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Cytotoxic and antioxidant properties *in vitro* of Functional beverages based on blackberry (*Rubus glaucus* B.) and soursop (*Annona muricata* L.) pulps

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Submission Date: July 30th, 2018**, Acceptance Date:** November 28th, 2018, **Publication Date:** November 30th, 2018

Citation: Zambrano A., Raybaudi-Massilia R., Arvelo F., Sojo F. Cytotoxic and antioxidant properties in vitro of Functional beverages based on blackberry (*Rubus glaucus* B.) and soursop (*Annona muricata* L.) pulps. *Functional Foods in Health and Disease* 2018; 8(11): 531-547. DOI: https://doi.org/10.31989/ffhd.v8i11.541

ABSTRACT

Background: There are many kinds of tropical fruit available in Venezuela. Two of these fruits are the focus of our study: blackberry ("mora") and soursop ("guanábana"). These fruits have extraordinary bioactive components. For example, blackberry has antioxidant compounds such as anthocyanins, which are characteristic of the Rosaceae family. Acetogenins present in the Annonaceae family have been shown to possess cytotoxic properties that act against different types of tumor cells. In previous research, we have discovered how lyophilized soursop pulp has an elevated cytotoxic effect with an IC_{50} of 7.1940±1.06 in human cervix carcinoma cells (HeLa) and 0.8460±1.29 in human prostate carcinoma cells (PC3).

Objective: This study focused on the health benefits and properties of the soursop and blackberry. Our focus was to determine the antioxidant and cytotoxic properties in a formulated beverage containing blackberry, soursop, and yogurt containing probiotics and prebiotics.

Methods: The research includes the study of soursop pulp (SP), blackberry pulp (BP), and two formulations of the functional beverage selected through a sensorial analysis, F2 (BP + SP + Yogurt + Truvía® + Sacarose) and F3 (BP + SP + Yogurt + Truvía® + Sacarose + Sodium tripolyphosphate). Cell viability of prostate carcinoma cells (PC3), breast carcinoma without over-expression of the HER2/c-erb-2 gene (MCF-7), breast carcinoma in which the HER2/c-erb-2 gene is over-expressed (SKBr3) and healthy cells of human connective tissue used as control

(Fibroblasts). The previous indicated samples were assessed using MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide). The antioxidant activity of the functional beverage was also done using a fresh preparation of 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Results: The BP demonstrated the highest cytotoxicity for both lines of breast cancer cell lines, MCF-7 and SKBR3. The values of the minimum concentration required to inhibit 50 % of the cell population (IC_{50}) was 0.12 ± 1.10 and 1.81 ± 1.68% v / v respectively, followed by SP in MCF-7 and PC3 with values of 1.40 ± 1.03 and 1.34 ± 1.06 respectively. At the same time, the effectiveness of the formulations used found that 3.60 ± 1.04% v / v of F2 beverage was necessary to achieve 50 % inhibition of cell viability of MCF-7 line. For the formulation F3, it was necessary to use a concentration of 5.21 ± 1.04% v / v for that tumor cell line. However, the F2 and F3 formulations demonstrated IC_{50} values of 3.69 ± 1.08% v / v and 2.50 ± 1.08% v / v respectively for the PC3 cell line. On the other hand, the antioxidant capacity of BP and SP reached elevated values at 30 minutes of exposure to DPPH, obtaining a rate of 85.28 ± 0.11 and 80.94 ± 0.07 % respectively by using a concentration of 12.5 %, F2 and F3 formulations also reached values of 83.97 ± 0.46 and 85.62 ± 0.11 % at 100 % concentration of both drinks respectively.

Conclusion: We discovered that the cytotoxic activity of both formulations prepared, as well as the pulps were fairly good, revealing highly effective consequences for the inactivation of breast tumor cells MCF-7 and prostate tumor cells PC3. Moreover, BP and SP demonstrated a high antioxidant activity, with a synergistic effect accomplished by the mixture on F2 and F3.

Keywords: Functional beverage, cytotoxic, antioxidant, soursop, blackberry, yogurt.

INTRODUCTION

In recent times, fruits and vegetables have been promoted as healthy foods due to their demonstrated antioxidant and anticancer properties [1, 2, 3, 4]. Organizations such as the Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), World Cancer Research Fund, and Global Alliance for the Promotion of Fruit and Vegetable Consumption "5 a day" (AIAM5) have recommended the consumption of fruits and vegetables as a key component of a healthy diet for the prevention of chronic diseases [5]. In particular, FAO and WHO have promoted incomes for farmers by increasing the production and supply and the consumption of fruit and vegetables [6, 7]. The Harvard University indicated that at least half of the meal should be based on fruits and vegetables in order to be considered a healthy meal. In the last decade, the demand for “healthy” foods and beverages has increased in many parts of the world [9, 10]. From 1980, these type of “healthy” foods and beverages have been called “functional foods,” a term which originated in Japan [11]. The demand has increased due to the prevalence of chronic diseases, busy lifestyles, low consumption of convenience foods, and insufficient exercise [12, 10].

In 1991, the Ministry of Health of Japan introduced rules for the approval of a specific health-related food category called FOSHU (Food for Specified Health Uses) which included the establishment of specific health claims for this type of food [11, 13, 14, 15, 16]. In 2015, the Functional Food Center defined functional food as a natural or processed food that contains known or unknown biologically-active compounds. Foods in a defined or adequate amount provide a

clinically proven and documented health benefit for the prevention or treatment of chronic disease [17].

