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Effects of elderflower extract enriched with polyphenols on antioxidant defense of salmon leukocytes

Paula Andrea Santana [a,1], Carlos Jara-Gutiérrez [b,1], Marco Mellado [c], Juan Carlos Forero [d], Fanny Guzmán [d], Andrés Barriga [e], Fernando Albericio [f,g], Claudio Andrés Álvarez [h,i,*]

[a] Instituto de Ciencias Químicas Aplicadas, Facultad de Ingeniería, Universidad Autónoma de Chile, Santiago, Chile
[b] Centro de Investigaciones Biomédicas, Escuela de Kinesiología, Facultad de Medicina, Universidad de Valparaíso, Valparaíso, Chile
[c] Instituto de Química, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[d] Núcleo Biotecnología Curauma, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[e] Unidad de Espectrometría de Masas, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile
[f] Department of Organic Chemistry and CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, University of Barcelona, Barcelona, Spain
[g] School of Chemistry, University of KwaZulu-Natal, Durban, South Africa
[h] Laboratorio de Fisiología y Genética Marina, Centro de Estudios Avanzados de Zonas Áridas, Coquimbo, Chile
[i] Facultad de Ciencias del Mar, Universidad Católica Del Norte, Coquimbo, Chile

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ABSTRACT

Background: In fish farming, the plant extracts containing antioxidant compounds have been added to the diet for enhancing pathogen resistance. *In vitro* studies evaluating the antioxidant effect of herbal extracts on fish cell models have focused on ROS production and the respiratory burst mechanism. However, the effects on enzymatic antioxidant defense on salmon leukocytes have not been evaluated. This study aims to evaluate the enzymatic antioxidant defense and ROS-induced cell damage in Salmon Head Kidney-1 (SHK-1) cell line exposed to polyphenol-enriched extract from *Sambucus nigra* flowers.

Results: Firstly, the Total Reactive Antioxidant Power (TRAP) assay of elderflower polyphenol (EP) was evaluated, showing 459 and 489 times more active than gallic acid and butyl hydroxy toluene (BHT), respectively. The toxic effect of EP on salmon cells was not significant at concentrations below 120 μg/mL and no hemolysis activity was observed between 20 and 400 μg/mL. The treatment of SHK-1 cell line with EP decreased both the lipid peroxidation and protein oxidation induced by H_2O_2, which could be associated with decreasing oxidative stress in the SHK-1 cells since the GSH/GSSG ratio increased when only EP was added.

Conclusions: These results suggest that plant extracts enriched with polyphenols could improve the enzymatic antioxidant defense of salmon leukocytes and protect the cells against ROS-induced cell damage.

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1. Introduction

Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are the most important commercial fish species of world aquaculture, with Norway and Chile the largest producers [1]. During cultivation, high mortalities occur due to the presence of pathogens mainly viruses and bacteria [2]. Therefore, the salmon industry has chosen to use antibiotics against these microorganisms, although bacterial strains may be resistant [3]. Medicinal plants are a plentiful source to identify new antimicrobial agents whose phenolic compounds may directly eliminate pathogens or strengthen the immune system for preventing aquatic diseases in fish farming [2,4,5,6]. Current reviews have detailed the various herbal extracts that are directly applied to the fish diet to explore their biological properties, as alternative treatments for aquaculture pathogens [7,8,9]. Tilapia fed with pellets containing different amounts of two Chinese medicinal herbal extracts (*Astragalus membranaceus* and *Lonicera japonica*) significantly enhanced the

* Corresponding author.
E-mail address: claudio.alvarez@ceaza.cl (C.A. Álvarez).
[1] These authors contributed equally to this work.

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phagocytic activity [2]. However, the aquaculture feed supplementation industry has not followed the typical scheme of medicinal plant drug discovery and development, which require cell-based bioassays as the first step [10]. Thus, research on the safety and action mechanisms of herbs needs to be more thoroughly pursued.

One medicinal plant with interesting properties is the elder tree (*Sambucus nigra)* [11]. Several studies have focused on the beneficial effects of *S. nigra*, mainly examining the berries, although flowers also have shown to be a rich source of bioactive flavonoids and phenolic acids [11]. Due to their high antioxidant activity, *S. nigra* extracts have been used in traditional medicine and healing [12,13], but the benefits of this extract are still under research to expand their field of application. In this regard, our research group identified antimicrobial peptides in *Sambucus nigra* flowers and proved their antibacterial activity against Gram-negative bacterial strains, including recurrent pathogens of Chilean salmon aquaculture [14]. Hence, *S. nigra* flower extracts can be explored for cell-based *in vitro* bioassay in an appropriate model of fish cells.

