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The anthocyanin-enriched extract of *Vaccinium corymbosum* exerted in vitro and ex vivo antiproliferative and anti-inflammatory activities

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Abstract

Background: *Vaccinium corymbosum* berries represent a source of anthocyanins and polyphenols studied and tested for their healthy potential. The present study was aimed at isolating, characterizing, and quantifying the anthocyanin-enriched fraction obtained from blueberry and at assessing its biological and protective effects against head and neck cell lines and under inflammatory-related conditions. *V. corymbosum* berries extract was subjected to colorimetric analysis, antioxidant evaluation, and HPLC-DAD analysis. This extract was characterized by chlorogenic acid and 12 glycosylated anthocyanins, being the most abundant delphinidin-3-O-galactoside, delphinidin-3-O-arabinoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside, and malvidin-3-O-arabinoside. The blueberry extract (BL) was further used in extensive biological evaluation. Head and neck cell lines, namely CAL27 and A253, were treated at different concentrations in order to evaluate cell migration ability and ErbB receptors and antioxidant enzymes gene expression.

Results: The gene expression analysis highlighted the capability of BL to reduce ErbB receptors expression in CAL27 cell lines. The same treatments induced an opposite effect in A253 cell line for ErbB2/ErbB3 receptors. Moreover, in A253 cell line, BL increased SOD2 levels and reduced cell migration. The action of BL was also studied in an *ex vivo* experimental model of colon inflammation and was effective both in reducing the lipopolysaccharide-induced gene expression of different proinflammatory biomarkers involved in colon inflammation, among which tumor necrosis factor α, interleukin (IL)-6, and in stimulating the gene expression of the anti-inflammatory cytokine IL-10.

Conclusion: The content of specific anthocyanins and chlorogenic acid can be considered responsible of such biological activities providing a new food supplement as coadjuvant of standard therapies.

Giuseppe Falcone and Mariangela Mazzone contributed equally to this work.

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KEYWORDS
anthocyanins, blueberry, head and neck cancer, HPLC-DAD analysis, IBD, *Vaccinium corymbosum*

INTRODUCTION

Vaccinium corymbosum L. is a globally widespread deciduous shrub belonging to the Ericaceae family.[1] Besides, it is the most common commercially grown blueberry in North America, northern high shrub adapted itself to different areas characterized by woods and moist acidic soils. Production and consumption of its sweet fruit, raw or processed, conferred the status of "super fruit"[2] due to the high nutritional and economic value. Recently, it was developed as food supplement for disease prevention[3–5] and several studies highlighted the valuable content in bioactive components (organic acids as citric acid, tannins, polyphenols as stilbenoids, anthocyanins and flavonoids, sugar, minerals, vitamins, fibers, and pectins).[6] Daily intake of blueberries is thus highly recommended for their antioxidant properties, and they can serve as natural colorants and preservatives in foods. As for other natural matrices, processing and extraction procedures can profoundly affect the composition[7,8] and, therefore, the biological potential of this edible fruit.

Despite the large plethora of articles related to the beneficial effects,[9,10] our interest focused on the recently investigated anti-proliferative/anti-inflammatory properties of *V. corymbosum*.[11–14] More in detail, most of the proposed healthy properties were correlated to the high content of anthocyanins in blueberry. These derivatives of 2-phenylbenzopyran were investigated as chemo-preventive and antiproliferative/inflammatory agents involving pleiotropic mechanisms of action, alone or in combination to improve the sensitivity to currently used chemotherapeutic drugs.[15–17]

Head and neck cancer (HNC) is the seventh most common cancer worldwide and it accounts ~900,000 cases and over 400,000 deaths every year.[18] Over 90% of HNC are squamous cell carcinoma (SCC) and the highest frequency site of occurrence is the oral cavity[19]. At present, the main treatments are surgery, radiation, and chemotherapy, but their use is not 100% effective and is mostly associated with adverse toxic effects. The ErbB family of receptor tyrosine kinases which includes epidermal growth factor receptor (EGFR, ErbB1), ErbB2, ErbB3, and ErbB4, regulates a complex signaling network that impacts several cellular processes such as proliferation, survival, angiogenesis, and metastasis in many cancers, including HNC. EGFR is overexpressed in HNC, especially in SCC, and is correlated with decreased survival, resistance to radiation, local treatment failure, and increased distant metastasis.[20] It has been proposed that oxidative damage facilitates tumor initiation and progression by enhancing mutation rates and triggering oncogenic pathways through increased reactive oxygen species (ROS) levels that disrupt cellular redox balance.[21,22] Moreover, elevated ROS levels, together with downregulation of cellular antioxidant enzymes, result in malignant transformation. Since a few effective treatment options are available for patients with recurrent or unresectable tumors, finding a cure for HNC remains a challenge for experimental and clinical oncology. Recently, we have seen a resurgence of natural compounds (NCs) for new drug discovery in particular for cancer treatment due to their mild or no side effects and their easy availability. Several studies reported the capability of colorful fruits, such as blueberry, to reduce the risk of some types of human cancer and their anticancer effects in combination with chemotherapy agents due to their antioxidant potential.[23]

In addition, the protective effects exerted by blueberry were also evaluated against the inflammatory damage produced on intestinal cells during Crohn's disease (CD) and ulcerative colitis (UC), the two main types of inflammatory bowel disease (IBD): a group of inflammatory conditions of the colon and small intestine.[24] IBD and oxidative stress (OS), a phenomenon characterized by the imbalance between the production of ROS and antioxidant defenses, are highly correlated: OS plays a pivotal role in the initiation and progression of IBD with overproduction of ROS during inflammation due to the high number of cytokines and chemokines secreted by inflammatory cells.[25–27] Furthermore, the increase in ROS determines malabsorption and intestinal atony and to avoid these unpleasant problems, scientific research is focusing on understanding the close relationship between OS and the onset of IBD.[28]

Based on these premises and given that the insufficient levels of anthocyanins in the most common foods also limit their optimal benefits, the research was aimed at isolating, purifying, quantifying, and characterizing the anthocyanin-enriched fraction obtained from a local Italian blueberry using of an eco-friendly, easily reproducible at industrial scale, procedure and at assessing its pharmacological effects against head and neck cell lines and under inflammatory-related conditions. We chose to use two different HNC cell lines, namely CAL27 and A253, because their response to *V. corymbosum* extracts treatment, to the best of our knowledge, has not been investigated yet. Finally, the use of an enriched extract will allow a better correlation between the chemical content and biological activities avoiding interferences of sugars and other components abundant in the natural matrix.