Fruits and vegetables are rich sources of many diverse nutrients and bioactive compounds, capable of modulating metabolic processes and resulting in the promotion of better health. They exhibit beneficial effects such as antioxidant activity, inhibition or induction of enzymes, inhibition of receptor activities, and induction and inhibition of gene expression [18]. A combination of fruits has been demonstrated to exhibit additive or synergistic effects on enhancing the antioxidant effectiveness and status in human subjects [19, 20, 21].

Edible berries like blackberries from the Rosaceae family, genus Rubus, are known for curing and preventing a wide variety of ailments, such as colitis, in folk medicine [22]. Apart from vitamins, minerals, sugars, and fibers they are rich in bioactive compounds including phenolics, flavonoids, and tannins. These phytochemicals have been reported to possess anticancer and antioxidant activities [23]. Studies have also indicated that the antioxidant effect of berries are directly associated with its anticancer potential [12, 13] and anti-inflammatory properties [25, 26]. Digested metabolites from wild blackberries (*R. brigantinus* and *R. vagabundus*) in human plasma could protect neuronal cells against oxidative damage, which is an influential factor for neurodegeneration [27].

In vitro cell culture studies demonstrated how hull blackberry extract inhibited HT-29 colon tumor cell growth in a concentration-dependent manner with 49.2 μg of total anthocyanins/mL without cytotoxicity [28]. The content of the total polyphenols and total anthocyanins are significantly related to the antioxidant activity that can prevent diseases generated by the action of free radicals [29, 30, 31, 32, 33, 34]. Blackberries are rich in anthocyanins and flavonoid glycosides [35, 36]. These compounds provide natural pigmentation and exhibit a wide range of antioxidant protection and therapeutic benefits including the integrity of genomic DNA, potent cardioprotective, neuroprotective, anti-inflammatory, and anticarcinogenic properties [21, 37, 38, 39, 19, 40].

The fruit soursop (*Annona muricata* L.) which comes from the Annonaceae family (also called "guanábana" or "chirimoya"), has attributed various health claims, in addition to being a fruit used in products like jellies, yogurts, and cakes. Soursop pulp has high antioxidant potential, with a half-maximal inhibitory concentration (IC_{50}) of 2.0 mg / mL determined by the method of free radical 1,1-diphenyl - 2 picrylhydrazyl (DPPH), which has been linked to the high content of phenolic compounds (368 mg / 100 g) [41, 42]. Another health and wellness property of soursop is their cytotoxic activity against various types of cancers; acetogenins of Annonaceaes have cytotoxic activity against several types of cancer cells such as prostate cancer (PC3), breast (MCF-7), lung (A-549) [42]. Soursop pulp and seed also have higher cytotoxic activity on the PC3 (0.0024 to 1.275 µg/mL) and HeLa (0.0011 to 7.194 µg/mL) cell lines with low impact on healthy cells (fibroblasts, as control) [43].

In this way, it is possible to incorporate a large number of antioxidants and natural bioactive components in the human body through the consumption of blackberries and soursop. Many researchers report that functional foods represent one of the most interesting areas of research and innovation in the food field [44]. Thus, the aim of this study was to determine the antioxidant and cytotoxic properties of formulations of a beverage made from blackberry, soursop, and yogurt with

probiotics and prebiotics incorporated in order to demonstrate its properties, welfare, and consumer health by including antioxidants, anti-cancer, and gastrointestinal properties to promote general health.

MATERIALS AND METHODS

Fruit

Annona muricata L. (soursop) and *Rubus glaucus* B. (blackberry) at commercial ripeness (stage of ripeness for consumption) were selected in a local supermarket (Caracas, Venezuela) and maintained at 7° C until processing.

Preparation of functional beverage

Different formulations were developed containing 40% of liquid yogurt prepared from a skim powdered milk reconstituted and homogenized at 17.0% with the addition of inulin (3.0 %), and then heat-treated (95° C x 10 min.) before the incorporation of starter cultures (CHR HANSEN - lactic culture with *Bifidobacterium* BB-12 ®) and kept at 41 °C until pH 4.3. The rest of the formulation (60%) consisted of an elaborated mixture from pulps of soursop and blackberries combined with drinking water, Truvía® sweetener, and sucrose. Two formulations of the beverage ranging only in content of water, sweeteners, and in the addition of a stabilizing salt (sodium tripolyphosphate). Mixtures were previously evaluated and selected through sensory analysis including 80 consumers from 18 to 60 years old.

Human Tumor Cell Lines

Human tumor cell lines from MCF-7 (breast carcinoma, without over-expression of the HER2/c-erb-2 gene), SKBr3 (breast carcinoma, in which the HER2/c-erb-2 gene is overexpressed), and PC3 (prostate carcinoma) were provided by Marie-France Poupon from Laboratory of Molecular Cytogenetic and Oncology of the Curie Institute (Paris, France). Human dermis fibroblasts, used as control cells, were obtained from Laboratory of Tissue Culture and Tumor Biology of Instituto de Biología Experimental (Caracas, Venezuela). All cell lines were used to determine the cytotoxic activity from the functional beverage. MCF-7, SKBr3, and fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamax (Gibco), 100 units/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco). PC3 was grown in Roswell Park Memorial Institute medium (RPMI 1640; Gibco) supplemented with 10% (v/v) heat inactivated FBS, 2 mM glutamax (Gibco), 100 units/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). For treatments, exponentially growing cells were collected, counted, re-suspended in fresh culture medium, and incubated in 96 sterile well plates.