Oxidative stress is a well-known cause of persistent chronic inflammation in mammals. This condition induces cell damage because progressive regulation of the inflammatory response is lost, as a result of altered intracellular signaling in leukocytes [15]. Therefore, the antioxidant defense system is key to modulate the pro-inflammatory response. The evidence about the effects of polyphenols on immune function is abundant; however, the mechanisms involved are not fully understood, due to their dependence on the type of polyphenol. A recent study in grass carp revealed that the dietary supplementation of polyphenols positively affects the innate immune response and oxidative status. In that study, the activity of catalase and peroxidase significantly increased in carp fingerlings treated with polyphenol enriched diets [16]. Similarly, the findings of a separate study suggest polyphenol-administered fish generate lower levels of intestinal pro-inflammatory cytokines [17].

Leukocyte cell cultures have been widely used to evaluate the effects of both purified polyphenols and polyphenol plant extracts in mammals. The antioxidant effects on mammal leukocytes involve reactive oxygen species (ROS) scavenging, oxidative stress protection, and attenuation of membrane lipid peroxidation [18]. Jurkat T lymphocyte cells and mouse macrophage cell line RAW 264.7 are the most common cells reported. For example, polyphenolic compounds of black tea (*Camellia sinensis*) decreased DNA oxidative damage and affected the antioxidant activity of glutathione peroxidase (GPx) in Jurkat T cell line [19], while French red wine polyphenol extract induced apoptosis in Jurkat cells by a redox-sensitive mechanism associated with the intracellular formation of superoxide anions [20]. Studies on RAW 264.7 have shown the antioxidant properties of different fruits, such as dried plum (*Prunus domestica* L) and Korea berries (*Rubus coreanus*), and herbal polyphenol extracts from *R. chalepensis* L. and *Cornus officinalis*, mainly through reduction of malondialdehyde (MDA) production and/or by increasing the activities of antioxidative enzymes, including superoxide dismutase (SOD), catalase (CAT), and GPx [21,22,23,24,25]. Also, elderberry polyphenols and elderflower polyphenols were investigated for their effect on lipopolysaccharide (LPS)-activated RAW 264.7 macrophages, showing that phenolic compounds are strong inhibitors of ROS production [26]. Therefore, leukocyte cells are a sound model of study to understand the antioxidant effects of polyphenols in teleost immune cells.

Previous work supports the use of SHK-1 cell line as a good model of salmonid leukocyte response with demonstrated cytokine expression profile, phagocytosis, respiratory burst cell response, and oxidative stress [27,28,29]. Besides, fish nutrition additives have been tested in SHK-1 cells [30], and in ROS production after pathogen infection [31]. Based on this background information, the purpose of this study was to investigate both the enzymatic antioxidant defense and ROS-induced cell damage in the SHK-1 cell line by the treatment with a polyphenol-enriched extract obtained from flowers of *S. nigra.* This information should be valuable for the development of antioxidant feed additives for fish aquaculture.

2. Materials and methods

2.1. Plant materials and polyphenol extraction

Flowers of Elder tree (*S. nigra*) were collected in the south of Chile (forest near to Temuco city, coordinate 38°45′00″ S 72°40′00″ W) in September and October of 2017 (elder flowering). The plant material was processed as previously described [14]. 80 g of ground flowers was homogenized in a mixture methanol (MeOH)/dichloromethane (DCM)/). After that, the liquid extract was filtered and transferred to a separating funnel. Then, ultrapure water (UPW) was added, and the aqueous layer was concentrated on a rotary evaporator. Finally, the extract was freeze-dried and weighed.

For the polyphenol extraction, 10 mg of freeze-dried flower extract was suspended in 10 mL of UPW and loading in DPA-6S polyamide SPE pre-packed cartridges (Sigma-Aldrich, St. Louis, MO, USA). Different concentrations of methanol were used for the elution of the analytes (30, 50, and 80% MeOH in UPW). Then, the MeOH was evaporated on a Speedvac centrifugal device and samples were freeze-dried and weighed.

2.2. Estimation of phenolic content and identification of elderflower polyphenols

Total phenolic compounds were quantified using the Folin Ciocalteu method as previously described [32]. All measurements were done in triplicate.

