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FULL ARTICLE

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The synergistic effect of cell wall extracted from probiotic biomass containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 on the anticancer activity of cranberry juice—HPLC fractions

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Abstract

Anticancer effects were evaluated on three HPLC fractions obtained from a concentrated cranberry juice and cell wall constituents extracted from a probiotic biomass containing *Lactobacillus acidophilus* CL1285, *Lactobacillus casei* LBC80R, and *Lactobacillus rhamnosus* CLR2. The samples were tested at increasing concentrations for the antiproliferative assay using HT-29 cells' line and for the quinone reductase (QR) assay using Hepa-1c1c7 murine hepatoma cells. Fraction 1 (F1) which is highly concentrated with phenolic acids inhibited the growth of the HT-29 cells' line with IC_{50} values of 14.80 µg Gallic acid equivalent (GAE)/ml. The fraction 3 (F3) which is highly concentrated in flavonols had potency as QR inducer. Furthermore, the results showed that all cranberry fractions combined with cell wall constituents extracted from the probiotic biomass were more effective in inhibiting the growth of HT-29 as compared to the cranberry fractions tested alone, indicating a possible synergy effect between these bio-functional compounds.

Practical applications

This study strongly evidenced that cranberry juice fractions combined with cell wall constituents extracted from the probiotic biomass can be used as a potent preventive functional compound against colorectal cancer. Therefore, this research proposes a natural dietary compound to prevent mutagenesis and carcinogenesis of colorectal cancer. Furthermore, the industry can formulae products containing probiotic and phenolic compounds as colon cancer cell growth preventive and anticancer products.

KEYWORDS

cell proliferation, colon cancer, cranberry juice, HPLC fractionation, probiotic cell wall, QR induction

Abbreviations: BCA, bicinchoninic acid protein; BSA, bovine serum albumin; CRC, colorectal cancer; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FBS, fetal bovine serum; GST, glutathione-S-transferase; HPLC, high-performance liquid chromatography; IC_{50}, inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADP, nicotinamide adenine dinucleotide phosphate; QR, quinone reductase; SDS, sodium dodecyl sulphate; UGT, uridine diphospho glucuronyl transferase.

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most predominant cancer in both men and women (Haggar & Boushey, 2009) and is strongly related to dietary habits. Many epidemiological and clinical studies associate its high incidence to obesity, diet low in fruits, vegetables, and whole grains but rich in fat as well as red and processed meat (Rasool, Kadla, Rasool, & Ganai, 2013; Siegel, Ma, Zou, & Jemal, 2014). Besides the diet, there is also a link between CRC and the intestinal microbiota (Sambi, Bagheri, & Szewczuk, 2019). Some studies reported a difference between the microbiome of healthy subjects and patients with colon cancer (Shen et al., 2010; Sobhani et al., 2011). Furthermore, recent studies showed that the presence of some commensal bacteria in the gut could trigger inflammation which can subsequently generate colonic cancer cells (Yang, Owen, Lightfoot, Kladde, & Mohamadzadeh, 2013). Taking into account this information, an emphasis should be made on using natural dietary agents such as fruits, vegetables or probiotics to prevent the mutagenesis and carcinogenesis of CRC.

Berries and small soft-flesh colored fruits are consumed worldwide for their numerous health benefits. They are getting much attention recently due to the presence of a diverse array of bioactive phytochemical components including anthocyanins, phenolic acids, stilbenes, tannins, and carotenoids. Berries, including cranberries, are healthy fruits contributing color, flavor, nutritional value, and functionality. Cranberry belongs to the genus *Vaccinium* in the *Ericaceae* family and is commonly consumed as a part of the Western diet in fresh or processed form (Stoner, Wang, & Casto, 2008). It is known for its beneficial health effects toward bacterial infection involving the urinary tract disorders, dental decay, as well as stomach ulcers, and chronic disease such as cancer and diabetes (Howell et al., 2010). Those biological activities are mainly due to the presence of various phenolic compounds, including anthocyanins, flavonols, flavanols, phenolic acids, and proanthocyanidins (Paredes-López, Cervantes-Ceja, Vigna-Pérez, & Hernández-Pérez, 2010). Several in vitro studies have shown the anticancer effects of berries and/or berries extracts by strongly inhibiting cellular growth, inducing apoptosis or enzyme metabolism (Flis et al., 2012).

Over the past years, scientists have identified an increasing number of mechanisms that help to explain the anticancer properties of cranberries. The known mechanisms include blocked expression of MMPs (matrix metalloproteinases); inhibition of ODC (ornithine decarboxylase enzymes); stimulation of QRs (Quinone reductase enzymes); inhibition of CYP2C9s (Phase I detoxification enzymes); and triggering of apoptosis (programmed cell death) in tumor cells. The cancer-preventive benefits of cranberries are now known to extend to breast, colon, lung, and prostate cancers (Rasool et al., 2013).

Probiotics are defined as live microorganisms which when consumed in an adequate number exert beneficial effects in the host (Hill et al., 2014). There are numerous health benefits attributed to probiotics such as protection against gastrointestinal pathogens, modulation of the immune system, reduction of high level of cholesterol and blood pressure, and anticarcinogenic capacity (D'Aimmo, Modesto, & Biavati, 2007).

Their inhibitory effects against several colon cancer cell line growth have been demonstrated in many studies (Choi et al., 2006). Besides the whole bacterial structure, others bioactive components commonly found in probiotics such as cell walls (or its different components such as peptidoglycan and lipoteichoic acids) and proteins or secreted by them such as exopolysaccharides, bacteriocins, organic acids, nucleic acids and short-chain fatty acids, antimicrobial proteins are responsible for their health effects (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013).

Despite the great number of studies in the literature, the precise mechanisms by which probiotics may prevent CRC still remain not perfectly clear. However, it is conceivable that they include alteration of the intestinal microflora; inactivation of cancerogenic compounds; competition with putrefactive and pathogenic microbiota; improvement of the host's immune response; antiproliferative effects via the regulation of apoptosis and cell differentiation; fermentation of undigested food; and inhibition of tyrosine kinase signaling pathways (Uccello et al., 2012). Probiotics can enhance innate immune functions including the phagocytic activity of neutrophils and cytotoxic activity of NK cells. The activation of neutrophils and NK cells might be closely connected with the anti-infectious or anticancer abilities of probiotics (Kaminogawa & Nanno, 2004).

