Antioxidant and Oxidative Stress Modulation Properties of *Azorella pedunculata* Methanolic Extract on A549 Cancer Cells

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ABSTRACT

Species of the genus *Azorella* have been widely studied for the presence of secondary metabolites with biological activities. *Azorella pedunculata* is the most common species of *Azorella* genus in Ecuadorian moors. The present study evaluated the oxidative stress-protective and antioxidant effects of methanolic extract from *A. pedunculata* on A549 cells treated with hydrogen peroxide (for induction of oxidative stress). The DPPH assay (2,2-diphenyl-1-picrylhydrazyl) was used to assess the antioxidant activity of the extract. Cell viability for extract-treated cells was assessed by the 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The protective activity against oxidative stress in A549 cells pretreated with the extract and then exposed to H_2O_2 was evaluated by flow cytometry. In addition, gene expression analysis for oxidative stress-related genes superoxide dismutase 2 (*SOD-2*), and catalase (*CAT*) was performed with real-time quantitative reverse transcription PCR (RT-qPCR). The extract was cytotoxic at concentrations above 500µg/mL. Pre-treatment with methanolic extract at concentrations of 100 and 250µg/mL significantly decreased ROS (+) cell population, in comparison with the H_2O_2 treatment control. The mRNA levels of assessed genes were also modulated when cells were pretreated with the extract. In comparison with the H_2O_2 control, *SOD-2* was up-regulated when cells were pre-treated with the methanolic extract at 100 µg/mL, and *CAT* was down-regulated when cells were pre-treated with 100 and 250 µg/mL of extract. The methanolic extract of *A. pedunculata* might exert a protective effect against oxidative stress. Further studies are required to elucidate the molecular mechanisms involved. The findings of this study might be, however, of value for potential biological and medical applications.

Key Words: *Azorella pedunculata, Oxidative stress, Antioxidant capacity, DPPH.*

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INTRODUCTION

Reactive oxygen species (ROS) are generated inside the cells as a consequence of aerobic metabolism and are involved in important physiological processes, such as energy production, antimicrobial defense, signal transduction activation, and molecule biosynthesis [1]. ROS are responsible for the oxidative stress in various pathophysiological conditions [2]. ROS might be classified into two categories: 1) free radicals, e.g. superoxide ion, hydroxyl radicals, alkoxyl radicals, peroxyl radicals, and nitric oxide, and 2) non-radical species, e.g. hydrogen peroxide [3]. In general terms, there are endogenous and exogenous sources of ROS. Mitochondrial metabolism, peroxisomes, NADPH oxidase enzymes, and cytochrome P450 enzymes [1, 4, 5]

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contribute to the generation of endogenous ROS which carries out essential cellular functions. On the other hand, exposure to external factors, including cigarette smoking, ozone, ionizing radiation, xenobiotics, several drugs, pesticides, and environmental pollutants, among others, could increase the amount of intracellular ROS [4].

According to the recent findings, the roles of antioxidants as health-promoting factors are noteworthy [6]. The antioxidant compounds have the ability to induce endogenous antioxidant defense systems and scavenge reactive species [7]. In normal conditions, intracellular antioxidant defenses counteract the formation of free radicals. Various enzymatic and non-enzymatic elements, available in the animal's body, make an important defense mechanism against oxidative stress [8]. However, when this balance is not achieved, ROS production leads to a pathological condition named oxidative stress, that may inflict damage to important biomolecules (DNA, lipids, and proteins). For instance, it has been reported that the hydroxyl radical reacts with DNA components and causes mutations [9]. Furthermore, oxidative stress could initiate intracellular chemical reactions that contribute to the development of several disorders, such as cancer, inflammatory and cardiovascular diseases, Alzheimer´s, diabetes, atherosclerosis and rheumatoid arthritis [10, 11].

Suppression of endogenous ROS production in aerobic organisms could be achieved either by enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include, among others, superoxide dismutases (*SOD*), catalases (*CAT*), and glutathione-S-transferases (*GSTs*) [12], which are physiologically produced in cells to neutralize the harmful effects of free radicals [13]. The *SOD* enzyme converts O_2^{-} into H_2O_2 and molecular oxygen (O_2). In humans, there are three types of SOD: 1) Cu-SOD, 2) Zn-SOD, both present in the extracellular fluid and/or cytosol, and 3) Mn-SOD or SOD-2, present in mitochondria. Subsequently, H_2O_2 is transformed into water and oxygen by catalases, located in the peroxisomes [9]. The function of *GSTs* is to catalyze the conjugation of glutathione to a variety of hydrophobic and electrophilic compounds and to remove them from cells [14]. Tumor suppressor *p53*, considered as a protector of genome integrity, protects DNA from oxidation and activates the expression of several antioxidant enzymes, e.g. catalase and SOD-2 [15]. On the other hand, non-enzymatic antioxidants could be divided into two classes: endogenous antioxidants, and exogenous antioxidants (from foods) [16]. Some examples of non-enzymatic antioxidants include vitamin E (α-tocopherol), vitamin C (ascorbic acid), carotenoids, reduced glutathione (GSH), and plant polyphenols [17]. The administration of a mixture of non-enzymatic antioxidants reduced oxidative stress in a Wistar rat-carcinosarcoma model with appropriate concentrations and intake periods, as reported in the study by Grigorescu and co-workers [18]. Cell models are also suitable biological sources to assess the effect of non-enzymatic antioxidants under oxidative stress conditions, including aging [19, 20].

Natural sources of antioxidants might ameliorate the negative consequences of oxidative cell damage [21]. Thus, studies on new biomolecules and active principles that reduce the production of ROS and modulate the responses to oxidative stress at the cellular level will be significant for devising novel biomedical and pharmaceutical approaches in oxidative stress-related diseases. Many such biologically active molecules are being discovered and studied as a result of ongoing bioprospection initiatives from natural sources, mostly medicinal plants [22, 23].

