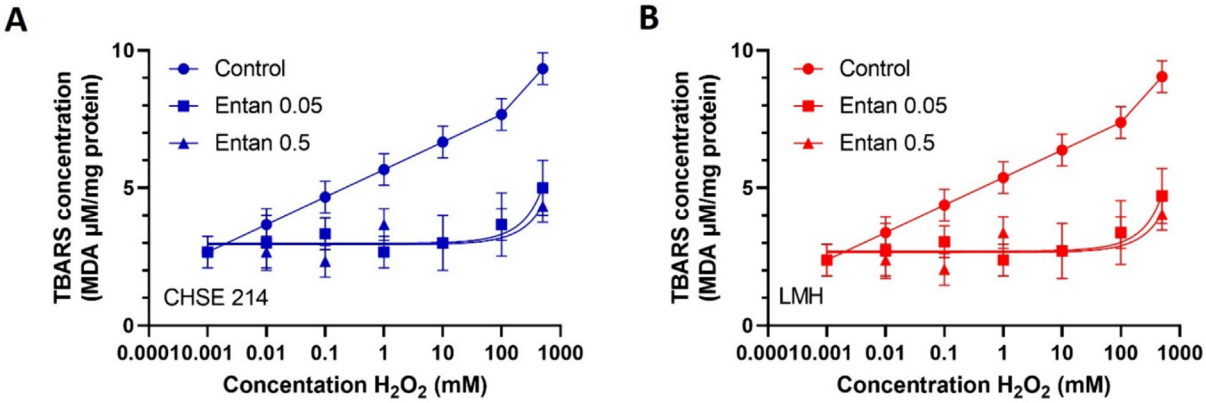

Fig. 4. ENTAN effects on TBARS. In (A), a curve for the TBARS concentration in CHSE-214 cells for increasing peroxide concentrations in the presence or absence of ENTAN at concentrations of 0.05 and 0.5 g/L. (B) a curve for the TBARS concentration in LMH cells for increasing peroxide concentrations in the presence or absence of ENTAN at concentrations of 0.05 and 0.5 g/L. The curves represent 3 independent experiments, each with 3 replications, and the points represent the mean ± SEM.

recommended doses is an important characteristic, indicating that using ENTAN does not present significant risks to cells.

Conflict of interest

I Jorge Parodi Rivera, the author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

CRediT authorship contribution statement

Pamela Olivares-Ferretti: Writing – review & editing, Methodology. **Ekaitz Maguregui:** Methodology, Funding acquisition. **Viviana Chavez:** Resources, Project administration, Funding acquisition. **Jorge Parodi:** Writing – review & editing, Writing – original draft, Conceptualization.

Supplementary material

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Data availability

Data will be made available on request.

References

[1] Chen S, Li Z, Zhang S, et al. Emerging biotechnology applications in natural product and synthetic pharmaceutical analyses. Acta Pharmaceutica Sinica B 2022;12(11):4075–97. https://doi.org/10.1016/j.apsb.2022.08.025. PMid: 36386468.

[2] Bergin A, Carvell J, Butler M. Applications of bio-capacitance to cell culture manufacturing. Biotechnol Adv 2022;61:. https://doi.org/10.1016/j.biotechadv.2022.108048. PMid: 36208846108048.

[3] Parodi J. Something old, something new and something used in Alzheimer's; the idea of pore, ethanol and the use of oocytes to understand the disease. Curr Chem Biol 2019;13(2):105–9. https://doi.org/10.2174/2212796812666180710125905.

[4] Olivares P, Sanchez R, Carmona E, et al. Nucleotides and effect over starving condition on fish SHK-1 cells model. J Aquac Res Dev 2016;7(9):448. https://doi.org/10.4172/2155-9546.1000448.

[5] Olivares-Ferretti P, Sanchez R, Ochoa de la Paz L, et al. A synergy of the nutritional additives taurine and silymarin in salmon farming: Evaluation with the CHSE-214 cellular model. Fish Physiol Biochem 2020;46:945–52. https://doi.org/10.1007/s10695-020-00760-5. PMid: 32350676.

[6] Kamimura N, Wolf AM, Yokota T, et al. Transgenic type2 diabetes mouse models for in vivo redox measurement of hepatic mitochondrial oxidative stress. Biochimica et Biophysica Acta (BBA) - General Subjects 2023;1867(3):. https://doi.org/10.1016/j.bbagen.2022.130302. PMid: 36577487130302.

[7] Alyethodi RR, Sirohi AS, Karthik S, et al. Role of seminal MDA, ROS, and antioxidants in cryopreservation and their kinetics under the influence of ejaculatory abstinence in bovine semen. Cryobiology 2021;98:187–93. https://doi.org/10.1016/j.cryobiol.2020.11.002. PMid: 33476643.

[8] Tsai MC, Huang LT. Thiobarbituric acid reactive substances (TBARS) is a state biomarker of oxidative stress in bipolar patients in a manic phase. J Affect Disord 2015;173:22–6. https://doi.org/10.1016/j.jad.2014.10.045. PMid: 25462391.