MATERIALS AND METHODS

Ethanol, methanol, and acetonitrile (HPLC-grade) were obtained from Merck Science Life s.r.l (Milan, Italy). Bidistilled water was purchased from Carlo Erba s.r.l. (Milan, Italy). Fresh blueberries (*Vaccinium corymbosum*), kindly cultivated, authenticated, and provided by "Naturagri" (San Giorgio Lucano, MT, Italy), were collected in May 2021 and stored at −20°C until processing.

Extraction and purification methods

Frozen berries were divided into four parts and dried for 72 h using a freeze-dryer (Labconco, Kansas City, MO, USA). After being freeze-dried, the berries were pulverized using a mortar and pestle ensuring a homogenized material. The crushed berries were stored at −20°C until preliminary extraction with different solvents (methanol, ethanol, and water) to assess the best choice for this natural matrix in terms of

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percent recovery of total anthocyanins from 15 to 120 min of extraction (each extraction was performed three times). Then, an aliquot of crushed berries (1 g) was extracted at room temperature and in the dark with 10 mL of ethanol, for 15 min under mechanical stirring and then for 30 min in an ultrasonic bath. Then, the suspension was centrifuged at 31,200 *g* for 15 min at 4°C using the Model J2-21 centrifuge (Beckman Coulter, Brea, CA, USA). The supernatant collected was purified using a reversed phase chromatographic technique. The column used was made of Pyrex glass, 30 cm long with a diameter of 1 cm. As a stationary phase, the content of four Resprep® SPE Cartridges (C18—25 mL–500 mg) (Restek Corporation, Bellefonte, PA, USA) was used. The column was activated with one volume of ethanol and conditioned with one volume of distilled water. The whole system was placed under pressure using nitrogen at a constant pressure of 1 atm. The supernatant collected from the centrifugation was loaded into the column and washed with distilled water to reduce the sugar content. Then, at the first appearance of the pink color, the water solution was selected, until the complete collection of the violet fraction (anthocyanin-enriched) was achieved. The anthocyanin-enriched violet fraction was stored ad −80°C until lyophilization, using the nitrogen current dryer TurboVap® LV (Caliper Life Science, Waltham, MA, USA) and the resulting lyophilized powder (BL) stored at −80°C until HPLC analysis.

pH and color analysis

The provided powder (BL) was dissolved in water (15 mg/mL) at different pH values (pH 2.5; pH 3.5; pH 4.5). pH was controlled by a pH-meter Mettler-Toledo GmbH (Greifensee, Switzerland). The lyophilized extract (BL) and the relative obtained solutions were submitted to CIE-L**a***b* analysis with a colorimeter X-Rite MetaVueTM (Prato, Italy) equipped with a full-spectrum LED illuminant and an observer angle of 45°/0° imaging spectrophotometer. The CIEL**a***b** parameters (*L**, *a**, *b**, Cab*, hab) and the reflectance percentages were calculated.[29] Results of color analysis are reported in Supplementary Materials (Figure S1).

HPLC-DAD analysis

The ethanolic extract (before SPE) and the anthocyanin-enriched violet fraction (obtained after SPE) were used after reconstitution in water at 1–10 mg/mL. The obtained solutions were filtered through a syringe with 0.45 μm pores (Whatman, Maidstone, UK) and analyzed with two different HPLC systems. An HPLC-DAD system Agilent Technologies 1200 Series (Agilent Technologies, Santa Clara, CA, USA) equipped with a C18 Phenomenex 5 μm, 100 Å, LC column 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) was used for the detection of anthocyanins. The column temperature was set to 24°C, the injection volume to 20 μL, and the flow rate to 1 mL/min. About 3% formic acid in water was used as solvent A, and acetonitrile/methanol/water (80:10:10, v/v/v) was used as solvent B. The gradient, according to Liu et al.,[30] was 0–45 min, linear gradient from 5% to 29% B; 45–46 min, linear gradient from 29% to 50% B; 46–48 min, 50% B isocratic; 48–49 min, linear gradient from 50% to 5% B; 49–55 min, 5% B isocratic. The mobile phases were filtered by 0.45 μm nylon membrane filters and degassed by ultrasonication. The detection wavelength was selected at 520 nm. Peaks were identified on the base of their UV–visible spectra and by comparison with the elution order reported in other published studies.[8,31,32] Anthocyanins were quantified as the sum of peaks areas recorded at 530 nm and expressed as Cyanidin-3-O-glucoside, by calibration line shown in Tables S1 and S2. An HPLC-DAD (Perkin Elmer, Milan, Italy), according to the method described by Garzoli et al.,[33] was used for the detection of phenolic acids and flavonoid fractions other than anthocyanins, at wavelengths of 280 and 360 nm, respectively. Analyses were performed on Luna RP-18, 3 μ column, with a linear gradient consisting of acetonitrile and acidified water (5% formic acid), from 100% aqueous phase to 85% in 15 min, 85% to 55% in 30 min and 55% to 40% in 20 min, at a flow rate of 0.8 mL/min. In both cases, analytes were identified by comparing retention times and UV–Vis absorption spectra to those of authentic standards (Tables S1 and S2, Figures S2 and S3). All determinations were performed at ambient temperature.

DPPH (2,2-diphenyl-1-picryl-hydrazyl) antioxidant activity

According to Cairone et al.,[34] with slight modifications, a 100 μM solution of DPPH was prepared in ethanol. Then, 2 mL of this solution was added to 1 mL of ethanol, stored in the darkness, and monitored by UV/VIS Lambda 25 spectrophotometer (Perkin Elmer Waltham, MA, USA), at the wavelength of 515 nm, until the absorbance value was stable. 0.5 mL of the sample solution (1 mg/mL of ethanolic extract and 0.1 mg/mL of BL were used) was added with 2 mL of the same DPPH solution and 0.5 mL of ethanol. The absorbance at 515 nm was controlled, following the same conditions described above, and the reduction of DPPH absorbance after 30 min was detected. A calibration curve (using gallic acid as reference) was used ($y = 0.6473e - 378.5x$), and the antioxidant capacity was expressed as mg of gallic acid equivalents/g of dry extract and as IC_{50} (mg of gallic acid/mL).

Cell lines culture and treatments

Human tumor cell line CAL27 (derived from a tongue squamous cell carcinoma) was kindly provided by Prof. L. Lo Muzio, University of Foggia, Italy and has been authenticated by short tandem repeat (STR) DNA genotyping (Figure S4), whereas A253 cell line (derived from a submaxillary salivary gland carcinoma) was purchased from American Type Culture Collection (ATCC #HTB-41, Rockville, MD, USA). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium and McCoys medium (EuroClone S.p.A., Pero (MI), Italy), respectively, supplemented with 10% fetal bovine serum (FBS). Tumor cells were cultured in complete cell culture medium supplemented with different concentrations of BL (50, 100, 200, and 500 μg/mL in water) for the

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time reported in the various experiments. Stock solutions of the BL were prepared in H_2O and stored at −80°C. The human submandibular carcinoma A253 cell line and the human tongue squamous cell carcinoma CAL27 cell line used in this study were selected for their different anatomical localization, low differentiation degree of the primary tumor (CAL27 has a G3 differentiation grade), and their proven invasive capacity.[35–37] Moreover, A253 has characteristics of therapy resistance.

Wound healing migration assay

The cell migration ability of CAL27 and A253 cells after BL treatment was assessed using the wound healing assay as previously described.[38] The wound healing scratch assay was performed both on untreated control cells and on cells treated with BL. The cells were cultured in 24-well plates and at 95% confluence a scratch wound was made on the cell culture followed by treatment. The plates were incubated, and cellular migration was recorded at 24 h. Wound closure was monitored by microscopy, and images of 10–15 random areas were captured using Leica DMi1 inverted microscope. The wound width was calculated by measuring the scratch along the border using the ImageJ 1.53a analysis software.

Ex vivo studies

Adult C57/BL6 mice (3-month-old, weight 20–25 g) were housed in Plexiglas cages (2–4 animals per cage; 55 × 33 × 19 cm) and maintained under standard laboratory conditions (21 ± 2°C; 55 ± 5% humidity) on a 14/10 h light/dark cycle, with ad libitum access to water and food. Housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 63/2010) on the care of animals for scientific research. According to the recognized principles of "Replacement, Refinement and Reduction in Animals in Research," colon specimens were obtained as residual material from vehicle-treated animals randomized in our previous experiments, approved by the local ethical committee ('G. D'Annunzio' University, Chieti, Italy) and the Italian Health Ministry (Project no. 885/2018-PR). Isolated colon specimens collected from euthanized mice were maintained in a humidified incubator with 5% CO2 at 37°C for 4 h (incubation period), in RPMI buffer with added *Escherichia coli* lipopolysaccharide (LPS) (50 μg/mL) and in the presence of BL (50, 100, 200, and 500 μg/mL).

Gene expression analysis for *in vitro* and *ex vivo* experiments

Extraction of total RNA was performed from the CAL27 and A253 untreated cells, from the following samples of CAL27 and A253 cells treated with BL (50, 100, 200, and 500 μg/mL) after 24 h and from the colon specimens, using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), in agreement with the manufacturer's protocol. For the *in vitro* experiments, RNA samples were assessed for purity and quantified by Nanodrop 1000 Spectrophotometer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The synthesis of complementary DNA (cDNA) was performed employing the GoTaq® 2 Step RT-qPCR Kit (Promega) according to the manufacturer's instructions. The mRNA levels were evaluated using IDT primers (Integrated DNA Technologies, Leuven, Belgium) (Table S2) by a SYBR green quantitative Real-time PCR (qRT-PCR) analysis using the StepOne™ 2.0 real-time PCR system (Applied Biosystems). The cycling conditions were performed as follows: 10 min at 95°C and 40 cycles of 15 s at 95°C, followed by 1 min at 60°C and final elongation of 15 s at 95°C. For each experimental condition, expression of EGFR, ErbB2, ErbB3, CAT, iNOS, and SOD2 genes was assessed performing three replicates in three independent experiments.

For the *ex vivo* experiments the gene expression of TNF-α, IL-6, and IL-10 was conducted as previously reported.[39] Total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (ThermoFischer Scientific, Waltman, MA, USA). Gene expression was determined by quantitative real-time PCR using TaqMan probes: TNF-α (Mm00443258), IL-6 (Mm00446190), and IL-10 (Mm0188386) and β-actin (Mm02619508). The mRNA amounts of the target genes of *in vitro* and *ex vivo* experiments were normalized by the ratio on the median value of the endogenous housekeeping β-actin. Relative quantification of gene expression was performed by the comparative $2^{-\Delta\Delta Ct}$ method.[40]

Statistical analysis

The software GraphPad Prism version 5.01 (Graphpad Software Inc., San Diego, CA, USA) was used to perform data analysis. Means ± SD were determined for each experimental group and analyzed by ANOVA, followed by Newman–Keuls multiple comparison for real-time PCR *ex vivo* analysis and Bonferroni test post hoc test for real-time PCR *in vitro* analysis, respectively. The limit of statistically significant differences between mean values was set at p-value <0.05.

RESULTS AND DISCUSSION

Extraction optimization and anthocyanins enrichment

To test the ability of an environmentally friendly anthocyanin-enriched extract to prevent different chronic diseases, a quality source of these valuable components was chosen. The blueberries were preliminary submitted to simple and efficient extraction protocols in order to afford a minimal impact associated to the best yield performance. Extraction conditions have been optimized after numerous attempts. After fresh picked fruits freeze-drying and pulverization steps, the analysis for the most appropriate extraction solvent was carried out. The extraction solvents chosen where methanol, ethanol, and distilled water (Figure S5). Testing the pre-extraction protocol by HPLC analysis, ethanol was

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found to be the best extraction solvent in terms of yield. A study was also conducted on the best combined extraction times of mechanical stirring and ultrasonic bath. Four extractions were carried out with different times for mechanical agitation and for ultrasonic bath, respectively of 15, 30, 60, and 120 min. In the protocol, the first extraction was done by mechanical stirring at room temperature and in the dark. Of the four times examined, after 15 min the quantity of extracted anthocyanins remained unchanged, so it was decided to stop the first extraction at 15 min, then the solution thus obtained was subjected to an ultrasound bath extraction. Of the four times examined, after 30 min the quantity of extracted anthocyanins remained unchanged, so it was decided to stop the second extraction at 30 min. After setting up the extraction protocol, we moved on to the purification protocol. The applied purification step, based on solid-phase extraction method, produced an extract highly enriched in violet pigments, namely the anthocyanin components. The optimization of the chromatographic technique confirmed our previous experience that the violet component enriched in anthocyanins was the first fraction collected after washing with distilled water. The obtained anthocyanin-enriched fraction, through a single step purification, was a highly purified nontoxic water solution (as further confirmed by the HPLC analysis) ready to be directly freeze-dried (BL) and stored until further analysis. Our attention was then focused on the specific bioactive class of anthocyanins, which are responsible of many beneficial properties.

HPLC-DAD analysis and DPPH activity

The composition analyses performed at 520 nm showed the presence of 12 different anthocyanins (Figure 1). Nine main peaks were tentatively assigned through comparison with literature data and our previous results.[8,32] These were identified as delphinidin-3-O-galactoside (1), cyanidin-3-O-galactoside (2), delphinidin-3-O-arabinoside (4), petunidin-3-O-galactoside (6), cyanidin-3-O-arabinoside (7), petunidin-3-O-arabinoside (9), malvidin-3-O-galactoside (10), malvidin-3-O-glucoside (11), and malvidin-3-O-arabinoside (12). As provided by the purification by solid-phase extraction, the anthocyanins content of BL results about 20-fold more concentrated with respect to the firstly obtained ethanolic extract. In fact, the anthocyanins sum, expressed as mg equivalent of cyanidin-3-O-glucoside/g of enriched extract (BL), affords to about 20 (19.2) mg/g enriched extract, compared with about 1 mg/g of ethano lic extract. Further analyses performed at 280 and 360 nm showed the only presence of chlorogenic acid. This is coeluted with anthocyanins in reverse phase, as well as other flavonoid components were not revealed. Chlorogenic acid content, resulted of 25.5 mg/g enriched extract, about 25-fold concentrated after SPE purification of ethanolic extract (about 1 mg/g of ethanolic extract).

The DPPH assays were also applied to evaluate the radical scavenging activity of the ethanolic extract and of the obtained purified powder BL. This showed an about 20-fold potentiated antiradical activity (from 3.9 ± 0.1 mg of gallic acid equivalents/g dry ethanolic extract, results congruent with literature (about 4–8 mg of gallic acid equivalents/g dry extract)[41] to 69.7 ± 0.3 mg of gallic acids equivalents/g dry matter in BL.[42,43] IC_{50} values (19.5 ± 0.30 mg/mL in dry ethanolic extract; 1.08 ± 0.03 mg/mL in BL purified extract).

Considering an about 100–200 mg mean content of total anthocyanins and about 30–100 mg of chlorogenic acid in 100 g of fresh blueberries[44–47] quantitatively extracted with ethanol and concentrated twenty and twenty-five folds with SPE, respectively, a content of about 20–40 mg/g of dry anthocyanin extract and 15–25 mg/g of dry chlorogenic acid extract can be achieved. Anthocyanins and chlorogenic acid represent together the almost whole potential healthy properties of blueberries, as shown by the DPPH results, which show a twenty-fold potentiated anti-radical activity of the SPE purified extract with respect to the initial ethanolic one.

Analysis of the effects of BL on A253 and CAL27 cell migration

To determine whether BL treatment affects cell migration, an event representative of cell invasion ability and tumor metastasis, we

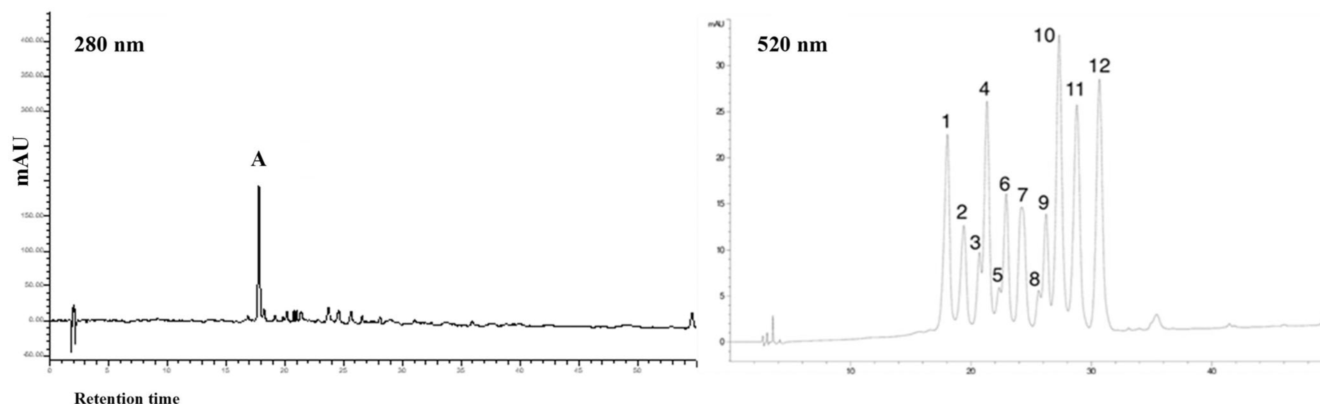

FIGURE 1 Chromatogram of the enriched lyophilized extract (BL) recorded at 280 (left) and 520 (right) nm (chlorogenic acid (A), delphinidin-3-O-galactoside (1), cyanidin-3-O-galactoside (2), delphinidin-3-O-arabinoside (4), petunidin-3-O-galactoside (6), cyanidin-3-O-arabinoside (7), petunidin-3-O-arabinoside (9), malvidin-3-O-galactoside (10), malvidin-3-O-glucoside (11), and malvidin-3-O-arabinoside (12).

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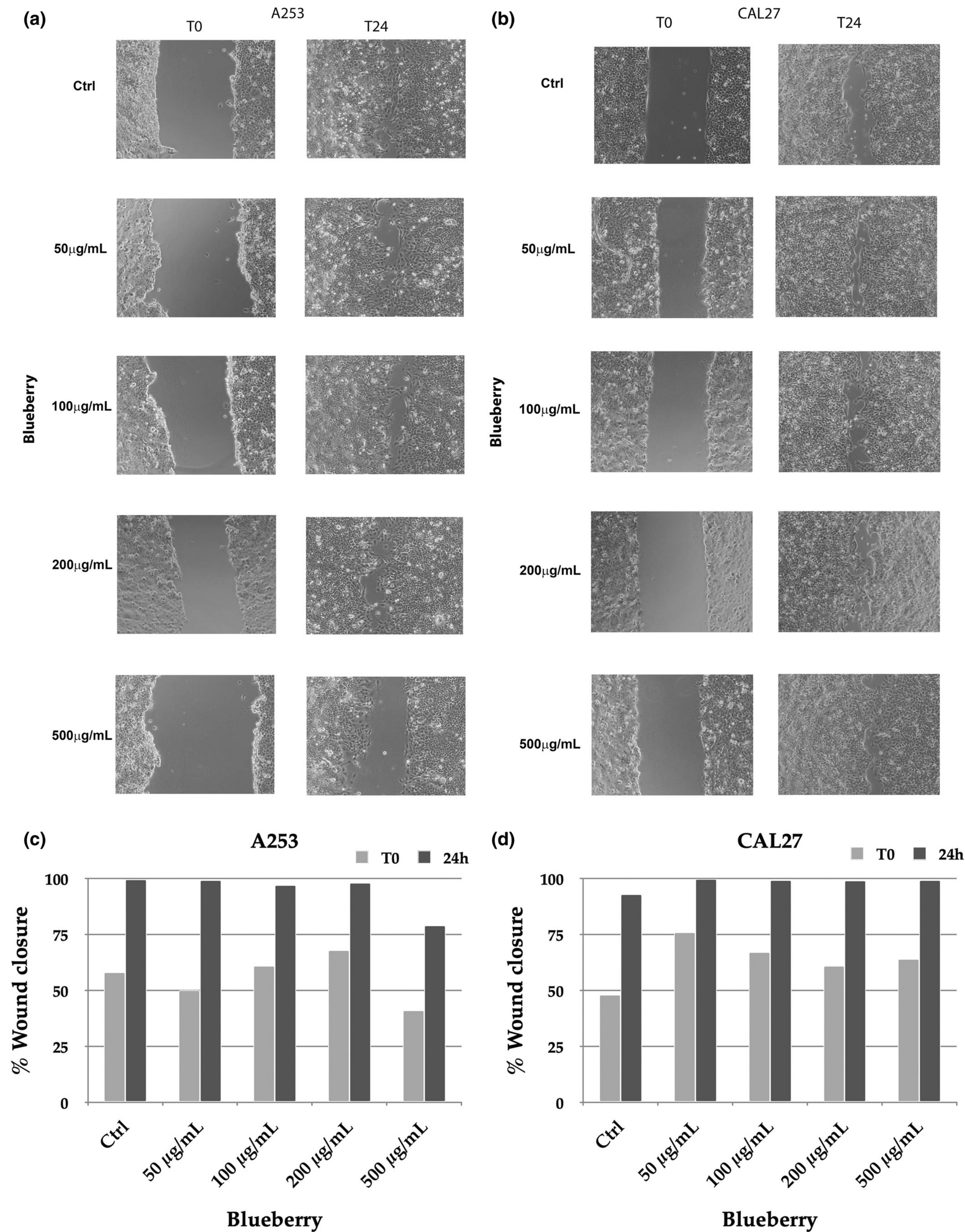

FIGURE 2 Legend on next page.

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measured the rate at which CAL27 and A253 cell lines migrate to fill a cell-free gap by using wound healing assay.[48] As shown in Figure 2a, in A253 cells the treatment with BL, dissolved in water at 500 μg/mL, caused a reduction in cell migration compared to untreated cells where the scratch was almost completely closed. This ability observed in A253 after BL treatment is not correlated to an increase of proliferation as assessed by MTS analysis (data not shown). In CAL27 cells, exposure to BL had no effect on cell migration (Figure 2b).

ErbB and antioxidant enzymes gene expression analysis in CAL27 and A253 cells

ErbB receptors combined expression has been reported in HNC as more predictive of reduced survival, in particular ErbB2 showed the greater correlation.[49] To investigate whether the extracts of BL were able to affect the expression of these receptors, a Real-time PCR was performed. Variations in gene expression levels observed in CAL27 and A253 after treatment with BL (50, 100, 200, and 500 μg/mL) are described in Figure 3a,b.

In particular, the analysis of EGFR, ErbB2, and ErbB3 expression levels performed in CAL27 cell line showed a significant reduction after treatment with BL at 50, 100, and 200 μg/mL. At dose of 500 μg/mL of BL, an increase in gene expression was observed for EGFR and ErbB2 receptors while ErbB3 expression remained unchanged. Our results showed that the mRNA expression levels of ErbB receptors analyzed in CAL27 decreased at lower concentrations of BL. This "paradoxical" behavior responds to the common biological, pharmacological, and toxicological phenomenon of hormoligosis, indicating a beneficial effect at lower doses of a specific substance that produces a damage at high doses.

In the A253 cell line, no statistically significant change in EGFR expression level was observed except for 500 μg/mL of BL which produced a significant increase, while the ErbB2 gene was significantly upregulated at all concentrations used, even if not in a dose-dependent manner. ErbB3 expression level showed a significant increase after 50 and 100 μg/mL of BL treatments but was significantly downregulated by 500 μg/mL BL treatment. These preliminary results highlight the capability of BL to reduce ErbB receptors expression in CAL27 cell lines at lower concentrations. The same treatments induced an opposite effect in A253 cell line for ErbB2 and ErbB3 receptors.

Phytochemicals are able to generate dual effects: in normal cells, they play a well-known cancer preventive role due to their antioxidant defense mechanism, while in cancer treatment, in combination with chemotherapeutic agents, behave in opposite way, increasing ROS production to toxic levels and thereby triggering cancer cells death. The ability of phytochemicals to exert antioxidant and/or pro-oxidant effects depends on several factors such as the different cancer cells environment, their concentration or the ability to activate p53.[50] Therefore, this peculiarity could represent a strategic opportunity in the ROS management in order to inhibit tumors in different conditions.

Therefore, it has been examined whether the expression of enzymes involved in the "classical" antioxidant defense system was modified by BL treatment. In particular, in CAL27 cell line, the mRNA expression levels of SOD2 and CAT genes were significantly decreased after 50 μg/mL ($p < 0.005$), 100 μg/mL ($p < 0.05$), 200 μg/mL ($p < 0.001$), 200 μg/mL ($p < 0.005$), and 500 μg/mL ($p < 0.01$), respectively even if not in a dose-dependent manner (Figure 3). On the contrary, in A253 cell line, only SOD2 expression level was modulated by BL treatment and, specifically, it significantly increased in a dose-dependent manner while, for CAT gene expression we did not observe any change (data not shown). iNOS gene was poorly expressed in CAL27 and A253 cells both in control and after treatment with BL (data not shown). Taken together, these data demonstrated BL differential biological effects in the two cell lines analyzed.

Several studies showed the relevance of oxidative stress in HNC, highlighting a decreased antioxidants expression in blood and tissue of patients at various clinical stages accompanied by an increase of ROS.[51] More in detail, a significant reduction of SOD levels is reported in HNC samples than controls, probably due to oxidant–antioxidant system imbalance in cancer cells attributable to its involvement in counteracting the excessive circulating free radicals. Therefore, SOD could represent a potential biomarker to determine cancer progression.[52–54]

A study published in 2015 on transgenic mouse model investigated the role of upregulated ErbB2 on mitochondrial function and antioxidant defense mechanisms in the heart, demonstrating that ErbB2 overexpression could play a role in regulating antioxidant defenses by upregulating and activating "protective pathways."[55] According to our results, it is interesting to speculate that the increase of ErbB2 and EGFR in A253 cell line at 500 μg/mL of BL treatment could favor the activation of antioxidant defense, in particular of SOD2 enzyme.

Finally, ROS would seem to be able to affect cell migration in both direct and indirect ways.[56] Indeed, in the A253 cell line 500 μg/mL of BL increased SOD2 levels and reduced cell migration. In the light of the above, these results suggest that SOD2 overexpression in A253 cell line potentially reduce ROS levels and, as a result, reduce cell migration. Further studies are needed to clarify this correlation. The results obtained in A253 and CAL27 cell lines are clearly context dependent, exhibiting a different behavior after BL treatments.

FIGURE 2 Effects of Blueberry on cell migration, by wound closure assay in A253 (a) and CAL27 (b) cells. Images with 10× magnification were captured at 0 and 24 h after wounding in media without (Ctrl) or with Blueberry (50–500 μg/mL in water), with a Leica DMi1 microscope with a digital camera; (c, d) percentages of wound closure at time 0 and after 24 h under each conditions are plotted. The data are representative of three independent experiments.

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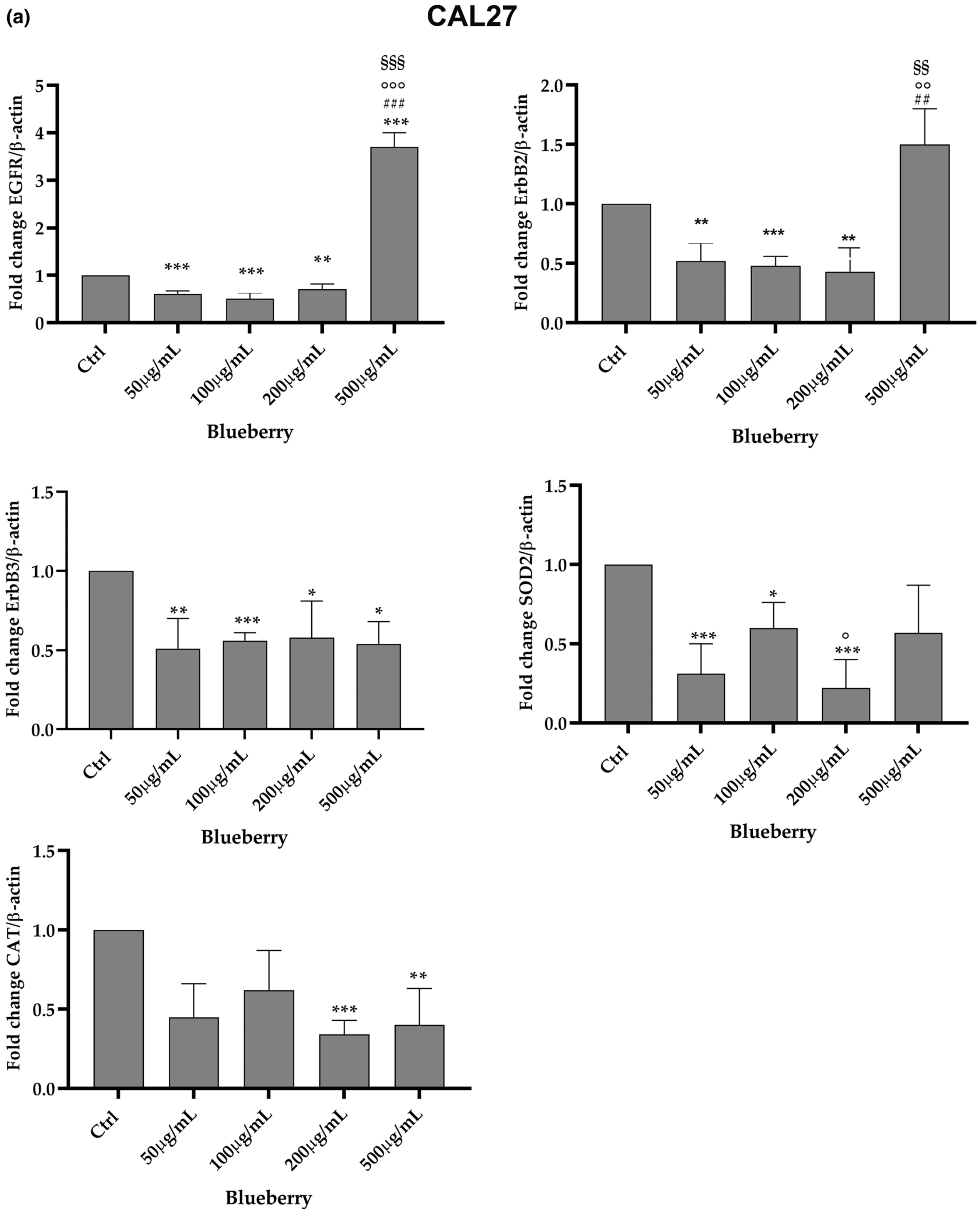

FIGURE 3 Assessment of EGFR, ErbB2, ErbB3, CAT, and SOD2 gene expression shown in CAL27 (a) and A253 (b) cell line treated with Blueberry (50–500 μg/mL in water). The relative expression of each gene was then measured by real-time PCR and normalized to that of the β-Actin housekeeping gene, according to the $2^{-\Delta\Delta CT}$ method. Data are expressed as means ± SD. and analyzed by 1-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ versus CTRL; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus CTRL 50 μg/mL BL;$^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ versus CTRL 100 μg/mL Blueberry; $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.005$ versus CTRL 200 μg/mL Blueberry.

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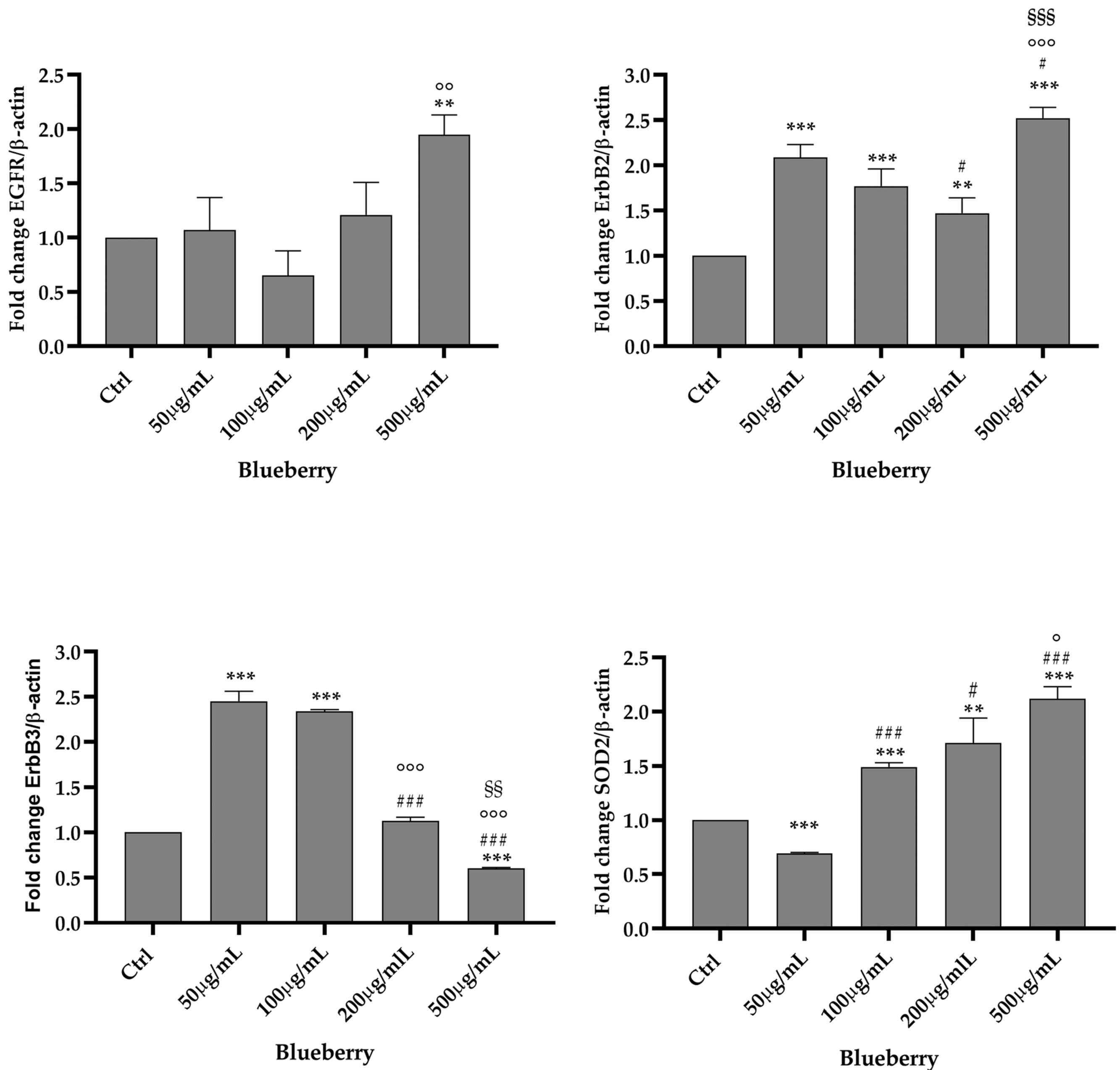

FIGURE 3 (Continued)

Results of the *ex vivo* study

BL, dissolved in water (50, 100, 200, and 500 μg/mL), was the subject of study in the *ex vivo* experimental model already previously illustrated. The results were determined as a significant variation on the increased gene expression, induced by the proinflammatory stimulus (LPS) and the inflammatory cytokines examined, such as IL-6 and TNF-α (Figure 4a,b).

New promising findings have emerged in this regard: BL has proven capable of significantly and dose-dependently decreasing the gene expression of both TNF-α and IL-6. These data must be linked to the high presence of anthocyanins and agree with numerous preclinical studies attesting that these phytonutrients, belonging to the flavonoid family, possess anti-inflammatory properties and improve IBD.[57] In Kim and colleagues' research, the anthocyanin-rich extract acted as a regulator of intestinal inflammation pathways by exerting beneficial effects following LPS-induced inflammatory damage and in significantly recovering intestinal epithelial cells in a Caco-2 intestinal and RAW 264.7 macrophages co-culture model.[58] Also, another study conducted on Caco-2 cells exposed to TNF-α anthocyanin-rich

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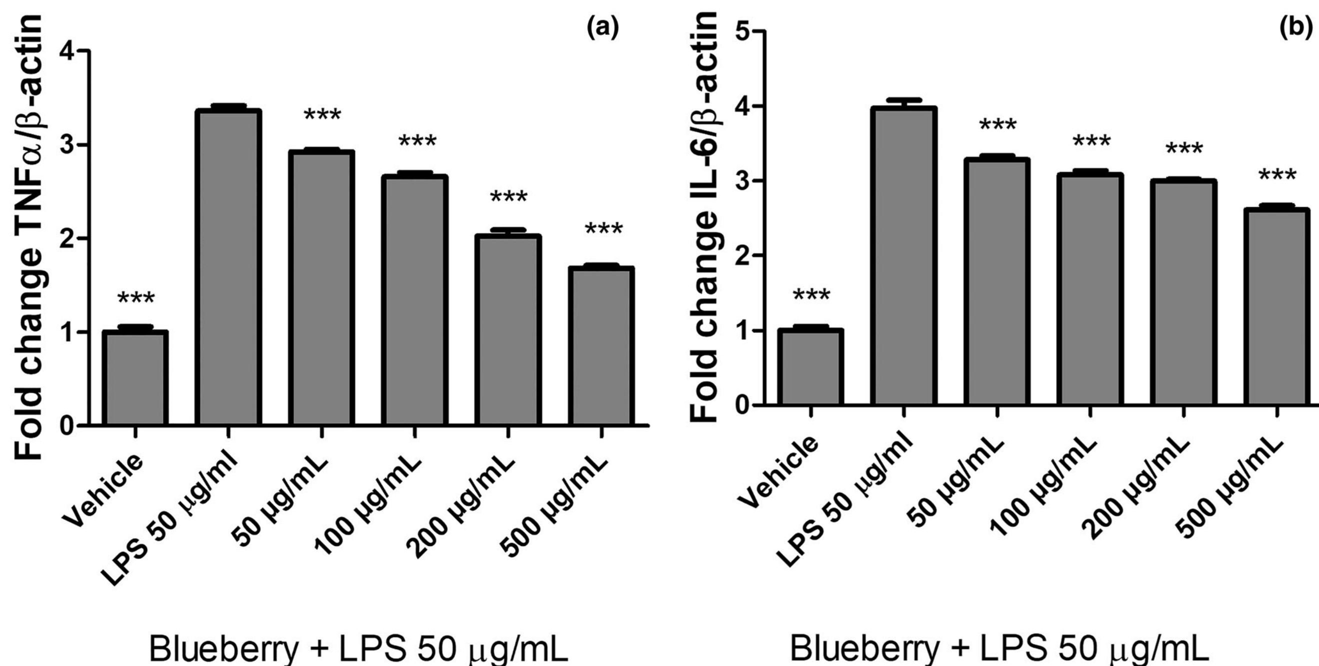

FIGURE 4 (a) Inhibitory effect of the Blueberry (50–500 μg/mL in water) from berries of *Vaccinium corymbosum* on TNF-α gene expression in isolated mouse colon specimens exposed to LPS (50 μg/mL). ***$p < 0.001$ versus LPS group. (b) Inhibitory effect of the Blueberry solution 50–500 μg/mL from berries of *V. corymbosum* on IL-6 gene expression in isolated mouse colon specimens exposed to LPS (50 μg/mL). ***$p < 0.001$ versus LPS group.

extracts was efficient in exhibiting protective actions on IBD.[59] Meanwhile, in vivo, and clinical studies have been conducted and significant positive effects exerted by anthocyanins on intestinal inflammation and enhanced colon health.[60,61]

In parallel, BL exhibited stimulatory effects on IL-10, the most important cytokines with anti-inflammatory properties, gene expression after LPS treatment (Figure 5); this action could be further attributable to the anthocyanins present in the BL solution. The findings obtained agree with previous studies which have demonstrated that IL-10 contributes in a fundamental way in antagonizing the production of factors associated to inflammation and preserving intestinal epithelial barrier integrity, thereby reducing the onset and progression of IBD.[62]

The results obtained agree with many previous studies attesting the beneficial effects exerted by anthocyanins in inflammation through inhibiting nuclear factor-kappaB activation, TLR4 protein expression, and MAPKs signaling pathway with consequent reduction of expression of proinflammatory factors as well as also nitric oxide (NO), ROS and prostaglandin E_2 (PGE_2), and the increase of anti-inflammatory cytokines.[63–65]

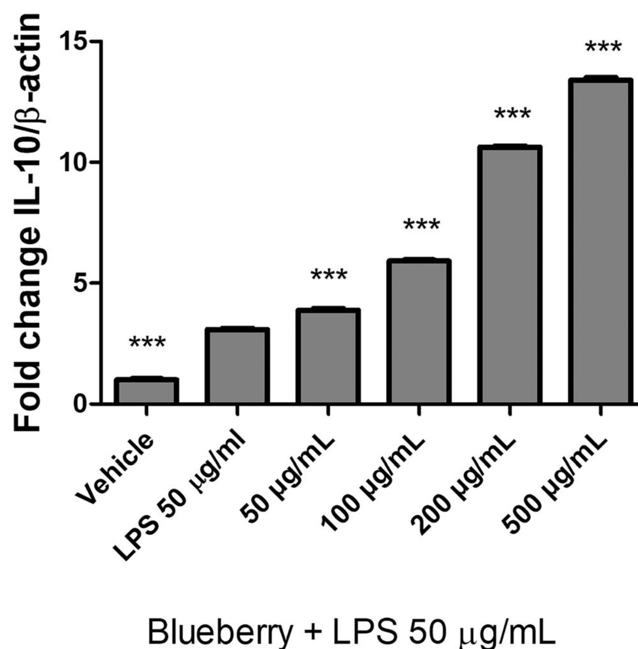

FIGURE 5 Stimulatory effect of the Blueberry (50–500 μg/mL in water) from berries of *Vaccinium corymbosum* on IL-10 gene expression in isolated mouse colon specimens exposed to LPS (50 μg/mL). ***$p < 0.001$ versus LPS group.

CONCLUSION

Given their high antioxidant activity, the anthocyanins and polyphenols consumption has been associated with healthy benefits. Unfortunately, due to human selection and natural evolution, anthocyanins are found only in insufficient levels in foods limiting their optimal benefits. Thus, an anthocyanin and chlorogenic-enriched extract from *V. corymbosum* berries was obtained by an SPE purification allowing

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to obtain a significant concentration of the bioactives and a twenty-fold potentiated activity in terms of antiradical effects. The result is congruent with the complete collection of all the bioactive molecules initially contained in the ethanolic extract. This was characterized by HLPC at three different wavelengths, as well as pH and color analyses deepened its physical–chemical properties knowledge. The antioxidant activity and biological assays *in vitro* (two HNC cell lines) and *ex vivo* (murine colon samples incubated with inflammatory stimulus) correlated with the presence of bioactive and precious compounds, such as specific anthocyanins in synergistic activity with chlorogenic acid for their healthy properties, which can be exploited as food supplements and pharmaceuticals. The outcomes of the treatments with BL led to counteract oxidation and inflammation, common hallmarks at the basis of several human diseases. Future studies must be also addressed regarding the limited bioavailability and chemical stability of these NCs to scale up this production industrially.

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CONFLICT OF INTEREST STATEMENT

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 STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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