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Bifurcaria bifurcata extract exerts antioxidant effects on human Caco-2 cells

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Abstract:	The present research study investigated the potential protective effect of Bifurcaria bifurcata extract on cell viability and antioxidant defences of cultured human Caco-2 cells submitted to oxidative stress induced by tert-butylhydroperoxide (tert-BOOH). Aqueous extracts were firstly characterized in terms of total phenolic contents. Concentrations of reduced glutathione (GSH) and malondialdehyde (MDA), generation of reactive oxygen species (ROS), nitric oxide (NO) production, antioxidant enzymes activities [NADPH quinone dehydrogenase 1 (NQO1) and glutathione S-transferase (GST)], caspase 3/7 activity and gene expression linked to apoptosis, proinflammation and oxidative stress signaling pathways were used as markers of cellular oxidative status. B. bifurcata extract prevented the cytotoxicity, the decrease of GSH, the increase of MDA levels and the ROS generation induced by tert-BOOH. B. bifurcata extract prevented the significant decrease of NQO1 and GST activities, and the significant increase of caspase 3/7 activity induced by tert-BOOH. B. bifurcata extract also caused an over-expression of GSTM2, Nrf2 and AKT1 transcriptors, as well as reduced ERK1, JNK1, Bax, BNIP3, NFκB1, IL-6 and HO-1 gene expressions induced by tert-BOOH suggesting an increase in cellular resistance against oxidative stress. The results of the biomarkers analyzed show that treatment of Caco-2 cells with B. bifurcata extract enhance antioxidant defences, which imply an improved cell response to an oxidative challenge. B. bifurcata extract possesses strong antioxidant properties and may be a potential effective alternative to oxidant agents in the functional food industry.

Bifurcaria bifurcata extract exerts antioxidant effects on human Caco-2 cells

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ABSTRACT

The present research study investigated the potential protective effect of *Bifurcaria bifurcata* extract on cell viability and antioxidant defences of cultured human Caco-2 cells submitted to oxidative stress induced by *tert*-butylhydroperoxide (*tert*-BOOH). Aqueous extracts were firstly characterized in terms of total phenolic contents. Concentrations of reduced glutathione (GSH) and malondialdehyde (MDA), generation of reactive oxygen species (ROS), nitric oxide (NO) production, antioxidant enzymes activities [NADPH quinone dehydrogenase 1 (NQO1) and glutathione S-transferase (GST)], caspase 3/7 activity and gene expression linked to apoptosis, proinflammation and oxidative stress signaling pathways were used as markers of cellular oxidative status. *B. bifurcata* extract prevented the cytotoxicity, the decrease of GSH, the increase of MDA levels and the ROS generation induced by *tert*-BOOH. *B. bifurcata* extract prevented the significant decrease of NQO1 and GST activities, and the significant increase of caspase 3/7 activity induced by *tert*-BOOH. *B. bifurcata* extract also caused an over-expression of GSTM2, Nrf2 and AKT1 transcriptors, as well as reduced ERK1, JNK1, Bax, BNIP3, NFκB1, IL-6 and HO-1 gene expressions induced by *tert*-BOOH suggesting an increase in cellular resistance against oxidative stress. The results of the biomarkers analyzed show that treatment of Caco-2 cells with *B. bifurcata* extract enhance antioxidant defences, which imply an improved cell response to an oxidative challenge. *B. bifurcata* extract possesses strong antioxidant properties and may be a potential effective alternative to oxidant agents in the functional food industry.

KEYWORDS: Antioxidant defences*; B. bifurcata* extract; Phenolic and carbohydrate derivatives; Oxidative stress biomarkers.

Abbreviations:

^{1}H-NMR	proton nuclear magnetic resonance spectroscopy
^{13}C-NMR	carbon-13 nuclear magnetic resonance
AKT1	V-akt murine thymoma viral oncogene homolog 1
ANOVA	analysis of variance
Bax	Bcl2-associated X protein
Bcl2	B-cell lymphoma 2
BNIP3	Bcl2 interacting protein 3
BSA	bovine serum albumin
CD_3OD	methanol-d4
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
COSY	correlated spectroscopy
D_2O	deuterium oxide
DAF-2	4,5-diaminofluorescein
DAF-FM-DA	4-amino-5-methylamino-2,7-difluorofluorescein diacetate
DCFH	2,7-dichlorofluorescein
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DEVD	aspartic acid-glutamic acid-valine-aspartic acid
DMEM	Dulbecco's modified eagle medium
DMEMF12	Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECACC	European Collection of Authenticated Cell Cultures
EDTA	ethylenediaminetetraacetic acid
ERK1	extracellular signal-regulated kinase 1

EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione reduced
GST	glutathione S-transferase
GSTs	glutathione S-transferases
GSTM2	glutathione-S-transferase mu 2
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HMBC	heteronuclear multiple bond correlation
HO-1	heme oxygenase 1
HSQC	heteronuclear single quantum coherence
ICN	International Code of Nomenclature for algae, fungi, and plants
IL-6	interleukin 6
JNK1	c-Jun N-terminal kinase 1
LDH	lactate dehydrogenase
MDA	malondialdehyde tetrabutylammonium salt
mRNA	messenger RNA
MTT	3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NaCl	sodium chloride
NADPH	β-nicotinamide adenine dinucleotide phosphate
NFκB1	nuclear transcription factor kappa B subunit 1
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NO	nitric oxide
NQO1	NADPH quinone dehydrogenase 1
Nrf2	nuclear factor-E2-related factor 2

$ONOO^-$	peroxynitrite
OPT	*o*-phthalaldehyde
PCR	polymerase chain reaction
PUFAs	polyunsaturated fatty acids
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
tert-BOOH	*tert*-butyl hydroperoxide

1. Introduction

Macroalgae (or seaweeds) are critical habitat-structuring species in coastal ecosystems (**Reisewitz et al., 2006; Bertocci et al., 2015**) and some species are important sources of specific components such as antioxidants, pigments, oils, and vitamins (**Hemantkumar and Rahimbhai, 2019; Niccolai et al., 2019**). Seaweeds have been widely used in the food industry as a stabilizer, emulsifier, gelling and thickening agent to improve the shelf life, nutritional, textural, and organoleptic properties of different food products (**Roohinejad et al., 2017)**. Seaweed consumption has been associated with a range of health benefits, such as anticancer, immune-modulatory, antiviral, antibacterial and antioxidant activities which are attributed to seaweed constituents, like sulphated polysaccharides, carrageenans, fucoidans, terpenoids, polyphenols and polyunsaturated fatty acids (PUFAs) (**Smit, 2004; Erpel et al., 2020; Peñalver et al., 2020; Besednova et al., 2022; Matulja et al., 2022**). Some seaweed species collected along the European coasts are used as feedstock for the extraction of food, pharmaceutical, biomedical or biotechnological grades of the hydrocolloids alginate and agar (**Kraan, 2012; Peteiro, 2018**). Algae biomass is also used in feed for aquaculture (**Makkar et al., 2016; Wan et al., 2019**) and recently explored as a feed supplement for cattle to improve the weight gain while reducing enteric methane emissions (**Machado et al., 2014**; **Roque et al., 2019**; **Kinley et al., 2020**). Further uses include fertilizer and plant bio-stimulant applications as well as a source of high quality and high-value bioactive products for cosmetics and nutraceuticals (**Thomas and Kim, 2013; Chatterjee et al., 2017**). The global production of seaweeds is mostly used for human consumption (80%), and about 20% for hydrocolloid (agar, alginates and carrageenan) production as functional ingredients in commercial applications as stabilizers, emulsifiers, thickening agents and texture modifiers (**EUMOFA, 2018**). The economic potential of the algae industry products is widely recognized and their bioactivities have recently gained a considerable interest due to their multiple applications in the food and/or pharmaceutical industries.

Marine resources are considered as a very promising source of bioactive molecules, and macroalgae in particular have gained special attention, due to their structurally diverse composition. Amongst the macroalgae species (close to 10,000), only a limited number have been the object of extensive research studies due to their unique composition and consequent diversity of biological activities or health benefits. Particular interest has been devoted to the brown macroalga *Bifurcaria bifurcata*, due to their abundance in bioactive linear diterpenes. Many diterpenes have been demonstrated to possess antimicrobial and antifungal activities (**Gupta and Abu-Ghannam, 2011; Pais et al., 2019**). Because of *B. bifurcata* extract reveals to be a promising source of compounds with potential against oxidative stress, the aim of the present research study was to evaluate, in human colon carcinoma Caco-2 cells, the underlying mechanisms of the potential protective effect of different *B. bifurcata* extract concentrations against an oxidative stress chemically induced by the potent pro-oxidant, *tert*-butyl hydroperoxide (*tert*-BOOH**).** To this end, cell viability (MTT) and lactate dehydrogenase (LDH) assays; concentration of reduced glutathione (GSH); determination of malondialdehyde (MDA) levels as marker lipid peroxidation; generation of reactive oxygen species (ROS); determination of nitric oxide production (NO); evaluation of the activity of antioxidant enzymes: NADPH quinone dehydrogenase 1 (NQO1) and glutathione S-transferase (GST); evaluation of caspase 3/7 activity as a marker of apoptosis and messenger RNA (mRNA) expressions linked to apoptosis/oxidative mediators were investigated. Caco-2 cells are widely used for biochemical and nutritional research studies as a cell culture model of human colonocytes since they retain their morphology and most of their function in culture (**Sambuy et al., 2005; Phelan et al., 2009**).

2. Material and Methods

2.1. Chemicals and reagents

The compounds *tert*-butyl hydroperoxide (*tert*-BOOH), 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), menadione, dicoumarol, malondialdehyde tetrabutylammonium salt (MDA), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), 2-thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), Tween 20, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reduced (GSH), *o*-phthalaldehyde (OPT), ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid and primer genes were purchased from Sigma-Aldrich, St Louis, MO 63103, USA. Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEMF12) and Dulbecco's modified eagle medium (DMEM) were obtained from Biowhitaker Lonza (Walkersville, MD, USA). Penicillin and streptomycin were obtained from Invitrogen (Madrid, Spain). The fluorescent probes 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM-DA) were acquired from Sigma-Aldrich and Invitrogen, respectively. The kit Caspase-Glo® 3/7 assay was purchased from Promega Corporation, Madison, WI 53711-5399, USA. All other chemicals were of the highest quality grade and obtained from commercial sources.

2.2. Macroalga collection, identification and preparation

The seaweed *Bifurcaria bifurcata* R. Ross 1958 was collected during the summer season of 2017 (23-24th August) from Comillas, Cantabria, Spain (43° 23′ N and 4° 17′ W) in the Atlantic coast of northern Spain. Sample collections were done at the same rocky shore site in the middle intertidal zone for *B. bifurcata*. The Cantabrian coast (northern Spain) is included in the warm-temperate area of the Atlantic region. *B. bifurcata* grows without problems in zones with high presence of sediments in the Coast of Cantabria, particularly in the Comillas area where the water quality is an important aspect to be considered. *B. bifurcata* can be considered a good

indicator of undisturbed habitats, quickly deteriorate when contaminants are introduced in the environment (**Díez et al., 2003**). Adult fresh specimens were selected and carefully washed with fresh seawater to remove sand or remaining debris, and any epiphytes and animals attached to the algal surface. They were then wrapped in sterile cloths moistened with seawater and kept dark and cool with ice packs (<15°C) to preserve the algae alive and healthy until transport them to the laboratory where they were stored until further use. The proper identification of seaweed specimens was based on morphological characters using specialized taxonomic keys of Iberian Fucales (**Gómez Garreta et al., 2000**). The currently accepted scientific names of seaweed species, with recent synonyms in brackets, are given according to Algaebase (**Guiry and Guiry, 2022**) and following the rules of the International Code of Nomenclature (ICN) for algae, fungi, and plants (**Turland et al., 2018**). The brown algae (class *Phaeophyceae*) used in this research study is member of the order Fucales (also known as fucoids), which is commonly dominant seaweed in cold-temperate rocky shores of North Atlantic. The seaweed *B. bifurcata* is restricted to the Atlantic coasts from the British Isles to Senegal (**Fletcher, 1987; Gómez Garreta et al., 2000**); this perennial fucoid is particularly abundant along northern Atlantic coasts of Spain. This macroalga often grows in the form of large and dense monospecific stands (the so-called brown algal beds or forests) that are available in exploitable quantity.

2.3. Preparation of seaweed extract

The homogenized sample was analyzed in triplicates. Samples of 3.5 ± 0.01 g were placed in an amber tube and soaked in the extraction solvent (hexane: ethanol, 1:1, v/v) in an orbital homogenizer for 60 min. The extracts were centrifuged at 5000 rpm for 10 min at 4 °C, and the extractions were repeated until a colorless supernatant was observed. The pellet was re-extracted three times with 30 mL in a basic solution (pH 10) during 60 min. in an orbital homogenizer at room temperature. The extracts were centrifuged at 5000 rpm for 10 min. at 4°C, and the supernatants were pooled and lyophilized. The residue was stored at -20 °C until the analysis.

2.4. Characterization of B. bifurcata extract

To perform the total phenolic and carbohydrate content 100 mg of the obtained residue were dissolved in 1 mL water. The total polyphenolic content of algal extracts was determined according to the method of **Wang et al. (2012)** with a minor modification. 100 µL of the basic extract were mixed with 50 µL Folin-Ciocalteu reagent (Sigma-Aldrich, Germany), 450 µL water and 500 µL of 12.5% (w/w) sodium carbonate. The solution was incubated at 45 °C for 30 min. 250 µL were transferred to a microplate and the absorbance was measured at 760 nm in a Varioskan spectrophotometer (Thermo Fischer Scientific, USA) using phloroglucinol as standard reference (Sigma-Aldrich, Germany). The phenolic content of the extract was 2.60 µg phenolics/mg residue expressed as phloroglucinol.