Among all the mechanisms involved in the chemopreventive strategy, xenobiotic-detoxification is considered as one of the major mechanisms. The enzymes responsible for the detoxification are phase II-xenobiotic enzymes such as glutathione-S-transferase, uridine diphosphate-glucuronosyltransferase and quinone reductase (QR) (Talalay, 2000). QR catalyzes a wide variety of reactions that serve to protect cells against the toxicities of electrophiles and reactive oxygen species by converting them into less toxic products (Talalay, 2000). QR catalyzes the reduction of toxic oxygen metabolites leading to their deactivation and could protect the exposed tissue from oxidative stress (Chen & Kunsch, 2004). It also has the ability to reduce oxidative stress (Kang & Pezzuto, 2004). This enzyme can be used as a key in vitro bioassays to evaluate chemopreventive properties of diverse biomolecules/bioactive components/chemical substances (Su et al., 2004).

Thus, the aims of this study were to evaluate: (a) the effect of fractions from a cranberry concentrated juice and cell walls extracted from a probiotic biomass on the in vitro antiproliferative properties against one colon cancer cells line, (b) their ability to induce the phase II detoxifying enzymes QR and, (c) the effect of the combination of cranberry fractions with the probiotic cell walls on the growth of colon cancer cells and the ability of liver cells to induce QR activity.

2 | MATERIALS AND METHODS

2.1 | Chemical reagents

Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin–Ciocalteu, menadione,

glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (EDTA), flavin adenine dinucleotide (FAD), digitonin, bovine serum albumin (BSA), β-naphtoflavone (BNF), sodium dodecyl sulphate (SDS), and Tween 20 were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Cancer cell culture media (DMEM-Ham's F12 and MEMEBSS), foetal bovine serum (FBS), and other supplements were purchased from Fisher Scientific Limited (Ottawa, ON, Canada).

2.2 | Raw materials

The cranberry concentrate juice (50 °Brix) was graciously provided by Atoka Cranberries Inc. (Manseau, QC, Canada) and the probiotic biomass by the Bio-K Plus International Inc. (Laval, QC, Canada).

2.3 | HPLC-DAD fractionation of concentrated cranberry juice and total phenol determination

2.3.1 | Fractionation of concentrated cranberry juice

The HPLC analyses were performed on a ProStar 230 from Varian Canada Inc. (Mississauga, ON, Canada), equipped with a ternary pump delivery system, a Rheodyne injection valve, and a ProStar 330 diode-array UV-Vis detector from Varian Canada Inc. Integration and data elaboration were performed using Star Chromatography Workstation software. A Zorbax SB-C18, 5 µm, 9.4 × 250 mm column purchased at Agilent Technologies Canada Inc. (Mississauga, ON, Canada) was used. All solvents were filtered with a 0.20 µm Whatman paper from Millipore Canada Ltd. (Etobicoke, ON, Canada). An elution gradient was carried out using the following solvent system: Mobile phase A, Milli-Q water/formic acid (97/3, v/v); mobile phase B methanol only (100 v/v). The linear gradient elution was 85% A–15% B. A volume of 1 ml of each sample was injected after filtration through a 0.2 µm filter disk. The flow rate was 3 ml/min and the detection was achieved by photodiode array (250–550 nm). Three fractions were recovered (Table 1). The solvent was then removed by evaporation using the SpeedVac automatic evaporation system (Savant System, Holbrook, NY, USA). Finally, the dry matter was obtained by freeze-drying the fractions for at least 48 hr using a Labconco freeze-drying system (Kansas City, MO, USA) and stored at −20°C until used (Caillet et al., 2012).

2.3.2 | Total phenol concentration

Total phenolic compounds' content in each cranberry fraction was determined by spectrophotometry according to the Folin–Ciocalteu method (Bagheri, Madadlou, Yarmand, & Mousavi, 2013; Singleton & Rossi, 1965). The total phenolic content was estimated using a calibration curve (r^2 = .9994) by plotting the known solution of gallic acid (7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1,000 µg/ml). The total phenolic content of each fraction was expressed as microgram gallic acid equivalent (GAE) per mg powder (µg GAE/mg of powder). Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid which is soluble in water, is commonly used as a standard for determining the phenol content of various analyses (Fiuza et al., 2004).

2.4 | Probiotic cell wall constituents extraction

2.4.1 | Physical treatment

The concentrated biomass of probiotic *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 fermented in MRS broth was centrifuged at 9,000× *g* for 30 min at 4°C. The resulting pellets were washed with phosphate buffer 10 mM pH 7.2 then suspended in the same buffer at a ratio of 1:4 (w/v). The suspension was heated at 65°C for 40 min, then submitted to ultrasound in 500 W, 20 kHz (Q500, QSONICA, Newtown, CT, USA) for 30 min (1 s on and 1 s off) with 40% acoustic power (amplitude) at 4°C. The sonicated samples were centrifuged at 800× *g* for 30 min. The supernatant was recovered and subjected to ultracentrifugation at 50,000× *g* for 30 min. The pellet was collected and suspended in 10 ml of 100 mM Tris–HCl buffer pH 7.5 (Kim, Woo, Kim, & Lee, 2002; Signoretto, Boaretti, & Canepari, 1998).

2.4.2 | Enzymatic treatment

The pellet obtained in the previous step was first incubated under agitation with 50 µl of α-amylase (20 mg/ml) for 2 hr at 37°C. Then, 50 µl of $MgSO_4$ (1 M), 100 µl of DNase (1 mg/ml), and 100 µl of

TABLE 1 Time of elution of cranberry concentrate juice fractions obtained by HPLC-DAD fractionation, total phenolic content of cranberry fractions, and IC_{50} values of the cranberry fractions for the inhibition of HT-29 growth. HT-29 cells incubated with different concentrations (156.25–2500 µg/ml) of cranberry fractions for 48 hr

Fraction	Time of elution (min)	Phenolic composition	µg GAE/mg of powder	IC_{50} (µg GAE/ml)
F1	0–12	Phenolic acids	10.15 ± 2.59[a]	14.80 ± 1.64[a]
F2	13–28	Anthocyanins	70.75 ± 3.55[b]	200.91 ± 1.58[c]
F3	29–45	Flavonols	136.41 ± 8.94[c]	129.30 ± 2.18[b]

Note: Data are presented as mean ± *SD*. Different letters are significantly different ($p \leq .05$). F1, F2, and F3 collected by fractionation of cranberry concentrated juice by HPLC. F1: composed of phenolic acids, F2: constituted of anthocyanins, and F3: contained flavonols.