According to the World Health Organization (WHO), lung cancer is one of the top causes of cancer-related deaths, with a reported 1.69 million cases worldwide in 2015 [24]. Lung cancer and its interaction with oxidative stress have been extensively investigated. The presence of toxic compounds that induce ROS production, namely cigarette smoking as well as second-hand smoking, is a known risk factor for lung cancer.

Azorella pedunculata (family Apiaceae) is a cushion-forming plant that grows in the Andean region of Ecuador and Colombia [25]. Species of the genus *Azorella* contain secondary metabolites, including mulinane and azorellane diterpenoids. These compounds display unique skeletons, with potential biological activities such as antibacterial [26], antiplasmodial [27], hypoglycemic [28], and trichomonicidal activity [29]. In addition, the presence of polyphenols with antioxidant activity in *Azorella* genus has also been described [30].

Free radical scavenging studies have been applied to report the antioxidant activity of several *Azorella* species [31]. Nevertheless, there is a lack of studies on the bioactivity of *A. pedunculata* extracts and no assessment of the mechanism of action has been done at the cellular level. Thus, the aims of this study were to determine the antioxidant activity of *A. pedunculata* extract and evaluate its effect on oxidative stress modulation and antioxidant gene expression in A549 cells (*human non-small-cell lung cancer* cell line).

MATERIALS AND METHODS

Reagents

Phosphate buffered saline (PBS), penicillin/streptomycin solution, trypsin-EDTA, trypan blue, MTT cell proliferation assay kit, PureLink® RNA Mini Kit, TURBO DNA-free™ Kit, Power SYBR® Green RNA-to-CT™ 1-Step Kit, TaqMan™ RNA-to-CT™ 1-Step Kit, and ultra-pure DEPC-treated water, were purchased from Thermo Fisher Scientific. Hydrogen peroxide (H_2O_2), F-

12K culture medium, and DPPH were obtained from Sigma-Aldrich. Muse Oxidative Stress Kit, Muse Count & Viability Assay Kit, fetal bovine serum (FBS), and methanol were purchased from Merck.

Preparation of *Azorella pedunculata* extract

Azorella pedunculata was gathered in Quilloac, Cañar (Ecuador). Voucher specimens were deposited at Herbario Azuay, affiliated to Universidad del Azuay, Cuenca (Ecuador). Plant material was cleaned and selected before air-drying it in aluminum shelves, for seven to ten days at room temperature. Aerial parts were ground and extracted via maceration with methanol (Merck, Darmstadt, Germany) in darkness, for 48 hours. This procedure was performed by triplicate. After that, extracts were concentrated *"in vacuo"*, with a rotatory evaporator, at the Organic Synthesis Laboratory, Natural Resources Chemistry Institute, Universidad de Talca, Chile.

DPPH radical-scavenging activity

The free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Chemical Co, St. Louis, MO, USA) was used to estimate the antioxidant activity of the crude extract from *Azorella pedunculata*, according to the method described by Molyneux (2004) [31]. Briefly, three solutions (10, 50, and 100 µg /mL) and one blank (80% methanol v/v) were mixed with a DPPH solution. After a vigorous homogenization, the reaction mixture was placed in darkness at room temperature for 30 minutes. The absorbance of the samples and the control was measured spectrophotometrically at a wavelength of 515 nm, in a Thermo Scientific Genesys 150 UV-Vis spectrophotometer. The percentage of reduction of DPPH was obtained from the following expression: $\%\ DPPH\ reduction = 100 * (1 - \frac{A_E}{A_B})$;

where AE is the absorbance of the reaction mixture with the extract solution and AB is the absorbance of the blank.

Cell culture

The A549 cell line was kindly provided by Dr. Javier Camacho (CINVESTAV-IPN, Mexico). Cells were cultivated in Kaighn's Modification of Ham's F-12 Medium (F-12K) (Sigma-Aldrich, St. Louis), supplemented with 10% FBS (Merck, Temecula) and 1% penicillin/streptomycin (Gibco, Grand Island), and maintained at 37°C and 5% CO_2 in a humidified atmosphere. The medium was changed every 48 hours until the cells reached 90% of confluence. Then, cells were harvested with trypsin/EDTA solution (Gibco, Burlington), stained with trypan blue (Gibco, Grand Island), and counted on a Neubauer chamber. Finally, cells were seeded in cell culture plastic flasks at specific concentrations depending on the experiment.

Cytotoxicity assessment of *Azorella pedunculata* extract

A549 cells were plated at a seeding density of 5×10^3 cells/mL in 96-well plates and allowed to grow for 24 hours. The extracts were diluted in complete culture medium to the exposure concentrations of 50, 100, 250, 500, and 750 y 1000 µg/mL. Next, the culture medium was replaced with the extracts, and cells were exposed to the treatments for 24 hours. For each extract, six technical replicates were performed. Cytotoxicity was then assessed by an MTT (Invitrogen, Eugene) assay, which is based on the conversion of the MTT reagent to insoluble formazan. In brief, after removing supernatants, 10 µL of MTT solution (12mM) was added to each well, followed by incubation for 4 hours at 37 °C. Formazan products were then solubilized by the addition of 100 µL of SDS-HCl solution to each well and further incubation at 37 ºC for 4 hours. Finally, absorbance was measured at 570 nm with a plate reader (Perlong, Beijing). Cell viability was calculated with non-exposure control representing 100% of cell viability.

Oxidative stress cell model

To induce oxidative stress in A549 cells, H_2O_2 (Sigma Aldrich, St. Louis) at different concentrations were used. Cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well and maintained in incubation for 48 hours. Next, stock H_2O_2 (8.82 M) was diluted in complete culture medium to obtain different treatments (1, 2, 3, 4, and 5 mM). Cells were treated with H_2O_2 for 3 hours. Non-exposure control was included. After that, cells were visualized under an inverted microscope (Olympus, Japan). Then, cells were trypsinized and centrifuged. The measurement of cell viability and ROS generation was then performed on a *Muse™ Cell Analyzer* (Merck KGaA, Germany).

