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Eleganolone, a diterpene isolated from seaweed *Bifurcaria bifurcata* (Phaeophyceae, Fucales), protects neuronal cells from oxidative stress-induced damage

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ABSTRACT

The brown seaweed *Bifurcaria bifurcata* has gained particular interest in recent years due to its abundance in bioactive linear diterpenes with potential high-value applications. Evidences suggests that oxidative stress is an important mediator of neurodegenerative disorders. In this research study, the aim was to evaluate the potential protective effect of eleganolone, diterpene isolated from the brown seaweed *B. bifurcata,* on oxidative stressed human neuroblastoma SH-SY5Y cells by *tert*-butyl hydroperoxide (*tert*-BOOH). The protective effects of eleganolone on the oxidative stressed SH-SY5Y cells were measured by cell viability, cytotoxicity, oxidative stress biomarkers and antioxidative enzyme activity assays, as well as associated intracellular signaling pathways. The mRNA expression of apoptosis, inflammation, oxidative stress and neuronal development signaling pathway-related genes was analyzed by real-time RT-PCR. Eleganolone prevented the elevation of oxidative stress

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Abbreviations: ^{1}H NMR, Proton nuclear magnetic resonance spectroscopy; ^{13}C NMR, Carbon-13 nuclear magnetic resonance; AcOEt, Ethyl acetate; AcOH, Acetic acid; ANOVA, Analysis of variance; APAF1, Apoptosis protease-activating factor-1; ATCC, American Type Culture Collection; BAX, Bcl2-associated X protein; BCL2, B-cell lymphoma 2; BNIP3, Bcl2 interacting protein 3; BSA, Bovine serum albumin; CAMK2, Calcium/calmodulin-dependent protein kinase type II; CAMK2A, Calcium/calmodulin-dependent protein kinase type II subunit alpha; CAMK2B, Calcium/calmodulin-dependent protein kinase type II subunit beta; CAMK2D, Calcium/calmodulin-dependent protein kinase type II subunit delta; CAMK2G, Calcium/calmodulin-dependent protein kinase type II subunit gamma; $CDCl_3$, Deuterated chloroform; cDNA, Complementary DNA; CDNB, 1-chloro-2,4-dinitrobenzene; CNS, Central nervous system; COSY, Correlated spectroscopy; DAF-2, 4,5-diaminofluorescein; DCF, Highly fluorescent dichlorofluorescein; DCFH, 2,7-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMEM, Dulbecco's modified eagle medium; DMEM/F12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; DMSO, Dimethyl sulfoxide; DNA, Deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic acid; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GPx, Glutathione peroxidase; GSH, Reduced glutathione; GR, Glutathione reductase; GSSG, Glutathione oxidized; GST, Glutathione S-transferase; GSTM2, Glutathione-S-transferase mu 2; H_2O_2, Hydrogen peroxide; HEPES, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); HMBC, Heteronuclear multiple bond correlation; HO-1, Heme oxygenase-1; HPLC, High performance liquid chromatography; HRESIMS, High-resolution electrospray ionization mass spectrometry; HSQC, Heteronuclear single quantum coherence; IC_{50}, Inhibitory concentration 50; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; KCN, Potassium cyanide; LDH, Lactate dehydrogenase; MDA, Malondialdehyde; MeOH, Methanol; mRNA, Messenger RNA; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NaCl, Sodium chloride; NADPH, β-nicotinamide adenine dinucleotide phosphate; NEUROD1, Neuronal differentiation 1; NFKB1, Nuclear transcription factor kappa B subunit 1; NMR, Nuclear magnetic resonance; NO, Nitric oxide; NQO1, NADPH quinone dehydrogenase 1; OPT, *O*-phthalaldehyde; p38, Mitogen-activated protein kinase 14; p53, Tumor protein 53; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PUFA, Polyunsaturated fatty acids; qPCR, Quantitative PCR; RNA, Ribonucleic acid; ROS, Reactive oxygen species; RT-PCR, Reverse transcription polymerase chain reaction; SEM, Standard error of the mean; SOD2, Superoxide dismutase 2; SYN1, Synapsin I; SYN2, Synapsin II; SYN3, Synapsin III; SYNs, Synapsins; TBARS, Thiobarbituric acid reactive substance; *tert*-BOOH, *Tert*-butyl hydroperoxide; TNF-α, Tumor necrosis factor-α; Tween 20, Polysorbate 20; WNT5A, Wnt family member 5A; WNT7A, Wnt family member 7A; XO, Xanthine oxidase; XTT, 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

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markers, such as reactive oxygen species, malondialdehyde, nitric oxide and caspase 3/7 activity, as well as induced an increase in reduced glutathione and antioxidant enzyme activities in a dose-dependent manner on oxidative stressed SH-SY5Y cells. Additionally, our data showed that eleganolone downregulated the expression of genes associated with apoptosis (BAX, BNIP3, p53, p38, APAF1), inflammation (NFKB1, TNF-α, IL-6, IL-1β), oxidative stress (HO-1) and neuronal development (CAMK2A, WNT5A, WNT7A) pathways in SH-SY5Y cells exposed to oxidative stressor *tert*-BOOH. These findings suggest that eleganolone offers neuroprotective potential in ameliorating diverse pathological aspects associated with oxidative stress.

1. Introduction

Macroalgae, commonly known as seaweeds, play a vital role as primary producers in oceanic aquatic food webs, thereby supporting marine ecosystems (Tait and Schiel, 2018). Most marine macroalgae are predominantly found attached to substrates in coastal zones, which are characterized by variable environmental conditions due mainly to tidal cycles that vary in intensity according to geographic location. Seaweeds have evolved adaptive mechanisms to manage biotic and abiotic stressors through a range of physiological responses, leading to the production of a wide variety of chemical compounds different from those found in terrestrial environments. This chemical diversity offers a valuable resource for the development of new bioactive compounds. Primary and secondary metabolites from different macroalgae such as proteins and peptides, polysaccharides, polyphenols, polyunsaturated fatty acids (PUFA) or pigments have been described as having different biological activities (antioxidant, antimicrobial, antifungal, anticancer or anti-inflammatory, among others) (Rubiño et al., 2022).

Among the macroalgae species (about 10,000), only a very limited number have been the subject of broad research studies due to their composition and related biological activities. Special interest has been given to the brown macroalga *Bifurcaria bifurcata*, on account of its content of PUFA (Alves et al., 2016) and linear diterpenes (Muñoz et al., 2013; Ortalo-Magné et al., 2005; Pais et al., 2019). From the many species of macroalgae, the seaweed *B. bifurcata*, usually found along the Atlantic coast (France, Spain and Portugal) contains several linear diterpenes in its organic extracts that are not commonly present in other species of brown macroalgae (Smyrniotopoulos et al., 2017). In recent years, the brown alga *B. bifurcata* is of great concern due to its high content of bioactive linear diterpenes. Several *in vitro* biological effects have been reported for *B. bifurcata* extracts including antimicrobial, antitumour and antioxidant activities (Alves et al., 2016; Martínez et al., 2023), which could be associated with the content of these diterpenes (Culioli et al., 2004; Daoudi et al., 2001). Attention has focused on the search for natural substances with neuroprotective potential that can scavenge free radicals and protect cells from oxidative damage (Chen et al., 2020; Silva et al., 2021). Since *B. bifurcata* extracts have shown to be a potential source of compounds against oxidative stress (Martínez et al., 2023), in the present research study, in human dopaminergic neuroblastoma SH-SY5Y cells, we explored mechanisms supporting the neuroprotective effect of eleganolone, a diterpene isolated from the brown algae *B. bifurcata*, against chemically induced oxidative stress by the potent pro-oxidant *tert*-butyl hydroperoxide (*tert*-BOOH), an organic hydroperoxide extensively used as a reference compound for inducing oxidative stress. Among the different compounds that induce oxidative stress *in vitro*, *tert*-BOOH is the most widely used in short-term cell culture experiments (Aherne and O'Brien, 2000; Sestili et al., 1998, 2002). SH-SY5Y cells are widely used as an *in vitro* model to investigate oxidative stress and neurodegenerative processes (Iwata, 2018; Martínez et al., 2020a; Xicoy et al., 2017).

2. Materials and methods

2.1. Chemicals and reagents

The compounds *tert*-BOOH, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), menadione, dicoumarol, malondialdehyde (MDA), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), 2-thiobarbituric acid, sodium dodecyl sulfate, sodium chloride (NaCl), β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide, bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), Tween 20, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reduced (GSH), glutathione oxidized (GSSG), *o*-phthalaldehyde (OPT), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), potassium cyanide (KCN), xanthine oxidase (XO), trichloroacetic acid, the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and primer genes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and Dulbecco's modified eagle medium (DMEM) were obtained from Biowhitaker Lonza (Walkersville, MD, USA). Penicillin, streptomycin and the fluorescent probe 4-amino-5-methylamino-2,7-difluorofluorescein diacetate were obtained from Invitrogen (Madrid, Spain). The kit Caspase-Glo® 3/7 assay was purchased from Promega Corporation (Madison, WI, USA). All other chemicals were of the highest quality grade and obtained from commercial sources.

2.2. Macroalga collection, identification and preparation

The fucoid *B. bifurcata* is a dominant and widely distributed perennial macroalga found in the middle to lower intertidal rocky zone along the European Atlantic coast. It features numerous fronds reaching up to 30 cm in length and forms large and dense monospecific stands, commonly also referred to as beds, on rocky shores (Fletcher, 1987; Gómez Garreta et al., 2000). The untapped resources of *B. bifurcata* in Europe have not been utilized to date, in contrast to other fucoids such as *Ascophyllum nodosum* (Linnaeus) Le Jolis 1863 and *Fucus* spp. which are already commercially exploited for several industrial applications (Araújo et al., 2021). Additionally, it has been observed that *B. bifurcata* has shown an expansion in its wild populations along the northern coast of Spain in the current context of global warming (Ramos et al., 2020).

The present research study focused on the brown macroalga *B. bifurcata* R. Ross 1958 (Class Phaeophyceae, Order Fucales, Family Sargassaceae). The scientific name of the species and its taxonomic classification are based on Algaebase (Guiry and Guiry, 2024) and following the rules of the International Code of Nomenclature for algae, fungi, and plants (Turland et al., 2018). This fucoid seaweed (i.e. members of the Fucales) was collected during the autumn season (October 10, 2021) from La Maruca, Santander (43° 28′ 56″ N, 3° 50′ 27″ W) on the Atlantic coast of northern Spain. Healthy specimens (whole adult individuals) were hand-selected and harvested from the intertidal rocky zone during low tide. The collected specimens were immediately transported under refrigeration to the laboratory, where they were meticulously washed with sterile seawater to remove sediments, and cleaned from visible epiphytes and animals associated with the seaweed. They were then dried in an air-circulated oven at 40 °C for 48 h until reaching a constant weight, after which they homogenized using a grinder. Finally, the dried samples were stored in hermetic plastic bottles in a dry and dark place until subsequent analysis.