RNase (50 mg/ml) were added and the solution was incubated at 37°C under agitation for 2 hr. Finally, 100 µl of trypsin (10 mg/ml) and 10 µl of 1 M $CaCl_2$ were added and the sample was incubated at 37°C overnight under agitation. After the incubation, 3.5 ml of 4% SDS (w/v) was added to 10 ml of samples and heated at 100°C for 15 min. The samples were centrifuged at 30,000× *g* for 30 min. The newly obtained pellet was washed with distilled water (4X) and then with different solutions including LiCl (8 M), EDTA (100 mM), and acetone. Each solution was used twice to wash the pellet. The washed pellets were mixed with 10 ml of $(NH_4)_2CO_3$ and 250 µl of alkaline phosphatase (1 mg/ml) and incubated overnight at 37°C under agitation. Then, the sample was heated at 100°C for 5 min and centrifuged at 30,000× *g* for 45 min. The obtained pellet was washed with distilled water (2X), suspended in water, and lyophilized. The obtained powder was weighed and kept at −20°C until used (Signoretto et al., 1998).

2.5 | Measurement of cell proliferation

2.5.1 | Cells culture

The HT-29 cells (ATCC HTB-38) and the Hepa-1c1c7 murine hepatoma cells (ATCC CRL-2026) were purchased from America Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in complete DMEM-Ham's F12 and MEM-EBSS medium, respectively, containing 10% of fetal bovine serum. They were maintained in a humidified atmosphere incubator with 5% CO_2 and 37°C.

2.5.2 | Preparation of samples

Evaluation of cell viability assay and QR induction assay was done, measured for cranberry fractions and cell wall extract. These compounds were serially twofold diluted using 10% (v/v) DMSO solution to have serial concentrations. The concentrations of cranberry concentrate juice fractions are 20, 39, 78, 155, 310, 625, 1,250, and 2,500 µg/ml, while concentrations of 8, 16, 32, 63, 125, 250, and 1,000 µg/ml were considered for cell wall extract. For the QR assay, the concentrations tested were 8, 16, 32, 63, 125, 250, and 500 µg/ml for both samples.

2.5.3 | Antiproliferative assay

The antiproliferative effect of samples was measured using MTT color assay. The HT-29 cells were seeded at 2 × 10^4 cells in 200 µl of complete growth medium in 96 wells microplate Corning® CELLBIND® Surface microplate (Costar, Corning Inc., Corning, NY, USA). After 24 hr of incubation at 37°C in 5% CO_2, the medium was replaced with 90 µl of fresh medium containing 10 µl of serial concentration of samples. Control wells received the same amount of medium with 10 µl of DMSO 10% and blank wells received 100 µl of medium with no cells. After 48 hr of incubation, the growth medium containing the sample is decanted and replaced with 200 µl of new growth medium containing 25 µl of MTT. The microplate was incubated for 4 hr. Then, 200 µl of DMSO with 25 µl of Sorensen buffer was added to the microplate. Absorbance was measured at 562 nm with a microplate reader (EL800 BioTek Winooski, VT, USA). The absorbance of the control samples was set at 100% of cell proliferation. At least five replications for each sample were used to determine the cell proliferation. The cell proliferation was determined by the ability of the metabolic active cells to cleave the tetrazolium salt to purple formazan crystals (Vistica et al., 1991). The inhibition rate was calculated as follows:

$$\text{Inhibition } (\%) = 100 - [\,(\text{OD treated cells/OD control cells}) \times 100]$$

IC_{50} is the fraction concentration under which the proliferation of 50% of the cells is inhibited.

2.6 | Quinone reductase assay

2.6.1 | Assay procedure

Hepa-1c1c7 cells were seeded in 96-well microplate in 200 µl of MEM/EBSS medium at a concentration of 1 × 10^4 cells. After 24 hr of incubation at 37°C in 5% CO_2, humidified incubator, the medium was decanted and replaced with 190 µl of fresh medium containing 10 µl of serial concentrations of samples. After 48 hr of incubation, the medium was decanted and the cells were incubated for 10 min with 50 µl of 0.8% digitonin and 2 mM EDTA solution, then the microplates were agitated for 10 min in an orbital shaker and 200 µl of a reaction mixture were added in each well. A stock solution was prepared as described for each series of assays: 20.5 ml of distilled water, 1.1 ml of 500 mM Tris–HCl buffer pH 7.4, 14.7 mg of BSA, 146.7 µl of 1.5% (w/v) Tween 20 solution, 14.7 µl of 7.5 mM FAD^+, 146.7 µl of 150 mM glucose 6-phosphate solution, 13.2 µl of 50 mM $NADP^+$ solution, 44 µl of glucose 6-phosphate dehydrogenase solution, 6.6 mg MTT, and 22 µl of 50 mM menadione solution. The microplates were incubated for 5 min at room temperature then the absorbance was measured at 595 nm with a microplate reader (Chang, Shim, Cha, & Chee, 2010).

2.6.2 | Protein determination

The protein quantification was performed using the BCA assay kit from Fisher Scientific Limited according to the manufacture's specifications. A 20 µl aliquot of lysed cells was transferred to a new microplate and 300 µl of BCA was added to each well and incubated for 30 min at a humidified incubator under 5% CO_2 at 37°C. Then, absorbance was measured at 595 nm using a microplate reader. Protein concentration was determined using BSA as a standard. Specific activity was calculated and expressed as nM MTT formazan

formed per min and per mg of protein (Desrouillères, Millette, Vu, Touja, & Lacroix, 2015). The induction of the activity of the QR enzyme for the treated groups was calculated by dividing the specific activity of the treated sample to the specific activity for the control (cells + DMSO 10%).

2.7 | Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate the differences between the samples by SPSS Base 16.0 software (Stat-Packets statistical analysis software, SPSS Inc., Chicago IL, USA). Significant differences ($p \leq .05$) were evaluated using the Duncan's comparisons test.

3 | RESULTS AND DISCUSSION

3.1 | Cranberry concentrate juice HPLC fractionation

The HPLC analysis of the cranberry concentrate juice allowed the separation of the phenolic compounds present in the juice according to their polarity. The first eluted compounds (F1) are the most polar ones with the smallest molecular weight. The previous study was performed in our laboratory on phenolic compounds analysis by HPLC, provided the retention time of phenolic compounds (Table 1) (Caillet et al., 2012). By considering these findings, we can describe the phenolic content of each fraction as follow; fraction 1 (F1) mainly contained the phenolic acids, while fraction 2 (F2) is mostly constituted of anthocyanins and fraction 3 (F3) is dominantly composed of flavonols.

3.2 | Total phenol content

The total phenol content of each fraction obtained from the cranberry concentrate juice is shown in Table 1. The highest phenolic content belongs to F3, the fraction enriched with flavonols. These findings were in agreement with previous studies (Vu et al., 2012) on the extracts of cranberries fruits, puree, depectinized puree, and pomace. It was observed that the extracts containing polar phenolic compounds such as flavonols (quercetin, kaempferol, and myricetin), flavan-3-ols (catechin and epicatechin), and proanthocyanidins have the highest phenolic content (Côté et al., 2011).

3.3 | Antiproliferative assay

"Normal cells" are sensitive to environmental growth inhibitors unlike "cancer cells." They do not respond to the inhibitory growth signals and also can grow without the stimulatory growth factors which are indispensable for normal cell proliferation (Karp, 2009). Generally, to verify the cancer cells' ability to grow indefinitely, using substances (natural and/or chemical) to inhibit their growth is one of the chemoprevention strategies against cancer. Here, the fractions of cranberry extracts and probiotic cell wall constituents were evaluated for their antiproliferative activity against the cancer cells.

3.3.1 | Antiproliferative activity of cranberry fractions against HT-29

The effect of cranberry fractions on HT-29 cells' growth and the IC_{50} values are shown in Figure 1 and Table 1, respectively. As the IC_{50} represents the concentration required to inhibit the growth of 50% of the cancer cells, it should be mentioned that the lower IC_{50} value means the higher efficiency of fraction in inhibiting the growth of cancer cells (Shoemaker, 2006). Results showed a concentration-dependant response for all three fractions concerning the inhibitory effect against HT-29 cells. The fraction 1(F1) which contains mainly phenolic acids has an IC_{50} value of 14.80 ± 1.64 µg GAE/mL. This fraction is the most effective at inhibiting HT-29 cells' growth. The phenolic acids usually found in cranberry are *p*-coumaric, sinapic, caffeic, and ferulic acids (Zuo, Wang, & Zhan, 2002). These compounds have demonstrated their antiproliferative effects on cancer cells in in vitro studies. For example, ferulic and *p*-coumaric acids present in whole grains cereals bran were able to inhibit the growth of Caco-2 cell lines by 43 and 57%, respectively, compared to control after 2–3 days of treatment at a concentration of 1,500 µM. Janicke, Önning, & Oredsson, 2005 evaluated the effect of caffeic acid on HT-29 cells' viability at different concentrations (1.25 to 80.0 µM) from 0.5 to 96 hr. They found that caffeic acid had inhibitory effect against HT-29 cells' growth. Therefore, the obtained results concerning the effect of phenolic acids on colon cancer cells proliferation correspond with previous studies.

Fraction 2 (F2) mainly composed of anthocyanins, presents the highest IC_{50} values, 200.91 ± 1.58 µg GAE/ml. At the concentration tested, F2 did not have any effect on HT-29 cells' proliferation. Anthocyanins are colored pigments especially abundant in berries such as blueberries, blackberries, strawberries, black currants, grapes, cranberries, etc. (Murad, Soares, Brand, Monteiro, & Teodoro, 2015). The anthocyanins available in cranberry are mostly cyanidin 3-galactoside, cyanidin 3-arabinoside, and peonidin (Côté, Caillet, Doyon, Sylvain, & Lacroix, 2010). Many studies demonstrated the inhibitory effect of anthocyanins from different fruits against cancer cells. For example, it was demonstrated that fractions of anthocyanins from strawberry had an inhibitory effect of 60% on HT-29 growth compared to the control at a concentration of 0.25 mg/ml (Zhang, Chen, Li, Chen, & Yao, 2008; Zhang, Chen, Li, Yao, & Chen, 2008). However, anthocyanins from cranberry seem to have little to no effect on cancer cells growth. For instance, purified cyanind-3-galactoside was tested on eight tumors cell lines growth (Murphy et al., 2003). They found that even at the highest concentration, the inhibition rate was less than 50% for all the cancer cells. Furthermore, anthocyanins' subfractions

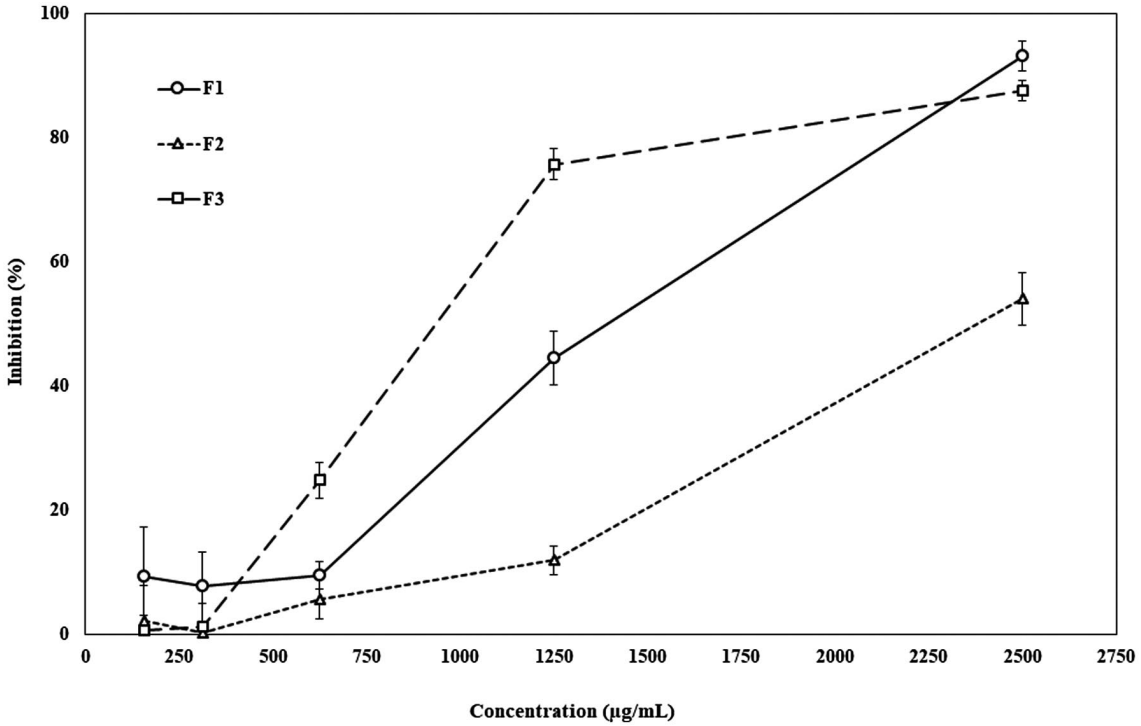

FIGURE 1 Effect of HPLC fractions obtained from a cranberry concentrated juice against HT-29 proliferation. HT-29 cells were exposed to different concentrations of cranberry fractions (156.25 to 2,500 µg/ml). F1, F2, and F3 were collected by HPLC fractionation of cranberry concentrated juice. Data are presented as mean ± *SD*. F1: composed of phenolic acids, F2: constituted of anthocyanins, and F3: contained flavonols

from cranberry were also tested against several tumors cell lines: Prostate, colon, and oral (Seeram, Adams, Hardy, & Heber, 2004). They found that the anthocyanins were able to limit prostate cancer cells growth by 50%–70% but not oral and colon tumor cells proliferation.

Our results are in good agreement with these previous studies. That antiproliferative activity of anthocyanins subfractions from cranberry might depend on some bioactive components and their synergistic effects. Further investigations are needed to evaluate the antioxidant capacity and antiangiogenic effect of F2 and also to determine its active compounds.

Fraction 3 (F3) is mainly composed of flavonols and presented also a noticeable inhibitory effect with an IC_{50} of 129.30 ± 2.18 µg GAE/ml. It is known that the main flavonols in cranberry are quercetin, kaempferol, and myricetin. In vitro and in vivo studies demonstrated several potential roles for quercetin and kaempferol in cancer prevention (Zhang, Chen, Li, Chen, et al., 2008). Li and his team (Li, Du, Wang, Wang, & Zhang, 2009) confirmed the ability of kaempferol to inhibit the proliferation of HCT116 cells in a dose-dependent manner. The antiproliferative activity was stronger for the cell line wild-type p53. Another study designed by Zhang et al. (2012) compared the anticancer activity of quercetin and quercetin-5′, 8-disulfonate against colorectal cancer cell lines LoVo. They found that both compounds were able to inhibit the growth of the cells in a concentration-dependant manner with IC_{50} values of 40.02 and 20.08 µM, respectively (Zhang et al., 2012). Those results suggested that quercetin-5′, 8-disulfonate is more effective against cancer cell proliferation. Our results are in agreement with previous studies. However, further investigation is needed to determine if the inhibitory effect is mainly caused by a single compound in each fraction or it is rather the combination of all compounds, and also to define the mechanism by which phenolic compounds distraught the proliferation of colon cancer cells.

3.3.2 | Antiproliferative activity of cranberry fractions combined with the extract of probiotic cell wall constituents against HT-29

The effect of the combination of cranberry fraction and probiotic cell wall extracts on HT-29 cells' growth is shown in Figure 2. By combining the cranberry fractions with the probiotic cell wall, it was observed an increase of the inhibitory effect of each sample against HT-29 cells' proliferation as compared to the fractions alone. Fractions F1 and F3 presented an inhibition activity against HT-29 cells' proliferation around 59.99 ± 2.30% and 23.72 ± 3.55%, respectively, at 625 µg/ml. When Fractions F1 and F3 combined with the cell wall extracts (100 µg/ml), the inhibitory effect increased nearly by 12% and 20%, respectively. The fraction F2 alone at a concentration of 2000 µg/ml inhibited the HT-29 cells' proliferation by 23.72 ± 1.66%, but in the presence of cell wall, an increment of 28% in inhibition rate was observed. We can hypothesize that probiotic bacteria and phenolic compounds can act as complementary bioactive compounds.

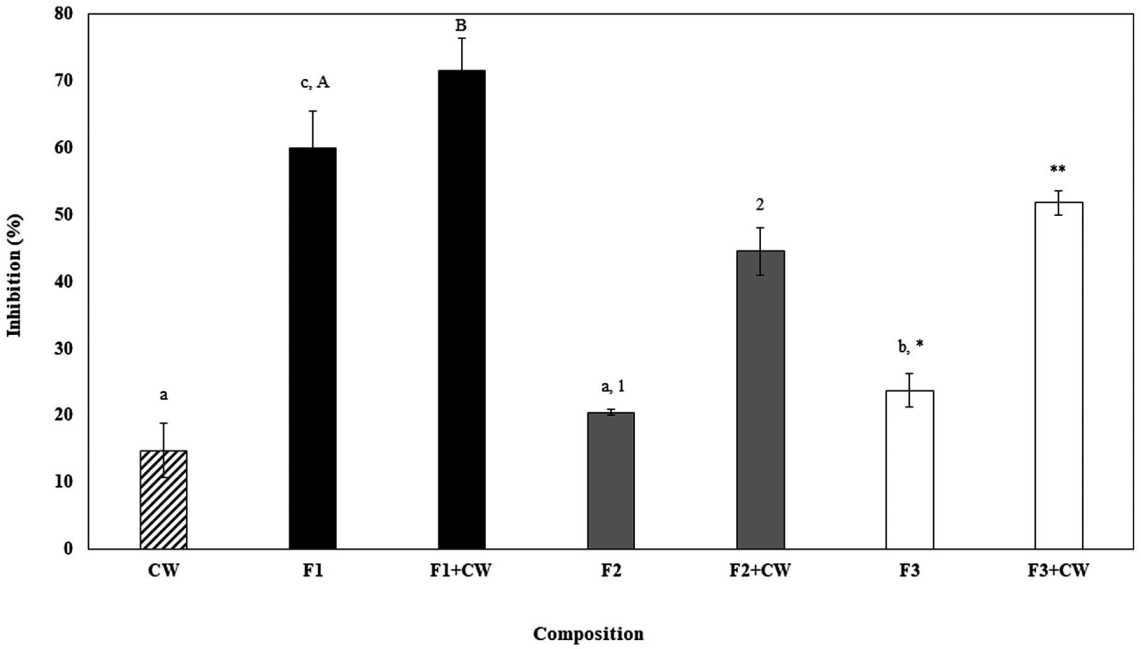

FIGURE 2 Effect of the cranberry fractions combined with probiotic cell walls on HT-29 proliferation. HT-29 cells were exposed to cranberry fractions combined with extract of cell wall constituents. F1, F2, and F3 were collected by fractionation of cranberry concentrated juice by HPLC and CW constituents were extracted from a probiotics biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2. The samples are tested at different concentrations F1: 625 µg/ml, F2: 2000 µg/ml, F3: 625 µg/ml and CW: 100 µg/ml. Data are presented as mean ± standard deviation. Different letters are significantly different ($p \leq .05$). F1: composed of phenolic acids, F2: constituted with mostly anthocyanins, and F3: contained mainly flavonols and CW: cell wall constituents. Different letters represent significant differences between cranberry fractions and cell wall extract (a, b, c). Different symbols (A,B), (1, 2), (*, **) represent significant differences between cranberry fractions alone (F1, F2, and F3) and in combination with cell wall extract (F1 + CW, F2 + CW, and F3 + CW) at ($p < .05$)

Many studies showed that phenolic compounds from berries could act as antimicrobial substances against food pathogens such as *E. coli* (Lacombe, Wu, Tyler, & Edwards, 2010). Millette, Luquet, and Lacroix (2007) also found that the soluble fraction of the fermented milk produced by *L. acidophilus* and *L. casei* was also able to inhibit the growth of food pathogens. Those studies showed that phenolic compounds from berries and compounds produced by probiotic bacteria have the same antimicrobial properties. Additionally, in another research reported that the grape phenolic extracts and their phenolic compounds did not exert an inhibitory effect on *L. acidophilus* growth at a maximum concentration of 5,000 µg/disk in agar diffusion assays (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009). Furthermore, in the presence of grape pomace phenolic extract, a stimulatory trend in bacterial growth was observed (Hervert-Hernández et al., 2009). Other studies also demonstrated that phenolic compounds present in berries could act as growth promoting factors for probiotics. They are able to activate bacterial growth but also increase the bacterial count in a culture medium (Tabasco et al., 2011). Recently, Peres, Hernandez-Mendonza, Bronze, Peres, and Malcata (2015) showed that the olive bioactive phytochemicals (such as phenolic compounds) and probiotic strain combination can inhibit (26%–50%) proliferation of human colon adenocarcinoma cells. Their results approved the potential synergism between olive phenolic compounds and probiotic bacteria, which could be considered to stimulate antiproliferation or probiotics activity at pathogen control. Our results are in agreement with the results of the recent study.

In accordance with our results, the increment observed in the inhibition rate of combined compounds could be explained by an interaction between the phenolic compounds and the component of the probiotic cell wall (peptidoglycans, teichoic acid, and cell wall-associated polysaccharides). This interaction presented a synergic effect (12%–28%) on antiproliferation activity against cancer cell lines. The combination may have an effect on the cancer cell proliferation process by either modifying the cell morphology or affecting some growth factors that could lead to cell death. Additionally, this phenomenon could again be explained by the interference of phenolic compounds acting on the cell membrane; they can attack the cell wall and penetrate the cell, where they react with the cytoplasm and cellular proteins (Tahmourespour & Kermanshahi, 2011). As for the probiotic cell walls, our previous research reported that cell wall extracted from the biomass of three Lactobacillus strains (*L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2) showed a chemopreventive activity (Desrouillères et al., 2016). Furthermore, other studies reported that probiotic cell walls prevent gut mutagenicity and colon cancer by modifying the immune system (Kolling, Salva, Villena, Marranzino, & Alvarez, 2015) and binding the mutagenic compounds (Sreekumar & Hosono, 1998). Moreover, another study reported a correlation between increasing the patient response to chemotherapies and immunotherapies and the composition of the

gut microbiome, thereby implicating a more involved role for probiotics (Sambi et al., 2019). Further studies are needed to determine the nature of the interaction between phenolic compounds and cell walls and the precise mechanism by which this interaction can affect cancer cell growth.

3.4 | Quinone reductase assay

3.4.1 | Effect of cranberry fraction on QR activity

One of the well-known strategies of cancer prevention involves the suppression of carcinogen metabolic activation or blocking the formation of ultimate carcinogens. In particular, the induction of phase II enzymes, such as QR can offer protection against toxic and reactive chemical species (Tabasco et al., 2011). QR elevation with in vitro and in vivo systems has been shown to correlate with the induction of other protective phase II enzymes and can approve the potential chemoprotective effect of molecules against cancer (Pezzuto, 1995).

The interest in berry studies is due to its high content of phenolic compounds, which are plant secondary metabolites, well-known for their health-protecting attributes. Berries are known for their antioxidant properties and their ability to help the organism to protect itself from free radicals that tend to provoke damages in DNA and cellular membrane (Kahkonen, Hopia, & Heinonen, 2001).

For this purpose, β-naphtoflavone (BNF) was used as a positive control at a concentration of 20 μM, which had an induction of 2.45 ± 0.1 folds (results not shown). Cranberry fractions, F1 and F2 had no effect on the induction of QR activity (Figure 3). The results concerning F1 are consistent with previous studies. Phenolic acids such as caffeic and ferulic acids, the dominant compounds present in F1, were found to be a weak inducer of QR activity in some in vitro studies (Yang & Liu, 2009). Furthermore, Ngamdee and coworkers also observed the QR-inducing activity was enriched in the phenolic acid-rich fraction and reduced in the anthocyanin-rich fraction extracted from rice bran (Ngamdee, Jiamyangyuen, & Parkin, 2016). These findings are in good agreement with F2 (anthocyanin-rich fraction) results. Indeed, the studies on the potency of anthocyanins to induce QR activity are contradictory. For instance, another study confirmed the capacity of anthocyanins extracted from sorghum seedlings to induce QR activity (Shih et al., 2007), while Srivastava and coworkers informed that QR activity was not induced by natural anthocyanins extracted from Georgia-Grown Blueberries (Srivastava, Akoh, Fischer, & Krewer, 2007).

It is also interesting to mention that the contents of the phenolic compounds among different berry genera varied considerably, as anthocyanins were the main phenolic constituents in bilberry and cranberry, but in cowberries, flavanols, and procyanidins predominated (Kahkonen et al., 2001). Furthermore, more studies showed that the extraction method and variety of berries harvested in

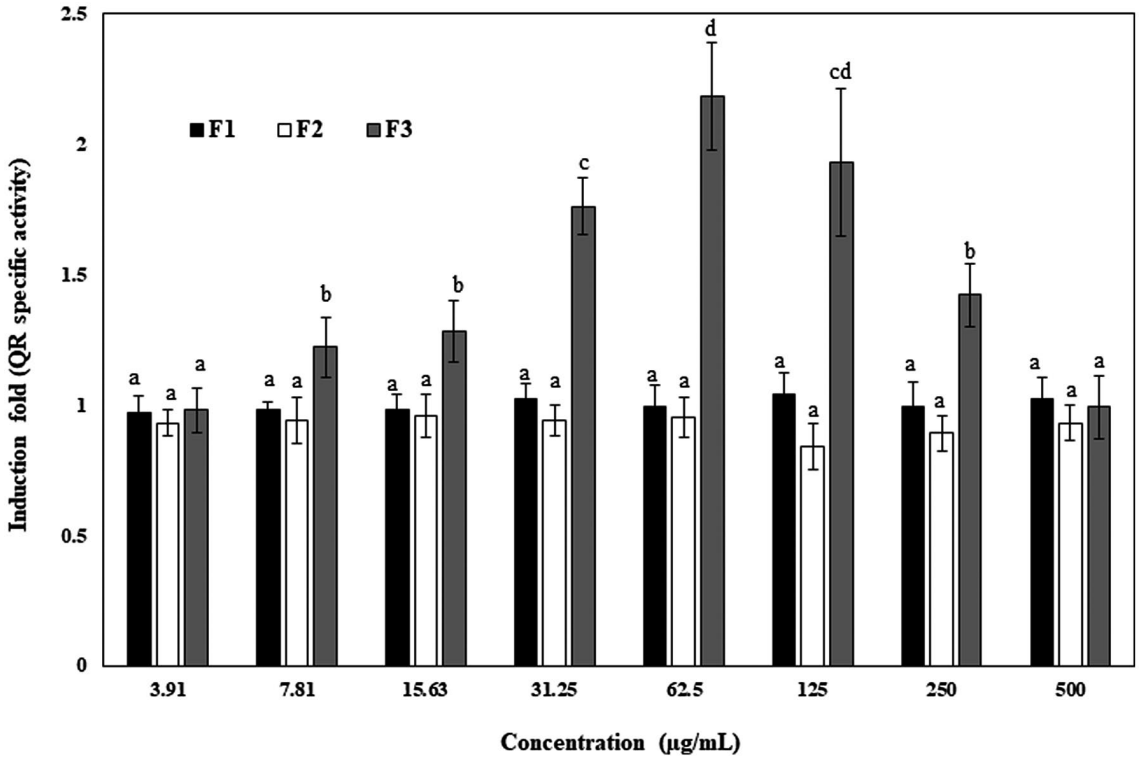

FIGURE 3 Effect of HPLC fractions obtained from a cranberry concentrated juice on the induction of quinone reductase activity. Murine hepatoma (Hepa-1c1c7) cells were exposed to different concentrations of cranberry fractions (3.91 to 500 μg/ml). F1, F2, and F3 were collected by fractionation of cranberry concentrated juice by HPLC. Data are presented as mean ± *SD*. F1: composed of phenolic acids, F2: constituted with mostly anthocyanins, and F3: contained mainly flavonols. Different letters represent significant differences between HPLC fractions obtained from a cranberry concentrated juice on QR activity ($p < .05$)

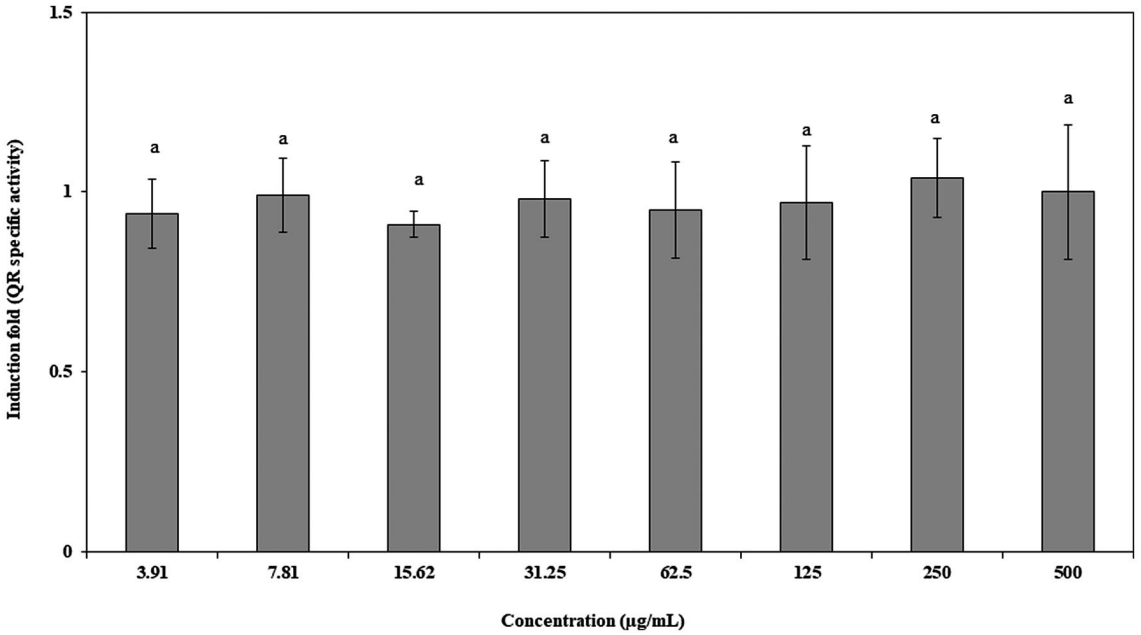

FIGURE 4 Effect of cell wall extracted from a probiotic biomass on the induction of quinone reductase activity. Murine hepatoma (Hepa-1c1c7) cells were exposed to different concentrations of extract of cell wall constituents (3.91 to 500 µg/ml). CW constituents were from a probiotics biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2. Data are presented as mean ± *SD*. CW: cell wall constituents. Different letters represent significant differences between cell wall extracts on QR activity ($p < .05$)

different locations, affected remarkably both the phenolic composition and the antioxidant activity (Kahkonen et al., 2001). This diverging results of F2 can be explained by phenolic compounds extraction method, variety and harvesting location of berries.

F3, mainly composed of flavonols, is the only fraction able to induce QR activity. The greater induction was observed at 62.5 µg/ml and it was of 2.18 ± 0.206 folds. Previous studies showed that flavonols like quercetin, myricetin, and kaempferol were effective QR inducers with maximal induction within the range 1.6 to 3.6 (Uda, Price, Will iamson, & Rhodes, 1997). Our results are in good agreement with previous results. According to the obtained results, the relative potency of QR induction of cranberry juice fractions was flavonols > phenolic acids > anthocyanins.

3.4.2 | Effect of extract of probiotic cell wall constituents on QR activity

As mentioned above, probiotic bacteria have the ability to inhibit colon cancer using diverse mechanisms like the enhancement of the host immune response, alteration of intestinal microbiota, production of beneficial compounds, degradation of potential carcinogens, alterations of the metabolic activities of the intestinal microflora and the production of antitumorigenic compounds in the colon (Ewaschuk, Walker, Diaz, & Madsen, 2006; Hirayama & Rafter, 2000). Since the induction of phase II enzyme such as QR is considered as one of the major mechanisms to inhibit the first stage of colon carcinogenesis, therefore, we investigated the effect of mixed cell walls extracts from *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 on QR activity. The obtained results showed that the cell wall extract did not have any effect on QR activity at the concentration tested (Figure 4). Chang and his team (Chang et al., 2010) demonstrated that cell extracts from four strains of lactobacilli (*L. acidophilus* KFRI342, *L. casei* KFRI707, *L. acidophilus* LA72712, and *L. casei* KFRI809) were able to induce QR activity and KFRI342 being the strain with the greater activity. These findings do not coincide with our observation.

It should be noted that our findings in another study showed that probiotics and their cell walls extracts could individually exert a cancer-preventing effect on colon carcinogenesis in male F344 rats treated with N, N-dimethylhydrazine (DMH), by reducing and eventually inhibiting the formation of aberrant crypt foci (ACF) compared to the positive control (PC) group (Desrouillères et al., 2016). As it was observed the rats fed with the fermented milk pellet which contain the above-mentioned probiotics, induced significantly ($p \leq .05$) the QR enzyme activity compared to the PC group.

By considering these results, it can be concluded that the presence of whole probiotic cells (including whole cells, cell walls or other cellular components), as well as their viability in the pellet fraction, can demonstrate better QR induction activities in comparison to probiotic cell wall extracts. Kim and his team (Kim et al., 2002) also mentioned heat-killed whole cells of different lactic acid bacteria (LAB) tested for in vitro cytotoxicity, had significant antiproliferative activities against several cancer cell lines. They also found that the prostate carcinoma cell line was highly affected by only heat-killed whole cells of Llac (*Lactococcus lactis* ssp. *lactis*). These findings also indicated the anticancer activities of probiotic against cancer cell lines, depending on their strains and also on the cancer cell lines.

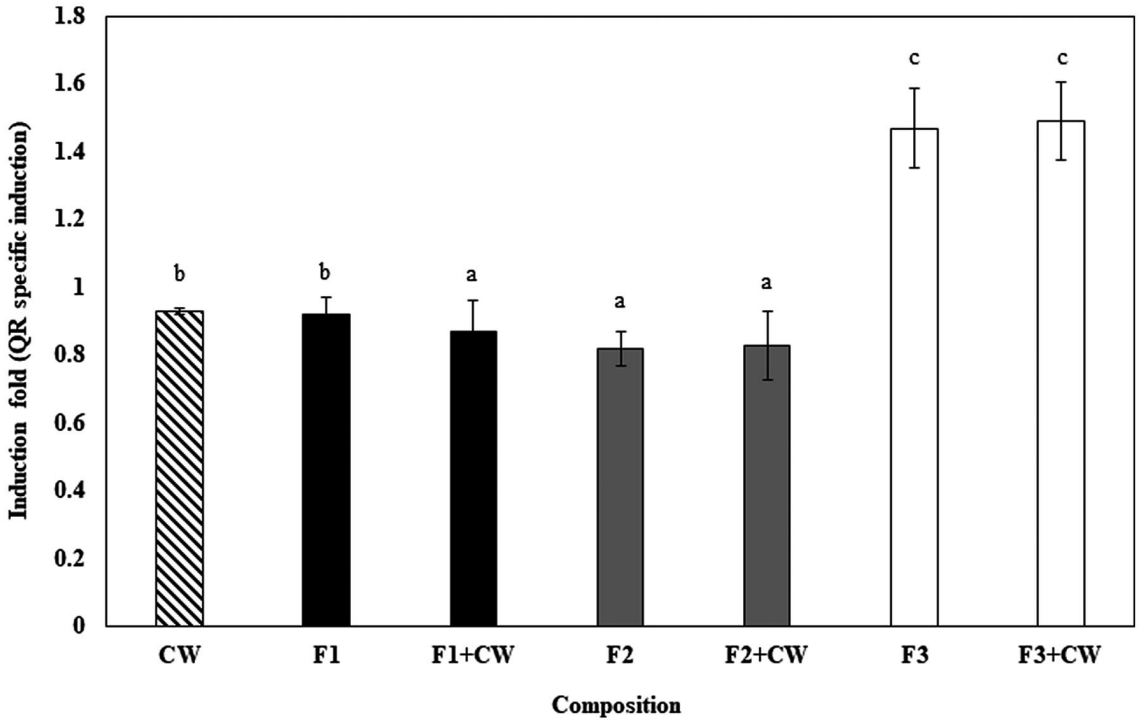

FIGURE 5 Effect of the cranberry fractions combined with probiotic cell walls on the induction of quinone reductase activity: Murine Hepatoma (Hepa-1c1c7) cells were exposed to cranberry fractions combined with extract of cell wall constituents. F1, F2, and F3 were collected by the fractionation of cranberry concentrated juice by HPLC and CW constituents were extracted from a probiotics biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2. The samples are tested at a concentration of 100 µg/ml. Data are presented as mean ± *SD*. Different letters are significantly different ($p \leq .05$). F1: composed of phenolic acids, F2: constituted with mostly anthocyanins, F3: contained mainly flavonols, and CW: cell wall constituents

3.4.3 | Effect of cranberry fraction combined with the extracts of probiotic cell wall constituents on QR activity

The QR activity induced by the cranberry fraction did not change when it was combined with the extracted cell walls from the probiotic biomass (Figure 5). As, it was observed, the chemical interaction between the probiotic cell wall components and the phenolic compounds favored an increase in the antiproliferative effect but the combination of these compounds did not affect the QR activity neither positively nor negatively. As, the cell wall extracts had no effect on QR activity at the concentration tested, so their combination with cranberry fraction, show no more effective act.

4 | CONCLUSION

A concentration-dependant response was observed for all three fractions concerning the inhibitory effect against HT-29 cells. It was found that fractions 1 and 3 obtained from cranberry concentrate juice were able to inhibit the growth of HT-29 cell line. The fraction 1 (F1); which contains mainly phenolic acids, is the most effective at inhibiting HT-29 cells' growth. Anthocyanins-rich fraction (F2) was the fraction the least effective at inhibiting the growth of HT-29. Furthermore, these results suggest that F2 fraction has a different mechanism of protection against cancer proliferation that is not related to its antioxidant capacity. The antiproliferative activity of anthocyanins subfractions from cranberry might depend on some bioactive components and their synergistic effects. Cell wall constituents extracted from a probiotic biomass containing *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2 tested alone did not affect the growth of colon cancer cells but when combined with the cranberry fractions, a significant increase in the inhibition rate was observed. Only flavonols-rich fraction (F3) was able to induce QR activity. The total phenolic content of the cranberry fractions did not have a direct correlation with their antiproliferative effect and their ability to induce phase II enzymes activity. According to these findings, probiotics and their cell walls extracts could probably have the ability to act as anticancer compounds but not able to act as cancer-preventing agents such as chemopreventive compounds.

Furthermore, the results obtained in this study confirm the potential of cranberry as a chemopreventive agent against cancer but also prove that probiotics components and phenolic compounds present in berries could act as a synergetic manner to prevent the colon cancer cells growth and also acts as anticancer compounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in this study.

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