Cytotoxicity Test

The evaluation of the cytotoxic activity was done with previous centrifugation (5.000 rpm x 15 min.) of the samples to obtain the water phase and then filtrated (20 µm). Cell viability was assessed using the MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) test, which is based on the ability of viable cells to metabolically reduce a yellow tetrazolium salt (MTT; Sigma, USA) to purple crystals of formazan [45]. This reaction takes place when mitochondrial reductases are active. Cells were seeded in 96-well plates (5×10^5 cells/well) and incubated at 37°C

for 72 hours with the samples (functional beverage and each of the fruit pulps selected) at concentrations of 0.00; 0.04; 0.20; 0.40; 0.80; 1.70; 2.70; 5.00; 7.50; 10.00% v/v, diluted in bidistilled water respectively, in a humidified atmosphere with 5% CO_2. After incubation, the medium was removed, and the cells were treated with 100 μL MTT for 3 hours at 37° C. Subsequently, the MTT was removed and 100 μL dimethyl sulfoxide (DMSO) was added. The formazan product was quantified with the help of a microplate reader TECAN-Sunrise™ at 570 nm (Tecan Group LTD, Männedorf, Switzerland). Taxol (Bristol-Myers Squibb, USA) was used as a positive control in the test. The experiment was carried out in triplicate.

Selectivity Index for Cytotoxicity Test

After the cytotoxicity test was calculated, the selectivity index (SI) as the IC_{50} (control cells)/IC_{50} (tumor cell line) ratio was conducted. A selectivity index > 1 indicates that the cytotoxicity on tumor cell lines surpassed that on the healthy non-tumor cells [46].

Determination of Antioxidant Activity

The antioxidant activity of the functional beverage was determined through the method described by Celep *et al.* 2012. The sample was centrifuged at 15.000 rpm x 15 minutes to obtain the water phase, then were prepared in solutions of 3.15; 6.25; 12.50; 25.00; 50.00; 75.00; 100.00% v/v, diluted in methanol, and those were mixed with 2.8 mL of freshly prepared 0.06 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, USA) solution in methanol. The mixture was shaken with a vortex and incubated in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm (the extract was added just after 30 min of dark incubation). Ascorbic acid (176 μg/mL) was used as reference substance (control). The absorbance was measured at 517 nm. The antioxidant activity expressed as DPPH radical-scavenging activity was calculated as follows (Equation 1):

$$DPPH\ radical - scavenging\ activity\ (\%) = \left(\frac{Asorbance\ control - \ Absorbance\ sample}{Absorbance\ control}\right)$$

Statistical Analysis

The methods described before were repeated at least three times. The values of cytotoxicity, expressed in IC_{50}, of formulated beverages were determined by a non-linear regression of individual experiments using the program GraphPad Prism v.5.02 (GraphPad Software, San Diego, CA, USA). To detect significant statistically differences (*p-values)* analyses of variance (ANOVA) were carried out on both cytotoxic and antioxidant activity of the functional beverage; and between their concentrations using statistic package Statgraphics Centurion XVI (StatPoint Technologies). Multiple range tests, using the Fisher's LSD method, were then applied to determine which beverage and concentrations had significantly different p-values.

RESULTS AND DISCUSSION

Cytotoxic Activity

Table 1, 2, 3, and 4 show the cytotoxic effect of the two formulated functional beverages (F2 and F3) and pasteurized pulps, soursop (SP), and blackberry (BP) on human epidermal fibroblasts

(control cells) and tumor cells (MCF-7, SKBr3 and PC3). A progressive decrease in cell viability was observed, depending on the product (F2, F3, SP and BP) and concentration, in a dose-dependent manner. These results showed *p-values* differences between the percentages of viability; to compare between products and concentrations evaluated individually, fruit pulps evaluated were more unfavorable on the viability of the cells in relation to both beverages.

Thereby, through evaluating fibroblast cell viability (Table 1) it became clear using concentrations less than or equal to 5 % of fruit pulp, viability remained close to 50 %, with values of 54.31 ± 0.22 % and 47.53 ± 0.26 % for the pulp of blackberry and soursop respectively, while viability at this concentration was kept at 100 ± 1.90 % when using the F2 formulation and 98.75 ± 0.34 % by adding F3.

A greater effect was observed on human tumor cell lines. Specifically, breast cancer cell line MCF-7 viability percentages (Tables 2) were 43.52 ± 0.32% and 59.31 ± 1.85% for 5% of F2 and F3 drinks respectively. In contrast, for soursop and blackberry pulps viability was 36.25 ± 0.19 and only 9.10 ± 0.61 % respectively. Moreover, by using blackberry pulp in breast cancer cell line SKBr3 (Table 3), we found a greater cytotoxic effect on cell viability through a percentage viability of 8.35 ± 0.36% at a concentration of 5% of the pulp. Followed by a 30.00 ± 1.30% of viability for soursop pulp and 72.34 ± 0.79% and 92.04 ± 0.23% for F2 and F3 formulations respectively.

Table 4 has the results of the prostate cancer (PC3) cell line. Formulations F2 and F3 show a viability percentage of 44.38 ± 0.29% and 56.05 ± 0.68% at 5% concentration. We obtained a greater reduction using soursop and blackberry pulps, with values of 9.64 ± 0.69% and 11.70 ± 0.29% at the same concentration.

Table 1. Percentage of cell viability of fibroblast employing two functional beverages, F2 (Yogurt + Soursop and blackberry pulps + Sweeteners), F3 (Yogurt + Soursop and Blackberry pulps + Sweeteners + pH regulator), soursop and blackberry pulps (SP and BP) in different concentrations.