2.3. *Characterization of eleganolone from B. bifurcata*

Air-dried *B. bifurcata* (54.9 g) was subjected to extraction with acetone at room temperature and was concentrated to give a dark gum (1.8 g). C-18 reversed-phase flash chromatography of the crude extract gave fraction 5 (1.2 g; 1:4 H_2O/MeOH) and fraction 6 (641.1 mg; 100 % MeOH) containing eleganolone, as stated by their proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectra.

Fraction 5 was chromatographed by molecular exclusion LH-20 (MeOH) to give a sub-fraction of interest: 5_1 (485.5 mg). Fraction 6 was further chromatographed by molecular exclusion LH-20 (MeOH) to give one sub-fraction of interest, 6_1 (631.0 mg). These two fractions were chromatographed separately by normal-phase high performance liquid chromatography (HPLC) using a gradient from hexane-ethyl acetate (AcOEt) (7:3) to AcOEt (100 %), to afford 180.4 mg of compound **1** (named as eleganolone, 180.4 mg; t_R 27 min). Compound **1** was characterized by comparing its nuclear magnetic resonance (NMR) spectroscopic data with those of the compound isolated from *B. bifurcata* (Göthel et al., 2012). Compound **1** (eleganolone):

^{1}H (500 MHz, $CDCl_3$) δ 1.60 (3H, s, H-19), 1.61 (3H, s, H-18), 1.68 (3H, s, H-20), 1.88 (3H, H-17), 2.02 (2H, m, H-8), 2.03 (2H, m, H-4), 2.11 (2H, m, H-5), 2.13 (2H, m, H-9), 2.14 (3H, s, H-16), 3.03 (2H, s, H-12), 4.15 (2H, d, 7.0, H-1), 5.12 (1H, dd, 6.1, 6.1, H-6), 5.24 (1H, dd, 8.2, 8.2, H-10), 5.41 (1H, dd, 6.8, 6.8, H-2), 6.11 (1H, s, H-14); ^{13}C NMR (125 MHz $CDCl_3$) δ 16.2 (CH_3, C-19), 16.4 (CH_3, C-18), 16.6 (CH_3, C-20), 20.8 (CH_3, C-16), 26.4 (CH_2, C-5), 26.9 (CH_2, C-9), 27.9 (CH_3, C-17), 39.5 (CH_2, C-8), 39.7 (CH_2, C-4), 55.5 (CH_2, C-12), 59.6 (CH_2, C-1), 123.0 (CH, C-14), 123.6 (CH, C-2), 124.2 (CH, C-6), 129.3 (CH, C-10), 129.7 (C, C-11), 135.2 (C, C-7), 139.9 (C, C-3), 155.8 (C-15), 199.7 (C, C-13). High-Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) *m/z* $[M + Na]^+$ 327.2299 (calcd. for $C_{20}H_{32}O_2Na$, 327.2300).

The HRESIMS data were obtained on a Waters LCT Premier XE spectrometer. ^{1}H NMR and carbon-13 nuclear magnetic resonance (^{13}C NMR), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) and correlated spectroscopy (COSY) spectra were performed using a Bruker AMX 500 instrument operated at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. All ^{13}C and ^{1}H NMR spectra were referenced to the residual solvent signal ($CDCl_3$: δ_C 77.0 ppm, δ_H 7.25 ppm). Two-dimensional NMR spectra were obtained using the standard Bruker software. HPLC analyses were conducted on an Agilent 1200 Series Quaternary LC system equipped with a UV detector (DAD G1315D) and a Jaigel-Sil semipreparative column (10 μm, 20 cm × 250 mm) with hexane-AcOEt mixtures. Size-exclusion chromatography used Sephadex LH-20 as stationary phase and MeOH (100 %) as solvent system. The spray reagent used to develop thin-layer chromatography plates was H_2SO_4 - H_2O - AcOH (1:4:20).

NMR spectra were included in the Supplementary section: ^{1}H NMR (Fig. S1), ^{13}C NMR (Fig. S2), COSY NMR (Fig. S3), HSQC NMR (Fig. S4) and HMBC NMR (Fig. S5) of eleganolone in $CDCl_3$. Also, HRESIMS data of eleganolone **1** is included in the Supplementary section (Fig. S6).

2.4. *Cell culture*

The experiments were carried out on human dopaminergic neuroblastoma SH-SY5Y cells (ATCC® CRL-2266™) purchased from the American Type Culture Collection (Manassas, VA, USA). SH-SY5Y cells were cultured in DMEM/F12 medium supplemented with 10 % (v/v) heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. SH-SY5Y cells were maintained at 37 °C in a 5 % CO_2-humidified atmosphere. The cell culture medium was renewed daily to eliminate non-attached and dead cells. For the experiments, SH-SY5Y cells were cultured in 96-well microplates at a density of 8×10^4 cells per well or in 25 cm^2 culture flasks at a concentration of 2×10^5 cells/cm^2. Cells were adhered for 24 h before treatment with eleganolone. In the assays, cell culture plates with FBS were replaced with medium without FBS to minimize analytical interferences and ensure more replicable experimental conditions. All SH-SY5Y cells used in this research study had a low passage number (<13), and experiments were conducted when cells reached 80–90 % confluence.

2.5. *Cell treatment*

To study the direct effect of the eleganolone compound, SH-SH5Y cells were incubated with different eleganolone concentrations (0.05, 0.1, 1, 5, 10, 25 and 50 μM dissolved in 0.1 % DMSO) for 24 h. Following the incubation period, cell cultures were processed according to the experiment protocols.

Before the eleganolone protection effect assays, to produce a cellular oxidative stress condition, SH-SH5Y cells were submitted to *tert*-BOOH (Fallarini et al., 2009; Lombardi et al., 2002). The *tert*-BOOH-induced oxidative damage model was established with MTT assay by measuring the cell viability, intracellular reactive oxygen species (ROS) generation and caspase 3/7 activity, after which cell cultures were processed according to the specific assay. SH-SY5Y cells were treated with *tert*-BOOH (10–400 μM) for 1, 3 and 9 h (MTT assay), for 15, 30, 60, 90 and 120 min (ROS assay) and for 1, 3 and 5 h (caspase 3/7 activity).

In the experiments to evaluate the protective effect of eleganolone against an oxidative insult, cells were pre-treated with 0.05–10 μM of eleganolone for 24 h. Finally, oxidative stress was induced by exposing the cells to *tert*-BOOH (200 μM) for 3 h (cell viability assay), 90 min (ROS assay) or 3 h (caspase 3/7 activity assay), after which the cell cultures were processed depending on the assay.

Moreover, SH-SY5Y cells were submitted to 200 μM *tert*-BOOH for 3 h to test the protective effect of eleganolone on LDH release, NO, MDA, GSH levels, antioxidant enzyme activities and PCR assay.

2.6. *Evaluation of cell viability (MTT assay) and cytotoxicity (LDH assay)*

MTT assay is widely used to assess the viability of cells by measuring their mitochondrial activity (Ghasemi et al., 2021, 2023). Cell viability was determined by using the MTT assay following the method previously described (Denizot and Lang, 1986). The cells seeded in 96-well microplate were pre-incubated for 24 h and then exposed to eleganolone, *tert*-BOOH and *tert*-BOOH plus eleganolone to examine the effect of them on SH-SH5Y cells viability. Shortly, MTT reagent (22 μL, final concentration of 0.5 mg/mL), was applied to each well and the plate was incubated for 2 h at 37 °C in a 5 % CO_2-humidified atmosphere. Reduction of MTT results in a purple formazan compound by metabolically active cells at 37 °C. The water-insoluble formazan was dissolved in DMSO and was measured at 540 nm using a microplate reader (SPECTROStar Nano, Ortenberg, Germany). Control cells treated with DMEM were taken as 100 % viability.

A common method for determining cytotoxicity is based on measuring the activity of cytoplasmic enzymes released by damaged cells. LDH is a stable cytoplasmic enzyme that is found in all cells. LDH is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cellular damage (Kumar et al., 2018). LDH activity was determined using a LDH Cytotoxicity Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. SH-SY5Y cells were pre-incubated for 24 h and then exposed to eleganolone and *tert*-BOOH plus eleganolone to examine the effect of them on SH-SH5Y cells cytotoxicity. A colorimetric absorbance was recorded at 490 nm with a reference wavelength of 620 nm using a microplate reader for colorimetric detection (SPECTROStar Nano, Ortenberg, Germany). Total LDH (intracellular plus extracellular) was normalized to 100 %; then, the amount of LDH released to the extracellular medium was expressed as percentage of this total.

2.7. *Intracellular reactive oxygen species (ROS) measurement*

Cellular ROS generation was assessed by DCFH-DA as a fluorometric assay for hydrogen peroxide (Keston and Brandt, 1965). In the presence of ROS, 2,7-dichlorofluorescin (DCFH) is oxidized to highly fluorescent dichlorofluorescein (DCF) (LeBel et al., 1992). Therefore, the intracellular DCF fluorescence is used as an index to quantify the overall oxidative stress in cells (Wang and Joseph, 1999).

SH-SY5Y cells (8 × 10^4 cell/well) were cultured in 96-well plates with complete medium and replaced with the FBS-free medium containing *tert*-BOOH and eleganolone followed by *tert*-BOOH treatment. After 24 h, DCFH-DA (10 μM) was added to the cells for 30 min at 37 °C. Cells were then washed twice with phosphate buffered saline (PBS), plated in fresh medium without serum and ROS generation was assessed for the cytotoxic effect of *tert*-BOOH and the protective effect of eleganolone. ROS generation was measured in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (FLx800 Fluorimeter, Winooski, USA). Through quantification of the fluorescence over a period of 90 min, a precise estimation of ROS generated under the different conditions was obtained. This parameter gives a very good evaluation of the degree of cellular oxidative stress (Liang et al., 2018; Martínez et al., 2021).

2.8. *Determination of nitric oxide (NO) production*

Nitric oxide (NO) is an arginine-derived radical that plays a key role in numerous physiological and pathophysiological processes. 4,5-diaminofluorescein (DAF-2) and its derivate DAF-2 diacetate are fluorescent indicators that have been developed for NO detection. In the presence of NO, DAF-2 is transformed in a highly fluorescent triazole (Leikert et al., 2001). In the present research study, NO production was measured by spectrofluorometry following the optimized method for the detection of NO using lower DAF-2 concentrations (Leikert et al., 2001). Cells were cultured in 96-well multiwells (8 × 10^4 cells per well) and incubated with the different treatments (eleganolone plus *tert*-BOOH). Then, DAF-2 (2.5 μM) was added to the wells for 30 min at 37 °C. Cells fluorescence was tested using a fluorescent microplate at 495 nm excitation wavelength and 515 nm emission wavelength (FLx800 Fluorimeter, Winooski, USA).

2.9. *Lipid peroxidation assessment (malondialdehyde levels)*

In the present research study, MDA production for cytotoxic effect of *tert*-BOOH and the protecting effect of eleganolone were evaluated. MDA is the main and most studied product of PUFA peroxidation. MDA is a highly toxic molecule and is considered as a marker of lipid peroxidation (Del Rio et al., 2005). Intracellular MDA generation was quantified using thiobarbituric acid reactive substances (TBARS) assay kit (Cell Biolabs, CA, USA) (Martínez et al., 2021).

2.10. *Determination of reduced glutathione (GSH)*

As an index of the intracellular non-enzymatic antioxidant defenses, the concentration of GSH was measured in SH-SY5Y cells. GSH was shown to react specifically with OPT at pH 8.0, yielding a highly fluorescent product (Hissin and Hilf, 1976). In the present research study, the influence of eleganolone on GSH content and the protecting effect of eleganolone against *tert*-BOOH were analyzed. The content of GSH was quantitated following the Hissin and Hilf (1976) procedure. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the samples were referred to those of a standard curve of GSH (0.1–2 μg/μL).

2.11. *Determination of caspase 3/7 activity*

Apoptosis is a mode of programmed cell death that is coordinated by members of the caspase family of cysteine proteases. Among them, caspase 3/7 activation is widely considered the defining molecular marker of apoptotic cell death (Lakhani et al., 2006).

Apoptosis was quantified by measuring caspase-3 and -7 activities by bioluminescence using the commercially available Caspase Glo-3/7 Assay (Promega, WI, USA), according to the manufacturer's instructions. Briefly, SH-SY5Y cells were seeded in a 96-well plate with white walls at a density of 15,000 cells/well overnight. Then, culture medium was replaced with the FBS-free medium containing eleganolone, *tert*-BOOH and eleganolone followed by *tert*-BOOH treatment. Following 30 min at room temperature, 50 μL of Caspase-Glo 3/7 reagent was mixed with 50 μL of culture medium containing the pretreated cells in each well. Luminescence was measured with a plate reader (FLx800 Fluorimeter, Winooski, USA). To generate a cellular oxidative stress condition, SH-SY5Y cells were treated with *tert*-BOOH and caspase 3/7 activity was determined. The protective effect of eleganolone against the cytotoxic effect of *tert*-BOOH was also evaluated.

2.12. *Determination of antioxidant enzyme activities: NADPH quinone dehydrogenase 1 (NQO1), glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), and superoxide dismutase 2 (SOD2)*

To determine antioxidant enzyme activities, cells were washed twice with PBS, then scraped and collected in 1 mL PBS. They were homogenized in 200 μL of HEPES-buffered saline (50 mM HEPES plus 154 mM NaCl, pH 7.5), and subsequently centrifuged at 1000×*g* for 5 min at 4 °C. The supernatants were collected and stored at −80 °C until analysis. Protein content in cell lysates was estimated by the method of Lowry et al. (1951) using BSA. All enzyme activities were measured in the supernatants.

NADPH quinone dehydrogenase 1 (NQO1) activity was assessed according to the method of Tsvetkov et al. (2005), with slight modifications. This assay is based on the decline of NADPH absorbance at 340 nm (Martínez et al., 2021).

Glutathione S-transferase (GST) activity was measured by the method of Habig et al. (1974) which is based on the enzymatically catalyzed condensation of glutathione with the substrate CDNB. The resulting product, 2–4 dinitrophenyl glutathione, shows an absorption maximum at 340 nm. Values were expressed as μmol of CDNB-GSH conjugate formed per min per mg protein (Martínez et al., 2021).

Glutathione reductase (GR) activity was assessed by Staal et al. (1969) method, which is based on NADPH oxidation with GSSG as an acceptor. GR activity is defined as the amount of enzyme that is required to oxidize 1 μmol of NADPH per min at 340 nm. SH-SY5Y cells were incubated for 24 h with eleganolone followed by *tert*-BOOH treatment. Afterwards, supernatant (10 μL) was added to the reaction mixture. The reaction mixture (final volume 110 μL) contained buffer phosphate (0.1 M, pH 7.0), EDTA (1 mM), NADPH (0.2 mM), GSSG (1 mM) and Tween 20 (0.01 %). GR activity was assessed spectrophotometrically at a wavelength of 340 nm (SPECTROStar Nano, Ortenberg, Germany). Data are reported as consumed pmol of NADPH per minute per mg of protein.

Total glutathione peroxidase (GPx) activity was determined using a protocol adapted of Flohé and Günzler (1984). GSSG formed during GPx reaction is instantly and continuously reduced by an excess of GR activity providing for a constant level of GSH. The concurrent NADPH oxidation is measured at 340 nm. Data are presented as μmol NADPH oxidized per min per mg of protein. SH-SY5Y cells were incubated for 24 h with eleganolone followed by *tert*-BOOH treatment. Following, supernatant (10 μL) was added to the reacting mixture, which contained phosphate buffer (0.1 M, pH 7.0), EDTA (0.1 mM), GR (4.8 mU/mL), sodium azide (2 mM), GSH (1 mM), NADPH (0.3 mM) and H_2O_2 (0.25 mM). GPx activity was determined spectrophotometrically at a wavelength of 340 nm (SPECTROStar Nano, Ortenberg, Germany).

Total superoxide dismutase (SOD) activity was determined according to a protocol adapted of Ukeda et al. (1997) which is based on the inhibition of the reduction of tetrazolium salt XTT. After reduction with superoxide anion, the reduced form of XTT exhibits a maximum absorption at 470 nm. Cyanide-insensitive superoxide dismutase mitochondrial (SOD2) activity was measured adding KCN (Cano-Europa et al., 2008). SH-SY5Y cells were incubated for 24 h with the eleganolone followed by *tert*-BOOH treatment. Then, supernatant (10 μL) was added to the reaction mixture [final volume of 110 μL contained buffer potassium carbonate (54 mM, pH 9.4), EDTA (0.1 mM), xanthine (0.2 mM), XTT (55 μM), XO (0.10 μL) and Tween 20 (0.01 %)]. SOD and SOD2 enzyme activities were assessed spectrophotometrically at a wavelength of 470 nm (SPECTROStar Nano, Ortenberg, Germany). Data are expressed as % of formazan formation inhibition per min/mg protein.

2.13. *RNA extraction and real-time RT-PCR analyses*

In SH-SY5Y cells, the protecting effect of eleganolone was evaluated against cytotoxic effect of *tert*-BOOH (200 μM). Total RNA was obtained using the Trizol Reagent (Invitrogen, CA, USA) and purified using RNeasy MinElute Cleanup Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA concentration and purity were quantified with a NanoDrop 2000c spectrophotometer (Thermo-Fisher Scientific, Madrid, Spain) showing A260/A280 ratios between 1.9 and 2.1 in all the samples. RT^2 First Strand kit (Qiagen, Hilden, Germany) was used to synthesize complementary DNA (cDNA) from 5 μg total RNA. After cDNA synthesis, quantitative PCR (qPCR) amplification was performed on CFX96 real-time PCR detection system using RT^2 SYBR Green qPCR master mix (Qiagen, Hilden, Germany). Primer sequences were designed and were obtained from Sigma-Aldrich (St. Louis, MO, USA). The PCR conditions were as follows: 10 min at 95 °C for initial activation, 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Table 1 shows the primer sequences for the genes analyzed (BAX, BCL2, BNIP3, p53, p38, APAF1, NFKB1, TNF-α, IL-6, IL-1β, HO-1, GSTM2, CAMK2A, NEUROD1, WNT5A, WNT7A and SYN1). Relative expression level for each target gene was normalized to the housekeeping gene GAPDH. A negative control with non-genetic material was included to eliminate nonspecific reactions or contamination. Each sample was tested in triplicate and the method was performed to quantify the relative messenger RNA (mRNA) expressions (Pfaffl, 2001).

2.14. *Statistical analysis*

Four independent experiments were performed in triplicate, and data results were presented as mean ± standard error of the mean (SEM). Statistical differences between experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by the Tukey's *post-hoc* test. Statistical analysis was performed using GraphPad Prism version 6 software package. Statistical significance was set at $P < 0.05$.

Table 1
Primer sequences for real-time qPCR.

Gene name	Primer sequences	
	Forward	Reverse
BAX	CCCCCGAGAGGTCTTTTTCC	CCTTGAGCACCAGTTTGCTG
BCL2	TCTCATGCCAAGGGGGAAAC	TCCCGGTTATCGTACCCTGT
BNIP3	CCTCAGCATGAGGAACACGA	GCCACCCCAGGATCTAACAG
p53	GAACAAGTTGGCCTGCACTG	GAAGTGGGCCCCTACCTAGA
p38	TATGCGTCTGACAGGAACACC	GATCGGCCACTGGTTCATCA
APAF1	TCTTCCAGTGGTAAAGATTCAGTT	CGGAGACGGTCTTTAGCCTC
NFKB1	TTTTCGACTACGCGGTGACA	GTTACCCAAGCGGTCCAGAA
TNF-α	CTGGAAAGGACACCATGAGCA	TCTCTCAGCTCCACGCCATT
IL-6	CCAGTACCCCCAGGAGAAGA	CAGCTCTGGCTTGTTCCTCA
IL-1β	CCAGCTACGAATCTCCGACC	TATCCTGTCCCTGGAGGTGG
HO-1	GCTCAAAAAGATTGCCCAGA	GCGGTAGAGCTGCTTGAACT
GSTM2	GATCACCTTTGTGGATTTCATCGC	TGTGAACACAGGTCTTGGGA
CAMK2A	CATGGTTTGGGTTTGCAGGG	CCGGCTTTGATCTGCTGGTA
NEUROD1	TCTTCCACGTTAAGCCTCCG	CCATCAAAGGAAGGGCTGGT
WNT5A	AGCAGACGTTTCGGCTACAG	TGCCCCCAGTTCATTCACAC
WNT7A	GGAAAGTGAGCCACCGGATA	ACAGGCCGTGGAATGATACA
SYN1	TACAACGTACCCCGTGGTTG	TTTGGCATCGATGAAGGGCT
GAPDH	GAGAAGGCTGGGGCTCATTT	AGTGATGGCATGGACTGTGG

For MTT assay, the cytotoxic concentration (IC_{50} concentration) was calculated by concentration-response curves (Sigmoidal fitting) with Origin-Pro version 9 software package.

For quantitative Real-Time PCR assays, data are represented as mean ± SEM. Statistical analysis was carried out using one-way ANOVA followed by the Tukey's *post-hoc* test. Statistical significance was defined at $P < 0.05$.