[9] Kasianchuk N, Rzymski P, Kaczmarek Ł. The biomedical potential of tardigrade proteins: A review. Biomed Pharmacother 2023;158:. https://doi.org/10.1016/j.biopha.2022.114063. PMid: 36495665114063.

[10] Dai M, Wang L, Yang J, et al. LDHA as a regulator of T cell fate and its mechanisms in disease. Biomed Pharmacother 2023;158:. https://doi.org/10.1016/j.biopha.2022.114164. PMid: 36916398114164.

[11] Wang G, Yang F, Zhou W, et al. The initiation of oxidative stress and therapeutic strategies in wound healing. Biomed Pharmacother 2023;157:. https://doi.org/10.1016/j.biopha.2022.114004. PMid: 36375308114004.

[12] Ray A, Maharana KC, Meenakshi S, et al. Endothelial dysfunction and its relation in different disorders: Recent update. Health Sci Rev 2023;7:. https://doi.org/10.1016/j.hsr.2023.100084100084.

[13] Hou J, Yuan Y, Chen P, et al. Pathological roles of oxidative stress in cardiac microvascular injury. Curr Probl Cardiol 2023;48(1):. https://doi.org/10.1016/j.cpcardiol.2022.101399. PMid: 36103941101399.

[14] Moini Jazani A, Karimi A, Nasimi Doost Azgomi A. The potential role of saffron (*Crocus sativus* L.) and its components in oxidative stress in diabetes mellitus: A systematic review. Clin Nutr ESPEN 2022;48:148–57. https://doi.org/10.1016/j.clnesp.2022.01.022. PMid: 35331485.

[15] Martins MPSC, Oliveira ASSS, Martins MCC, et al. Effects of zinc supplementation on glycemic control and oxidative stress in experimental diabetes: A systematic review. Clin Nutr ESPEN 2022;51:28–36. https://doi.org/10.1016/j.clnesp.2022.08.003. PMid: 36184216.

[16] Sharma V, Mehdi MM. Oxidative stress, inflammation and hormesis: The role of dietary and lifestyle modifications on aging. Neurochem Int 2023;164:. https://doi.org/10.1016/j.neuint.2023.105490. PMid: 36702401105490.

[17] Olivares-Ferretti P, Chavez V, Maguregui E, et al. Exploring the metabolic and antioxidant potential of solergy: Implications for enhanced animal production. Biotechnol Rep 2024;41:. https://doi.org/10.1016/j.btre.2023.e00821. PMid: 38173966e00821.

[18] Aissia E, Blier PU, Fadhlaoui M, et al. Thermal modulation of mitochondrial function is affected by environmental nickel in rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 2023;257:. https://doi.org/10.1016/j.aquatox.2023.106451. PMid: 36868082106451.

[19] Sheik Abdul N, Marnewick JL. Green rooibos extract attenuates high glucose induced oxidative stress in a human derived (HepG2) liver cell line. S Afr J Botany 2022;151(part A):852–65. https://doi.org/10.1016/j.sajb.2022.11.002.

[20] Das D, Kabir ME, Sarkar S, et al. Antidiabetic potential of soy protein/peptide: A therapeutic insight. Int J Biol Macromol 2022;194:276–88. https://doi.org/10.1016/j.ijbiomac.2021.11.131. PMid: 34848240.

[21] Sambraus F, Fjelldal PG, Remo SC, et al. Water temperature and dietary histidine affect cataract formation in Atlantic salmon (*Salmo salar* L.) diploid and triploid yearling smolt. J Fish Dis 2017;40(9):1195–212. https://doi.org/10.1111/jfd.12594. PMid: 28188652.

[22] Hemre GI, Lock EJ, Olsvik PA, et al. Atlantic salmon (*Salmo salar*) require increased dietary levels of B-vitamins when fed diets with high inclusion of plant based ingredients. PeerJ 2016;4:e2493. https://doi.org/10.7717/peerj.2493. PMid: 27703849.

[23] Sanchez R, Olivares P, Carmona E, et al. Fish nutrition additives in SHK-1 Cells: Protective effects of silymarin. Adv Biosci Biotechnol 2016;7(2):55–62. https://doi.org/10.4236/abb.2016.72007.

[24] Remigante A, Spinelli S, Basile N, et al. Oxidation stress as a mechanism of aging in human erythrocytes: Protective effect of Quercetin. Int J Mol Sci 2022;23(14):7781. https://doi.org/10.3390/ijms23147781. PMid: 35887126.

[25] Olivares-Ferretti P, Hernandez K, Peredo-Parada M, et al. Polyphenols obtained from *Didymosphenia geminata* (Lyngbye) Schmith altered the viability and proliferation of salmonids cells lines SHK-1 and CHSE-214. Aquat Toxicol 2019;211:141–7. https://doi.org/10.1016/j.aquatox.2019.03.022.