	Celular Viability (%)			
[P]	**F2**	**F3**	**SP**	**BP**
0	100,00 ± 1,00 ^{Aa}	100,00 ± 1,00 ^{Aa}	100,00 ± 1,00 ^{Ba}	100,00 ± 1,00 ^{Ba}
0,04	99,48 ± 0,98 ^{Aa}	100,00 ± 0,12 ^{Aa}	91,53 ± 0,16 ^{Ba}	100,00 ± 0,91 ^{Ba}
0,2	98,06 ± 0,74 ^{Aab}	100,00 ± 0,15 ^{Aab}	77,67 ± 0,11 ^{Bab}	100,00 ± 1,38 ^{Bab}
0,4	97,34 ± 0,77 ^{Aab}	97,59 ± 0,60 ^{Aab}	84,93 ± 1,46 ^{Bab}	100,00 ± 1,14 ^{Bab}
0,8	96,44 ± 0,72 ^{Aab}	93,89 ± 0,87 ^{Aab}	83,77 ± 1,30 ^{Bab}	90,89 ± 0,48 ^{Bab}
1,7	95,98 ± 1,44 ^{Aab}	100,00 ± 0,12 ^{Aab}	77,35 ± 1,20 ^{Bab}	82,82 ± 0,50 ^{Bab}
2,7	100,00 ± 0,69 ^{Aab}	100,00 ± 0,66 ^{Aab}	64,72 ± 0,33 ^{Bab}	72,64 ± 1,42 ^{Bab}
5	100,00 ± 1,90 ^{Ab}	98,75 ± 0,34 ^{Ab}	47,53 ± 0,26 ^{Bb}	54,31 ± 0,22 ^{Bb}
7,5	94,24 ± 1,42 ^{Ac}	36,48 ± 0,70 ^{Ac}	22,39 ± 0,22 ^{Bc}	10,46 ± 0,17 ^{Bc}
10	70,88 ± 0,34 ^{Ac}	26,10 ± 0,15 ^{Ac}	3,13 ± 0,07 ^{Bc}	3,31 ± 0,35 ^{Bc}

[P]: concentration of each product (% v/v), F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp. Different capital letters (A y B) represent significant difference *p-values between products (F2, F3, PM and PG). Different lowercase letters (a, b, c) in the same column indicate significant differences (p-values) between products concentration (*0,04; 0,20; 0,40; 0,80; 1,70; 2,70; 5,00; 7,50 y 10,00%). *Values are mean of eight (8) determinations ± standard deviation.*

Table 2. Percentage of cell viability of MCF-7 (breast carcinoma, without over-expression of the HER2/c-erb-2 gene) employing two functional beverage, F2 (Yogurt + Soursop and blackberry pulps + Sweeteners), F3 (Yogurt + Soursop and Blackberry pulps + Sweeteners + pH regulator), soursop and blackberry pulps (SP and BP) in different concentrations.

	Celular viability (%)							
[P]	**F2**		**F3**		**SP**		**BP**	
0	100,00 ± 1,00	Aba	100,00 ± 1,00	Aa	100,00 ± 1,00	Ba	100,00 ± 1,00	Ca
0,04	97,82 ± 0,27	Abab	100,00 ± 0,42	Aab	87,55 ± 0,78	Bab	55,55 ± 0,81	Cab
0,2	85,96 ± 0,22	ABbc	98,45 ± 0,68	Abc	77,93 ± 0,83	Bbc	51,22 ± 0,76	Cbc
0,4	70,66 ± 0,33	ABcd	88,60 ± 0,92	Acd	63,27 ± 0,46	Bcd	40,77 ± 0,87	Ccd
0,8	70,05 ± 0,20	ABde	87,62 ± 0,74	Ade	51,28 ± 0,53	Bde	28,28 ± 0,81	Cde
1,7	58,06 ± 0,34	ABdef	84,05 ± 0,79	Adef	47,80 ± 0,20	Bdef	17,98 ± 0,70	Cdef
2,7	56,86 ± 0,16	ABef	79,13 ± 0,64	Aef	43,21 ± 0,59	Bef	11,92 ± 0,19	Cef
5	43,52 ± 0,32	ABfg	59,31 ± 1,85	Afg	36,25 ± 0,19	Bfg	9,10 ± 0,61	Cfg
7,5	40,41 ± 0,25	ABgh	36,64 ± 1,0	Agh	23,44 ± 0,27	Bgh	1,76 ± 0,32	Cgh
10	38,33 ± 0,35	ABh	0,00 ± 0,73	Ah	26,32 ± 0,46	Bh	1,76 ± 0,18	Ch

[P]: concentration of each product (% v/v), F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp. Different capital letters (A, B, C) represent significant difference *p-values between products (F2, F3, PM and PG). Different lowercase letters (a, b, c, d, e , f, g and h) in the same column indicate significant differences (p-values) between products concentration (*0,04; 0,20; 0,40; 0,80; 1,70; 2,70; 5,00; 7,50 y 10,00 %). *Values are mean of eight (8) determinations ± standard deviation*

Table 3. Percentage of cell viability of SKBr3 (breast carcinoma, in which the HER2/c-erb-2 gene is over-expressed) employing two functional beverages, F2 (Yogurt + Soursop and blackberry pulps + Sweeteners), F3 (Yogurt + Soursop and Blackberry pulps + Sweeteners + pH regulator), soursop and blackberry pulps (SP and BP) in different concentrations.