3. Results

3.1. *Cell viability and LDH release (cytotoxicity) of eleganolone*

The effect of eleganolone (0.05–50 μM, 24 h) on SH-SY5Y cell viability was estimated by the MTT assay and the results are displayed in Fig. 1A. No significant effect on cell viability (expressed as a percentage of control) was found when SH-SY5Y cells were exposed to 0.05–25 μM eleganolone for 24 h (Fig. 1A). However, eleganolone at higher concentrations (50–100 μM), significantly decreased cell viability in the range 78 %–98 %.

LDH is a stable cytoplasmic enzyme present in all cells. LDH is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cells undergoing apoptosis, necrosis, and other forms of cell damage (Kumar et al., 2018). As shown in Fig. 1B, LDH release (expressed as a percentage of control) increased

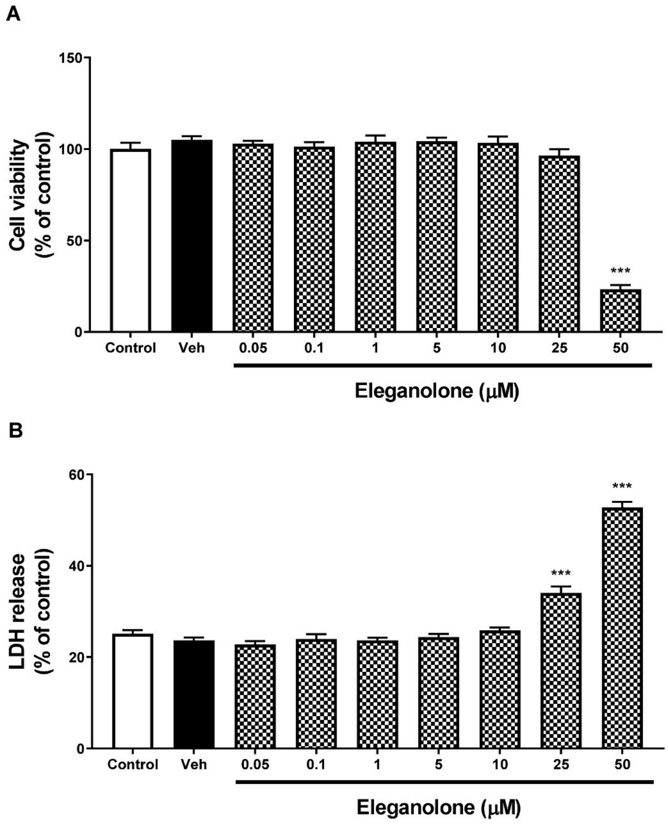

Fig. 1. Effect of eleganolone (0.05, 0.1, 1, 5, 10, 25, 50 μM) for 24 h on cell viability **(A)** and LDH release **(B)** in SH-SY5Y cells. Cell viability and LDH release were assayed as described in "Materials and methods". Data represent the mean ± SEM of 4 independent experiments in triplicate. ***$P < 0.001$ compared to vehicle (0.1 % DMSO), (black column).

about 44 % and 123 % in 25 or 50 μM eleganolone-treated cells for 24 h, respectively. The cytotoxicity remained unaffected when the cells were cultured with eleganolone (0.05–10 μM) (Fig. 1B). According to our results, it can be concluded that eleganolone (0.05–10 μM) did not induce cytotoxicity; these concentrations were used in subsequent experiments.

3.2. Protective effect of the eleganolone on oxidative stressed SH-SY5Y cells

3.2.1. Effects of eleganolone on cell viability and LDH release in SH-SY5Y cells with oxidative damage induced by tert-BOOH

The cytotoxic effects of the strong prooxidant *tert*-BOOH and the protecting effects of eleganolone were studied in SH-SY5Y cells. Cell viability was determined after incubation with *tert*-BOOH (10–400 μM) for 1, 3 and 9 h. The results showed that *tert*-BOOH reduced cell viability in concentration- and time-dependent manners (Fig. 2A). *Tert*-BOOH IC_{50} for cell death was 197.61 ± 42.50 μM at 3 h (Fig. 2B). Based on these findings, to generate cellular oxidative stress condition, SH-SY5Y cells were submitted to 200 μM *tert*-BOOH for 3 h to perform the subsequent analyses: cell viability, LDH release, NO, MDA and GSH levels, antioxidant enzyme activities and PCR assay.

In order to evaluate the protective effect of eleganolone against *tert*-BOOH-induced cell death, SH-SY5Y cells were pre-treated with eleganolone (0.05–10 μM) for 24 h, followed by incubation with *tert*-BOOH (200 μM) for 3 h. Cell viability and LDH release (cytotoxicity) were then assayed (Fig. 2C and D). As shown in Fig. 2C, eleganolone exerted a dose-response protective effect against *tert*-BOOH-induced cell death. In addition, eleganolone dose-dependently prevented LDH release induced by *tert*-BOOH (Fig. 2D). The results showed that eleganolone (1, 5, 10 μM) protects SH-SY5Y cells against *tert*-BOOH-induced cytotoxicity through its effects on increased viability and LDH release suppression.

3.2.2. Effects of eleganolone on intracellular ROS generation in SH-SY5Y cells with oxidative damage induced by tert-BOOH

Reactive oxygen species (ROS) generation plays a key role in human physiological and pathophysiological processes. In the present research study, to promote a cellular oxidative stress state, SH-SY5Y cells were exposed to the pro-oxidant *tert*-BOOH (10–400 μM), and ROS production at 15, 30, 60, 90 and 120 min was tested (Fig. 3A). *Tert*-BOOH, like other organic peroxides, may be degraded to other alkoxyl and peroxyl radicals that can produce ROS (Alía et al., 2005). The outcomes indicate that *tert*-BOOH (200 μM) produced the most marked increase (353 % over control) in ROS generation at 90 min (Fig. 3A). Moreover, Fig. 3B demonstrates that the ROS generation induced in SH-SY5Y cells by *tert*-BOOH (200 μM) at 90 min was significantly decreased (14–33 %) when the cells were pre-treated with eleganolone (1–10 μM) for 24 h. In this research study, eleganolone was shown to exert antioxidant effects against *tert*-BOOH-induced oxidative stress, mainly through its ability to scavenge ROS.

3.2.3. Effects of eleganolone on caspase 3/7 activity in SH-SY5Y cells with oxidative damage induced by tert-BOOH

In the present research study, in order to elucidate whether cell death generated by cellular oxidative stress was due to apoptosis, caspase 3/7 activity, as a biomarker of apoptosis, was measured in SH-SY5Y cells treated with *tert*-BOOH (10–400 μM). As shown in Fig. 4A, the presence of *tert*-BOOH (200 μM) caused activation of caspase 3/7 (403 % over control), which reached a maximum activation peak at 3 h. This stress condition was used to test the protective effect of eleganolone (Fig. 4B). Pre-treatment of cells with eleganolone (5 and 10 μM) for 24 h significantly reduced caspase 3/7 activity evoked by *tert*-BOOH (200 μM) (27

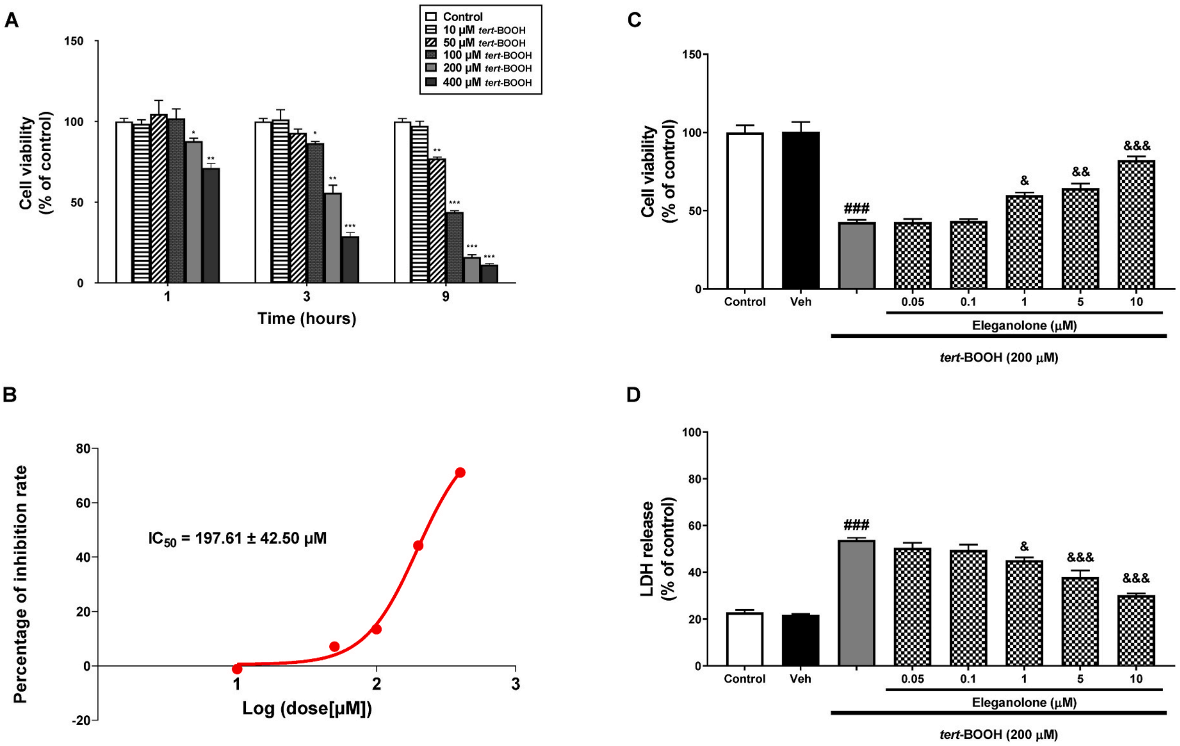

Fig. 2. (A) Cytotoxicity induced by *tert*-BOOH (10–400 μM) on SH-SY5Y cell viability after 1, 3 and 9 h incubation period. **(B)** Dose-response curve of cell viability reduction (%) used to generate IC_{50} value after *tert*-BOOH (10–400 μM) exposure for 3 h in SH-SY5Y cells. Eleganolone (0.05, 0.1, 1, 5, 10 μM) protective effect on **(C)** cell viability and **(D)** LDH release. [SH-SY5Y cells were pre-treated with eleganolone for 24 h, and then exposed to *tert*-BOOH (200 μM) for 3 h]. Cell viability and LDH release were assayed as described in "Materials and methods". Data represent the mean ± SEM of 4 independent experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control (white column). $^{\#\#\#}P < 0.001$ compared to vehicle (0.1 % DMSO), (black column). $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ compared to *tert*-BOOH (grey column).