For oxidative stress studies, cell pellets were dispersed at 1×10^6 cells/mL in the 1X Assay Buffer provided with the *Muse™ Oxidative Stress Kit* (Millipore, Hayward). ROS detection was performed according to the manufacturer´s protocol. In brief, Muse Oxidative Stress Reagent was diluted 1:100 with 1X Assay Buffer to make an intermediate solution. The intermediate solution was diluted 1:80 with 1X Assay Buffer and a working solution was obtained. Next, 190 µL of the working solution was added to 10 µL of cell suspension, and vortexed thoroughly. Samples were then incubated at 37 °C for 30 minutes and loaded finally onto the *Muse™ Cell Analyzer*.

Cell viability of the H_2O_2 treated-cells was also investigated. Cells in suspension were mixed with *Muse™ Count & Viability Reagent* (Millipore, Hayward), allowed to stain for a minimum of five minutes and then assessed in the *Muse™ Cell Analyzer*.

Oxidative stress modulation assessment of *Azorella pedunculata* extract

A549 cells were seeded into 6-well plates at a cell density of 1.5×10^5 per well. After a 24-hour culture period, cells were exposed (pre-treatment) to 100 and 250 µg/mL of the extract for 24 hours, followed by incubation with 3mM of H_2O_2 for 3 hours (treatment). Then, cells were harvested, and ROS status for the cell population was evaluated with the *Muse™ Cell Analyzer* as described above. Appropriate controls, (untreated, and H_2O_2-treated cells) were included in every experiment.

The quantitative real-time PCR analysis

Cell suspensions used for oxidative stress assays were also used for real-time PCR experiments. First, total RNA extraction was performed with the *PureLink® RNA Mini Kit* (Ambion, Carlsbad) in accordance with the manufacturer´s protocol. Next, samples were purified with the *TURBO DNA-free™ Kit* (Ambion, Carlsbad) to obtain RNA samples free of genomic DNA. The NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to assess the purity and quantity of extracted RNA. Purified RNAs were used as templates for one-step reverse transcription-quantitative PCR (RT-qPCR) assays. Sequences for the primers and probes used in this investigation are listed below (Table 1) [32–36].

The relative expression of superoxide dismutase 2 (*SOD-2*), and catalase (*CAT*) genes was assessed with the *Power SYBR® Green RNA-to-CT™ 1-Step Kit* (Applied Biosystems, Foster City). PCR reactions consisted of 1X Power SYBR® Green RT-PCR Mix, 0.2 µM of forward and reverse primer each, 1X RT Enzyme Mix, 10 ng RNA template, and DEPC-treated water (Invitrogen) to a final volume of 10 µL. RT-qPCR was performed in a LightCycler® 96 System (Roche Life Science, USA), using the following thermocycler program: 30 min at 48 °C for cDNA synthesis, 10 min of initial denaturation at 95 °C followed by 35 cycles for 15 s at 95 °C, 30 s at 55 °C (for primer annealing of *SOD-2* and *CAT*) or 58 °C (for *PPIA*), and 30 s at 60 °C. The specificity of PCR reactions was checked by melting curve analysis.

For expression analysis of glutathione-S-transferase (*GST*), and tumor suppressor (*p53*) genes the *TaqMan™ RNA-to-CT™ 1-Step Kit* (Applied Biosystems, Foster City) was used. PCR reactions consisted of 1X TaqMan® RT-PCR Mix, 0.5 µM of forwarding primer, 0.5 µM of reverse primer, 0.15 µM of TaqMan probes, 1X TaqMan® RT Enzyme Mix, 20 ng RNA template and DEPC-treated water (Invitrogen, Carlsbad) to a final volume of 10 µL. The thermal cycling program for RT-qPCR was set as follows: cDNA synthesis at 48 °C for 15 min followed by a polymerase activation step at 95 °C for 10 min. Then, 40 cycles of thermal amplification at 95 °C for 15 s, 50 °C or 56 °C for 15 s (for primer annealing of *ACTB* and *GST* genes respectively), and 45 s at 60 °C. For *p53* gene amplification, annealing and extension were performed in a single step at 61 °C for 60 s. Samples were assayed in three biological replicates. Relative expression levels for both genes were calculated according to the method described by Pfaffl (2001) [37], with *PPIA* or *ACTB* (reference genes) as normalizers.

Table 1. Primers Used In This Study

Gene name	Sequences (5'-3')	GenBank (accession number)	Amplicon size (bp)
Superoxide dismutase 2 (SOD2)	**Forward:** TGGACAAACCTCAGCCCTAA	NM_001322820.1	155
	Reverse: TTGAAACCAAGCCAACCC		
Catalase	**Forward:** ACAGCAAACCGCACGCTATG	NM_001752.3	105
	Reverse: CAGTGGTCAGGACATCAGCTTTC		
Peptidyl prolyl isomerase A (PPIA)	**Forward:** AGACAAGGTCCCAAAGAC	NM_021130.3	118
	Reverse: ACCACCCTGACACATAAA		
Glutathione-S-transferase (GST)	**Forward:** GATACTGGGGTACTGGGACATCC	NM_146421.2	130
	Reverse: CCACTGGCTTCTGTCATAATCAGG		
	Probe: 6-FAM-CCCACGCCATCCGCCTGCTCCT-TAMRA		
Tumor suppressor (p53)	**Forward:** TAACAGTTCCTGCATGGGCGGC	NM_000546.5	121
	Reverse: AGGACAGGCACAAACACGCACC		
	Probe: 6-FAM-CGGAGGCCCATCCTCACCATCATCA-TAMRA		
Actin Beta (ACTB)	**Forward:** CCTCGCCTTTGCCGA	NM_001101.3	171
	Reverse: TGGTGCCTGGGGCG		
	Probe: 6-FAM-CCGCCGCCCGTCCACACCCGCC-TAMRA		

Statistical analysis

The InfoStat software was used to analyze descriptive statistics of the data. For cytotoxicity results, confidence

intervals for the difference between the means were carried out using the *R package* (*The R Project for Statistical Computing*). Additionally, Wilcoxon signed-rank tests were applied to determine differences between treatments and control ($p < 0.05$). Lastly, to determine the effect of the *A. pedunculata* extracts on the relative expression of *SOD2*, *CAT*, *GST*, and *p53* genes, student´s t-tests were carried out ($p < 0.05$).