The carbohydrate concentration was determined by the phenol-sulfuric acid reaction, colorimetric method described by **DuBois et al. (1956**). 200 µL of extract was thoroughly mixed with 200 µL 5% phenol and 1 mL sulfuric acid (96%). Once the reaction was developed at 30°C for 15 min, 250 µL were transferred to a microplate. The absorbance was recorded at 490 nm in a Varioskan spectrophotometer (Thermo Fischer Scientific, USA) using glucose as standard reference (Sigma-Aldrich, Germany). The carbohydrate content of the extract was 5.84 µg carbohydrate/mg residue expressed as glucose.

The nuclear magnetic resonance spectroscopy (NMR) analysis of the extract *B. bifurcata* was also conducted. Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), correlated spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOESY) spectrum were measured employing a Bruker Avance II-500 spectrometer (Bruker, Fällanden, Switzerland) operating at 500 MHz for ^{1}H-NMR and at a 125.7 MHz for ^{13}C-NMR. Two-dimensional spectra were obtained with the standard Bruker software. 1D and 2D NMR spectra analysis of the extract of *B. bifurcata* in deuterium oxide

(D_2O) indicated that citric acid (**Pinto et al., 2021**) and α-hydroxybutyric acid (**Sciubba et al., 2020**) were major compounds present in this extract that dissolve in D_2O. The ^{1}H-NMR spectrum showed signals between 3.5 and 4.0 ppm that imply the presence of sacharides and signals in the aromatic area at $\delta = 6.79$; 6.86; 7.28 and 7.31 ppm that should correspond to phlorotannins although phloroglucinol (**Nazir et al., 2021**) has not been detected. Moreover, 1D and 2D NMR spectra analysis of the extract of *B. bifurcata* in methanol-d4 (CD_3OD) allowed us to identify the presence of 3-hydroxy-valine (**Seiple et al., 2014**) as well as acetic acid (**Sciubba et al., 2020**) in the extract. NMR spectra data of the extract *B. bifurcata* are presented in Supplementary Data.

2.5. Cell culture

Human Caco-2 cells from the European Collection of Authenticated Cell Cultures (ECACC 86010202, Sigma-Aldrich) were used in passages 14-30. Caco-2 cells were maintained in DMEMF12 medium supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For assays, Caco-2 cells were sub-cultured in 96-well plates at a seeding density of 8 x 10^4 cells per well. Cells were allowed to attach for 24 h before *B. bifurcata* extract treatment. The culture medium was changed every other day in order to remove the non-adhered and dead cells. In the assays, cell culture plates with FBS were changed to FBS-free medium to reduce analytical interference and provide more reproducible experimental conditions.

2.6. Cell treatment

To study a direct effect of the *B. bifurcata* extract, different concentrations of extract (10, 50, 100, 250 and 500 μg/mL), dissolved in serum-free culture medium, were added to the cell

plates for 24 h. At the end of the incubation period, cell cultures were processed depending on the assay.

In order to generate a condition of cellular oxidative stress, Caco-2 cells were also incubated with *tert*-BOOH, a strong pro-oxidant compound (**Sestili et al., 2002**; **O'Sullivan et al., 2012**). The protective effect of *B. bifurcata* extract against an oxidative stress induced by the pro-oxidant, *tert*-BOOH, was also determined.

2.7. Cell viability (MTT assay)

MTT assay is a common tool to measure cell proliferation/viability, drug cytotoxicity, and mitochondrial/metabolic activity of cells (**Stockert et al., 2018**). Cell viability was first measured for *B. bifurcata* extract (10, 50, 100, 250, 500 μg/mL) by quantitative colorimetric assay with MTT, as previously described (**Denizot and Lang, 1986**). Caco-2 cells were grown in a 96-well plate. After exposure (24 h) to *B. bifurcata* treatments, 22 μL of the MTT labeling reagent, at a final concentration of 0.5 mg/mL, was added to each well and the plate was placed in a humidified incubator at 37 ºC with 5% CO_2 and 95% air (v/v) for a period of 2 h. MTT tetrazolium salt is reduced to insoluble purple formazan compound by dehydrogenase enzyme present in the viable cells at 37 °C (**Ghasemi et al., 2021**). Formazan was dissolved with DMSO, and the colored product was measured at 540 nm using a microplate reader (BMG LABTECH GmbH SPECTROStarNano, Ortenberg, Germany). Control cells treated with DMEM were taken as 100% viability.

2.8. Evaluation of cytotoxicity: lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (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 cells undergoing apoptosis, necrosis, and other forms of cellular damage (**Kumar et al., 2018**). Cytotoxic effects of *B. bifurcata* extract (10, 50, 100, 250, 500 μg/mL) on

Caco-2 cells were evaluated by measuring LDH leakage into the extracellular fluid. After *B. bifurcata* extract exposure (24 h), samples were collected to estimate extracellular LDH as indication of cell death. LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche-Boehringer, Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; total LDH (intracellular plus extracellular) was normalized as 100%; then, the amount of LDH released to the extracellular medium was expressed as percentage of this total value. LDH activity was measured spectrophotometrically at 490–620 nm, using a microplate reader (BMG LABTECH GmbH SPECTROStarNano, Ortenberg, Germany).

2.9. Determination of reduced glutathione (GSH) and evaluation of malondialdehyde (MDA) levels.

As an index of the intracellular non-enzymatic antioxidant defenses, the concentration of GSH was measured. The content of GSH was quantitated by the fluorometric assay of **Hissin and Hilf (1976)**. The method takes advantage of the reaction of GSH with OPT at pH 8.0. After treatment with *B. bifurcata* extract (10, 50, 100, 250, 500 µg/mL) for 24 h, the culture medium was removed and cells were detached and homogenized by ultrasound with 5% (w/v) trichloroacetic acid containing 2 mM EDTA. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The content of GSH against cytotoxic effect of *tert*-BOOH and the protecting effect of *B. bifurcata* extract was also evaluated.

MDA is the main and most studied product of PUFA peroxidation. In this research study we evaluated, as a biomarker for lipid peroxidation, the cytoplasmic concentration of MDA induced by *B. bifurcata* extract (10, 50, 100, 250, 500 µg/mL) after 24 h incubation period. Cellular MDA levels were quantified by the thiobarbituric acid reactive substances (TBARS) assay kit (Cell Biolabs, Inc., San Diego, CA). The goal of the TBARS assay is to assess oxidative stress by measuring the production of lipid peroxidation products, primarily MDA,

using a spectrophotometric plate reader (BMG LABTECH GmbH SPECTROStarNano, Ortenberg, Germany) at 532 nm. MDA production for cytotoxic effect of *tert*-BOOH and the protecting effect of *B. bifurcata* extract was also evaluated. The assay has been previously described **(Martínez et al., 2021**).

2.10. Determination of reactive oxygen species (ROS)

Cellular ROS generation was quantified by the 2,7-dichlorofluorescein (DCFH) assay using a microplate reader (**Wang and Joseph, 1999**). After being oxidized by intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. For the assay of direct effect of *B. bifurcata* extract (10, 50, 100, 250, 500 µg/mL), Caco-2 cells were seeded in 96-well plates at a rate of 8 x 10^4 cells per well in medium containing FBS and replaced to FBS-free medium and the different extract concentrations. By quantifying fluorescence over a period of 60 min, a reliable estimation of the overall oxygen species generated under the different conditions was obtained. This parameter gives a very good evaluation of the degree of cellular oxidative stress. ROS formation for cytotoxic effect of *tert*-BOOH and the protecting effect of *B. bifurcata* extract was also evaluated. The assay has been previously described **(Martínez et al., 2021**).

2.11. Evaluation of nitric oxide (NO) production

NO is an arginine-derived radical playing a pivotal role in numerous physiologic as well as pathophysiologic processes. 4,5-Diaminofluorescein (DAF-2) and its membrane permeable derivate DAF-2 diacetate are fluorescent probes that have been developed to perform real-time biological detection of NO (**Leikert et al., 2001).** In the present research study, NO production was detected by spectrofluorometric following the optimized method for the detection of NO in the low-nM range developed for **Leikert et al. (2001).** Briefly, cells were seeded in 96-well multiwells (8 x 10^4 cells per well) and incubated with *B. bifurcata* extract (10, 50, 100, 250 and

500 µg/mL) for 24 h. After that, 2.5 µM DAF-2 was added to the wells for 30 min at 37 °C. Then, cells were washed twice with DPBS and replaced with FBS-free medium and incubated for an additional 15-30 min. The fluorescence of cells was evaluated in a fluorescent microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 515 nm (FLx800 Fluorimeter, BioTek, Winooski, USA).

2.12. Determination of NADPH quinone dehydrogenase 1 (NQO1) activity

NQO1 is a flavoenzyme that catalyzes two electron reduction of quinones and some other electrophiles by using NADPH as an electron donor. NQO1 activity was measured following the method of **Tsvetkov et al. (2005)** based on the decrease in NADPH absorbance at 340 nm. Caco-2 cells were incubated with *B. bifurcata* extract (10, 50, 100, 250, 500 µg/mL) for 24 h. Then, cells were mechanically homogenized in 200 µl of buffer saline HEPES (50 mM HEPES plus 154 mM NaCl, pH 7.5). The homogenate was centrifuged (2400 rpm for 5 min at 4 °C). The pellet was discarded and the supernatant was stored at -80° C until further analysis. The NQO1 activity against cytotoxic effect of *tert*-BOOH and the protecting effect of *B. bifurcata* extract was also evaluated. The assay has been previously described **(Martínez et al., 2021**). Protein contents were measured according to the method of **Lowry et al. (1951)** using BSA. All spectrophotometric measurements were recorded on BMG Labtech GmbH SPECTROStarNano spectrophotometer.

2.13. Determination of glutathione S-transferase (GST) activity

GST activity was assayed by the method of **Habig et al. (1974)** based on the enzymatically catalyzed condensation of glutathione with the substrate CDNB. The product formed 2–4 dinitrophenyl glutathione has a maximum absorption at 340 nm. The values were expressed as µmol of CDNB-GSH conjugate formed per min per mg of protein. Protein concentrations in the samples were measured according to the method of **Lowry et al. (1951)**. All spectrophotometric

measurements were recorded using a microplate reader (BMG LABTECH GmbH SPECTROStarNano, Ortenberg, Germany). The cells were incubated with *B. bifurcata* extract (10, 50, 100, 250, 500 µg/mL) for 24 h. Then cells were mechanically homogenized in 200 µL of buffer saline HEPES (50 mM HEPES plus 154 mM NaCl, pH 7.5). The homogenate was centrifuged (2400 rpm for 5 min at 4 °C). The pellet was discarded and the supernatant was stored at -80 °C until further analysis. 10 µL of supernatant was added to the reaction mixture. The reaction mixture in a final volume of 110 µL contained 0.1 M buffer phosphate (pH 6.5), 1 mM GSH, 1 mM CDNB and 0.01% Tween 20. The GST activity against cytotoxic effect of *tert*-BOOH and the protecting effect of *B. bifurcata* extract was also evaluated.

2.14. Determination of caspase 3/7 activity

Caspase-Glo 3/7 assay is a homogenous, luminescent assay that measures caspase-3 and -7 activities, key-caspases of apoptosis; detection of caspase activation may serve as an important tool in monitoring apoptosis *in vivo* and *in vitro* (**Liu et al., 2005**). The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD (aspartic acid-glutamic acid-valine-aspartic acid), in a reagent optimized for caspase activity, luciferase activity, and cell lysis. Caco-2 cells were exposed to *B. bifurcata* extract (10, 50, 100, 250, 500 µg/mL) for 24 h. Luminescence was measured using the plate reader (FLx800, BioTek, Winooski, USA). Caspase 3/7 activity for cytotoxic effect of *tert*-BOOH and the protecting effect of *B. bifurcata* extract were also evaluated. The precise protocol has been described elsewhere (**Martínez et al., 2021**).

2.15. RNA isolation and cDNA synthesis

Human colon carcinoma Caco-2 cells were grown in 25 cm^2 culture flasks at a concentration of 2×10^5 cells/cm^2 and allowed to adhere for 24 h. Then, cells were treated with *B. bifurcata* extract (100 and 500 µg/mL) for 24 h in FBS-free medium. These treatments 100

and 500 µg/mL *B. bifurcata* extract were tested because doses up to 100 µg/mL of *B. bifurcata* extract evoked no changes in Caco-2 cells submitted to *tert*-BOOH (200 µM). After 24 h, 200 µM *tert*-BOOH in FBS-free medium was added to the flasks for 3 h. FBS-free medium was removed, and cells were washed with DPBS. Subsequently, 1 mL of DPBS was added to the Caco-2 cells to detached and placed in a 1.5 mL collection tube. Cells were then centrifuged at a speed of 1700 rpm at 4°C for 5 min and the supernatant was removed and pellet was used for RNA extraction. Total RNA was extracted using the Trizol Reagent method (Invitrogen, Carlsbad, USA) and purified using RNeasy MinElute Cleanup Kit according to the manufacturer`s protocol (Qiagen, Hilden, Germany). The final RNA concentration and purity was determined using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Madrid, Spain) obtaining A260/A280 ratios between 1.9 and 2.1 in all the samples. First-strand complementary DNA (cDNA) was synthesized from 5 µg total RNA by reverse transcription using RT^2 First Strand kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, starting with a genomic DNA elimination step. At the end, cDNA was diluted 1:10 in nuclease-free water and stored at −80 °C for further analysis.