The determination of phenolic compounds eluted at 80% MeOH was performed by LC–MS (Esquire 4000 ESI-IT, Bruker Daltonics). For HPLC separation, a RP-18 column of 5 μm and 250 × 4.6 mm was used (Kromasil 100-5C18, Eka Chemicals AB, Sweden). The analysis was performed at room temperature injecting 20 μL of MeOH (blank) and EP, using a gradient system composed of two phases: (A) 0.1% formic acid in UPW and (B) acetonitrile. The flow rate was 1.0 mL/min and the UV detection was carried out at 280 nm. Nitrogen was used as nebulizer gas at 365°C, a pressure of 60 psi, and a flow rate of 10 L/min. Identification of the compounds present in the EP was done by comparison with a local library and employing the MassBank and Respect for Phytochemicals mass spectral database. (Bruker Data Analysis 3.2 and Compounds-AutoMSn option).

2.3. Radical scavenging assays using DPPH

The DPPH (2,2′-diphenyl-1-picrylhydrazyl radical) assay was performed as described by Madrid et al [33]. The polyphenol extracts or control (absolute ethanol) were mixed with DPPH solutions and then incubated for 15 min at room temperature. After that, the absorbance was measured at 517 nm and the DPPH radical scavenging was calculated by Equation (1):

$$DPPH\ radical\ scavenging\% = \frac{(A_{control} - A_{Sample})}{A_{control}} \times 100 \quad (1)$$

In addition, the IC50 values of the elderflower polyphenol extracts were determined by linear regression analysis.

2.4. Hydrogen peroxide inhibition

The hydrogen peroxide (H_2O_2) scavenging activity was determined according to the method described by Madrid et al [33]. The percent of H_2O_2 inhibition was calculated as Equation (2):

$$H_2O_2\ inhibition\% = \frac{(A_{control} - A_{Sample})}{A_{control}} \times 100 \quad (2)$$

The IC_{50} values of elderflower polyphenol extracts were determined according to Madrid et al. [33].

2.5. Total reactive antioxidant power (TRAP) assay

10 μL of 1.0 mg/mL of the elderflower polyphenol extracts was mixed with 990 μL of TRAP solution according to the method described by Leyton et al. [34]. After 50 s, the absorbance was measured at 734 nm. 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) solution was used as the blank. The absorbance values were interpolated in a Trolox standard curve (0–120 mg/L). All measurements were done in triplicate.

2.6. SHK-1 cell culture and cytotoxicity

The SHK-1 *Salmo salar* leukocyte cell line (ECACC No. 97111106) was maintained at 18°C in Leibovitz L-15 medium as previously reported [27,28]. The effects of EP on SHK-1 cells were performed in a biosafety cabinet. Firstly, SHK-1 cells were seeded in 96-well plates (1×10^4 cells/well) and allowed to adhere overnight. Resazurin assay was performed as previously reported [35]. Briefly, elderflower polyphenol was added to varying concentrations (from 0 to 120 μg/mL) in a final volume of 200 μL. After 24 h, 20 μL of 0.01% resazurin (0.01%) (Sigma-Aldrich, Germany) was added and incubated for 4 h. The fluorescence of resazurin was measured using an Appliskan plate reader (Thermo Scientific, Waltham, MA, USA) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The experiments were performed three times independently with three parallel measurements each.

2.7. Hemolysis assay

Red blood cells (RBC) were obtained from fresh salmon and immediately processed. Then, 2 mL of this RBC was spun at 2000×*g* per 10 min at 4°C. The supernatant was aspirated and the RBC pellet was washed three times with PBS. Then, 5% v/v suspension of erythrocytes (~$6x10^8$ cel/mL), and EP (at 20, 40, 200 and 400 μg/mL final concentration) were diluted with PBS. Aliquots of 65 μL of salmon blood suspension and EP solution were incubated at 20°C for 1 h. After that, the samples were centrifuged at 3000 × g for 5 min. Finally, aliquots of 80 μL were added to the wells of 96-well cell culture and the absorbance was determined at 540 nm (VERSA max microplate reader.) 0.5% (v/v) Triton X-100 solution was used as a positive control (100% hemolysis). PBS was used as a negative control (0% hemolysis). The assays were performed in triplicate to determine the percentage of hemolysis at different concentrations of EP according to Equation (3):

$$\%Hemolysis = \frac{A_{540}nm\ of\ EP - A_{540}nm\ of\ PBS}{A_{540}nmTritonX100 - A_{540}nm\ of\ PBS} \times 100 \quad (3)$$

2.8. Cell culture and treatment with EP

5×10^5 SHK-1 cells/well were placed in 100 mm cell culture plates. After 24 h, the cells were ready for EP exposure. Five conditions were carried out: negative control (cell with L-15 supplemented with 10% fetal bovine serum), SHK-1 cell exposure to absolute ethanol, 1.2 μg/mL of EP, 1.0 μM H_2O_2, and 1.2 μg/mL of EP in the presence of 1.0 μM H_2O_2. After 24 h, the cells were washed three times with PBS 1X and detached with 0.25% trypsin/EDTA (HyClone) solution for 2 min. Cells were then collected in sterile 15 mL tubes and centrifuged at 300 g for 10 min. The pellet was resuspended in lysis buffer (0.022 M Na_2HPO_4, 0.088 M NaH_2PO_4) diluted 1:15 in Milli Q water and sonicated at 35 watts. All measurements were done in triplicate.