	Celular viability (%)							
[P]	**F2**		**F3**		**SP**		**BP**	
0,00	100,00 ± 1,00	Aa	100,00 ± 1,00	Aa	100,00 ± 1,00	Ba	100,00 ± 1,00	Ca
0,04	97,42 ± 0,58	Aab	100,00 ± 0,61	Aab	94,03 ± 0,54	Bab	82,17 ± 0,50	Cab
0,20	96,67 ± 0,24	Aabc	100,00 ± 0,84	Aabc	93,8 ± 0,22	Babc	59,02 ± 0,94	Cabc
0,40	94,09 ± 0,66	Aabcd	100,00 ± 0,23	Aabcd	88,64 ± 0,89	Babcd	52,05 ± 1,88	Cabcd
0,80	93,77 ± 0,29	Abcd	100,00 ± 1,77	Abcd	84,22 ± 0,77	Bbcd	43,26 ± 0,40	Cbcd
1,70	91,72 ± 0,41	Acd	100,00 ± 0,00	Acd	65,47 ± 1,80	Bcd	31,07 ± 0,69	Ccd
2,70	89,82 ± 0,28	Ade	97,20 ± 1,29	Ade	57,72 ± 0,74	Bde	19,74 ± 1,35	Cde
5,00	72,34 ± 0,79	Ae	92,04 ± 0,23	Ae	30,00 ± 1,30	Be	8,35 ± 0,36	Ce
7,50	47,02 ± 0,89	Af	33,55 ± 0,87	Af	12,27 ± 0,45	Bf	7,5 ± 0,23	Cf
10,00	29,18 ± 0,58	Af	30,06 ± 1,56	Af	4,34 ± 0,71	Bf	2,12 ± 0,18	Cf

[P]: concentration of each product (% v/v), F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp. Different capital letters (A, B, C) represent significant difference *p-values between products (F2, F3, PM and PG). Different lowercase letters (a, b, c, d, e and f) in the same column indicate significant differences (p-values) between products concentration (*0,04; 0,20; 0,40; 0,80; 1,70; 2,70; 5,00; 7,50 y 10,00%). *Values are mean of eight (8) determinations ± standard deviation*.

Table 4. Percentage of cell viability of PC3 (human prostate carcinoma) employing two functional beverages, F2 (Yogurt + Soursop and blackberry pulps + Sweeteners), F3 (Yogurt + Soursop and Blackberry pulps + Sweeteners + pH regulator), soursop and blackberry pulps (SP and BP) in different concentrations.

	Viabilidad celular (%)			
[P]	**F2**	**F3**	**PG**	**PM**
0	100,00 ± 1,00 ^{Aa}	100,00 ± 1,00 ^{Aa}	100,00 ± 1,00 ^{Ba}	100,00 ± 1,00 ^{Ba}
0,04	90,67 ± 0,94 ^{Ab}	81,08 ± 0,19 ^{Ab}	86,15 ± 7,19 ^{Bb}	83,83 ± 0,68 ^{Bb}
0,2	86,03 ± 0,77 ^{Ab}	78,62 ± 0,82 ^{Ab}	83,92 ± 0,45 ^{Bb}	78,82 ± 0,17 ^{Bb}
0,4	78,05 ± 0,65 ^{Abc}	75,25 ± 0,40 ^{Abc}	80,17 ± 0,69 ^{Bbc}	72,22 ± 0,34 ^{Bbc}
0,8	75,19 ± 0,11 ^{Acd}	73,03 ± 0,65 ^{Acd}	61,19 ± 1,38 ^{Bcd}	63,15 ± 0,68 ^{Bcd}
1,7	69,4 ± 0,44 ^{Ade}	67,09 ± 0,22 ^{Ade}	49,82 ± 0,45 ^{Bde}	57,87 ± 0,49 ^{Bde}
2,7	65,54 ± 0,16 ^{Ae}	58,83 ± 0,58 ^{Ae}	44,55 ± 1,91 ^{Be}	44,43 ± 0,74 ^{Be}
5	56,05 ± 0,68 ^{Af}	44,38 ± 0,29 ^{Af}	9,64 ± 0,69 ^{Bf}	11,7 ± 0,29 ^{Bf}
7,5	38,52 ± 0,52 ^{Ag}	20,48 ± 0,55 ^{Ag}	0,00 ± 0,86 ^{Bg}	3,71 ± 0,22 ^{Bg}
10	8,04 ± 0,24 ^{Ag}	10,90 ± 0,41 ^{Ag}	0,00 ± 0,69 ^{Bg}	0,85 ± 0,10 ^{Bg}

[P]: concentration of each product (% v/v), F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp. Different capital letters (A and B) represent significant difference *p-values between products (F2, F3, PM and PG). Different lowercase letters (a, b, c, d, e. f and g) in the same column indicate significant differences (p-values) between products concentration (*0,04; 0,20; 0,40; 0,80; 1,70; 2,70; 5,00; 7,50 y 10,00 %). *Values are mean of eight (8) determinations ± standard deviation.*

In Table 5, the minimum concentration required to inhibit 50% of the cell population (IC_{50}) in each cancer cell line is shown. Blackberry pulp showed the highest cytotoxicity for both lines of breast cancer, MCF-7 and SKBR3, with values of 0.12 ± 1.10% and 1.81 ± 1.68% v / v respectively, followed by soursop pulp in MCF-7 and PC3 with values of 1.40 ± 1.03% and 1.34 ± 1.06% respectively. While the beverages used were effective, a higher concentration of the formula F2 (3.60 ± 1.04% v / v) was necessary to achieve 50% inhibition of cell viability of MCF-7 line. In contrast, for the formulation F3, it was necessary to use a concentration of 5.21 ± 1.04 % v / v for that cancer cell line. Surprisingly, the F2 and F3 formulations demonstrated IC_{50} values of 3.69 ± 1.08% v / v and 2.50 ± 1.08% v / v respectively for the PC3 cell line.

Table 5. Cytotoxic activity expressed in IC_{50} values of formulated beverages (F2 and F3), soursop and blackberry pulps (SP and BP) on various human tumor cell lines.