2.16. Measurement of mRNA expressions linked to apoptosis, proinflammation and oxidative stress signaling by Real-Time PCR

Quantitative Real-Time PCR assays for AKT1 (V-akt murine thymoma viral oncogene homolog 1), Bax (Bcl2-associated X protein), BNIP3 (Bcl2 interacting protein 3), ERK1 (extracellular signal-regulated kinase 1), GSTM2 (glutathione-S-transferase mu 2), HO-1 (heme oxygenase 1), IL-6 (interleukin 6), JNK1 (c-Jun N-terminal kinase 1), NFκB1 (nuclear transcription factor kappa B subunit 1) and Nrf2 (nuclear factor-E2-related factor 2), genes associated to apoptosis, proinflammation and oxidative stress, were performed to analyze mRNA expressions (**Table 1**). Reactions were run on a Real-Time PCR system, BioRad CFX96, using RT^2 SYBR Green qPCR master mix (Qiagen, Hilden, Germany) according to manufacturer's

protocol. Concentration of each primer was 400 nM and thermal protocol was as follows: 95 °C for 10 min, followed by 40 cycles composed of 15 s at 95 °C and 1 min at 60 °C. Sequences of primers are presented in Table 1. Relative changes in gene expression were calculated according to **Pfaffl (2001)**, using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as housekeeping gene (with tested no differences between groups) and extracting efficiencies from raw data using LinRegPCR free software (**Ramakers et al., 2003**).

(Insert Table 1)

2.17. Statistical analysis

To contrast groups, one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison were used. The level of significance was $P < 0.05$. A statistical program IBM SPSS Statistics 27 (IBM Corporation, Armonk, NY, USA) was used. Four independent experiments in triplicate were performed, and the presented results were representative of these replicates. Data are represented as mean values ± standard error of the mean (SEM).

3. Results and Discussion

There is considerable current interest in the cytoprotective effects of natural antioxidant products against oxidative stress and the different defense mechanisms involved. Recently, several studies have focused on the natural products of marine seaweeds, which are recognized to have health promoting effects. The biological and therapeutic properties of seaweeds have already been well known. Several studies showed that among the various natural marine sources of antioxidants, seaweeds have become a potential source of antioxidants because of their bioactive compounds such as polysaccharides, polyphenols, and carotenoids (**Biris-Dorhoi et al., 2020; Ismail et al., 2020**). Natural antioxidants have a potential role in the modification of stress-induced signaling pathways along with the activation of the oxidative defensive pathways (**Rengasamy et al., 2020**; **Begum et al., 2021**). The brown seaweed *B. bifurcata* can be a

promising source of bioactive compounds and functional ingredients with neuroprotective, antioxidant and anti-inflammatory activities **(Alves et al., 2016; Pinteus et al., 2017; Silva et al., 2019; Jaworowska and Murtaza, 2023)**. This research study demonstrates that *B. bifurcata* extract (with a phenolic content of 2.60 μg phenolics/mg residue expressed as phloroglucinol and a carbohydrate content of 5.84 μg carbohyrate/mg residue expressed as glucose) has the ability to protect human colon carcinoma Caco-2 cells against oxidative insult by modulating GSH concentration, ROS generation, NO production, MDA production, antioxidant enzyme activities and apoptosis, proinflammation and oxidative stress signaling pathways.

Previous research studies have demonstrated that *tert*-BOOH induced oxidative stress in Caco-2 cells being a useful model for evaluating the cytoprotective effect of natural antioxidants (**Rodríguez-Ramiro et al., 2011; Martínez et al., 2021**). In order to generate a condition of cellular oxidative stress, Caco-2 cells were submitted to 50, 100, 200, 400 μM *tert*-BOOH and (i) cell viability measured by MTT assay at 3, 9 and 15 h (**Fig. 1A)**, (ii) intracellular ROS production at 15, 30 and 60 min (**Fig. 1B)** and (iii) caspase 3/7 activity at 1, 3 and 5 h (**Fig. 1C)** were evaluated (**Fig. 1**). The results of this study indicated that treatment of Caco-2 cells with 200 μM *tert*-BOOH evoked a remarkable oxidative stress. *Tert*-BOOH (200 μM) induced a significant inhibition of cell viability (25-39%) at 3 h (**Fig. 1A)**, a significant increase (369%) in the cellular ROS production at 60 min (**Fig. 1B)** and a significant increase (29%) in caspase 3/7 activity at 3 h (**Fig. 1C)**. These conditions were used to study the potential protective effect of the *B. bifurcata* extract. Protective effect of *B. bifurcata* extract against an oxidative insult induced by *tert*-BOOH (200 μM) was evaluated in Caco-2 cells.

3.1. Effects of B. bifurcata extract on cell viability (MTT and LDH)

Treatment of Caco-2 cells for 24 h with *B. bifurcata* extract (10–500 μg/mL) evoked no changes in cell viability (MTT and LDH leakage) (**Fig. 2A and 2B**). In this research study, we also examined whether cell death induced by 200 μM *tert*-BOOH after 3 h incubation period

could be reverted with the *B. bifurcata* extract (10-500 μg/mL). The compound *tert*-BOOH (200 μM) reduced cell viability (39% inhibition) (**Fig. 2C**). The treatment of Caco-2 cells with the extract prevented the cell viability affected by *tert*-BOOH. *B. bifurcata* extract (100, 250 and 500 μg/mL) provided a significant increase of cell survival (35%, 38% and 52%, respectively) compared to *tert*-BOOH (**Fig. 2C**).

LDH leakage was used as an indicator of cytotoxicity. We examined whether the cytotoxic effect on LDH leakage induced by 200 μM *tert*-BOOH could be reverted with the *B. bifurcata* extract (10-500 μg/mL). The compound *tert*-BOOH (200 μM) produced a significant cytotoxic effect on LDH leakage (123% LDH release) (**Fig. 2D**). The treatment of Caco-2 cells with the extract prevented the cytotoxicity induced by *tert*-BOOH. *B. bifurcata* extract (100, 250 and 500 μg/mL) provided a significant protection (22%, 35% and 38%, respectively) against the cell insult evoked by 200 μM *tert*-BOOH (**Fig. 2D**).

3.2. Effects of B. bifurcata extract on reduced glutathione (GSH) and malondialdehyde (MDA)

GSH is the main nonenzimatic antioxidant defense within the cell, reducing different peroxides, hydroperoxides and radicals (**Jones, 2002**). The concentration of GSH decreases considerably when cells are exposed to oxidative damage (**Presa et al., 2018**). On the other hand, MDA, a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation (**Suttnar et al., 1997**). Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences (**Suttnar et al., 2001; Barrera et al., 2018**). In the present research article, levels of oxidative stress markers (GSH and MDA) were examined.

Treatment of Caco-2 cells for 24 h with *B. bifurcata* extract (10–500 μg/mL) for 24 h evoked no changes in GSH and MDA levels (**Fig 3A and 3B**). In order to generate a condition of cellular oxidative stress, Caco-2 cells were incubated with *tert*-BOOH (200 μM) and then

GSH and MDA levels were evaluated (**Fig 3C and 3D**). The compound *tert*-BOOH (200 μM) affected significantly the levels of GSH (47% inhibition) (**Fig. 3C**) and MDA (226% induction) (**Fig. 3D**). These remarkable decreases in GSH levels, as well as the significant increase in MDA levels in response to an oxidative stress induced by *tert*-BOOH, were significantly prevented by *B. bifurcata* extract (**Fig. 3C and 3D**). *B. bifurcata* extract (100, 250, 500 μg/mL) provided a significant increase of GSH levels (34%, 73% and 84%, respectively) compared to *tert*-BOOH (**Fig. 3C**). *B. bifurcata* extract (100, 250, 500 μg/mL) significantly decreased MDA levels (25%, 30% and 31%, respectively) compared to *tert*-BOOH (**Fig. 3D**). Our results demonstrated that the treatment of Caco-2 cells with *B. bifurcata* extract at concentrations ranging from 100 to 500 μg/mL prevented or delayed conditions which favor oxidative stress in the cell.

3.3. Effects of B. bifurcata extract on reactive oxygen species (ROS) generation, NO production and caspase-3/7 activity

Direct evaluation of the reactive oxygen species (ROS) yields a very good indication of the oxidative damage to living cells (**Wang and Joseph, 1999**). Activation of ROS leads to lipid peroxidation of cell membranes, and the products of peroxidation bind to biomolecules, leading to disease production (**Yuan et al., 2015**). Based upon the fact that nonfluorescent 2,7-dichlorofluorescin (DCFH) crosses cell membranes and is oxidized by intracellular ROS to highly fluorescent DCF (**LeBel et al., 1992**), the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells (**Wang and Joseph, 1999**). A prooxidant such as *tert*-BOOH can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus increasing fluorescence. Oxidative stress is associated with an imbalance between ROS and antioxidants in the human body. Excessive ROS accumulation can directly damage DNA, protein, and lipids, which disturbs normal cellular function (**Pizzino et al., 2017**; **Liu et al., 2018; Zheng et al., 2020**). This research study showed that *B. bifurcata* extract inhibits the generation of ROS induced by *tert*-

BOOH in cultured Caco-2 cells, thus preventing or delaying conditions which favor oxidative stress in the cells.

Findings of our study showed that *B. bifurcata* extract for 24 h evoked no changes in the cellular ROS generation levels (**Fig. 4A**). A significant increase in ROS production was observed in the presence of 200 μM *tert*-BOOH as compared to unstressed control (**Fig. 4C**). The compound *tert*-BOOH (200 μM) increased ROS generation (334% induction) (**Fig. 4C**). The treatment of Caco-2 cells with the *B. bifurcata* extract (100 – 500 μg/mL) significantly attenuated the ROS production induced by *tert*-BOOH (200 μM). *B. bifurcata* extract (100, 250, 500 μg/mL) provided a significant decrease of ROS levels (13%, 14% and 21%, respectively) against the cell insult evoked by *tert*-BOOH (**Fig. 4C**). *B. bifurcata* extract can play important roles in preventing ROS damage by scavenging free radicals.

Correlation between the protective effect of seaweeds and the *tert*-BOOH-induced production of NO was also examined. Reactive NO has been known to generate peroxynitrite ($ONOO^-$) that is a latent oxidizing agent, by the chemical reaction between NO and superoxide. Various negative effects of $ONOO^-$ have been described, such as oxidation and nitration of proteins (**Patel et al., 1999; Reiter et al., 2002; Lee et al., 2004; Seo et al., 2004**). Therefore, NO production is an important step in the regulation of NO-mediated diseases. We examined whether exposure to *B. bifurcata* extract at concentrations ranging from 10 to 500 μg/mL had an effect on the NO production in Caco-2 cells. Findings of our study showed that *B. bifurcata* extract for 24 h evoked no changes in the NO production (**Fig. 4B**).

Caco-2 cells were incubated with 200 μM *tert*-BOOH for 3 h, to test the protective effect of the *B. bifurcata* extract against the oxidative stress via NO production. It was examined whether NO production induced by 200 μM *tert*-BOOH after incubation period could be reverted with the *B. bifurcata* extract. The compound *tert*-BOOH (200 μM) increased NO production (56% induction) (**Fig. 4D**). The treatment of Caco-2 cells with the *B. bifurcata* extract significantly attenuated the NO production induced by *tert*-BOOH. *B. bifurcata* extract (100,

250, 500 μg/mL) providing a significant decrease of NO production (21%, 26% and 30%, respectively) against the cell insult evoked by *tert*-BOOH (**Fig. 4D**).

Several different types of biochemical events have been recognized as important in apoptosis, perhaps the most fundamental is the participation of members of a protease family known as caspases. Caspases are an evolutionary conserved family of cysteine proteases that are centrally involved in cell death and inflammation responses (**Opdenbosch and Lamkanfi, 2019**). Caspase-3 and -7 is the primary executioner of apoptosis death. During apoptosis, caspase 3/7 contributes to an accumulation of ROS production during intrinsic cell death (**Brentnall et al., 2013**). In the present research study, we evaluated the potential effects of the *B. bifurcata* extract exposure on caspase 3/7 activity. Treatment of Caco-2 cells with the *B. bifurcata* extract at increasing concentrations (10 – 500 μg/mL) for 24 h evoked no changes in the caspase 3/7 activity (**Fig. 5A**). Caco-2 cells were incubated, for a period of 3 h with 200 μM *tert*-BOOH. The compound *tert*-BOOH induced caspase 3/7 activation (40% induction) (**Fig. 5B**). The treatment of Caco-2 cells with the *B. bifurcata* extract (250, 500 μg/mL) significantly decreased caspase 3/7 activity (25% and 26%, respectively) against the cell insult evoked by *tert*-BOOH (**Fig. 5B**).

3.4. Effects of B. bifurcata extract on antioxidant enzyme (NQO1 and GST) activity.

Recent research data indicate the importance of the antioxidant functions of enzymes NQO1 and GST in combating oxidative stress (**Hayes et al., 2005; Dinkova-Kostova and Talalay, 2010; Allocati et al., 2018**). NQO1 has long been viewed as a chemoprotective enzyme involved in cellular defense against the electrophilic and oxidizing metabolites of xenobiotic quinones (**Ross, 2004; Ross and Siegel, 2017**). GST catalyzes the conjugation of endogenous and xenobiotic electrophiles with glutathione; GST proteins are crucial antioxidant enzymes that regulate stress-induced signaling pathways (**van Ommen et al., 1990; Singh and Reindl, 2021**). The present research study aimed to investigate *in vitro* effects of *B. bifurcata* extract on the activities of the enzymes NQO1 and GST. Treatment of Caco-2 cells with the *B. bifurcata*

extract (10–500 μg/mL) for 24 h evoked no changes in NQO1 and GST activities (**Fig. 6A and 6B**). The compound *tert*-BOOH (200 μM) significantly affected the activities of NQO1 (34% inhibition) (**Fig. 6C**) and GST (35% inhibition) (**Fig. 6D**). These remarkable decreases in the antioxidant defenses by *tert*-BOOH were significantly prevented by *B. bifurcata* extract. *B. bifurcata* extract (100, 250, 500 μg/mL) provided a significant increase of NQO1 activity (34%, 35% and 39%, respectively) (**Fig. 6C**) as well as a significant increase of GST activity (34%, 42% and 49%, respectively) (**Fig. 6D**).