2.9. Lipid peroxidation

Malondialdehyde (MDA) was measured using the thiobarbituric acid reactive substances (TBARS) according to Pheomphun et al. [36] with some modifications. Firstly, 1.0 mL of SHK-1 cell lysate was treated with 30% (w/v) trichloroacetic acid (TCA) and centrifuged for 15 min at 2,000 *g*. After that, the supernatant was mixed with 0.67 % (w/v) thiobarbituric acid (TBA). The samples were boiled at 95°C for 20 min. Finally, the absorbance spectrum was recorded at wavelengths between 400 and 600 nm (Rayleigh, UV-2601 spectrophotometer). The concentration of the TBA-MDA adduct was determined by extrapolation from a MDA calibration curve.

2.10. Protein carbonyl content

Protein carbonyl content assay was performed according Levine et al. [37]. For this assay, 10 μL of SHK-1 cell lysate was used.

2.11. Total antioxidant capacity

Total antioxidant capacity was carried out in 10 μL of SHK-1 cell lysate as previously described by Madrid et al. [33]. The antioxidant capacity was expressed as trolox equivalent antioxidant capacity (TEAC).

2.12. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity assay was performed according to Beauchamp and Fridovich [38]. Briefly, 5.0 μL of SHK-1 cell lysate was mixed with a solution A (0.5 mM xanthine mixed with 20 μM cytochrome C dissolved in phosphate buffer) and a solution B (xanthine oxidase and 0.1 mM EDTA 1:40). SOD activity was recorded at 550 nm.

2.13. Glutathione (GSH) and oxidized glutathione (GSS) ratio

GSH/GSSG assay was adapted to *S. nigra* flower extracts and SHK-1 cell lysate as described in Montenegro et al. [39]. The rate of yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) formation was followed at 412 nm and was proportional to the sum of the GSH and GSSG present. The rate was compared with a standard curve of GSH in buffer.

2.14. Total protein content

Total proteins were determined by Folin-Ciocalteau reagent [40]. Bovine serum albumin (BSA) was used as protein standard. Samples were measured at 650 nm (Rayleigh, UV-2601).

2.15. Statistical analysis

The data were reported as the mean values ± standard deviation (SD) with GraphPad Prism 6.0 software. Due to non-parametric data, a Kruskal-Wallis ANOVA was used with a confidence level of 95% ($\alpha = 0.05$). To verify these results statistically, we employed the Wilcoxon-Mann-Whitney test.

3. Results

3.1. Quantification and identification of polyphenolic compounds of EP

The polyphenol content of fractions from polyamide column chromatography separation with 30%, 50%, and 80% MeOH/H_2O was measured, obtaining the highest amount of polyphenol content in the 80% MeOH eluted fraction (139.65 mg gallic acid equivalents/g elderflower polyphenol-enriched extract (EP), Table S1).

The LC-MS analysis on EP was performed; obtaining flavonol glycosides with different hydroxylation degrees in the B ring, such as kaempferol, quercetin, and isorhamnetin (Table S2, Fig. S1). Among heterosides, the O-mono, O-di, and O-triglycosylated forms were identified.

3.2. Antioxidant capacity of EP

The antioxidant activity of the EP was measured using different *in-vitro* assays, based on different characteristics of the antioxidant activity, such as DPPH free radical inhibition, inhibition of hydrogen peroxide (H_2O_2), and total reactive antioxidant potential (TRAP). The DPPH assay showed that EP is 4.1 times less active than Trolox and 7.3 times less active than BHT (Table 1). In the case of inhibition of H_2O_2, it was observed that EP has 3.3 and 2.9 times less activity than the positive controls BHT and Trolox, respectively. While evaluating the antioxidant activity using the TRAP assay, EP fraction is 459 and 489 times more active than gallic acid and BHT, respectively (Table 1).

3.3. Evaluation of cytotoxicity of EP

A Resazurin assay was used to evaluate the cytotoxicity of EP against salmon leucocyte cells. The cytotoxic effects of EP on the SHK-1 line cell were examined at different concentrations (Fig. 1A). The results indicated that EP showed significantly cytotoxic effects at a high concentration (120 μg/mL). However, no significant cytotoxic effects were observed at low concentrations (20 and 40 μg/mL).