	Values of IC_{50} (% v/v)*			
Product	**Fibroblasts**	**MCF-7**	**SKBr3**	**PC3**
F2	11,43 ± 1,01	3,60 ± 1,04	7,92 ± 1,04	3,69 ± 1,08
F3	7,30 ± 1,01	5,21 ± 1,04	5,93 ± 1,00	2,50 ± 1,08
SP	3,54 ± 1,05	1,40 ± 1,03	5,16 ± 1,08	1,34 ± 1,06
BP	4,22 ± 1,02	0,12 ± 1,10	1,81 ± 1,68	1,33 ± 1,07

**Values are mean of eight (8) determinations ± standard deviation. IC_{50}: inhibitory concentration 50; F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp; Fibroblasts (healthy cells of animal connective tissue, used as control) MCF-7 (breast carcinoma, without over-expression of the HER2/c-erb-2 gene); SKBr3 (breast carcinoma, in which the HER2/c-erb-2 gene is over-expressed); PC3 (human prostate carcinoma).*

In contrast to the effectiveness observed in MCF-7 by F2 and F3 formulations, a greater IC_{50} in SKBr3 cell line was determined, reporting a value of 5.93 ± 1.00% v / v for F3 and 7.92 ± 1.04% v / v for F2, compared with 5.21 ± 1.04 and 3.60 ± 1.04 obtained for MCF-7. Compared to the trend observed in both cell lines MCF-7 and SKBr3 where blackberry pulp was more effective in inhibiting the growth of these cells, the results obtained in cell lines PC3 yielded IC_{50} values like; 1.34 ± 1.06 and 1.33 ± 1.07 for blackberry pulp and soursop pulp respectively, followed by the results obtained in the F2 and F3 formulations, 3.69 ± 1.08% and 2.50 ± 1.08% v / v.

By calculating the selectivity index (SI) [48] for each tumor line with each product studied, cancer cell lines were more affected compared to the control cells, with SI values greater than 1 (see Table 6). The largest SI was found in fruit pulps to MCF-7 cells, with a value of 35.17, being about 35 times more effective for the MCF-7 line compared to control cells, followed by the F2 formulation with a SI value of 3.18. The difference between both beverages in MCF-7 cells may be due to a pH which was adjusted in the F3 formulation with sodium tripolyphosphate, resulting in a pH 4.09 unlike pH 3.80 in F2. Meanwhile, the F2 and F3 formulations showed very similar values on SKBR3 and PC3 lines, 1.44 and 1.23 and 3.08 and 2.92 respectively.

Table 6. Determination of selectivity index (SI) in the functional beverages (F2 and F3) and pasteurized pulps from soursop (SP) and blackberry (BP) in different cell lines.

	SI		
Product	**MCF-7**	**SKBr3**	**PC3**
F2	3,18	1,44	3,08
F3	1,40	1,23	2,92
SP	2,53	0,68	2,64
BP	35,17	2,33	3,17

**Values are mean of eight (8) determinations ± standard deviation. SI: Selectivity index; F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp; Fibroblasts (healthy cells of animal connective tissue, used as control) MCF-7 (breast carcinoma, without over-expression of the HER2/c-erb-2 gene); SKBr3 (breast carcinoma, in which the HER2/c-erb-2 gene is over-expressed); PC3 (human prostate carcinoma).*

Studies by Raybaudi-Massilia et al. demonstrated that the use of soursop seed extracts by maceration and lyophilization techniques applied individually in breast cancer cell lines MCF-7 reached IC_{50} values of 70.15 ± 1.02 and 27.09 ± 1.03 mg / mL respectively. Moreover, the extraction by maceration of seeds also demonstrated cytotoxicity with an IC_{50} value of 20.50 ± 1.01 mg / mL in cell cultures SKBr3 [43]. In contrast, studies using lyophilized soursop pulp are only effective in inhibiting the growth of PC3 cells with an IC_{50} value of 0.846 ± 1.29 mg / mL.

Numerous studies have shown that acetogenins from Annonaceas are responsible for their cytotoxic activity, suggesting their potential use as an antitumor agent [49, 50, 51]. The mode of action of acetogenins is through inhibition of nicotinamide adenine dinucleotide-ubiquinone oxidoreductase (complex I) in mitochondria, in conjunction with suppressive inhibiting the adenosine triphosphate (ATP) production, especially in cancer cells with a high metabolic rate generating apoptosis [52, 53]. Additionally, Chiu et al. indicated that acetogenins of Annonaceaes as bullatacin induce apoptosis through reducing intracellular cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) values in human hepatoma cells 15 2.2 [53].

Among different medicinal uses attributed to blackberry, its anticarcinogenic, antiviral and antiallergic effects have currently been examined. Studies by Bowen-Forbesa et al. in which they employed a hexane extract of *Rubus Jamaican* species, determined the great potential to inhibit the growth of colon cancer cells, breast, lung, and gastric. Moreover, they discovered that hexane extracts were more effective than those made in methanol or ethyl acetate, suggesting that nonpolar components, such as fats and / or terpenes extracted from seeds of berries may be an effective anticancer agent. Likewise, the high content of anthocyanins of this fruit suggest that its consumption could generate health benefits useful in the production of functional foods [54]. Ross et al. at the James Hutton Institute suggests that ellagitanins of blackberries are particularly effective against cervical cancer cells (HeLa) *"in vitro,"* followed by blackberry extracts rich in anthocyanins, with an IC_{50} of 0.013 mg / mL and 0.067 mg / mL respectively [55].