3.5. B. bifurcata extract induces GSTM2, Nrf2 and AKT1 pathways

In view of the *in vitro* significant effects observed of *B. bifurcata* extract including antioxidant capacity, ROS reduction and NO production ability and antioxidant defenses activation, *B. bifurcata* extract could be related to a specific modulation of transcription factors. In the present research study, the potential effects of the *B. bifurcata* extract exposure on (i) GSTM2, (ii) Nrf2 and (iii) AKT1 mediators were examined by Real Time PCR assays:

(i) Research studies have demonstrated that glutathione S-transferases (GSTs) are an integral part of a dynamic and interactive defense mechanism that protects against cytotoxic electrophilic chemicals and allows adaptation to exposure to oxidative stress. GSTs have antioxidant and anti-inflammatory activities (**Strange et al., 2000; Hayes et al., 2005**). GSTM2 belongs to a large gene superfamily encoding glutathione S-transferases, a protein involved in detoxification of ROS (**Sharma et al., 2004**).

(ii) Nrf2 signaling pathway is an important mediator of cellular injury in response to oxidative stress. In general, activation of Nrf2 is necessary for antioxidant gene expression (**Loboda et al., 2016**). Nrf2 may be a key transcription factor regulating expression of genes affecting xenobiotic metabolism. Nrf2 regulates the metabolic fate of numerous pro-oxidants and electrophiles in the body (**Kensler et al., 2007**; **Ma, 2013**).

(iii) AKT, a serine/threonine protein kinase, plays a critical role in controlling the balance between apoptosis and cell survival in response to extra- and intracellular signaling. Three isoforms, AKT1, AKT2, and AKT3, which have high homology but are expressed from three separate genes. AKT1 is the predominant isoform in most tissue (**Nicholson and Anderson, 2002; Song et al., 2008**).

The results from this study demonstrate a protective activity of seaweed *B. bifurcata* extract via activation of GSTM2, Nrf2 and AKT1 signaling pathways (**Fig. 7A, 7B and 7C**). The *B. bifurcata* extract (100 and 500 μg/mL) caused a significant increase of mRNA levels for GSTM2 (1.52-fold and 1.81-fold, respectively) (**Fig. 7A**), for Nrf2 (1.56-fold and 1.72-fold, respectively) (**Fig. 7B**), and for AKT1 (1.37-fold only at dose 500 μg/mL) (**Fig. 7C**).

One important strength of the present research study is that *B. bifurcata* extract is an efficient inducer of GSTM2, Nrf2 and AKT1 gene expressions, results also reported from marine algae *Gongolaria baccata* (**Martínez et al., 2021**). Seaweeds and their constituents have been linked to beneficial activities, including antioxidant and life expansion activities (**Ratnayake et al., 2013; Snare et al., 2013; Wang et al., 2013**). **Bousquet et al. (2020)** showed that the marine green algae *Cymopolia barbata* and its major active component cymopol, activated the transcription factor Nrf2. The activation of the Nrf2 signaling pathway is a valid cancer-preventive strategy, and is a novel therapeutic approach for numerous oxidative stress-mediated diseases (**Kensler et al., 2007**). Alternatively, Nrf2 up-regulation could be directly associated with the AKT1 signaling pathway (**Yoon et al., 2021**). Overall, this research study suggests a protective action of *B. bifurcata* extract directly associated with the activation of GSTM2, Nrf2 and AKT1 signaling pathways.

3.6. Protective effects of B. bifurcata extract on signaling pathways (ERK1, JNK1, Bax, BNIP3, NFκB1, IL-6 and HO-1) affected by tert-BOOH.

A number of transcription factors have been linked to modulate signaling pathways involved in the regulation of apoptotic cell death. Signaling pathways ERK1, JNK1, Bax, BNIP3, NFκB1, IL-6 and HO-1 have been proposed to mediate integral actions in many areas including apoptotic, inflammatory and oxidant responses to external stresses. Substantial evidence supports that ERK1, JNK1, Bax, and BNIP3 signaling pathways are involved in promoting apoptosis (**Cowan and Storey, 2003**; **Prabhakaran et al., 2007**; **Dhanasekaran and Reddy, 2008**; **Youle and Strasser, 2008; Roskoski, 2019**). The transcription factor NFκB1 induces the expression of proinflammatory cytokines and serves as a pivotal mediator of inflammatory responses (**Liu et al., 2017**); IL-6, a pleiotropic cytokine plays a pathological effect on pro- and anti-inflammatory diseases (**Scheller et al., 2011**) and HO-1, inducible isoform whose expression can be upregulated by different stress conditions, pointing out its implications in cancer and other diseases development (**Consoli et al., 2021**). In this regard, the present research study analyzed the effect of the potent pro-oxidant *tert*-BOOH (200 μM) on gene expressions of apoptosis, proinflammation and oxidative stress mediators (ERK1, JNK1, Bax, BNIP3, NFκB1, IL-6 and HO-1) as well as the protective effectiveness of *B. bifurcata* extract (500 μg/mL) on the signaling pathways affected by *tert*-BOOH.

Data from this study demonstrated in Caco-2 cells that the potent pro-oxidant *tert*-BOOH significantly induced upregulation of ERK1 (3.07-fold), JNK1 (2.91-fold), Bax (2.64-fold), BNIP3 (2.34- fold), NFκB1 (2.86-fold), IL-6 (2.79-fold) and HO-1 (2.72-fold) gene expressions compared to control (**Table 2A**).

Quantitative gene expression analysis of a panel of 7 genes [4 genes linked to apoptosis signaling pathway (ERK1, JNK1, Bax, BNIP3), 2 genes linked to inflammatory signaling pathway (NFκB1, IL-6) and 1 gen linked to oxidative stress (HO-1)] revealed that *B. bifurcata* extract reduced the expression of these genes induced by *tert*-BOOH. *B. bifurcata* extract significantly reduced the gene expressions induced by *tert*-BOOH: ERK1 (from 3.07-fold to 1.31-fold), JNK1 (from 2.91-fold to 1.22-fold), Bax (from 2.64-fold to 1.46-fold), BNIP3 (from

2.34-fold to 1.43-fold), NFκB1 (from 2.86-fold to 1.47-fold), IL-6 (from 2.79-fold to 1.38 fold) and HO-1 (from 2.72-fold to 1.76-fold) (**Table 2B**). These results illustrated the ability of seaweed *B. bifurcata* extract to protect against *tert*-BOOH induced stress in Caco-2 cells. The addition of *B. bifurcata* extract to Caco-2 cells decreased ERK1, JNK1, Bax, BNIP3, NFκB1, IL-6 and HO-1 gene expressions in a range of 40-60 %, signaling pathways involved in the control of numerous processes including apoptosis, cell proliferation, the immune response, nervous system function, and RNA synthesis and processing. These results indicate that *B. bifurcata* extract can alleviate the *tert*-BOOH-induced oxidative stress injury of Caco-2 cells and that *B. bifurcata* extract may have a potential application prospect as a new functional food ingredient and/or health supplement.

4. Conclusion

Seaweeds are a group of marine multicellular algae has various health benefits and biomedical applications. Our research article establishes the effect of seaweed *B. Bifurcata* extract on the response of the antioxidant defense system to the oxidative stressor, *tert*-BOOH, in Caco-2 cells. The parameters evaluated included markers of the cell damage and oxidative stress such as cell viability, GSH and MDA levels, intracellular ROS generation, NO production, caspase 3/7 and particular antioxidant enzyme activities and gene expressions. Treatment with *tert*-BOOH yields an excellent model of oxidative stress in cell culture. *Tert*-BOOH evokes a consistent cellular stress in Caco-2 cells. The results of this study indicate that *B. Bifurcata* extract exerts an antiapoptotic effect protecting Caco-2 cells against an oxidative stress evoked by *tert*-BOOH. This antiapoptotic effect was associated with reduced ROS generation, reduced NO production and effective induction of antioxidant enzymes NQO1 and GST via GSTM2, Nrf2 and AKT1 activation. Moreover, pretreatment of Caco-2 cells with *B. bifurcata* extract increased GSH and decreased MDA levels in response to an oxidative stress induced by the compound *tert*-BOOH and thus protect cells against oxidative stress-induced apoptosis by

directly counteracting free radicals and by activating the antioxidant defense system. All these biological properties point to *B. bifurcata* extract as interesting candidate for cellular chemoprotection. Therefore, future studies with *B. bifurcata* extract-derived products may contribute to the protection against diseases in which oxidative stress has been implicated as a causal or contributory factor.

Credit author statement

María-Aránzazu Martínez: Conceptualization, Funding acquisition, Methodology, Validation, Investigation, Formal analysis, Writing – original draft. **Hugo Aedo, Bernardo Lopez-Torres, Jorge-Enrique Maximiliano and María-Rosa Martínez-Larrañaga:** Provide critical suggestions on manuscript preparation, Conducted the design of the study, Investigation, Methodology, Validation, Formal analysis. **Arturo Anadón and Marta Martínez:** Conceptualization, Investigation, Supervision, Writing – original draft & editing. **Cesar Peteiro:** Methodology, Investigation. **Mercedes Cueto:** Conceptualization, Funding acquisition, Methodology, Investigation. **Susana Rubiño:** Methodology, Investigation. **María Hortos:** Conceptualization, Funding acquisition, Methodology, Investigation. **Irma Ares:** Conceptualization, Investigation, Formal analysis, Supervision, Writing – original draft.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

References

Allocati, N., Masulli, M., Di Ilio, C., Federici, L., 2018. Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. Oncogenesis 7, 8. https://doi.org/10.1038/s41389-017-0025-3.

Alves, C., Pinteus, S., Simões, T., Horta, A., Silva, J., Tecelão, C., Pedrosa, R., 2016. *Bifurcaria bifurcata:* a key macro-alga as a source of bioactive compounds and functional ingredients. Int. J. Food Sci. Technol. 51 (7), 1638-1646. https://doi.org/10.1111/ijfs.13135.

Barrera, G., Pizzimenti, S., Daga, M., Dianzani, C., Arcaro, A., Cetrangolo, G.P., Giordano, G., Cucci, M.A., Graf, M., Gentile F., 2018. Lipid peroxidation-derived aldehydes, 4-hydroxynonenal and malondialdehyde in aging-related disorders. Antioxidants 7 (8), 102. https://doi.org/10.3390/antiox7080102.

Begum, R., Howlader, S., Mamun-Or-Rashid, A.N.M., Rafiquzzaman, S.M., Ashraf, G.M., Albadrani, G.M., Sayed, A.A., Peluso, I., Abdel-Daim, M.M., Uddin, M.S., 2021. Antioxidant and signal-modulating effects of brown seaweed-derived compounds against oxidative stress-associated pathology. Oxid. Med. Cell. Longev. 2021, 9974890. https://doi.org/10.1155/2021/9974890.

Bertocci, I., Araújo, R., Oliveira, P., Sousa-Pinto, I., 2015. Potential effects of kelp species on local fisheries. J. Appl. Ecol. 52 (5), 1216–1226. https://doi.org/10.1111/1365-2664.12483.

Besednova, N.N., Andryukov, B.G., Zaporozhets, T.S., Kuznetsova, T.A., Kryzhanovsky, S.P., Ermakova, S.P., Galkina, I.V., Shchelkanov, M.Y., 2022. Molecular targets of brown algae phlorotannins for the therapy of inflammatory processes of various origins. Mar. Drugs 20 (4), 243; https://doi.org/10.3390/md20040243.

Biris-Dorhoi, E.S., Michiu, D., Pop, C.R., Rotar, A.M., Tofana, M., Pop, O.L., Socaci, S.A., Farcas, A.C., 2020. Macroalgae-a sustainable source of chemical compounds with biological activities. Nutrients 12 (10), 3085. https://doi.org/10.3390/nu12103085.

Bousquet, M.S., Ratnayake, R., Pope, J.L., Chen, Q.Y., Zhu, F., Chen, S., Carney, T.J., Gharaibeh, R.Z., Jobin, C., Paul, V.J., Luesch, H., 2020. Seaweed natural products modify the host inflammatory response via Nrf2 signaling and alter colon microbiota composition and gene expresión. Free Radic. Biol. Med. 146, 306-323. https://doi.org/10.1016/j.freeradbiomed.2019.09.013.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R., Cepero, E., Boise, L.H., 2013. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 14, 32. https://doi.org/10.1186/1471-2121-14-32.

Chatterjee, A., Singh, S., Agrawal, C., Yadav, S., Rai, R., Rai, L.C., 2017. Role of algae as a biofertilizer, in: Rastogi, R.P., Madamwar, D., Pandey, A. (Eds.), Algal green chemistry: recent progress in biotechnology. Elsevier B.V., Amsterdam, pp. 189–200. https://doi.org/10.1016/B978-0-444-63784-0.00010-2.

Consoli, V., Sorrenti, V., Grosso, S., Vanella, L., 2021. Heme oxygenase-1 signaling and redox homeostasis in physiopathological conditions. Biomolecules 11 (4), 589. https://doi.org/10.3390/biom11040589.