The hemolysis assay is useful to establish potential cytotoxic activity related to cell membrane damage. Here, the hemolysis activity of EP on salmon blood samples was below 3% at the different concentration tested (20–400 μg/mL) (Fig. 1B).

Table 1
In vitro antioxidant properties of EP.

	IC_{50} DPPH (mg^*mL^{-1})	IC_{50} H_2O_2 (mg^*mL^{-1})	TRAP (mM TEAC)
EP	0.438 ± 0.015^a	8.229 ± 0.415^a	518.684 ± 7.872^a
TROLOX ®	0.106 ± 0.005^b	2.863 ± 0.032^b	n.a.
BHT	0.060 ± 0.001^c	2.526 ± 0.056^b	1.06 ± 0.010^b
Gallic Acid	n.a.	n.a.	1.13 ± 0.020^b

Values (mean ± SD) in the same row with the same superscript letter are not significantly different at $P > 0.05$. n.a. = Not applicable.

3.4. Determination of oxidative stress parameters in SHK-1 cell line response modulation

To evaluate the effect of EP on antioxidant cellular processes, SHK-1 cells were incubated for 24 h with 1.2 μg/mL of EP. Based on previous reports, prolonged incubation resulted in a severe loss of cell viability. In addition, H_2O_2 at final concentration of 1 μM has been reported as a positive control of antioxidant process in fish leukocyte [41].

In order to investigate whether lipid peroxidation could be activated under the experimental conditions, it was evaluated by measuring the extent of lipid degradation products, such as MDA.

The treatment of SHK-1 cells with H_2O_2 caused an approximate increase of 13% in lipid peroxidation with respect to the control cells, while the addition of EP decreased the lipid peroxidation induced by H_2O_2 by 24% (Fig. 2A).

The most commonly used marker type to assess protein oxidation are protein-bound carbonyls. As shown in Fig. 2B, basal levels of protein carbonyl content in crude extracts of SHK-1 cells exposed to H_2O_2 were higher (35%) than those in cells exposed to EP (17%) or H_2O_2 plus EP (15%).

It is interesting to note that TRAP decreased 25% in SHK-1 cells when EP was added. In fact, the simultaneous addition of H_2O_2 and EP extract decreased the TRAP to one half of that of non-treated cells (Fig. 2C).

The SOD activity decreased in the SHK-1 cells with different treatments. In the case of cells treated with EP, the SOD activity decreased 13.6% with respect to control cells, while the cells treated with H_2O_2 caused an approximate decrease of 22.7% in SOD activity. Besides, the simultaneous addition of H_2O_2 and EP extract strongly reduced the activity of SOD in SHK-1 line cell by 36.4% (Fig. 2D).

Finally, the GSH/GSSG ratio was used as an indicator of the overall redox state of the cell. The GSH/GSSG ratio was increased in SHK-1 cells treated with EP extract (Fig. 2E). In contrast, SHK-1 cells exposed to both H_2O_2 and EP decreased the GSH/ GSS ratio.

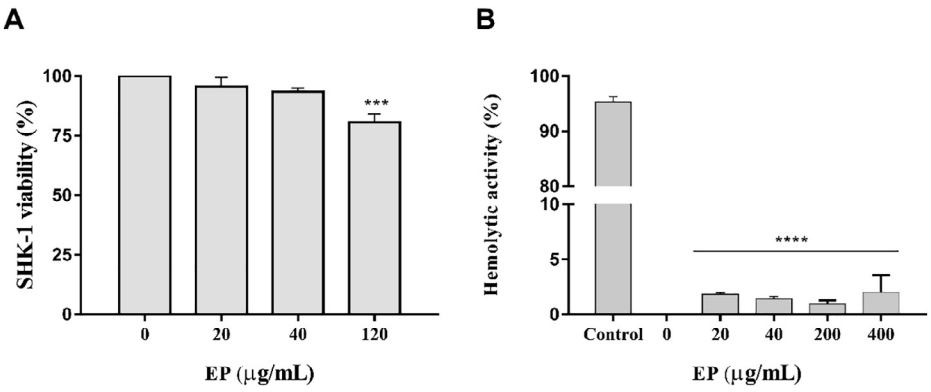

Fig. 1. Cytotoxic effects and hemolytic activity of EP. (A) Cytotoxic effects were performed on Salmon Head Kidney-1 (SHK-1) cell line. Cells were incubated for 24 h in the presence of different concentrations of EP (20–120 μg/mL). Cell viability was measured by the resazurin assay. (B) Hemolytic activity was determined on salmon red blood cells at different concentration of EP (20–400 μg/mL). 0.5% Triton X-100 was used as positive hemolytic control. Data are expressed as mean ± SD of three experiments performed in triplicate. **** $P < 0.0001$.

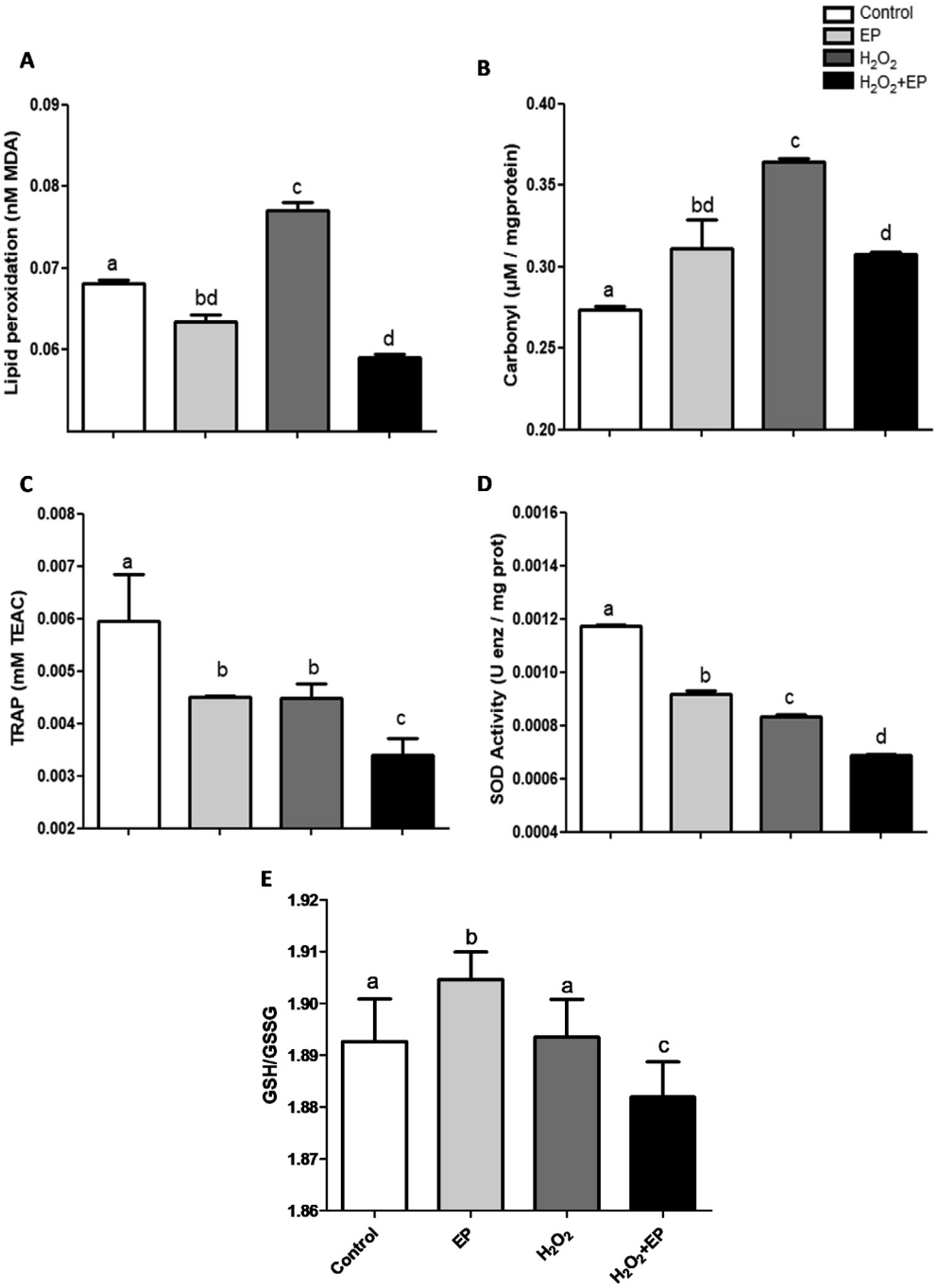

Fig. 2. Effect of EP extract on oxidative stress parameters in SHK- cell line. Oxidative damage was measured through malondialdehyde (MDA), a lipid peroxidation marker (A) and protein carbonyl content (B). While enzymatic antioxidant defenses were measured through the total reactive antioxidant potential (TRAP) (C), SOD activity (D) and glutathione complex (GSH/GSSG) ratio (E). SHK cells were incubated for 24 h without treatments (control) and with different exposures to 1.2 µg/mL of EP, 1 µM H_2O_2 and 1.2 µg/mL of EP in the presence of 1 µM H_2O_2. Data are expressed as mean ± SD of three independent experiments. Values (mean ± SD) with the same superscript letter are not significantly different at $P > 0.05$.

4. Discussion

Earlier reports revealed the presence of phytochemicals with antioxidant properties in elder flowers, berries and leaf extracts [11,42]. In this study, the polyphenol content in EP was analyzed by HPLC-MS, which revealed different glycoside compounds with cores of kaempferol, quercetin and isorhamnetin. Among them, kaempferol and quercetin aglycones have been found in different parts of *S. nigra*, as well as in other *Sambucus* species [43,44].

The *in-vitro* antioxidant activity of EP was measured by the inhibition of the free radical DPPH· obtaining a value of DPPH radical scavenging activity of *S. nigra* similar to that reported by Barros et al. [45]. Based on these results, it is concluded that EP has selectivity for peroxyl radical inhibition [46,47]. Although these tests demonstrate a high antioxidant power of EP, it was then determined if these properties were maintained in fish cells.

The EP showed no significant cytotoxic effect on salmon cells, as determined by hemolysis and a resazurin viability assay, at con-

centrations below 120 μg/mL. A previous report revealed the presence of cyanogenic glycosides (known as sambunigrin) in *Sambucus nigra* flowers and berries that could be toxic for the cells [48]. Interestingly, elderflowers produced four-fold lower levels of sambunigrin than obtained from leaves. We obtained the elderflower samples in springtime when temperature in the south of Chile is increasing. So, it is probable that the lack of detection of sambunigrin in our samples is associated with the high ambient temperatures [48], and could explain the lack of toxicity towards the SHK-1 cell line.

Reactive oxygen species (ROS) encompass a group of molecules derived from molecular oxygen, which are formed by reduction–oxidation (redox) reactions or by electronic excitation. In leukocytes, the respiratory burst generates ROS by an oxygen-dependent process during the phagocytosis of microbes. However, high concentrations of ROS result in cell injury by damaging key cellular molecules such as DNA, proteins, and lipids; therefore, the antioxidant defense network must control the levels of ROS to allow useful cell functions while minimizing oxidative damage [49,50,51]. For that reason, *in vitro* studies evaluating the antioxidant effect of both herbal extracts and pure polyphenols on fish cell models have focused on both ROS production and the respiratory burst mechanism [52]. For example, 6-gingerol and resveratrol pre-treatment decreased ROS production in PMA (Phorbol 12-myristate 13-acetate) stimulated salmon macrophages [53]. In addition, the treatment of gilthead seabream leukocytes with 0.1 mg/mL of *Origanum vulgare* leaf extracts significantly decreased respiratory burst in these cells [2]. However, to our knowledge, no reports are describing the protective effects of elderflowers extracts in salmon leukocytes.

Here, analysis of the H_2O_2-induced lipid damage revealed that EP decreased the lipid peroxidation in SHK-1 cell line, probably by scavenging the extracellular non-lipid radicals that initiated lipid peroxidation. It was described that quercetin present in EP diminished the arrest of cell proliferation, cell death, and lipid peroxidation triggered by H_2O_2 [54]. Moreover, studies were done in a linoleic acid emulsion model to assess the inhibitory action of elderflower extract from *S. nigra* on lipid peroxidation. In that study, the inhibitory potency of the extracts and constituents from elderflowers towards peroxidation of linoleic acid catalyzed by soybean 15-lipoxygenase was observed, demonstrating that they possess antiradical activity that could be applied in earlier and later stages of lipid oxidation [55]. Probably the presence of active sites for radical inactivation in these types of compounds could be associated with their action [56].

There are several studies with controversial results about some polyphenol antioxidants that may have pro-oxidant activity under certain conditions; type, dosage and matrix of these antioxidants may be determining factors impacting the balance between beneficial and deleterious effects of these natural compounds [57]. It has been described that flowers of *S. nigra* usually have higher antioxidant activity than berries and leaves. Also, the extract of elderflowers possesses better radical scavenging properties [58]. In this work, the levels of protein carbonyl content in SHK-1 cells exposed to EP or H2O2 were higher with respect to the control, and the SOD activity was decreased in the SHK-1 cells with the different treatments, probably due to the pro-oxidant activity of EP (Fig. 3). Further, the treatment with H_2O_2 decreased SOD activity, an expected effect since it has been reported that a high chemical reactivity of ROS by H_2O_2 triggers both lipid peroxidation and protein oxidation and degradation [59]. This is supported by the carbonyls assay (Fig. 2B). Pro-oxidant effects can be beneficial, since the levels of antioxidant defenses and xenobiotic-metabolizing enzymes might be raised by imposing a mild degree of oxidative stress, leading to overall cytoprotection [50].