Antioxidant Activity

Through the quantification of the absorbance decrease of the oxidant radical 1,1-difenil-2-picril-hidrazilo (DPPH) determined at 517 nm at 0 and 30 min, we obtained the antioxidant activity of the formulated beverages, F2 (Blackberry + Soursop + Yogurt + Truvía® + Sacarose) and F3 (Blackbery + Soursop + Yogurt + Truvía® + Sacarose + Sodium tripolyphosphate), and of the pasteurized fruit pulps, blackberry (BP), and soursop (SP). A higher antioxidant activity was expressed for both functional beverages, F2 and F3, without statistically significant differences (*p-values)* among them (Table 3), as well as differences between exposure times (0 or 30 min).

In this way, *p-values* for each beverage antioxidant activity were discovered, but when comparing the exposure times to the reagent with a value of 30.35 ± 0.66% at time zero and 83.97 ± 0.46% after thirty minutes exposure in F2 and 47.81 ± 0.11% and 85.62 ± 0.11% respectively for F3. Moreover, statistically significant differences for different concentrations of the formulations employed were also established, resulting in a direct proportional relationship, where the higher concentration of the product reached greater antioxidant capacity, from values of 14.98

± 0.15 and 24.57 ± 0.24% after 30 minutes of exposure using a 3.15% of the F2 and F3 formulations to values of 83.97 ± 0.46% and 85.62 ± 0.11% at 100% of both drinks respectively. The results obtained for the antioxidant capacity of blackberry and soursop pulp (Table 7) also reached elevated values at 30 minutes of exposure to DPPH, obtaining a rate of 85.28 ± 0.11% and 80.94 ± 0.07% respectively by using a concentration of 12.5%, values that are significantly higher than those obtained by using the F2 and F3 formulations. These differences are due to the proportions of blackberry and soursop pulp used in the formulated beverages.

Statistically significant differences were also determined by using different concentrations of each pulp, being in the case of blackberry pulp some interference of red-blue pigments, anthocyanins, which increased the absorbance reading. More importantly, this is a colorimetric method where the reduction of DPPH radical occurs from deep purple color, that in the presence of antioxidants pattern solutions such as vitamin C turns into a yellow color. Phenolic compounds have been attributed as antioxidants in soursop pulp by different authors [57, 41]. Previous studies with extracts of soursop by Raybaudi-Massilia et al. demonstrated that the antioxidant activity of a lyophilized soursop pulp, reached values of 68.53 ± 0.20% at time 0 and 69.77 ± 0.92% after 30 minutes of exposure to DPPH [43]. However, we had much better results in this study (88.68 ± 0.11% and 88.75 ± 0.07% respectively). This may be due to the type of processing subjected to the pulp, being in this research pasteurization, unlike a process of extraction and lyophilization in the case of the other study, which involved an initial extraction by cooking, followed by freezing and subsequent sublimation for obtaining lyophilized.

Table 7. Antioxidant activity expressed as DPPH radical-scavenging activity of formulated beverages (F2 and F3), soursop and blackberry pulps (SP and BP).

[P] (% v/v)	**DPPH radical-scavenging activity (%; v/v)**							
	F2		**F3**		**BP**		**SP**	
	0 min.	**30 min.**	**0 min.**	**30 min.**	**0 min.**	**30 min.**	**0 min.**	**30 min.**
100,00	30,35 ± 0,66$^{\alpha Aa}$	83,97 ± 0,46$^{\alpha Ba}$	47,81 ± 0,11$^{\alpha Aa}$	85,62 ± 0,11$^{\alpha Ba}$	68,04 ± 0,36$^{\beta Aa}$	68,55 ± 0,58$^{\beta Ba}$	88,68 ± 0,11$^{\beta Aa}$	88,75 ± 0,07$^{\beta Ba}$
75,00	23,82 ± 0,89$^{\alpha Aab}$	77,09 ± 0,45$^{\alpha Bab}$	42,98 ± 0,33$^{\alpha Aab}$	80,87 ± 0,30$^{\alpha Bab}$	68,94 ± 0,24$^{\beta Aab}$	69,28 ± 0,26$^{\beta Bab}$	87,25 ± 0,13$^{\beta Aab}$	88,53 ± 0,07$^{\beta Bab}$
50,00	19,35 ± 0,75$^{\alpha Aabc}$	60,80 ± 0,40$^{\alpha Babc}$	35,96 ± 0,57$^{\alpha Aabc}$	64,15 ± 0,26$^{\alpha Babc}$	76,26 ± 0,26$^{\beta Aabc}$	76,45 ± 0,11$^{\beta Babc}$	68,04 ± 0,26$^{\beta Aabc}$	81,89 ± 0,11$^{\beta Babc}$
25,00	18,09 ±0,78$^{\alpha Abcd}$	38,04 ± 0,40$^{\alpha Bbcd}$	31,55 ± 0,40$^{\alpha Abcd}$	44,83 ± 0,33$^{\alpha Bbcd}$	80,53 ± 0,11$^{\beta Abcd}$	80,68 ± 0,07$^{\beta Bbcd}$	62,30 ± 0,30$^{\beta Abcd}$	81,43 ± 0,11$^{\beta Bbcd}$
12,50	16,28 ± 0,89$^{\alpha Acde}$	20,25 ± 0,15$^{\alpha Bcde}$	24,64 ± 0,57$^{\alpha Acde}$	35,85 ± 0,17$^{\alpha Bcde}$	84,60 ± 0,30$^{\beta Acde}$	85,28 ± 0,11$^{\beta Bcde}$	52,75 ± 0,24$^{\beta Acde}$	80,94 ± 0,07$^{\beta Bcde}$
6,25	13,92 ± 0,40$^{\alpha Ade}$	15,18 ± 0,23$^{\alpha Bde}$	20,38 ± 0,47$^{\alpha Ade}$	28,38 ± 0,28$^{\alpha Bde}$	84,19 ± 0,17$^{\beta Ade}$	86,75 ± 0,23$^{\beta Bde}$	40,19 ± 0,07$^{\beta Ade}$	60,94 ± 0,11$^{\beta Bde}$
3,15	13,07 ± 0,68$^{\alpha Ae}$	14,98 ± 0,15 $^{\alpha Be}$	16,04 ± 0,43$^{\alpha Ae}$	24,57 ± 0,24$^{\alpha Be}$	83,92 ± 0,11$^{\beta Ae}$	85,02 ± 0,17$^{\beta Be}$	34,15 ± 0,13$^{\beta Ae}$	50,04 ± 0,26$^{\beta Be}$