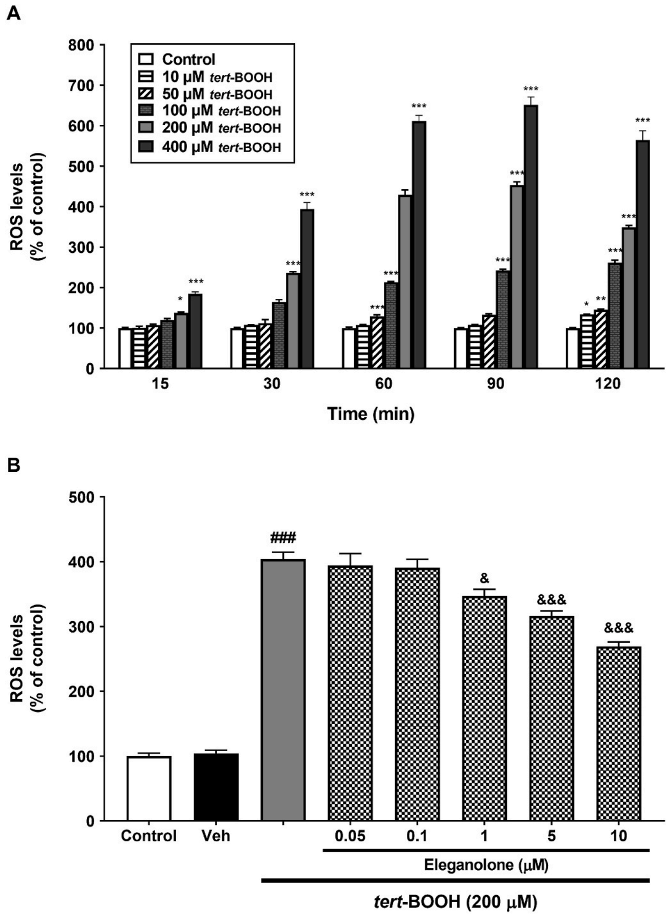

Fig. 3. (A) Effect of *tert*-BOOH on ROS generation in SH-SY5Y cells. Cells were exposed to *tert*-BOOH (10–400 µM) and intercellular ROS production was evaluated at 15, 30, 60, 90 and 120 min. **(B)** Protective effect of eleganolone against oxidative stress in SH-SY5Y cells. Cells were treated with eleganolone (0.05, 0.1, 1, 5, 10 µM) for 24 h, then submitted to *tert*-BOOH (200 µM) and ROS production was evaluated at 90 min. ROS production was assayed as described in "Materials and methods". Data represent the mean ± SEM of 4 independent experiments in triplicate. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to control (white column). $^{\#\#\#}P < 0.001$ compared to vehicle (0.1 % DMSO), (black column). $^{\&}P < 0.05$ and $^{\&\&\&}P < 0.001$ compared to *tert*-BOOH (grey column).

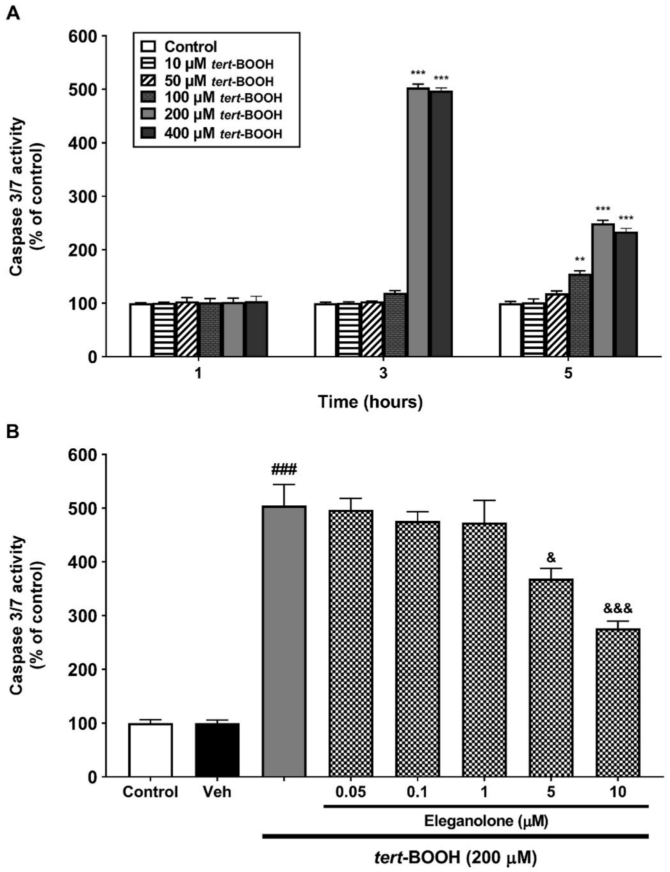

Fig. 4. (A) Effect of *tert*-BOOH on caspase 3/7 activity in SH-SY5Y cells. Cells were treated during 1, 3 and 5 h with *tert*-BOOH (10–400 µM) and caspase 3/7 activity was evaluated. **(B)** Protective effect of eleganolone against apoptosis in SH-SY5Y cells. Cells were treated with eleganolone (0.05, 0.1, 1, 5, 10 µM) for 24 h, then submitted to *tert*-BOOH (200 µM) and caspase 3/7 activity was evaluated at 3 h. Caspase 3/7 activity was assayed as described in "Materials and methods". Data represent the mean ± SEM of 4 independent experiments in triplicate. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control (white column). $^{\#\#\#}P < 0.001$ compared to vehicle (0.1 % DMSO), (black column). $^{\&}P < 0.05$ and $^{\&\&\&}P < 0.001$ compared to *tert*-BOOH (grey column).

and 45 %, respectively) (Fig. 4B). These results indicate that treatment of SH-SY5Y cells with eleganolone protected against induced apoptosis and cell death.

3.2.4. *Effects of eleganolone on NO, MDA, GSH levels in SH-SY5Y cells with oxidative damage induced by tert-BOOH*

NO, an unstable free radical, can be endogenously produced in different cell types and plays a wide range of biological roles, such as host defense, vascular regulation and neuronal communication (Mayer and Hemmens, 1997; Schmidt and Walter, 1994). Elevated NO concentrations may induce apoptotic cell death in different neuronal cell types, leading to neuronal injury (Hogg and Kalyanaraman, 1999; Wei et al., 2000). MDA is one of the primary products of lipid peroxidation. MDA levels have been found to be high in several common diseases related to free radical injury and has been used as an index of lipid peroxidation (Suttnar et al., 2001). In the present investigation, the protective effect of eleganolone (0.05–10 µM) on oxidative stressed SH-SY5Y cells induced by *tert*-BOOH (200 µM) was investigated. NO production and MDA levels (markers of oxidative stress, expressed as a percentage of control) were significantly increased (62 % and 216 %, respectively) after *tert*-BOOH exposure. Eleganolone pre-treatment (5 and 10 µM) significantly decreased NO production (18 % and 26 %, respectively) in SH-SY5Y cells exposed to *tert*-BOOH (200 µM) (Fig. 5A). Likewise, in SH-SY5Y cells exposed to *tert*-BOOH (200 µM), eleganolone pre-treatment (1, 5 and 10 µM) led to a decrease in MDA levels (16 %, 21 % and 30 %, respectively) (Fig. 5B).

GSH, a low-molecular-weight tripeptide thiol, is the primary scavenger of oxygen radicals (Berndt and Lillig, 2017) and the major antioxidant in the brain (Poladian et al., 2023). In the present investigation, it was observed that the cytoplasmic GSH levels in SH-SY5Y cells exposed to *tert*-BOOH (200 µM) dramatically decreased by 45 % (Fig. 5C). Eleganolone (1, 5, 10 µM) increased GSH levels by 20 %, 38 % and 63 %, respectively, compared to cells exposed to *tert*-BOOH (Fig. 5C).

In conclusion, in SH-SY5Y cells*,* *tert*-BOOH exposure caused a marked increase in lipid peroxidation rate evidenced by elevated NO and MDA levels. Moreover, *tert*-BOOH exposure caused a reduction in GSH levels. Eleganolone treatment decreased the effects caused by *tert*-BOOH. Treatment with eleganolone reduced NO and MDA formation and increased GSH levels. These results highlight the antioxidant role of eleganolone.

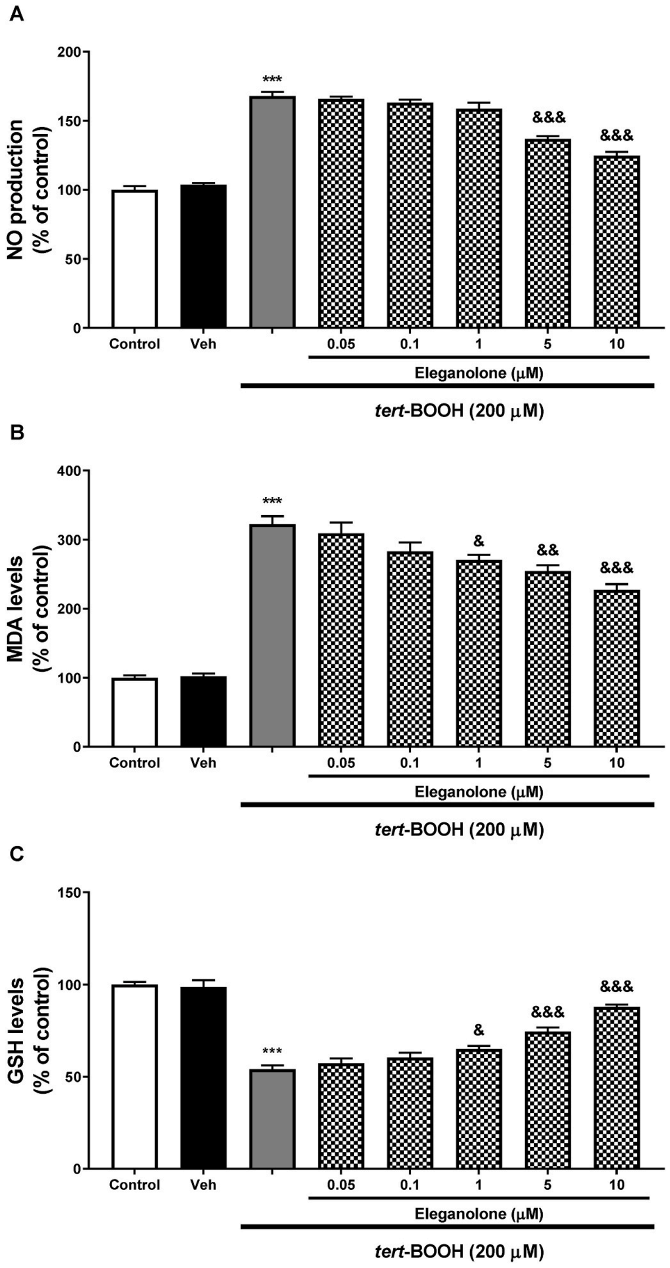

Fig. 5. Protective effect of eleganolone on NO production **(A)**, MDA levels **(B)** and GSH levels **(C)** in SH-SY5Y cells. Cells were treated with eleganolone (0.05, 0.1, 1, 5, 10 μM) for 24 h, then submitted to *tert*-BOOH (200 μM) and NO production, MDA and GSH levels were evaluated at 3 h. NO production, MDA and GSH levels were assayed as described in "Materials and methods". Data represent the mean ± SEM of 4 independent experiments in triplicate. $^{***}P < 0.001$ compared to vehicle (0.1 % DMSO), (black column). $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ compared to *tert*-BOOH (grey column).

3.2.5. Effects of eleganolone on antioxidant enzyme activities (NQO1, GST, GR, GPx and SOD2) in SH-SY5Y cells with oxidative damage induced by tert-BOOH

Antioxidants counteract free radicals and neutralize oxidants. The general endogenous antioxidant defense system consists of enzymatic antioxidants.

- NADPH quinone dehydrogenase 1 (NQO1) is an enzyme associated with cellular detoxification and protection against oxidative stress. NQO1 is involved in the regulation of neuronal function and synaptic plasticity, cellular adaptation to oxidative stress, neuroinflammatory and degenerative processes in the central nervous system (CNS) (Yuhan et al., 2024).
- Glutathione S-transferase (GST) is a family of isoenzymes located both in the cytosol and endoplasmic reticulum of the liver (Aniya et al., 1993). As a primary function, GST plays a key role in Phase II metabolism by protecting living cells against a wide variety of toxic molecules by conjugating them with the tripeptide glutathione (Mazari et al., 2023).
- Glutathione reductase (GR) is an essential enzyme that transforms oxidized glutathione to its reduced form. In its reduced form, glutathione plays a crucial role in the cellular control of ROS (Couto et al., 2016).
- Glutathione peroxidase (GPx) is a member of a phylogenetically related family of enzymes. Members of GPx have anti-oxidative functions at different cellular components (Brigelius-Flohé and Maiorino, 2013).
- Superoxide dismutase (SOD) enzymes regulate ROS and NO levels, reducing the toxicity of these molecules and regulating functions of cellular life (Wang et al., 2018).

In the present research study, in order to find out whether the eleganolone compound exhibited a protective effect against oxidative stress-induced by *tert*-BOOH in SH-SY5Y cells, the activities of antioxidant enzymes NQO1, GST, GR, GPx and SOD2 were evaluated. *Tert*-BOOH treatment alone reduced NQO1, GST, GR, GPx and SOD2 activities (Fig. 6). Notably, each of these *tert*-BOOH-induced effects were reversed in cells pre-treated with eleganolone (dose-dependent) (Fig. 6). These results clearly established that eleganolone suppressed *tert*-BOOH-induced oxidative stress.

3.3. Effects of eleganolone on the mRNA expression of apoptosis, inflammation, oxidative stress and neuronal development signaling pathway-related genes in SH-SY5Y cells with oxidative damage induced by tert-BOOH

3.3.1. Apoptosis related gene transcriptions

The BAX, BCL2, BNIP3, p53, p38 and APAF1 mRNA expression can be used to assess a cellular apoptotic process (Elmore, 2007). In the present research study in SH-SY5Y cells with oxidative damage (*tert*-BOOH-treated SH-SY5Y cells) an upregulated transcription of BAX, BNIP3, p53, p38 and APAF1 related genes was observed, while the expression of BCL2 was downregulated (Table 2A). The *tert*-BOOH-evoked apoptosis in SH-SY5Y cells, which can be inhibited by eleganolone (Table 2B and C). After the pretreatment with eleganolone, dose-dependent, a significantly downregulated transcription of BAX, BNIP3, p53, p38 and APAF1 related genes was observed, while the expression of BCL2 was upregulated, indicating that eleganolone possessed an inhibitory effect on *tert*-BOOH induced apoptosis. Overall, our research study has shown that the mechanism underlying the protective effects of eleganolone in *tert*-BOOH-injured SH-SY5Y cells might be related to the inhibition of apoptotic features.

3.3.2. Inflammation related gene transcriptions

The transcription factor NFKB1 and cytokines such as TNF-α, IL-6 and IL-1β, may support neurodegeneration or impairment of neuronal function (Rothwell, 1999). In the present research study, we measured the mRNA expression of the potent proinflammatory cytokines TNF-α, IL-6 and IL-1β and transcription factor NFKB1 by RT-qPCR. Our study demonstrated that the pro-oxidant *tert*-BOOH remarkably induced increases in the mRNA levels of transcription factor NFKB1 and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) in neuronal SH-SY5Y cells (Table 2A). Whereas, after pretreatment with eleganolone (1 and 10 μM), these genes were downregulated (Table 2B and C). These results indicate that eleganolone has anti-inflammatory activity in neuronal SH-SY5Y cells. Eleganolone might be a candidate for the treatment of neuroinflammatory diseases.

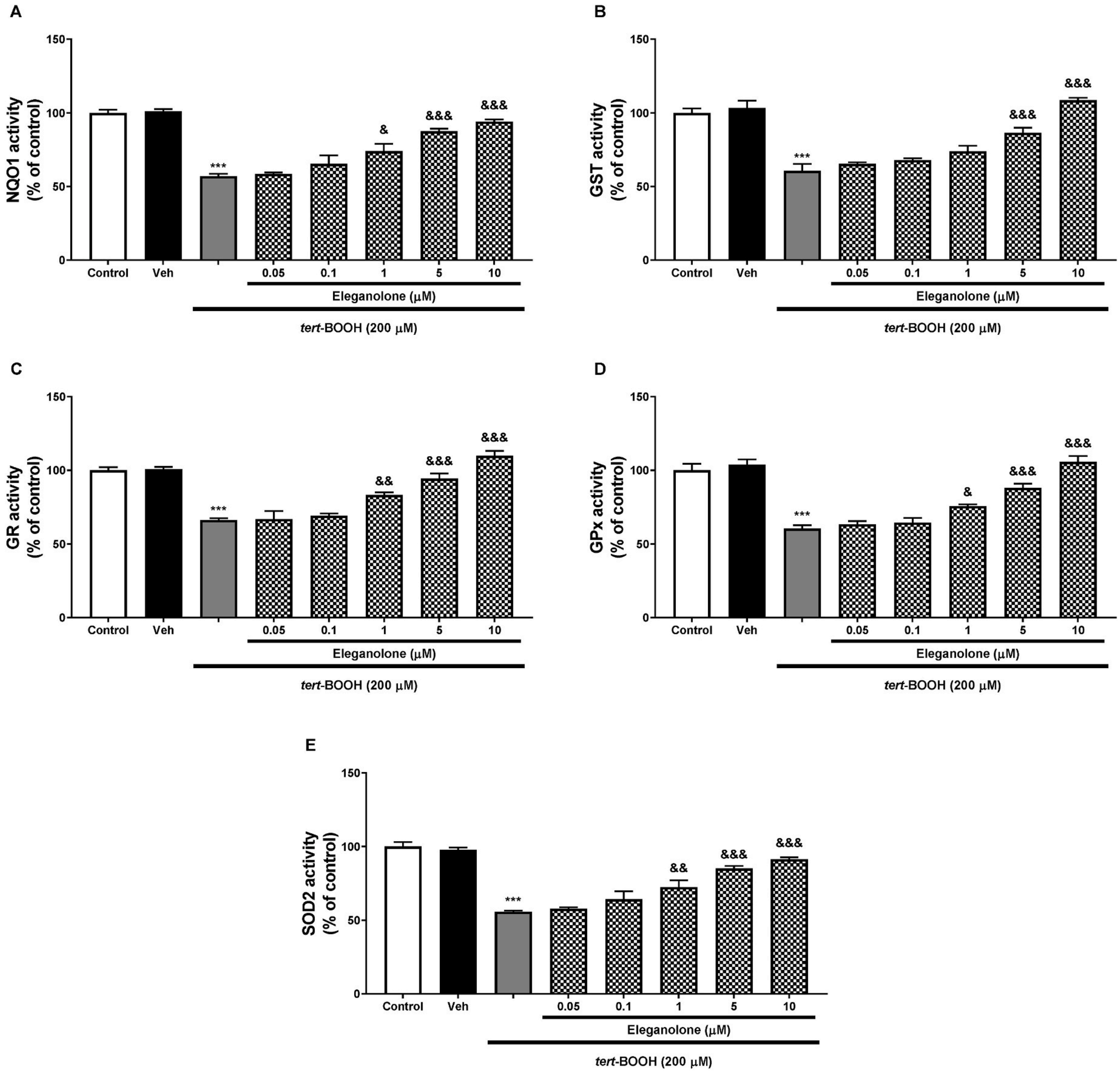

Fig. 6. Protective effect of eleganolone on the activity of antioxidant/detoxification enzymes. SH-SY5Y cells were treated with eleganolone (0.05, 0.1, 1, 5, 10 μM) for 24 h, then submitted to *tert*-BOOH (200 μM) for 3 h. NQO1 **(A)**, GST **(B)**, GR **(C)**, GPx **(D)** and SOD2 **(E)** activities were measured as described in “Materials and methods”. Data represent the mean ± SEM of 4 independent experiments in triplicate. $^{***}P < 0.001$ compared to vehicle (0.1 % DMSO), (black column). $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ compared to *tert*-BOOH (grey column).

3.3.3. Oxidative stress related gene transcriptions

The mRNA expression of key molecules, HO-1 and GSTM2, in response to cellular stress, was examined in SH-SY5Y cells with oxidative damage induced by *tert*-BOOH. Heme oxygenase-1 (HO-1) protein is a key molecule involved in nervous system response to damage. HO-1 up-regulation is recognized as a pivotal mechanism of cell adaptation to stress (Nitti et al., 2018). GSTM2 belongs to the GST family, which is involved in ROS detoxification (Sharma et al., 2004; Strange et al., 2000).

At the present research study, it was found that in *tert*-BOOH-treated cells, the mRNA levels of HO-1 were up-regulated (Table 2A). Conversely, eleganolone, in a dose-dependent manner, downregulated HO-1 mRNA levels (Table 2B and C), which prevent *tert*-BOOH-induced ROS production and cell death. These findings implicate that inhibiting HO-1 overexpression by eleganolone effectively suppressed oxidative stress-mediated *tert*-BOOH in SH-SY5Y cells.