RESULTS AND DISCUSSION

The antioxidant *in-vitro* activity of *Azorella* extract

In our study, polar solvent methanol was used to extract active compounds of *Azorella pedunculata*. Then, A549 cells were exposed to different concentrations of the *A. pedunculata* extract for 24 hours and the cytotoxic effect, along with the expression profiles of antioxidant-related genes were evaluated. DPPH radical-scavenging activity yielded 52.92% of DPPH reduction for the methanolic extract from *A. pedunculata*. The IC_{50} for the methanolic extract was 70.19 µg/mL. If we take into account the classification suggested by Troya et al. for antioxidant potential based on the IC_{50} value by the DPPH assay (IC_{50} < 30 µg/mL was considered as high antioxidant potential, between 30 µg/mL to < 100 µg/mL was considered as moderate, and IC_{50} > 100 µg/mL was considered as low antioxidant potential) [38], the methanolic extract possesses moderate antioxidant potential. This is in accordance with the findings by Abad, who through DPPH assays concluded that a methanolic extract of *A. pedunculata* possessed moderate antioxidant capacity [39].

The cytotoxic effect of *A. pedunculata* extract on A549 cells

A549 cells were exposed to different concentrations of *A. pedunculata* extracts (50, 100, 250, 500, 750, and 1000 µg/mL) for 24 hours. The MTT was then used to determine cytotoxic effects in terms of cell viability. Our results found that the methanolic extract showed cytotoxicity at the highest concentrations of 750 and 1000 µg/ml (Fig. 1). Previous studies [40, 41] have shown that extracts from non-polar solvents are more cytotoxic than extracts from polar solvents against cancer cell lines such us larynx carcinoma (HEp-2), breast carcinoma (MCF-7), and human myeloid leukemia (HL60), which is in agreement with our findings because no harmful effect was observed at lower concentrations of the methanolic extract.

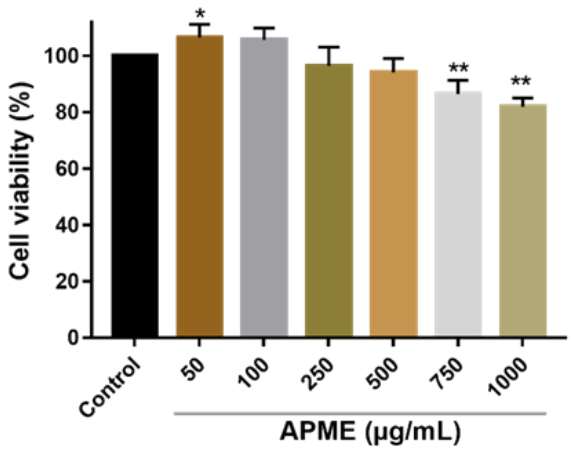


Fig. 1 Cytotoxicity of *A. pedunculata* extract on A549 cells. Cells were exposed to different concentrations of *A. pedunculata* methanolic extract for 24 hours. Data are presented as mean ± SD of six repeats from one independent experiment. *$p < 0.05$ or **$p < 0.01$ compared with untreated cells (control). APME: *Azorella pedunculata* methanolic extract.

Oxidative stress cell model

Before studying the effect of *A. pedunculata* extract on A549 cells, the optimum (high enough for keeping a large population of cells alive while inducing a significant rise in oxidative stress status) H_2O_2 concentration was screened. H_2O_2 is widely used to trigger oxidative stress in cell models. There is, however, a wide range of concentrations and exposure times for induction of ROS production in *in-vitro* assays in the literature. For A549 cells, H_2O_2 concentrations from as low as 100-500 µM [42–45] to up to 100 mM [46] with variable exposure times (from 15 min to 24 hours) have been reported. Franek and collaborators have suggested that the cytotoxic effect of H_2O_2 is affected by the number of cells used in the experiments [47]; therefore, the variability in concentrations and exposure times could be attributed to different cell densities at seeding.

In the present study, A549 cells were cultivated for 48 hours and then exposed to increasing concentrations of H_2O_2 (1, 2, 3, 4 and 5 mM) for 3 hours. After that, cells were observed under an inverted microscope. As shown in Fig. 2, non-treated cells showed epithelial-like morphology, whereas cells treated with 3, 4, and 5 mM, exhibited rounded shape. At higher concentrations of H_2O_2, cell detachment was also observed.