Cowan, K.J., Storey, K.B., 2003. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J. Exp. Biol. 206 (7), 1107-1115. https://doi.org/10.1242/jeb.00220.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89 (2), 271–277. https://doi.org/10.1016/0022-1759(86)90368-6.

Dhanasekaran, D.N., Reddy, E.P., 2008. JNK signaling in apoptosis. Oncogene 27, 6245-6251. https://doi.org/10.1038/onc.2008.301.

Díez, I., Secilla, A., Santolaria, A., Gorostiaga, J.M., 1999. Phytobenthic intertidal community structure along an environmental pollution gradient. Mar. Pollut. Bull. 38 (6), 463-472. https://doi.org/10.1016/S0025-326X(98)90161-8.

Dinkova-Kostova, A.T., Talalay, P., 2010. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch. Biochem. Biophys. 501 (1), 116-123. https://doi.org/10.1016/j.abb.2010.03.019.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356. https://doi.org/10.1021/ac60111a017.

Erpel, F., Mateos, R., Pérez-Jiménez, J., Pérez-Correa, J.R., 2020. Phlorotannins: from isolation and structural characterization, to the evaluation of their antidiabetic and anticancer potential. Food Res. Int. 137, 109589. https://doi.org/10.1016/j.foodres.2020.109589.

EUMOFA, European Market Observatory for Fisheries and Aquaculture Products, 2018. Blue bioeconomy. Situation report and perspectives. Directorate-General for Maritime Affairs and Fisheries, European Union, pp. 1–143. https://doi.org/10.2771/053734.

Fletcher, R.L., 1987. Seaweeds of the British Isles. Vol 3, Fucophyceae (Phaeophyceae), Part 1. British Museum (Natural History), London.

Ghasemi, M., Turnbull, T., Sebastian, S., Kempson, I., 2021. The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int. J. Mol. Sci. 22 (23), 12827. https://doi.org/10.3390/ijms222312827.

Gómez Garreta, A., Barceló Martí, M.C., Gallardo García, T., Pérez-Ruzafa, I.M., Ribera Sigual, M.A., Rull Lluch, J., 2000. Flora Phycológica Ibérica, Vol. 1. Fucales. Universidad de Murcia, Murcia.

Guiry, M.D., Guiry, G.M., 2022. AlgaeBase. World-wide electronic publication. http://www.algaebase.org. (Accessed 16 June 2022).

Gupta, S., Abu-Ghannam, N., 2011. Bioactive potential and possible health effects of edible brown seaweeds. Trends Food Sci. Technol. 22 (6), 315–326. https://doi.org/10.1016/j.tifs.2011.03.011.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. 45, 51-88. https://doi.org/10.1146/annurev.pharmtox.45.120403.095857.

Hemantkumar, J.N., Rahimbhai, M.I., 2019. Microalgae and its use in nutraceuticals and food supplments, in: Vítová, M. (Ed.), Microalgae - from physiology to application. IntechOpen, London, pp. 11. http://doi.org/10.5772/intechopen.90143.

Hissin, P.J., Hilf, R., 1976. A fluorimetric method for determination of oxidized and reduced glutathione in tissues. Anal. Biochem. 74 (1), 214–226. https://doi.org/10.1016/0003-2697(76)90326-2.

Ismail, M.M., Alotaibi, B.S., El-Sheekh, M.M., 2020. Therapeutic uses of red macroalgae. Molecules 25 (19), 4411. https://doi.org/10.3390/molecules25194411.

Jaworowska, A., Murtaza, A., 2023. Seaweed derived lipids are a potential anti-inflammatory agent: a review. 20 (1), 730. https://doi.org/10.3390/ijerph20010730.

Jones, D.P., 2002. Redox potential of GSH/GSSG couple: assay and biological significance. Methods Enzymol. 348, 93-112. https://doi.org/10.1016/S0076-6879(02)48630-2.

Kensler, T.W., Wakabayashi, N., Biswal, S., 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. Toxicol. 47, 89-116. https://doi.org/10.1146/annurev.pharmtox.46.120604.141046.

Kinley, R.D., Martinez-Fernandez, G., Matthews, M.K., de Nys, R., Magnusson, M., Tomkins, N.W., 2020. Mitigating the carbon footprint and improving productivity of ruminant

livestock agriculture using a red seaweed. J. Clean. Prod. 259, 120836. https://doi.org/10.1016/j.jclepro.2020.120836.

Kraan, S., 2012. Algal polysaccharides, novel applications and outlook, in: Chang, C.F. (Ed.), Comprehensive studies on glycobiology and glycotechnology. IntechOpen, London, pp. 489–532. https://doi.org/10.5772/51572.

Kumar, P., Nagarajan, A., Uchil, P.d., 2018. Analysis of cell viability by the lactate dehydrogenase assay. Cold Spring Harb. Protoc. 2018 (6), 465-468. https://doi.org/10.1101/pdb.prot095497.

LeBel, C.P., Ischiropoulus, H., Bondy, S.C., 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5 (2), 227-231. https://doi.org/10.1021/tx00026a012.

Lee, H.J., Kim, Y.A., Park, K.E., Jung, H.A., Yoo, J.S., Ahn, J.W., Lee, B.J., Seo, Y.W., 2004. Studies on screening of seaweed extracts for peroxynitrite and DPPH radical scavenging activities. Ocean and Polar Res. 26 (1), 59-64. https://doi.org/10.4217/OPR.2004.26.1.059.

Leikert, J.F., Rathel, T.R., Muller, C., Vollmar, A.M., Dirsch, V.M., 2001. Reliable i*n vitro* measurement of nitric oxide released from endothelial cells using low concentrations of the fluorescent probe 4,5-diaminofluorescein. FEBS Lett. 506 (2), 131–134. https://doi.org/10.1016/S0014-5793(01)02901-5.

Liu, J.J., Wang, W., Dicker, D.T., El-Deiry, W.S., 2005. Bioluminiscent imaging of TRAIL-induced apoptosis through detection of caspase activation following cleavage of DEVD-aminoluciferin. Canc. Biol. Ther. 4, 885–892. https://doi.org/10.4161/cbt.4.8.2133.

Liu, T., Zhang, L., Joo, D., Sun, S.C., 2017. NF-κB signaling in inflammation. Signal Transduct. Target. Ther. 2, 17023. https://doi.org/10.1038/sigtrans.2017.23.

Liu, Z., Ren, Z., Zhang, J., Chuang, C.C., Kandaswamy, E., Zhou, T., Zuo, L., 2018. Role of ROS and nutritional antioxidants in human diseases. Front. Physiol. 9, 477. https://doi.org/10.3389/fphys.2018.00477.

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., Dulak, J., 2016. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell. Mol. Life Sci. 73 (17), 3221-3247. https://doi.org/10.1007/s00018-016-2223-0.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193 (1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6.

Ma, Q., 2013. Role of Nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. Toxicol. 53, 401-426. https://doi.org/10.1146/annurev-pharmtox-011112-140320.

Machado, L., Magnusson, M., Paul, N.A., de Nys, R., Tomkins, N., 2014. Effects of marine and freshwater macroalgae on *in vitro* total gas and methane production. PLoS One 9 (1), e85289. https://doi.org/10.1371/journal.pone.0085289.

Makkar, H.P.S., Tran, G., Heuzé, V., Giger-Reverdin, S., Lessire, M., Lebas, F., Ankers, P., 2016. Seaweeds for livestock diets: a review. Anim. Feed Sci. Technol. 212, 1–17. https://doi.org/10.1016/j.anifeedsci.2015.09.018.

Martínez, M.A., Ares, I., Martínez, M., Lopez-Torres, B., Maximiliano, J.E., Rodríguez, J.L., Martínez-Larrañaga, M.R., Anadón, A., Peteiro, C., Rubiño, S., Hortos, M., 2021. Brown marine algae *Gongolaria baccata* extract protects caco-2 cells from oxidative stress induced by tert-butyl hydroperoxide. Food Chem. Toxicol. 156, 112460. https://doi.org/10.1016/j.fct.2021.112460.

Matulja, D., Vranješević, F., Kolympadi Markovic, M., Pavelić, S.K., Marković, D., 2022. Anticancer activities of marine-derived phenolic compounds and their derivatives. Molecules 27 (4), 1449; https://doi.org/10.3390/molecules27041449.

Nazir, N., Zahoor, M., Nisar, M., Khan, I., Ullah, R., Alotaibi, A., 2021. Antioxidants isolated from *Elaeagnus umbellata* (Thunb.) protect against bacterial infections and diabetes in

streptozotocin-induced diabetic rat model. Molecules 26 (15), 4464. https://doi.org/10.3390/molecules26154464.

Niccolai, A., Zittelli, G.C., Rodolfi, L., Biondi, N., Tredici, M.R., 2019. Microalgae of interest as food source: biochemical composition and digestibility. Algal Res. 42, 101617. https://doi.org/10.1016/j.algal.2019.101617.

Nicholson, K.M., Anderson, N.G., 2002. The protein kinase B/Akt signaling pathway in human malignancy. Cell. Signal. 14(5), 381–395. https://doi.org/10.1016/s0898-6568(01)00271-6.

Opdenbosch, N.V., Lamkanfi, M., 2019. Caspases in cell death, inflammation, and disease. Immunity 50 (6), 1352–1364. https://doi.org/10.1016/j.immuni.2019.05.020.

O'Sullivan, A.M, O'Callaghan, Y.C., O'Grady, M.N., Queguineur, B., Hanniffy, D., Troy, D.J., Kerry, J.P., O'Brien, N.M., 2012. Assessment of the ability of seaweed extracts to protect against hydrogen peroxide and *tert*-butyl hydroperoxide induced cellular damage in Caco-2 cells. Food Chem. 134 (2), 1137-1140. https://doi.org/10.1016/j.foodchem.2012.02.205.

Pais, A.C.S., Saraiva, J.A., Rocha, S.M., Silvestre, A.J.D., Santos, S.A.O., 2019. Current research on the bioprospection of linear diterpenes from *Bifurcaria bifurcata*: from extraction methodologies to possible applications. Mar. Drugs 17 (10), 556. https://doi.org/10.3390/md17100556.

Patel, R.P., McAndrew, J., Sellak, H., Roger White, C., Jo, H., Freeman, B.A., Darley-Usmar, V.M., 1999. Biological aspects of reactive nitrogen species. Biochim. Biophys. Acta 1411 (2-3), 385-400. https://doi.org/10.1016/S0005-2728(99)00028-6.

Peñalver, R., Lorenzo, J.M., Ros, G., Amarowicz, R., Pateiro, M., Nieto, G., 2020. Seaweeds as a functional ingredient for a healthy diet. Mar. Drugs 18 (6), 301. https://doi.org/10.3390/md18060301.

Peteiro, C., 2018. Alginate production from marine macroalgae, with emphasis on kelp farming, in: Rehm, B.H.A, Moradali, M.F. (Eds.), Alginates and their biomedical applications. Springer Singapore, Singapore, pp. 27–66. https://doi.org/10.1007/978-981-10-6910-9_2.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), 45. https://doi.org/10.1093/nar/29.9.e45.

Phelan, M., Aherne, S.A., Wong, A., O'Brien, N.M., 2009. Bioactive properties of wood knot extracts on cultured human cells. J. Med. Food 12, 1245–1251. https://doi.org/10.1089/jmf.2008.0125.

Pinteus, S., Silva, J., Alves, C., Horta, A., Fino, N., Rodrigues, A.I., Mendes, S., Pedrosa, R., 2017. Cytoprotective effect of seaweeds with high antioxidant activity from the Peniche coast (Portugal). Food Chem. 218, 591-599. https://doi.org/10.1016/j.foodchem.2016.09.067.

Pinto, D., Sut, S., Dall'Acqua, S., Delerue-Matos, C., Rodrigues, F., 2021. *Actinidia arguta* pulp: phytochemical composition, radical scavenging activity, and *in vitro* cells effects. Chem. Biodivers. 18 (3), e2000925. https://doi.org/10.1002/cbdv.202000925.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., Bitto, A., 2017. Oxidative stress: harms and benefits for human health. Oxid. Med. Cell Longev. 2017, 8416763. https://doi.org/10.1155/2017/8416763.

Prabhakaran, K., Li, L., Zhang, L., Borowitz, J.L., Isom, G.E., 2007. Upregulation of BNIP3 and translocation to mitochondria mediates cyanide-induced apoptosis in cortical cells. Neuroscience 150 (1), 159-167. https://doi.org/10.1016/j.neuroscience.2007.07.033.

Presa, F.B., Marques, M.L.M., Viana, R.L.S., Nobre, L.T.D.B., Costa, L.S., Rocha, H.A.O., 2018. The protective role of sulfated polysaccharides from green seaweed *Udotea flabellum* in cells exposed to oxidative damage. Mar. Drugs 16 (4), 135. https://doi.org/10.3390/md16040135.

Ramakers, C., Ruijter, J.M., Deprez, R.H., Moorman, A.F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339 (1), 62–66. https://doi.org/10.1016/S0304-3940(02)01423-4.

Ratnayake, R., Liu, Y., Paul, V.J., Luesch, H., 2013. Cultivated sea lettuce is a multiorgan protector from oxidative and inflammatory stress by enhancing the endogenous antioxidant defense system. Cancer Prev. Res. 6 (9), 989-999. https://doi.org/10.1158/1940-6207.CAPR-13-0014.

Reisewitz, S.E., Estes, J.A., Simenstad, C.A., 2006. Indirect food web interactions: sea otters and kelp forest fishes in the Aleutian archipelago. Oecologia 146 (4), 623–631. https://doi.org/10.1007/s00442-005-0230-1.