Otherwise, it has been found that several flavonoids like kaempferol, quercetin and isorhamnetin, show an inhibitory effect on xanthine oxidase (XO). The activity of XO is associated with the oxidation of hypoxanthine to xanthine and xanthine to uric acid during the immune system response [60]. Therefore, if the activity of XO decreases, the superoxide anion production also decreases, which is an important substrate for the activation of SOD, an inducible oxidoreductase [61]. This could be the cause of the decrease in SOD activity in SHK-cell lines exposed to EP.

GSH is another important defense molecule against oxidative stress. Oxidative stress has a strong effect on the cellular thiol balance and can lead to a decreased GSH/GSSG ratio in many body organs. Therefore, GSH/GSSG status is frequently measured in physiological and pathophysiological conditions [62]. In the present study, EP showed to increase the GSH/GSSG ratio, decreasing

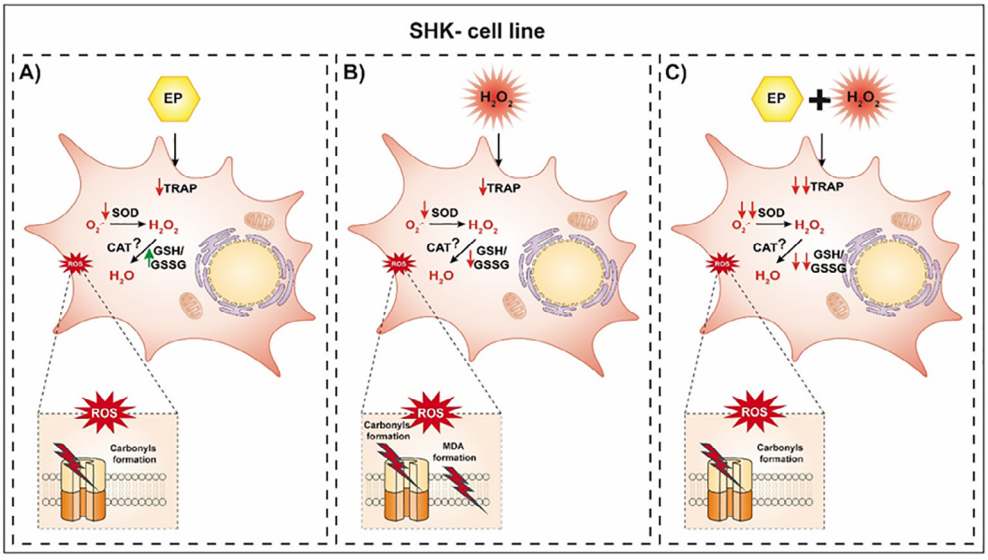

Fig. 3. Schematic representation of redox parameters modulation in the exposure of SHK-cell line to different experimental treatments. The figure summarizes the major redox mechanisms found in this study. SHK-cell line exposure to (A) EP, (B) H_2O_2, and (C) both EP and H_2O_2. EP = Elderflowers' polyphenol extract; H_2O_2 = Hydrogen peroxide; TRAP = Total reactive antioxidant power; SOD = Superoxide dismutase; CAT = Catalase; GSH/GSSG = Ratio of reduced and oxidized glutathione; ROS = Reactive oxygen species; MDA = Malondialdehyde; Red arrows: decrease; Green arrow: increase.

oxidative stress in the SHK cell line when EP was added alone. This effect could be explained because relatively low concentrations of quercetin stimulated transcription of the catalytic subunit of ɣ-glutamylcysteine synthetase (ɣGCS), improving cellular redox status [63]. Furthermore, for some antioxidant compounds, such as phenols, the presence of H_2O_2 induces compound-peroxidation, giving rise to new peroxides following the activation of oxidation pathways and oxidation products such as GSSG [50]. This could explain the result obtained from the decrease in the GSH / GSSG ratio when EP and H_2O_2 are simultaneously applied.

The results of this study suggest that plant extracts enriched with polyphenols could improve the enzymatic antioxidant defense of salmon leukocytes and to protect the cells against ROS-induced cell damage. In addition, flavonol glycosides from elderflower have antiradical activity, which may protect fish macrophages from lipid peroxidation. Nevertheless, because plant polyphenol extracts are used as feed additives, further research is needed to evaluate if the fish ingestion of elderflower polyphenols affects the biological properties of these compounds.

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Conflict of interest

None.

Supplementary material

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