[P]: concentration of each product (% v/v), F2: Yogurt + Soursop and blackberry pulps + Sweeteners, F3: Yogurt + Soursop and blackberry pulps + Sweeteners + pH regulator, SP: soursop pulp, BP: blackberry pulp. Different Greek letters (α y β) represent significant difference *p-values between drinks (F2 and F3) and blackberry and soursop pulps (PM and PG). Different capital letters (A and B) in the same row indicate significant difference p-values between times by each product. Different lowercase letters (a, b, c, d and e) in the same column indicate significant differences (p-values) between products concentration* 100; 75; 50; 25; 12,5; 6,25; 3,15 %). *Experiments were made in triplicate ± standard deviation.*

Studies by Hassimotto et al. reported a content of 120 ± 8 mg gallic acid equivalent (AGE) of total phenols per 100 grams of frozen soursop pulp [57]. Moreover, the authors Vijayameena et al. note that the acetogenins from Annonaceae family are also responsible for antioxidant power, related to the ability to inactivate reactive oxygen species such as singlet oxygen and peroxide radicals. They also attributed the high antioxidant properties of the soursop pulp to its high content of vitamin C [58]. The United States Department of Agriculture in 2010 described that soursop pulp provides a total of 20.60 mg / 100 g of vitamin C.

Berries are generally rich in organic acids such as citric acid, malic acid, tartaric acid, and fumaric acid, some vitamins (ascorbic acid and folic acid), and phytochemicals such as phenolic compounds. Phenolic compounds belong to a broad and heterogeneous group of chemical compounds that have one or more aromatic rings and one or more hydroxyl groups; they tend to donate an electron or a hydrogen atom to a free radical making it a harmless molecule. Phenolic compounds occur in free and conjugated forms with sugars, acids, and other water-soluble biomolecules (phenolic acids, flavonoids, and quinones) or insoluble like tannins. Blackberries antioxidant power is attributed to its content of phenolic compounds including flavonoids such as anthocyanins (cyanidin glycosides and glycosides pelargonidin), flavonoids (quercetin, myricetin and kaempiferol), and flavonoids (catechin and epicatechin). In turn, phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) and hydrolysable tannins are ellagitannins like. These components, either individually or in combination, are primarily responsible for the health benefits of this berry, in addition to its antioxidant properties [34]. Studies by Rodriguez et al. in 2010 reported polyphenol content between 95 and 135 mg equivalent gallic acid per 100 grams of blackberry (*Rubus glaucus* B.).

CONCLUSION

Blackberry pulp demonstrated the highest cytotoxic activity against tumor cells MCF-7, compared to soursop pulp and elaborated functional beverages. However, it was found that the cytotoxic activity of both formulations prepared (F2 and F3) as pulps were fairly good, being highly effective for the inactivation of breast tumor cells MCF-7 and prostate tumor cells PC3. Moreover, blackberry pulp and soursop pulp demonstrated a high antioxidant activity, with a synergistic effect accomplished for the mixture on F2 and F3. Therefore, the drinks have a favorable content on health claims. More advanced studies would help generate "*in situ"* fair evidence to confirm these promising benefits.

DISCUSSION

Exposing cells in a cell line to various combinations of fruit pulp and the formulated beverages are not equivalent to a clinical trial in which humans consume these beverages. Human digestion and absorption likely alter anti-cancer and antioxidant activity. While the findings in this area were interesting, they represent a very preliminary step in establishing the usefulness of these compounds in human health.

List of Abbreviations: HeLa, human cervix carcinoma cells; PC3, human prostate carcinoma cells; MCF-7, breast carcinoma without over-expression of the HER2/c-erb-2 gene; SKBr3, breast

carcinoma in which the HER2/c-erb-2 gene is over-expressed, MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide); DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl (DPPH); SP, soursop pulp; BP, blackberry pulp; F2, formulation two of the functional beverage (BP + SP + Yogurt + Truvía® + Sacarose); F3, formulation tree of the functional beverage (BP + SP + Yogurt + Truvía® + Sacarose + Sodium tripolyphosphate); pH regulator, Sodium tripolyphosphate; $IC_{50,}$ half maximal inhibitory concentration; SI, the selectivity index as a result of IC_{50} control cells/IC_{50}tumor cell line (derived from dividing the IC_{50} value in control cells between calculated for each tumor line with each product studied individually); cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; ATP, adenosin triphosphate; AGE, gallic acid equivalent.

Authors' Contributions: RR, AZ, FA, and FS participated in research design. AZ, RR, and FS participated in the writing of the paper. AZ, RR, FA, and FS participated in the performance of the research; AZ, RR, FS, and FA participated in data analysis.

Competing Interests: There are no conflicts of interest to declare.

Acknowledgements and funding: All the research was self-financed. Special thanks to Universidad Central de Venezuela for their support and invaluable help to Prof. Alexis Zambrano, Prof. Adelaida Durán, Dr. Rosa Raybaudi-Massilia, Ph.D. Francisco Arvelo, B. Sc. Felipe Sojo, MSc. Raúl Martínez Valdivieso, and B. Sc. Fabiana Barrios.

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