Reiter, R.J., Tan, D.X., Burkhardt, S., 2002. Reactive oxygen and nitrogen species and cellular and organismal decline: amelioration with melatonin. Mech. Ageing Dev. 123 (8), 1007-1019. https://doi.org/10.1016/S0047-6374(01)00384-0.

Rengasamy K.R.R., Mahomoodally, M.F., Aumeeruddy, M.Z., Zengin, G., Xiao, J., Kim, D.H., 2020. Bioactive compounds in seaweeds: an overview of their biological properties and safety. Food Chem. Toxicol. 135, 111013. https://doi.org/10.1016/j.fct.2019.111013.

Rodríguez-Ramiro, I., Martín, M.A., Ramos, S., Bravo, L., Goya, L., 2011. Comparative effects of dietary flavanols on antioxidant defences and their response to oxidant-induced stress on Caco2 cells. Eur. J. Nutr. 50, 313–322. https://doi.org/10.1007/s00394-010-0139-2.

Roohinejad, S., Koubaa, M., Barba, F.J., Saljoughian, S., Amid, M., Greiner, R., 2017. Application of seaweeds to develop new food products with enhanced shelf-life, quality and health-related beneficial properties. Food Res. Int. 99, 1066–1083. https://doi.org/10.1016/j.foodres.2016.08.016.

Roque, B.M., Brooke, C.G., Ladau, J., Polley, T., Marsh, L.J., Najafi, N., Pandey, P., Singh, L., Kinley, R., Salwen, J.K., Eloe-Fadrosh, E., Kebreab, E., Hess, M., 2019. Effects of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage. Anim. Microbiome 1 (1), 1–14. https://doi.org/10.1186/s42523-019-0004-4.

Roskoski Jr., R., 2019. Targeting ERK1/2 protein-serine/threonine kinases in human cancers. Pharmacol. Res. 142, 151-168. https://doi.org/10.1016/j.phrs.2019.01.039.

Ross, D., 2004. Quinone reductases multitasking in the metabolic world. Drug Metab. Rev. 36 (3-4), 639-654. https://doi.org/10.1081/DMR-200033465.

Ross, D., Siegel, D., 2017. Functions of NQO1 in cellular protection and CoQ_{10} metabolism and its potential role as a redox sensitive molecular switch. Front. Physiol. 8, 595. https://doi.org/10.3389/fphys.2017.00595.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stammati, A., Zucco, F., 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture related factors on Caco-2 cell functional characteristics. Cell Biol. Toxicol. 21, 1–26. https://doi.org/10.1007/s10565-005-0085-6.

Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim. Biophys. Acta 1813 (5), 878-888. https://doi.org/10.1016/j.bbamcr.2011.01.034.

Sciubba, F., Di Cocco, M.E., Angori, G., Spagnoli, M., De Salvador, F.R., Engel, P., Delfini, M., 2020. NMR-based metabolic study of leaves of three species of *Actinidia* with different degrees of susceptibility to *Pseudomonas syringae* pv. *Actinidiae*. Nat. Prod. Res. 34 (14), 2043-2050. https://doi.org/10.1080/14786419.2019.1574784.

Seiple, I.B., Mercer, J.A.M., Sussman, R.J., Zhang, Z., Myers, A.G., 2014. Stereocontrolled synthesis of *syn*-β-hydroxy-α-amino acids by direct aldolization of pseudoephenamine glycinamide. Angew. Chem. Int. Ed. Engl. 53 (18), 4642–4647. https://doi.org/10.1002/anie.201400928.

Seo, Y., Lee, H.J., Park, K.E., Kim, Y.A., Ahn, J.W., Yoo, J.S., Lee, B.J., 2004. Peroxynitrite scavenging constituents from the brown alga *Sargassum thunbergii*. Biotechnol. Bioprocess Eng. 9, 212-216. https://doi.org/10.1007/BF02942295.

Sestili, P., Diamantini, G., Bedini, A., Cerioni, L., Tommasini, I., Tarzia, G., Cantoni, O., 2002. Plant-derived phenolic compounds prevent the DNA single-strand breakage and

cytotoxicity induced by tert-butylhydroperoxide via an iron-chelating mechanism. Biochem. J. 364 (1), 121-128. https://doi.org/10.1042/bj3640121.

Sharma, R., Yang, Y., Sharma, A., Awasthi, S., Awasthi, Y.C., 2004. Antioxidant role of glutathione S-transferases: protection against oxidant toxicity and regulation of stress-mediated apoptosis. Antioxid. Redox Signal. 6 (2), 289-300. https://doi.org/10.1089/152308604322899350.

Singh, R.R, Reindl, K.M., 2021. Glutathione s-transferases in cancer. Antioxidants. 10 (5),701. https://doi.org/10.3390/antiox10050701.

Silva, J., Alves, C., Freitas, R., Martins, A., Pinteus, S., Ribeiro, J., Gaspar, H., Alfonso, A., Pedrosa, R., 2019. Antioxidant and neuroprotective potential of the brown seaweed *Bifurcaria bifurcata* in an in vitro Parkinson's disease model. Mar. Drugs 17 (2), 85. https://doi.org/10.3390/md17020085.

Smit, A.J., 2004. Medicinal and pharmaceutical uses of seaweed natural products: a review. J. Appl. Phycol. 16, 245–262. https://doi.org/10.1023/b:japh.0000047783.36600.ef.

Snare, D.J., Fields, A.M., Snell, T.W., Kubanek, J., 2013. Lifespan extension of rotifers by treatment with red algal extracts. Exp. Gerontol. 48 (12), 1420-1427. https://doi.org/10.1016/j.exger.2013.09.007.

Song, Y.S., Narasimhan, P., Kim, G.S., Jung, J.E., Park, E.H., Chan, P.H., 2008. The role of Akt signaling in oxidative stress mediates NF-kappaB activation in mild transient focal cerebral ischemia. J. Cereb. Blood Flow Metab. 28 (12), 1917-1926. https://doi.org/10.1038/jcbfm.2008.80.

Strange, R.C., Jones, P.W., Fryer, A.A., 2000. Glutathione S-transferase: genetics and role in toxicology. Toxicol. Lett. 112-113, 357-363. https://doi.org/10.1016/S0378-4274(99)00230-1.

Stockert, J.C., Horobin, R.W., Colombo, L.L., Blázquez-Castro, A., 2018. Tetrazolium salts and formazan products in cell biology: viability assessment, fluorescence imaging, and labeling

perspectives. Acta Histochem. 120 (3). 159-167. https://doi.org/10.1016/j.acthis.2018.02.005.

Suttnar, J., Cermák, J., Dyr, J.E., 1997. Solid-phase extraction in malondialdehyde analysis. Anal. Biochem. 249 (1), 20-23. https://doi.org/10.1006/abio.1997.2157.

Suttnar, J., Másová, L., Dyr, J.E., 2001. Influence of citrate and EDTA anticoagulants on plasma malondialdehyde concentrations estimated by high-performance liquid chromatography. J. Chromatogr. B Biomed. Sci. Appl. 751 (1), 193-197. https://doi.org/10.1016/S0378-4347(00)00453-9.

Thomas, N.V., Kim, S.K., 2013. Beneficial effects of marine algal compounds in cosmeceuticals. Mar. Drugs 11 (1), 146–164. https://doi.org/10.3390/md11010146.

Tsvetkov, P., Asher, G., Reiss, V., Shaul, Y., Sachs, L., Lotem, J., 2005. Inhibition of NAD(P)H: quinone oxidoreductase 1 activity and induction of p53 degradation by the natural phenolic compound curcumin. Proc. Natl. Acad. Sci. U.S.A. 102 (15), 5535–5540. https://doi.org/10.1073/pnas.0501828102.

Turland, N.J., Wiersema, J.H., Barrie, F.R., Greuter, W., Hawksworth, D.L., Herendeen, P.S., Knapp, S., Kusber, W.H., Li, D.Z., Marhold, K., May, T.W., McNeill, J., Monro, A.M., Prado, J., Price, M.J., Smith, G.F., 2018. International code of nomenclature for algae, fungi, and plants (Shenzhen Code), Koeltz Botanical Books, Glashütten. https://doi.org/10.12705/Code.2018.

van Ommen, B., Bogaards, J.J., Peters, W.H., Blaauboer, B., van Bladeren, P.J., 1990. Quantification of human hepatic glutathione S-transferases. Biochem. J. 269 (3), 609–613. https://doi.org/10.1042/bj2690609.

Wan, A.H.L., Davies, S.J., Soler-Vila, A., Fitzgerald, R., Johnson, M.P., 2019. Macroalgae as a sustainable aquafeed ingredient. Rev. Aquac. 11 (3), 458–492. https://doi.org/10.1111/raq.12241.

Wang, H., Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic. Biol. Med. 27 (5-6), 612–616. https://doi.org/10.1016/s0891-5849(99)00107-0.

Wang, T., Jónsdóttir, R., Liu, H., Gu, L., Kristinsson, H.G., Raghavan, S., Olafsdóttir, G., 2012. Antioxidant capacities of phlorotannins extracted from the brown algae *Fucus vesiculosus*. J. Agric. Food Chem. 60 (23), 5874–5883. https://doi.org/10.1021/jf3003653.

Wang, R., Paul, V.J., Luesch, H., 2013. Seaweed extracts and unsaturated fatty acid constituents from the green alga *Ulva lactuca* as activators of the cytoprotective Nrf2–ARE pathway. Free Radic. Biol. Med. 57, 141-153. https://doi.org/10.1016/j.freeradbiomed.2012.12.019.

Yuan, C., Li, Z., Peng, F., Xiao, F., Ren, D., Xue, H., Chen, T., Mushtaq, G., Kamal, M.A., 2015. Combination of selenium-enriched green tea polysaccharides and Huo-ji polysaccharides synergistically enhances antioxidant and immune activity in mice. J. Sci. Food Agric. 95 (15), 3211-3217. https://doi.org/10.1002/jsfa.7287.

Yoon, J.H., Youn, K., Jun, M., 2021. Protective effect of sargahydroquinoic acid against $A\beta_{25-35}$-evoked damage via PI3K/Akt mediated Nrf2 antioxidant defense system. Biomed. Pharmacother. 144, 112271. https://doi.org/10.1016/j.biopha.2021.112271.

Youle, R.J., Strasser, A., 2008. The BCL-2 protein family: opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. 9, 47-59. https://doi.org/10.1038/nrm2308.

Zheng, F., Gonçalves, F.M., Abiko, Y., Li, H., Kumagai, Y., Aschner, M., 2020. Redox toxicology of environmental chemicals causing oxidative stress. Redox Biol. 34, 101475. https://doi.org/10.1016/j.redox.2020.101475.

FIGURE LEGENDS

Fig. 1. Effect of *tert*-BOOH (50, 100, 200 and 400 μM) on cell viability, ROS generation and caspase 3/7 activity in Caco-2 cells. **(A)** Cells were treated during 3, 9 and 15 h with *tert*-BOOH and cell viability was measured by MTT assay. **(B)** Cells were exposed to *tert*-BOOH and intracellular ROS production was evaluated at 15, 30 and 60 min. **(C)** Cells were treated during 1, 3 and 5 h with *tert*-BOOH and caspase 3/7 activity was determined.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Values are expressed as a percent relative to the control condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Fig. 2. *B. bifurcata* extract (10, 50, 100, 250 and 500 μg/mL) did not affected cell viability **(A)** and cytotoxicity **(B)** in Caco-2 cells. Cell viability and cytotoxicity were measured as MTT reduction and LDH release, respectively.

Protective effect of *B.bifurcata* extract (10, 50, 100, 250 and 500 μg/mL) in Caco-2 cells against *tert*-BOOH (200 μM) on MTT reduction **(C)** and LDH release **(D)**.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Values are expressed as a percent relative to the control condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Fig. 3. *B. bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) did not affected GSH **(A)** and MDA **(B)** levels in Caco-2 cells..

Protective effect of *B. bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) in Caco-2 cells against *tert*-BOOH (200 µM) on GSH **(C)** and MDA **(D)** levels.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Values are expressed as a percent relative to the control condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Fig. 4. *B. bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) did not affected ROS generation **(A)** and NO production **(B)** in Caco-2 cells.

Protective effect of *B. bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) in Caco-2 cells against *tert*-BOOH (200 µM) on ROS generation **(C)** and NO production **(D)**.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Values are expressed as a percent relative to the control condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Fig. 5. *B. bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) did not affected caspase 3/7 activity **(A)** in Caco-2 cells.

Protective effect of *B.bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) in Caco-2 cells against *tert*-BOOH (200 µM) on caspase 3/7 activity **(B)**.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Values are expressed as a percent relative to the control condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Fig. 6. *B. bifurcata* extract (10, 50, 100, 250 and 500 µg/mL) did not affected the antioxidant enzymes NQO1 **(A)** and GST **(B)** activities in Caco-2 cells.

Protective effect of *B.bifurcata* extracts (10, 50, 100, 250 and 500 µg/mL) in Caco-2 cells against *tert*-BOOH (200 µM) on NQO1 **(C)** and GST **(D)** activities.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Values are expressed as a percent relative to the control condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Fig. 7. *B. bifurcata* extract (100 and 500 µg/mL) induces GSTM2, Nrf2 and AKT1 expressions. Real-time PCR determination of GSTM2 **(A)**, Nrf2 **(B)** and AKT1 **(C)** mRNA levels in the presence of *B. bifurcata* extract in Caco-2 cells.

Data represent the mean ± SEM of 4 independent experiments in triplicate. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Table 1

Sequences of forward and reverse primers for apoptosis, inflammation and oxidative stress related genes.