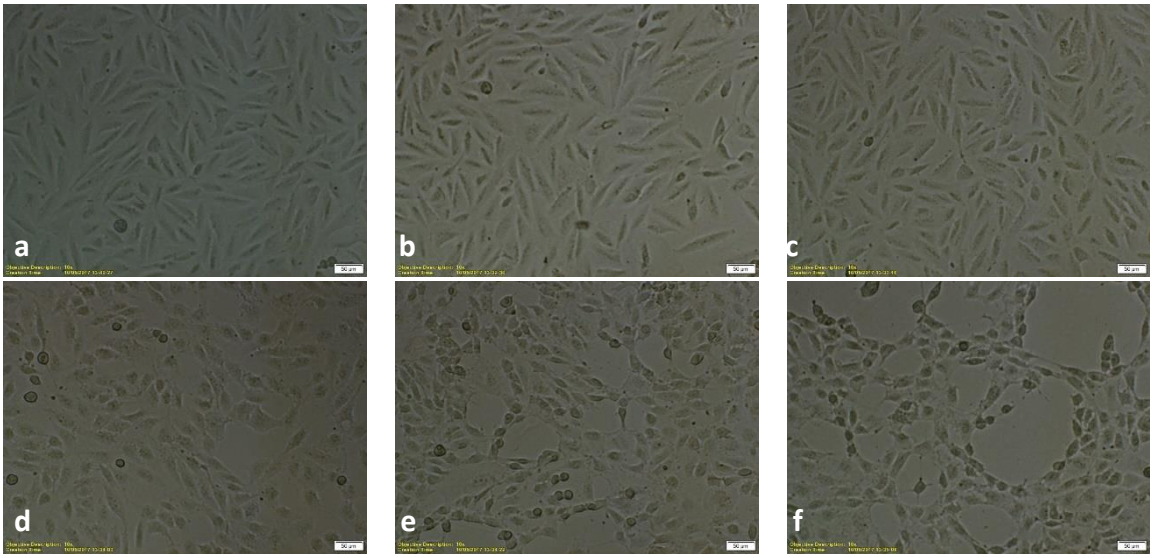
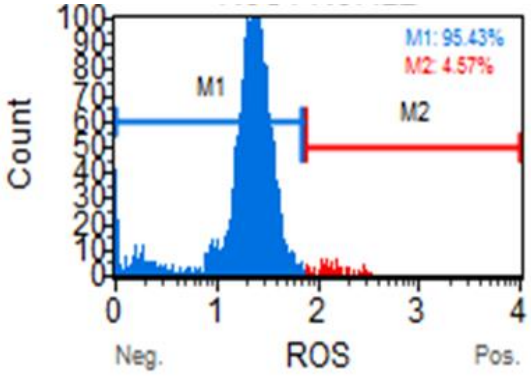


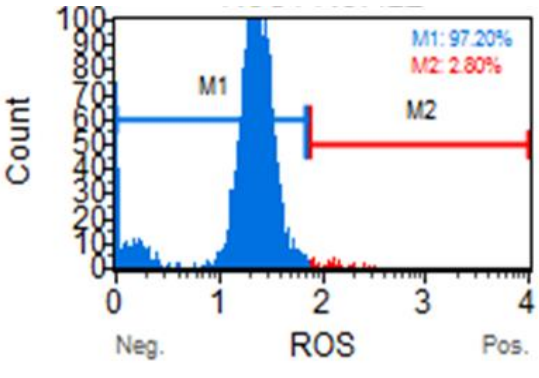
Fig. 2 A549 cells treated with increasing concentrations of H_2O_2: (a) 0 mM (untreated), (b) 1 mM, (c) 2 mM, (d) 3 mM, (e) 4 mM, and (f) 5 mM, were observed using an inverted microscope (original magnification X 100).

Treatment of A549 cells with several concentrations of H_2O_2 caused a dose-dependent increase of ROS and a decrease of cell viability (Fig. 3 and Fig. 4). Compared with the negative non-exposure control, the exposed cells showed an increase in ROS (+) cell populations of up to approximately 46%. A correlation between the concentration of H_2O_2 and cell viability was also found. As expected, when peroxide concentrations increased, cell viability diminished. The percentages of dead cells observed with treatments of 3, 4, and 5 mM of H_2O_2 were 48.5%, 56.0%, and 48.7%, respectively. Earlier findings in the literature have reported that H_2O_2 exposition decreased cell viability in a dose-dependent manner [48], and induced apoptosis in A549 cell line [47], which is consistent with our results (Fig. 4).

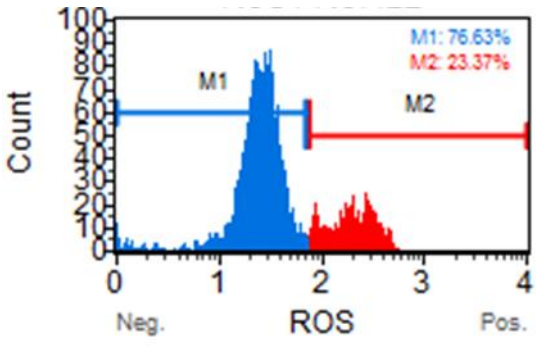
Although no significant differences in ROS (+) cell populations and cell viability were observed with the three highest concentrations, 3 mM of H_2O_2 was the concentration chosen to evaluate the oxidative stress modulation of *A. pedunculata* extracts, due to fewer cell detachment and less severe morphology alterations.



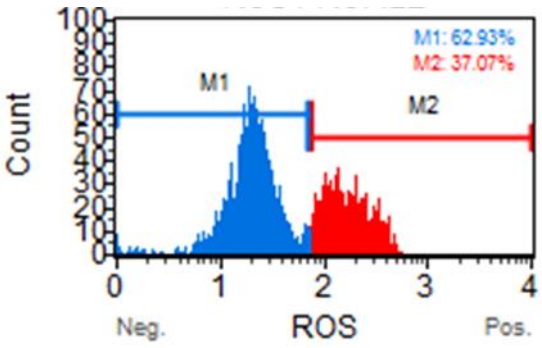
(a) Negative control



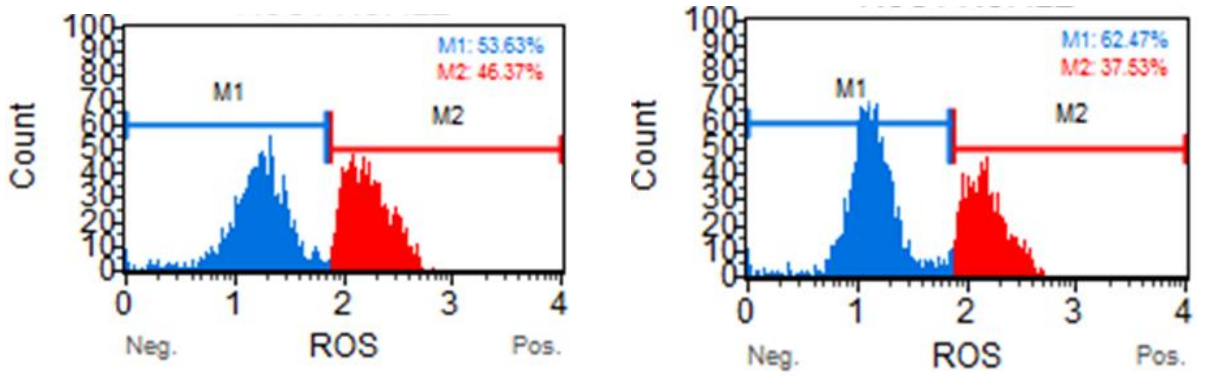
(b) 1mM H_2O_2



(c) 2mM H_2O_2

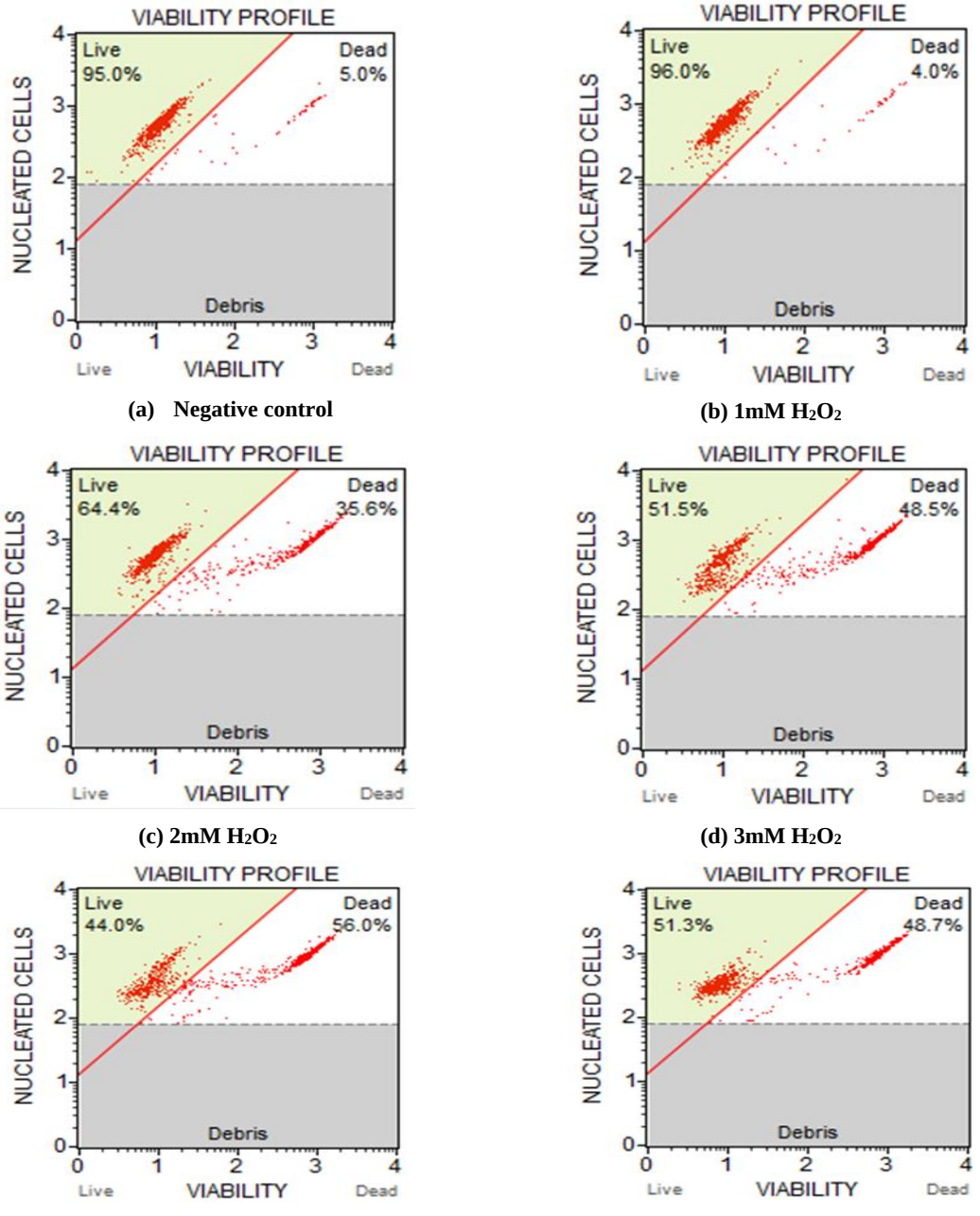


(d) 3mM H_2O_2



(e) 4mM H_2O_2 **(f) 5mM H_2O_2**

Fig. 3 Flow cytometry ROS histogram plots of A549 cells treated with H_2O_2 for 3 hours. Negative control: untreated cells. M1 and M2: percentage of ROS (-) and ROS (+) cells, respectively.



(a) Negative control **(b) 1mM H_2O_2**

(c) 2mM H_2O_2 **(d) 3mM H_2O_2**

(e) 4mM H_2O_2 **(f) 5mM H_2O_2**

Fig. 4 Viability dot plots of A549 cells treated with H_2O_2 for 3 hours. Negative control: untreated cells. Viable cells are displayed on the left of the graph; dead cells are displayed on the right.

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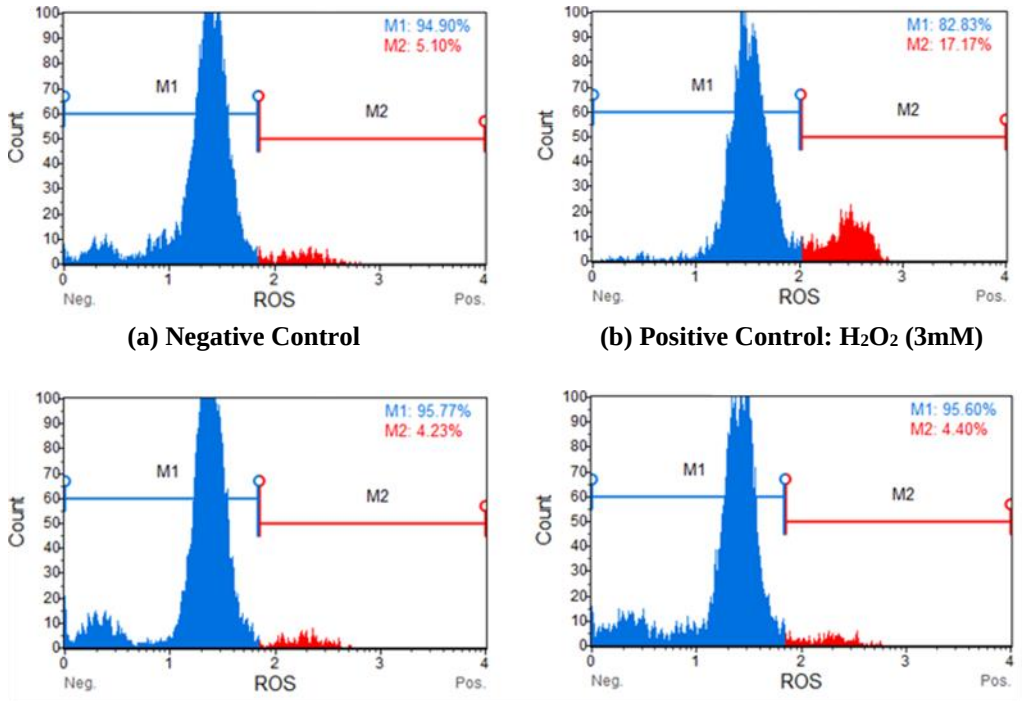
Oxidative stress modulation assessment of *Azorella pedunculata* extract

To determine whether the *A. pedunculata* extract inhibited ROS generation induced by H_2O_2, A549 cells were pretreated with different concentrations of methanolic extract for 24 hours. Then, cells were exposed to H_2O_2 (3 mM) for 3 hours. The intracellular levels of ROS were measured on the Muse Cell Analyzer using the Muse Oxidative Stress Kit® that is based on a cell-permeable probe called dihydroethidium (DHE) that is oxidized to ethidium bromide by superoxide radicals, resulting in red fluorescence. This probe is widely used to examine the redox state in tumor cells [49].

These results are presented in Fig. 5 through histograms, which show the percentages of ROS (+) (cells in oxidative stress) and ROS (-) (cells with no oxidative stress) populations. The percentage of ROS (+) cells increased to 17.17% after treatment with H_2O_2 (3 mM) (oxidative stress-positive control). However, pre-treatment with the methanolic extract inhibited ROS production. As illustrated in Fig. 5, ROS production decreased from 17.17% to 4.23% and to 4.40% when cells were pretreated with 100 and 250 µg/mL of the methanolic extract, respectively, which confirm that the extract has a moderate antioxidant activity.

Previous reports have described the biological activity of extracts obtained from several members of the genus *Azorella*. Some species possess the capacity to neutralize free radicals *in vitro* [50, 51], whereas others possess antibacterial activity against plant pathogens [52]. Tumová and collaborators have described that the free radical scavenging activity of *Azorella* species is due to its total contents of phenols, flavonoids, and tannins [53]. The assessment of the antioxidant activity of *A. pedunculata* in a cell model is reported for the first time in this study.



(a) Negative Control **(b) Positive Control: H_2O_2 (3mM)**

(c) 100 µg/ml + H_2O_2 (3mM) **(d) 250 µg/ml + H_2O_2 (3mM)**

Fig. 5 Flow cytometry histograms showing the effect of *A. pedunculata* methanolic extracts on ROS production induced by H_2O_2 in A549 cells. Cells were exposed to H_2O_2 after pretreatment with methanolic extracts at different concentrations (100 and 250 µg/mL). Negative control: untreated cells. M1 and M2: percentage of ROS (-) and ROS (+) cells, respectively.

Gene expression

Phytochemical compounds have been found to decrease intracellular levels of ROS by two mechanisms: (a) by inhibiting the expression or activity of enzymes that generate ROS such as NADPH oxidase and xanthine oxidase or, (b) increasing the expression and activity of intracellular antioxidant enzymes [54]. Antioxidant enzymes such as *SOD-2*, catalase, and glutathione-S-transferase, are very important mechanisms in mammalian cells that counteract the effects of free radicals [55].

Oxidative stress has been associated with an overproduction of ROS or deficiency in the intracellular antioxidant defense [13]. In the present study, mRNA levels of oxidative stress-related genes (*SOD-2*, *CAT*, and *GST*) and *p53* gene were measured in A549 cells pre-treated with *A. pedunculata* extract and then exposed to H_2O_2. Our results showed that the mRNA levels of *SOD-*

2 and *CAT* in cells treated with H_2O_2 were reduced (Fig. 6a and 6b). However, pre-treatment with 100 µg/mL of methanolic extract significantly increased the expression of *SOD-2* compared to the H_2O_2 control, which is in agreement with Masella et al. [56] who reported that phytochemicals such as phenolic compounds, modulate the intracellular response against oxidative stress by increasing the expression of antioxidant and phase 2 detoxifying enzymes.

On the other hand, expression of the *CAT* gene significantly decreased when cells were pre-treated with 100 or 250 µg/mL of the methanolic extract (Fig. 6b), compared to cells treated with H_2O_2. Our results are consistent with those of a previous study that found that trans-Resveratrol (RES), a molecule with a high content of phenolic compounds, increased the expression and activity of *SOD-2* and inhibited the activity of *CAT* on human lung fibroblasts [57].

Gene expression analysis showed that in cells exposed to 3 mM H_2O_2 for 3 hours, the expression of the *GST* gene significantly increased in comparison with untreated cells (Fig 6c). Since *GST* is a critical family of enzymes capable of conjugating GSH with electrophilic compounds to detoxify cells from these contaminants [58], the up-regulation of this gene could suggest a protective response to avoid cell damage induced by H_2O_2 exposure [55, 59]. Nevertheless, the samples that were pretreated with the methanolic extract and then exposed to H_2O_2, did not show significant variations in the levels of *GST* (Fig. 6c). This result has further strengthened our hypothesis that *A. pedunculata* extract could possess free radical scavenging activity through non-enzymatic mechanisms also, modulating the oxidative stress status, and rendering no significant differences between non-treated and treated cells.

The *p53* gene has been widely identified as a promotor of apoptosis in cells [60]. For instance, various investigations have demonstrated that H_2O_2 cause apoptosis in cells by the induction of p53, p73, and caspase-3 protein levels [61, 62]. Park (2018) proved that H_2O_2 induced a cytotoxic effect on A549 and Calu-6 cells by activation of both necrosis and apoptosis pathways [63]. It is known that H_2O_2 increases endogenous ROS, which in turn increases the expression levels of *p53*. In our study, A549 cells treated with 3 mM H_2O_2 for 3 hours showed a statistically significant increase in the expression of *p53* mRNA, compared to the untreated control (Fig. 6d), as well as decreased cell viability (Fig. 4d). We also found that pre-treatments with *A. pedunculata* extracts slightly up-regulate *p53* expression. These differences, however, were not statistically significant in comparison with the untreated control cells. Thereby, our results suggest a protective effect of *A. pedunculata* extracts against oxidative damage.

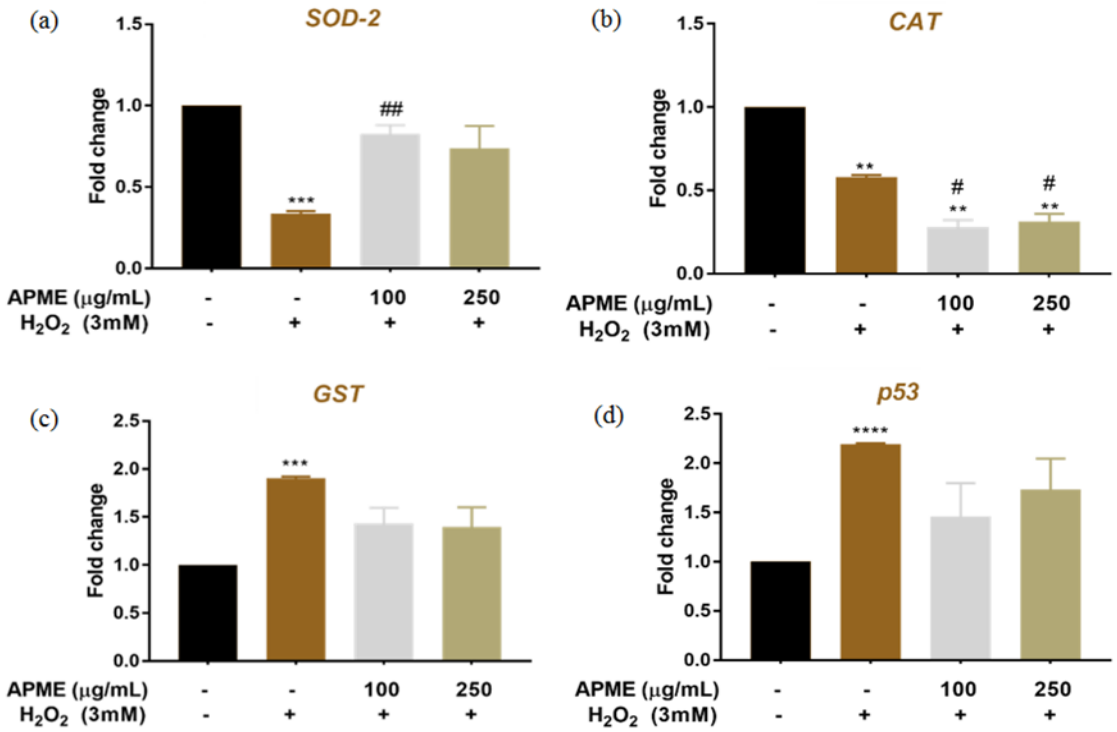


Fig. 6 Effect of methanolic extracts of *A. pedunculata* on the expression of (a) *SOD-2*), (b) *CAT*, (c) *GST* and (d) *p53* in A549 cells. Data are presented as mean ± SD for each experiment in triplicate. ** p <0.01 or *** p <0.001 or ****p <0.0001 with respect to negative control (untreated cells), # p <0.05 or ## p <0.01 compared to the control treated with H_2O_2. APME: *Azorella pedunculata* methanolic extract.

CONCLUSIONS

In this study, we assessed the antioxidant and oxidative stress modulation properties of *A. pedunculata* methanolic extract on A549 cells. The DPPH assay categorized the methanolic extract as having a moderate antioxidant activity. Flow cytometry assays showed that the extract exerted a protective effect to oxidative stress while gene expression assays demonstrated that this extract modulates the intracellular response against oxidative stress by significantly increasing the expression of the *SOD-2* gene. Mechanistic studies are needed for clarifying the found biological activities of the extract. The evidence from this study, however, suggests that the methanolic extract from *A. pedunculata* might be useful for pharmacological applications.

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

Author Contributions

MG, MEC, and LS conceptualized the study. NG, MJV, LS, and AJB performed experiments. NG and LC analyzed data. NG, LS, and MG wrote the paper. All authors approved the final manuscript version.

Supplementary Materials

Supplementary data associated with this article will be provided upon written request to Dr. Marcelo Grijalva, (rmgrijalva@espe.edu.ec).

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