This research study showed that the pro-oxidant *tert*-BOOH compound led to a significant decline of GSTM2 transcription (Table 2A). In contrast, *tert*-BOOH and eleganolone pre-treatment significantly upregulated the transcription of GSTM2 compared with only *tert*-BOOH treatment (Table 2B and C). These observations suggested that *tert*-BOOH exposure obviously induced the overproduction of ROS (Fig. 3B) and the reduction of GSH content (Fig. 5C) through downregulating the expression of GSTM2 redox gen (Table 2A), while eleganolone effectively attenuated this oxidative damage (Table 2B and C). These findings provide valuable information for further studies to recognize the antioxidant ability and neuroprotective potential of eleganolone compound.

3.3.4. Neuronal development related gene transcriptions

The present research study evaluated, in SH-SY5Y cells, whether eleganolone has a protective effect on the expression of selected neuronal development target genes (CAMK2A, NEUROD1, WNT5A, WNT7A, SYN1).

CAMK2A is a serine/threonine protein kinase in neurons that causes neuronal damage when it is abnormally activated (Liu et al., 2020). In this research study, in SH-SY5Y cells, our results showed that the pro-oxidant *tert*-BOOH induced activation of CAMK2A transcript (Table 2A) and eleganolone could protect SH-SY5Y cells against

Table 2

Effects of eleganolone on mRNA expression of genes encoding proteins related to apoptosis (a), inflammation (b), oxidative stress (c) and neuronal development (d) on oxidative stressed SH-SY5Y cells induced by *tert*-BOOH.

Gene symbol	A Fold change *Tert*-BOOH (200 μM)	B Fold change Eleganolone (1 μM) + *tert*-BOOH (200 μM)	C Fold change Eleganolone (10 μM) + *tert*-BOOH (200 μM)
	$^{**}P < 0.01$ and $^{***}P < 0.001$ compared to vehicle	$^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ compared to *tert*-BOOH	$^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ compared to *tert*-BOOH
BAX^{a}	(+) 2.06***	(−) 1.22$^{\&}$	(−) 1.52$^{\&\&\&}$
BCL2^{a}	(−) 1.53**	(+) 1.52$^{\&\&}$	(+) 1.75$^{\&\&\&}$
BNIP3^{a}	(+) 2.73***	(−) 1.37$^{\&\&}$	(−) 1.54$^{\&\&\&}$
p53^{a}	(+) 2.55***	(−) 1.29$^{\&}$	(−) 1.63$^{\&\&\&}$
p38 ^{a}	(+) 2.46***		(−) 1.42$^{\&\&\&}$
APAF1^{a}	(+) 2.56***	(−) 1.49$^{\&\&}$	(−) 1.88$^{\&\&\&}$
NFKB1^{b}	(+) 1.96***	(−) 1.23$^{\&}$	(−) 1.31$^{\&\&}$
TNF-α^{b}	(+) 2.37***		(−) 1.45$^{\&\&}$
IL-6^{b}	(+) 2.89***	(−) 1.29$^{\&}$	(−) 1.52$^{\&\&}$
IL-1β^{b}	(+) 1.98***		(−) 1.27$^{\&\&}$
HO-1^{c}	(+) 2.66***	(−) 1.24$^{\&\&}$	(−) 1.53$^{\&\&\&}$
GSTM2^{c}	(−) 2.04***	(+) 1.54$^{\&}$	(+) 2.20$^{\&\&\&}$
CAMK2A^{d}	(+) 2.24***	(−) 1.19$^{\&}$	(−) 1.35$^{\&\&\&}$
NEUROD1^{d}	(−) 2.18***		(+) 1.47$^{\&\&}$
WNT5A^{d}	(+) 1.89***		(−) 1.40$^{\&\&\&}$
WNT7A^{d}	(+) 2.17***	(−) 1.23$^{\&}$	(−) 1.40$^{\&\&\&}$
SYN1^{d}	(−) 1.91***		(+) 1.50$^{\&\&\&}$

(+) Significantly up-regulated genes.

(−) Significantly downregulated genes.

tert-BOOH-induced neurotoxic injury (Table 2B and C).

NEUROD1 is cell-intrinsically required for the survival and maturation of adult-born neurons (Gao et al., 2009; Pataskar et al., 2016). In this research study, we focus on the gene expression of NEUROD1, a member of the NEUROD family. In SH-SY5Y cells, our results showed that *tert*-BOOH treatment caused a significant downregulation of mRNA expression of the neurogenic gen/transcription factor NEUROD1 (Table 2A), effect which was offset by eleganolone pretreatment (Table 2C). *Tert*-BOOH could act to reduce cellular differentiation by inhibiting important transcription factors in cell fate determination, such NEUROD1. Eleganolone caused an increase in the gene expression of the NEUROD1 in a concentration-dependent manner (Table 2C). These findings suggest a neuroprotective and antioxidant role for eleganolone in *tert*-BOOH-induced cell injury.

WNT proteins are critical for neuronal development and maturation. The WNT signaling pathway takes a role not only during early neuronal connectivity but also in synaptic modulation in the adult brain (Salinas and Zou, 2008). We assessed whether eleganolone protects SH-SY5Y cells against WNT5A, and WNT7A up-regulation induced by the pro-oxidant *tert*-BOOH. The results of this study revealed that the pro-oxidant *tert*-BOOH induced a significant increase in WNT5A, and WNT7A gene expressions (Table 2A). Eleganolone treatment inhibited WNT5A, and WNT7A upregulation induced by *tert*-BOOH (Table 2B and C), suggesting that eleganolone could be a potential agent for preventing neurodegenerative diseases associated with neurodevelopmental disorders.

SYN1 is a synaptic protein essential for neurotransmitter release and synaptic transmission (Greengard et al., 1993). In the present research study, the expression of the neuronal marker SYN1 was evaluated. Decreased mRNA levels of SYN1 were detected in SH-SY5Y cells with oxidative damage induced by *tert*-BOOH (Table 2A). The decrease in SYN1 gene expression observed after *tert*-BOOH exposure could affect neuronal function leading to a deficient neuronal signaling system. However, concentration-dependent eleganolone pretreatment markedly increased SYN1 mRNA expression, which had been downregulated by *tert*-BOOH (Table 2C). These results indicated that eleganolone induces the expression of the SYN1 gene, which is associated with neural function, enhances axonal growth and alleviates oxidative damage induced by *tert*-BOOH.

3.3.5. *Effect of eleganolone treatment on tert-BOOH induced changes of mRNA expression profiles in SH-SY5Y cells. Heat map of differentially expressed genes*

Expression profiling of 17 genes related to apoptosis, inflammation, oxidative stress, and neuronal development pathways in response to the pro-oxidant *tert*-BOOH (200 μM) and eleganolone (1 and 10 μM) was performed using a Real-Time PCR assay in SH-SY5Y cells.

Heat map analysis revealed differentially expressed genes in three treatment groups.

- 200 μM *tert*-BOOH;
- 200 μM *tert*-BOOH +1 μM eleganolone and
- 200 μM *tert*-BOOH +10 μM eleganolone.

The heat map of differentially expressed genes was generated by the hierarchical cluster analysis, and the gene expression is reported in the heat map as highly expressed (red color), and low expressed (blue color) (Fig. 7). The heat map and hierarchical clustering analysis of 17 genes revealed different expression profiles of modulated genes from the three different groups (Fig. 7). In this research study, 13 genes were upregulated (red color) and 4 genes were downregulated (blue color) in the *tert*-BOOH (200 μM) group (Fig. 7). Whereas the eleganolone groups (200 μM *tert*-BOOH + 1 μM eleganolone and 200 μM *tert*-BOOH + 10 μM eleganolone), showed significant differential expressions of genes compared to 200 μM *tert*-BOOH group. From the heat map, 13 genes were downregulated (blue color) and 4 genes were upregulated (red color) (Fig. 7), which indicated that the effect of eleganolone on pro-oxidant *tert*-BOOH-treated SH-SY5Y cells may occur through the regulation of gene expression patterns. Gene modulation overview is presented in Fig. 7.

4. Discussion

Macroalgae have been shown to be particularly rich in bioactive substances, which can be exploited as functional foods or in cosmetic and pharmaceutical formulations (Holdt and Kraan, 2011; Mayer and Hamann, 2002; Shannon and Abu-Ghannam, 2019; Wijesinghe and Jeon, 2011). Particular attention has been given to brown macroalgae, especially *B. bifurcata*, due to the availability of bioactive molecules, such as linear diterpenes, which enhance the cellular response to an oxidative challenge (Martínez et al., 2023; Pais et al., 2019). In this research study, we demonstrate that the compound eleganolone, isolated from the brown macroalgae *B. bifurcata*, has the ability to protect human neuroblastoma SH-SY5Y cells against oxidative damage by regulating ROS generation, MDA and NO production, GSH concentration, antioxidant enzyme activities and mRNA expression of genes encoding proteins related to apoptosis, inflammation, oxidative stress and neuronal development.

Reactive oxygen species (ROS) and some non-radical oxygen derivatives are generated as common cellular metabolic by-products in cells. However, cells can produce excessive levels of ROS under conditions of stress, which can cause cellular damage and death (He et al., 2017). Exposure to high concentrations of ROS can cause non-specific damage to proteins, lipids and nucleic acids, whereas low to middle concentrations of ROS exert their effects mainly through the regulation of cell signaling pathways (Brieger et al., 2012). Research studies have shown that excessive ROS production can damage the cell membrane structure, induce lipid peroxidation, and led to accumulation of corresponding by-products, such as MDA (Xie et al., 2020). In this research study, we demonstrate that the compound eleganolone has the ability to

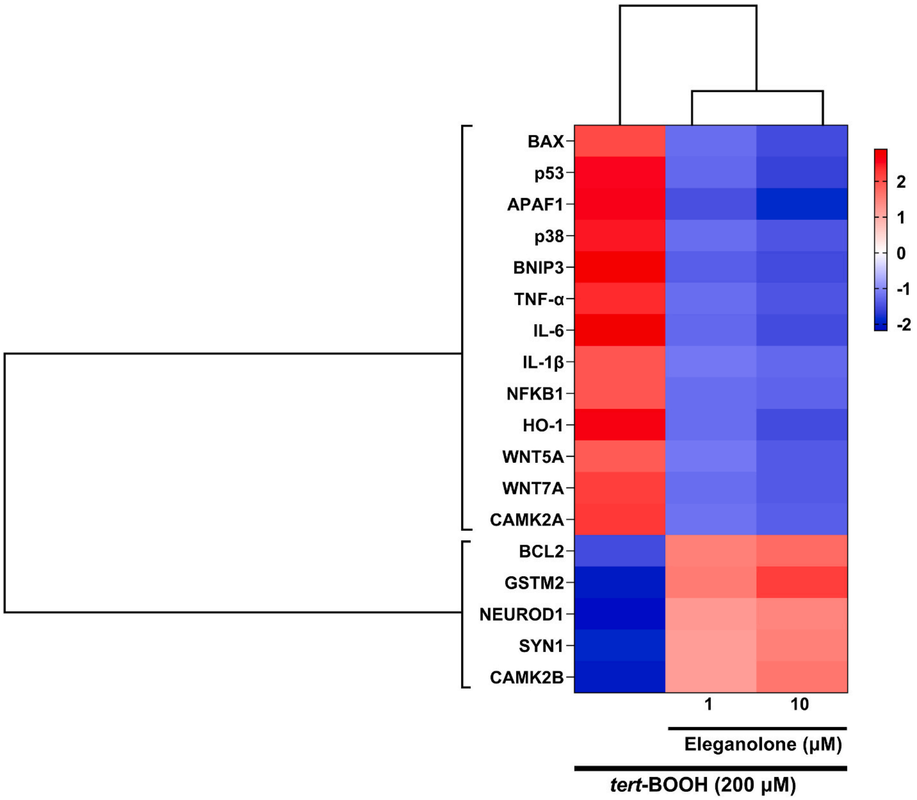

Fig. 7. Heat map and hierarchical cluster analysis of differentially expressed genes between *tert*-BOOH and eleganolone plus *tert*-BOOH-treated SH-SY5Y cells. SH-SY5Y cells were treated with eleganolone (1 and 10 μM) for 24 h, then submitted to *tert*-BOOH (200 μM) for 3 h. Red color represents upregulated genes and blue color represents downregulated genes (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protect human neuroblastoma SH-SY5Y cells against oxidative damage by decreasing ROS generation, MDA (primary product of lipid peroxidation), NO production (Wei et al., 2000), and increasing GSH concentration, primary scavenger of oxygen radicals (Berndt and Lillig, 2017).

Assessment of endogenous antioxidant enzymes activity is considered an important biomarker for the study of cellular response to oxidative stress. Antioxidant enzymes, NQO1, GST, GR, GPx and SOD2, are important components in cellular defense against the generation of free radicals and in the prevention and repair of molecular damage produced. Antioxidant enzymes are important for maintaining oxidant homeostasis and have been extensively studied for their capacity to scavenge oxygen free radicals *in vivo* (Lee and Park, 2021; Wheeler et al., 1990). The results of the present study on NQO1, GST, GR, GPx and SOD2 activities demonstrated that eleganolone suppressed *tert*-BOOH-induced oxidative stress.

Activation of caspase 3/7 activity is a critical event that integrates upstream signals in the final process of cell death. This converts caspase 3/7 into an interesting therapeutic target, and the search for caspase 3/7 inhibitors is an important task that many pharmaceutical companies are taking up in the search and develop of highly selective caspase inhibitors as potential pharmaceutical drug candidates (Lee et al., 2000; Nuttall et al., 2001; Okun et al., 2006). In the present research study, eleganolone resulted in a reduction of the oxidative stress condition (*tert*-BOOH) and an anti-apoptotic effect (decreased caspase 3/7 activity). Eleganolone may be a useful lead for the rational design of caspase-specific inhibitors as therapeutic agent. Caspases have been strongly implicated in playing an essential role in apoptosis. Apoptosis concerns the morphological characteristics of programmed cell death. Apoptosis provides important biological functions in cell development and homeostasis, as well as in the pathogenesis and expression of pathological processes. Apoptosis is regulated via the extrinsic pathway by the death receptor and the intrinsic pathway by the mitochondria (Saikumar et al., 1999). In this research study, we demonstrated that eleganolone reduces the activation of caspase 3/7 induced by *tert*-BOOH, as well as decreases the expression of the pro-apoptotic BAX BNIP3, p53, p38 and APAF1 genes, along with increasing those of the anti-apoptotic BCL2 mRNA levels. On these bases, we can assume that, at least in part, eleganolone prevents the apoptosis elicited by the prooxidant *tert*-BOOH through the protection of mitochondria.

Moreover, in this research study we examined eleganolone effects on modulation of the gene expression related to inflammation (NFKB1, TNF-α, IL-6, IL-1β), oxidative stress (HO-1, GSTM2) and neuronal development (CAMK2A, NEUROD1, WNT5A, WNT7A, SYN1) after treatments with *tert*-BOOH. Inflammation is a neuroprotective mechanism. However, chronic inflammation in the CNS causes neurotoxicity and neurodegeneration. Several factors affecting neuroinflammation and the main inflammatory signaling pathways involved in the pathogenicity of neurodegenerative diseases have been described (Zhang et al., 2023). We demonstrate that eleganolone markedly reduces the expression of proinflammatory cytokines TNF-α, IL-6 and IL-1β and transcription factor NFKB1 in SH-SY5Y cells, indicating an interesting anti-inflammatory role of this natural molecule.

Another important aspect of eleganolone is its potential antioxidant activity. Oxidative stress from excessive ROS production plays a role in the development of chronic diseases (Rigoulet et al., 2011). Regulation of intracellular ROS is critical for maintaining a healthy life, and research on antioxidants is relevant to find natural compounds with protective effects against oxidative stress (Kim et al., 2019). Heme oxygenase-1 (HO-1) protein is well known as a protective enzyme under a variety of cellular stress situations (Alam et al., 2004). HO-1 protein activation in neurons is highly protective against oxidative damage and cell death (Chen et al., 2000; Le et al., 1999). Moreover, its upregulation is clearly linked to the late phase of neurodegeneration and has been considered as a biomarker for neurodegenerative diseases (Liu et al., 2023; Schipper, 2007). On the other hand, GSTM2 protein has been implicated in cellular anti-oxidative stress defense to protect cells from oxidative stress-associated damage and cell death (Zhou et al., 2008). The present study supports that eleganolone downregulated HO-1 mRNA levels following treatment with *tert*-BOOH. These results suggest that the inhibition of HO-1 overexpression by eleganolone effectively suppressed *tert*-BOOH-induced oxidative stress in SH-SY5Y cells. As demonstrated by real-time RT-PCR, eleganolone induced the expression of the GSTM2 in SH-SY5Y cells exposed to *tert*-BOOH. These findings provide valuable information for further studies to recognize the antioxidant ability and neuroprotective potential of eleganolone compound.

Mammalian neurogenesis can be described as a process of functional neurons generation. The brain capacity to generate new neurons is linked to different development stages. Human studies have demonstrated the persistence of neurogenesis throughout life in several brain areas (Aimone et al., 2014; Tobin et al., 2019). Transcriptional regulation is critical for proper cellular functions through development and postnatal life. The natural compounds with neuronal-inducing properties are of high value for neuronal regenerative medicine and offer strategies for the prevention and treatment of neurological disorders (An et al., 2022). Neuronal damage is the primary endpoint of neurological diseases. Regulation of neurogenesis and neuronal differentiation is relevant for developing therapies to promote neuronal repair or reconstruction of the synaptic network. Neuronal differentiation is highly complex because it can involve different cell types and can be stimulated by a variety of inducers. The present research study evaluated whether eleganolone has a protective effect on the mRNA levels of selected neuronal development target genes (CAMK2A, NEUROD1, WNT5A, WNT7A, SYN1).

CAMK2 is a calcium-activated kinase, which has been shown to be modulated by oxidation. CaMK2 induces oxidative stress participating in pathophysiological processes and causing cell damage (Qu et al., 2019). Recent discoveries reveal the role of CAMK2 genes linked to neurodevelopmental disorders (human cognitive and adaptive functions). CAMK2 is encoded by four distinct genes, known as CAMK2A, CAMK2B, CAMK2G, and CAMK2D; CAMK2A gene is the one most commonly expressed in the mammalian brain (Proietti Onori and van Woerden, 2021).

Transcription factors of the NEUROD family are crucial in the development of various regions of CNS. Recent findings indicate a link between the dysfunction of these transcription factors and the onset of various neurological diseases (Tutukova et al., 2021). NEUROD1 is essential for eliciting the neuronal development program and possesses the ability to reprogram other cell types into neurons.

WNT proteins regulate critical functions such as neuronal migration, neuronal polarization, axon guidance, dendrite development, and synapse formation which are essential for correct brain function (Inestrosa and Arenas, 2010). Evidence supports the role of WNT proteins in neurodevelopment and neurodegenerative diseases (De Ferrari and Moon, 2006; Libro et al., 2016). Developmental neurotoxicity is defined as any adverse effect of a toxicant on the normal development of the nervous system before or after birth. Human neuroblastoma SH-SY5Y cells can be used to detect chemical effects on cellular events of neuronal development, such as neurite outgrowth (Martínez et al., 2020a, 2020b; Radio and Mundy, 2008).

Synapsins (SYNs) are phosphoproteins implicated in neuronal development and neurotransmitter release. In mammals, SYNs are encoded by three distinct genes SYN1, SYN2, and SYN3. Alterations in the balanced expression of SYNs genes induce critical deregulations in the functions of brain networks causing psychiatric or neurological disorders (Longhena et al., 2021).

The present data highlight that eleganolone has antiapoptotic, anti-inflamatory, antioxidative, and neuronal development properties, reducing *tert*-BOOH induced neuronal damage. Collectively, our results emphasize the potential of eleganolone as a promising molecule that strongly protects the cells against an oxidative insult.

5. Conclusions

The brown seaweed *B. bifurcata* is a relevant source of bioactive compounds with a wide range of biological activities, including antioxidant properties. In the present research study, bio-guided fractionation of the seaweed *B. bifurcata* extract led to the isolation of eleganolone, the main diterpenoid described in *B. bifurcata*. This research study showed that treatment of human neuroblastoma SH-SY5Y cells with eleganolone strongly protected the cells against *tert*-BOOH-induced oxidative damage. The protective effects of eleganolone were related to the induction of the antioxidant system as well as via regulation of mRNA expression related to apoptosis, inflammation, oxidative stress and neuronal development signaling pathways. Our results provide new evidence for the molecular mechanisms implicated in the protective effects exerted by eleganolone and confirm its potential role as an antioxidant agent in the prevention of oxidative stress injury.

CRediT authorship contribution statement

María-Aránzazu Martínez: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Bernardo Lopez-Torres:** Validation, Methodology, Investigation, Formal analysis. **Jorge-Enrique Maximiliano:** Validation, Methodology, Investigation, Formal analysis. **Marta Martínez:** Validation, Methodology, Investigation, Formal analysis. **María-Rosa Martínez-Larrañaga:** Validation, Methodology, Investigation, Formal analysis. **Irma Ares:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Arturo Anadón:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Cesar Peteiro:** Writing – original draft, Investigation, Conceptualization. **Teresa Aymerich:** Methodology, Investigation, Funding acquisition, Conceptualization. **Andrea Casal-Silva:** Methodology, Investigation. **Mercedes Cueto:** Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2025.177977.

Data availability

Data will be made available on request.

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