Genes	Primer forward sequence	Primer reverse sequence
Housekeeping gene		
GAPDH	GAGAAGGCTGGGGCTCATTT	AGTGATGGCATGGACTGTGG
Apoptosis related genes		
AKT1	GAAGGACGGGAGCAGGC	TGTACTCCCCTCGTTTGTGC
ERK1	GGCCCGAAACTACCTACAGTC	GAAGATGAGCTCCTTCAGCCG
JNK1	CTGAAGCAGAAGCTCCACCA	GCCATTGATCACTGCTGCAC
Bax	CCCCCGAGAGGTCTTTTTCC	CCTTGAGCACCAGTTTGCTG
BNIP3	CCTCAGCATGAGGAACACGA	GCCACCCCAGGATCTAACAG
Proinflammation and oxidative stress related genes		
GSTM2	GATCACCTTTGTGGATTTCATCGC	TGTGAACACAGGTCTTGGGA
Nrf2	CTGGTCATCGGAAAACCCCA	TCTGCAATTCTGAGCAGCCA
NFκB1	TTTTCGACTACGCGGTGACA	GTTACCCAAGCGGTCCAGAA
IL-6	CCAGTACCCCCAGGAGAAGA	CAGCTCTGGCTTGTTCCTCA
HO-1	GCTCAAAAAGATTGCCCAGA	GCGGTAGAGCTGCTTGAACT

Table 2

Effect of *B. bifurcata* extract on upregulation of apoptosis, proinflammation and oxidative stress transcripts induced by *tert*-BOOH

Gene symbol	**(A)** Fold of increase by *tert*-BOOH (200 µM)	**(B)** Fold of increase by *tert*-BOOH (200 µM) + *B. bifurcata* extract (500 µg/mL)
ERK1*	3.07[a]	1.31[b]
JNK1*	2.91[a]	1.22[b]
Bax*	2.64[a]	1.46[b]
BNIP3*	2.34[a]	1.43[b]
NFκB1**	2.86[a]	1.47[b]
IL-6**	2.79[a]	1.38[b]
HO-1**	2.72[a]	1.76[b]

Note:

*Genes related to apoptosis.

**Genes related to both proinflammation and oxidative stress.

Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Figure 1

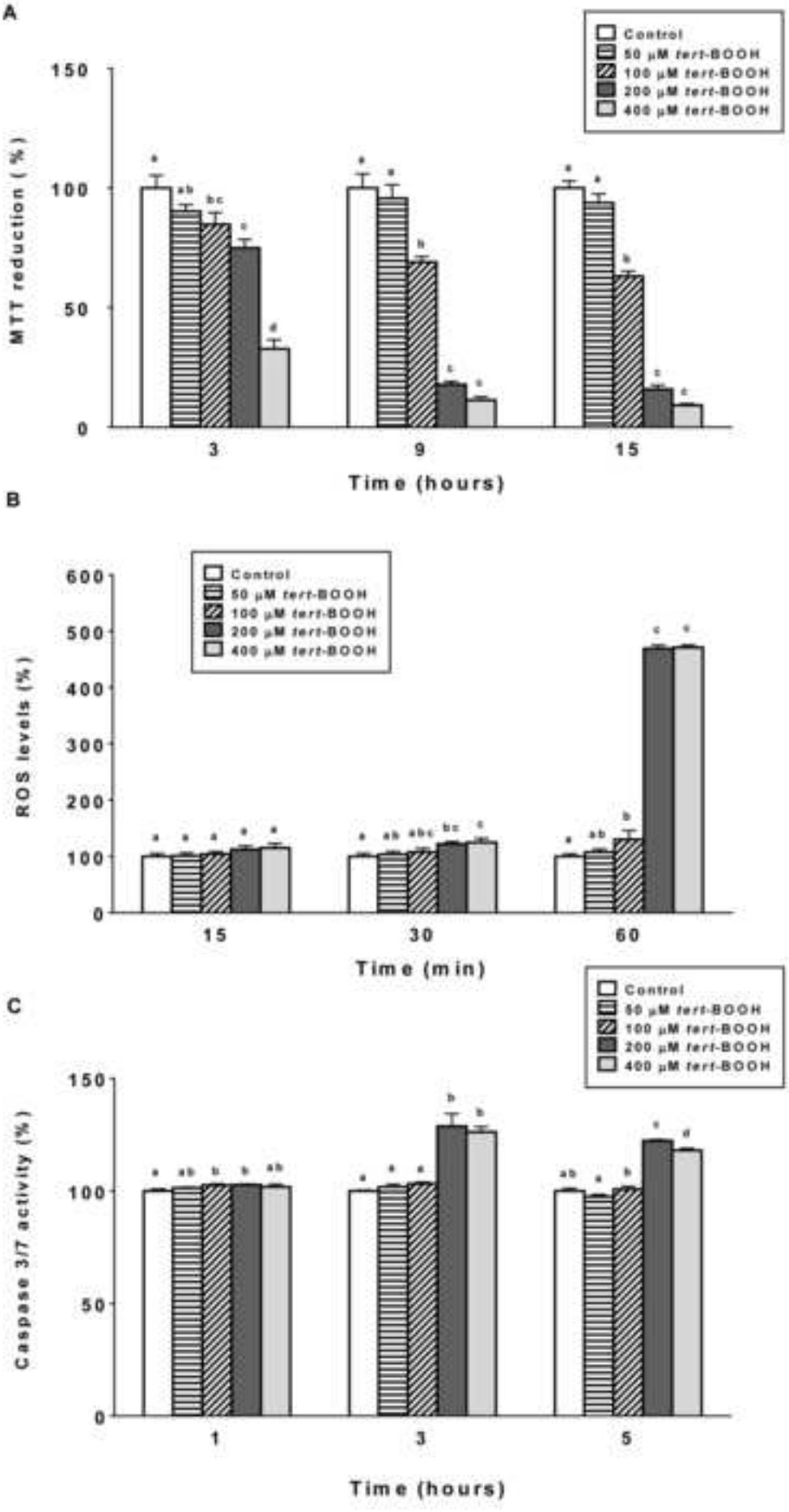


Figure 2

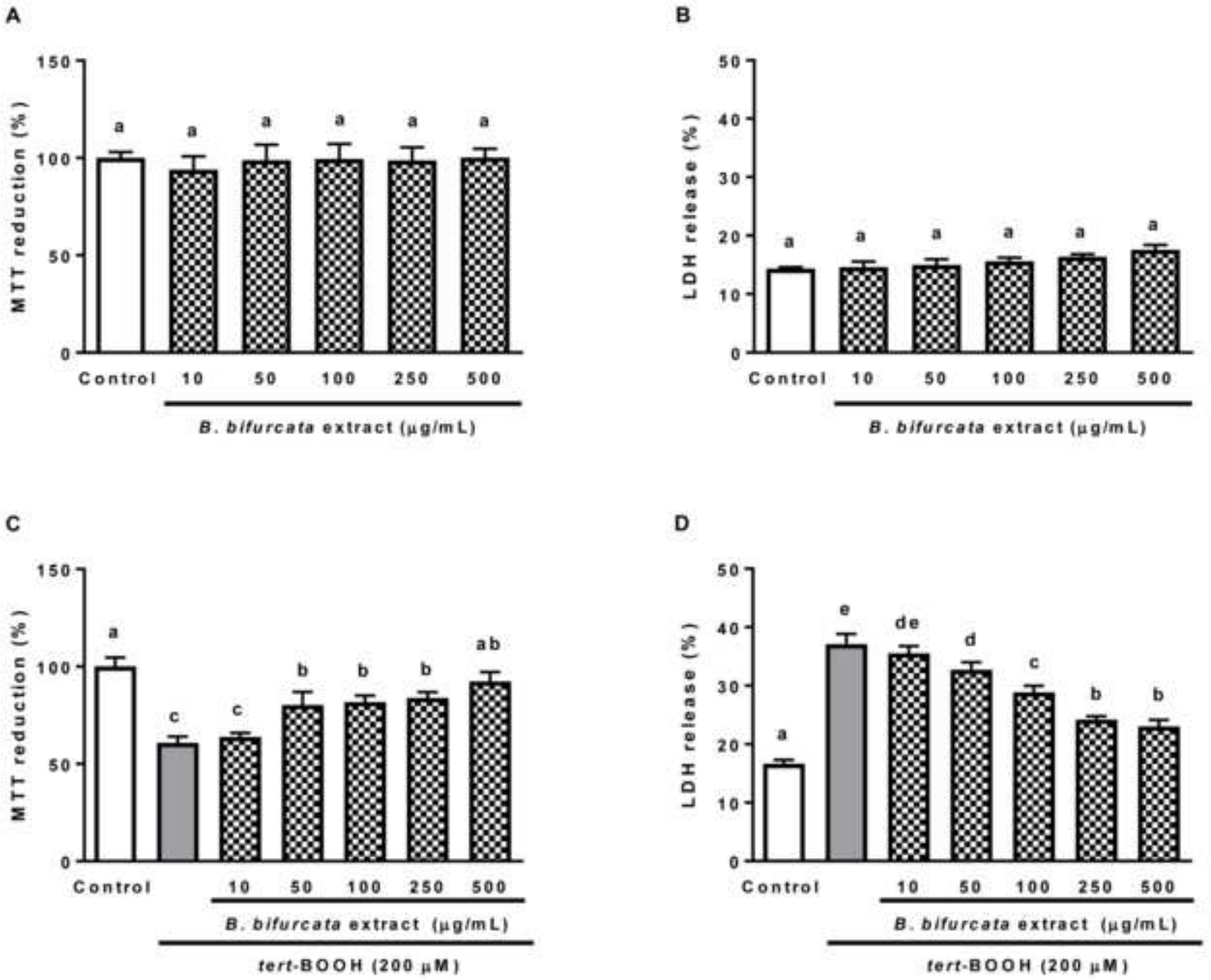
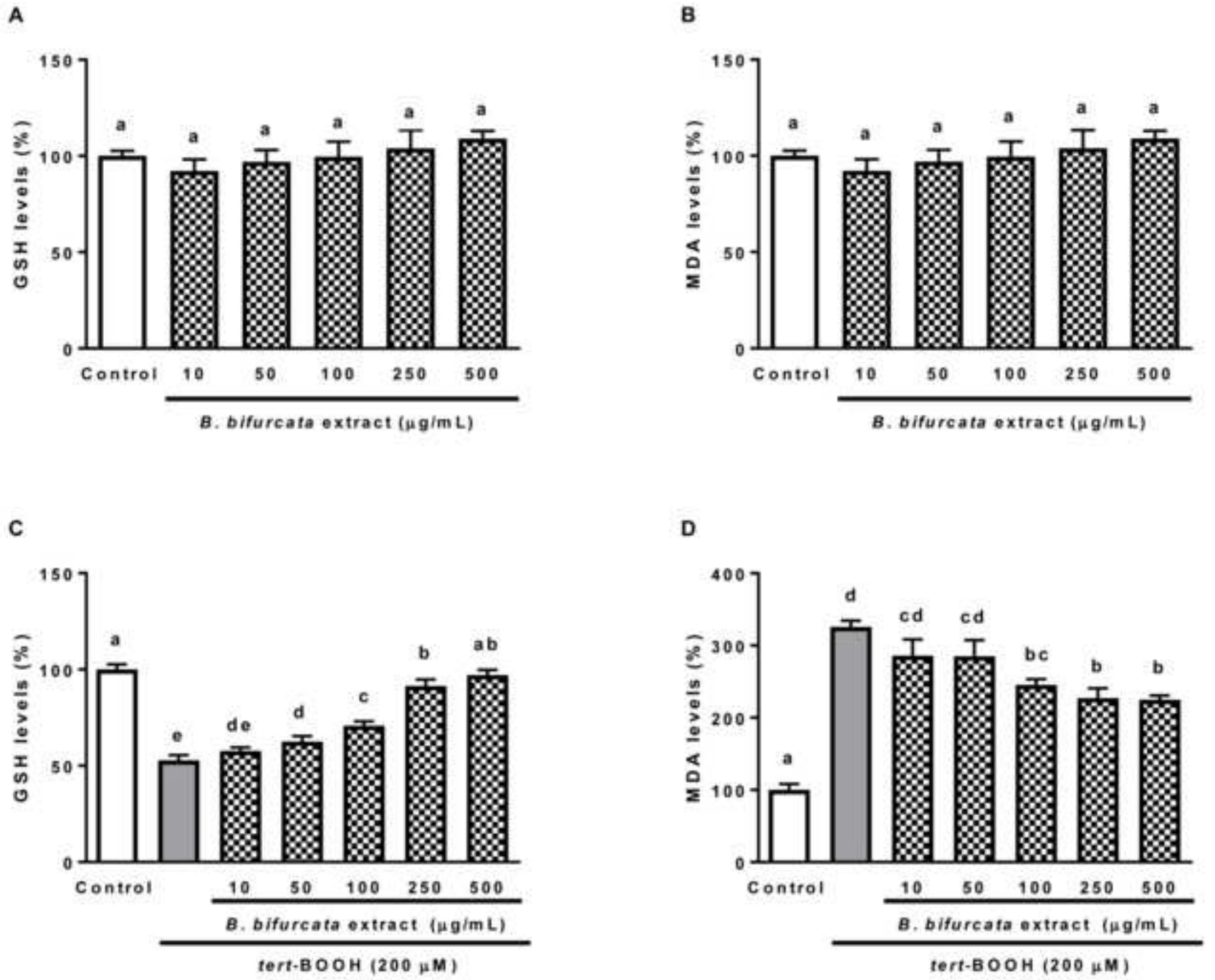


Figure 3



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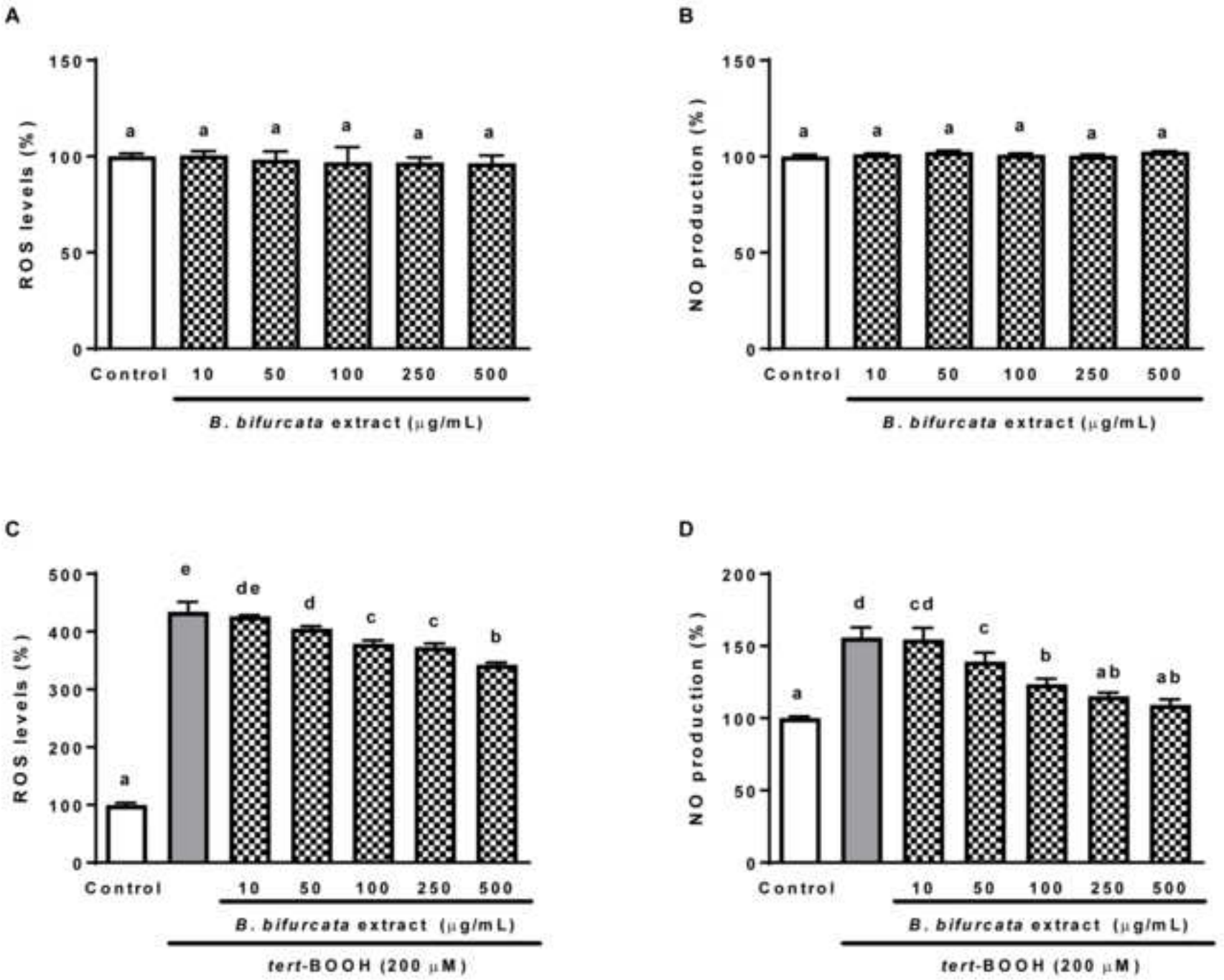

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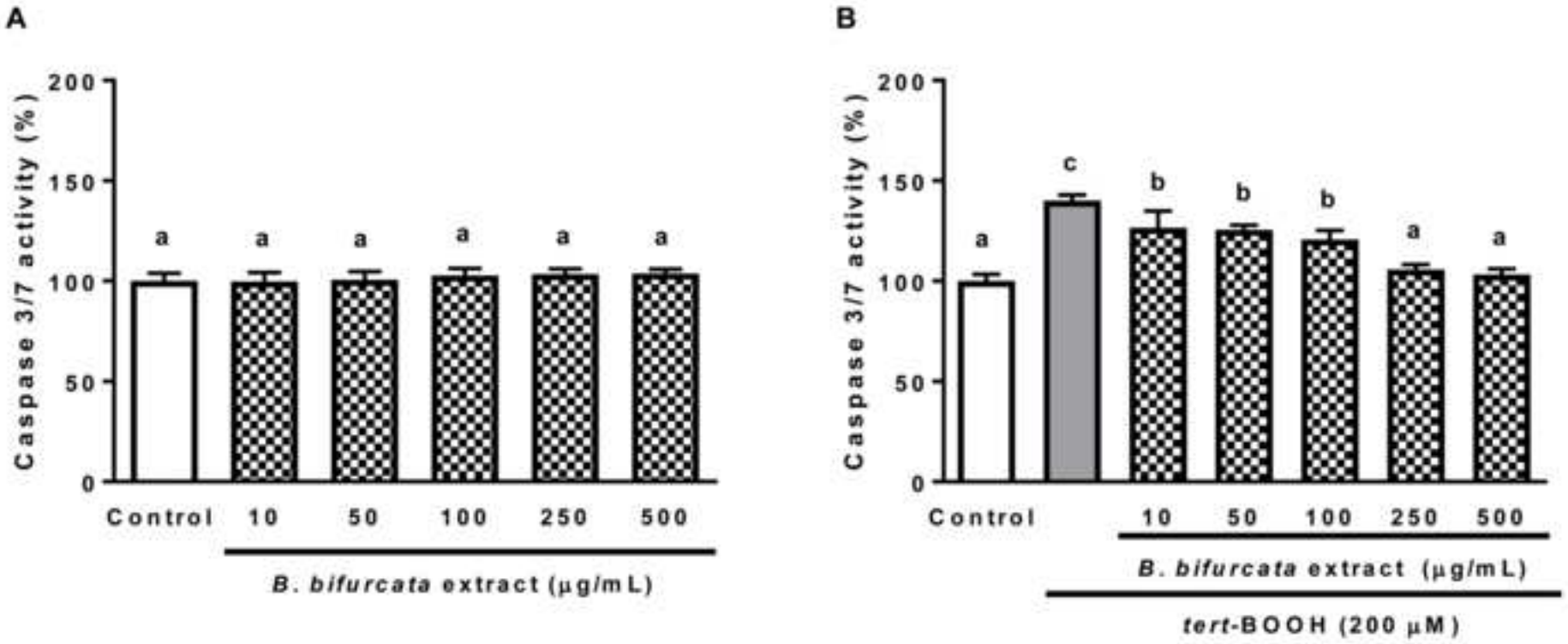

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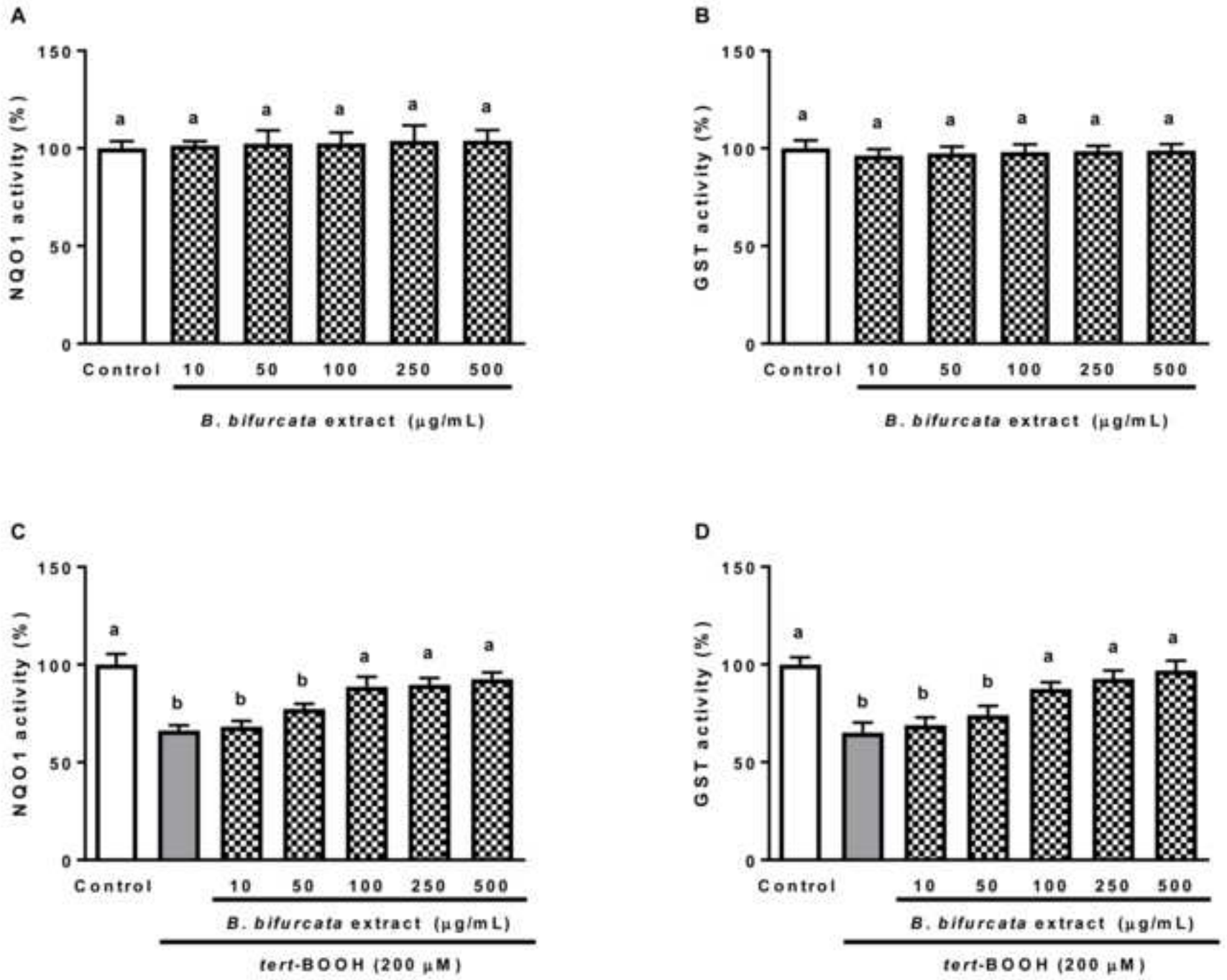
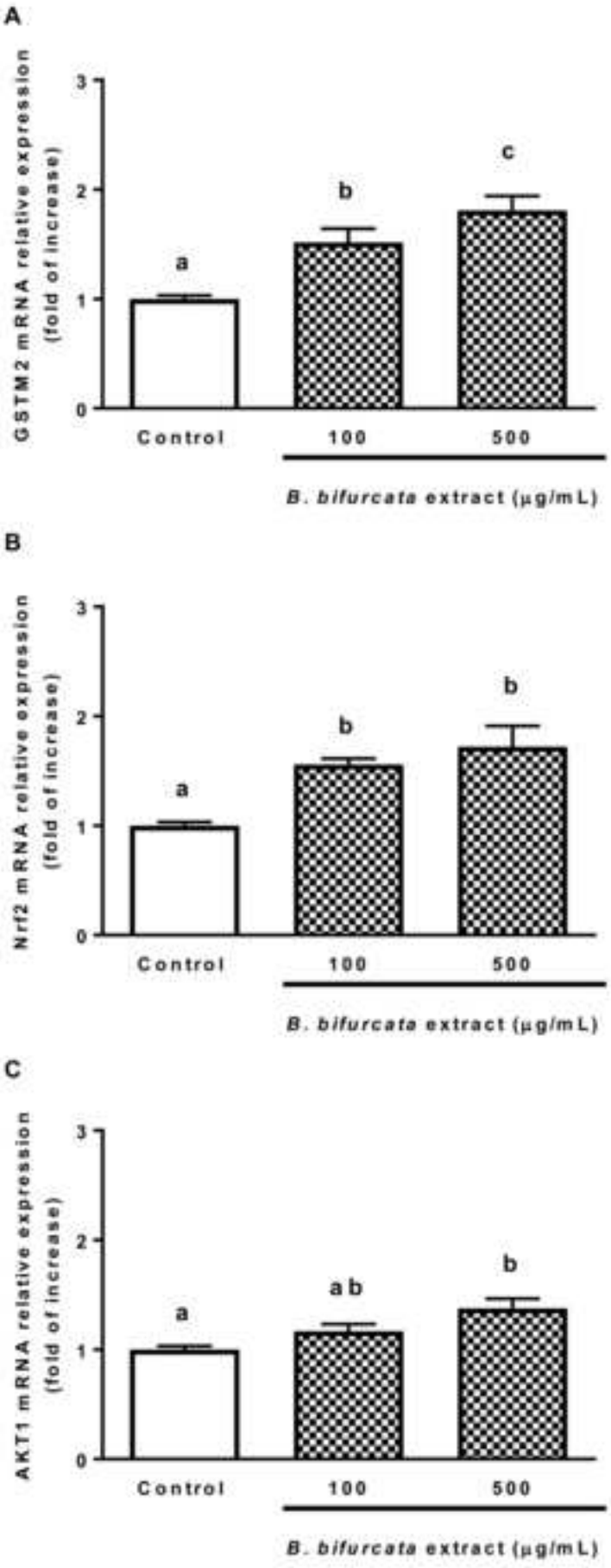


Figure 7



Credit author statement

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Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: