Bioactive Compounds and in Vitro Anti-Inflammatory Properties in a Fruit-Smoothie Processed by High Hydrostatic Pressure

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Abstract

A fruit-smoothie was analyzed in order to evaluate the effect of High Hydrostatic Pressure (HHP) on the release of Bioactive Compounds (BC) after different pressure and time treatments (500 and 600 MPa for 45 and 90 s), conditions commonly used in the food industry. Vitamin C, bioactive compounds (BC; phenolic compounds, carotenoids) and Dietary Fiber (DF) content, as well as, Antioxidant Capacity (AOX) and anti-inflammatory properties were analyzed in the samples. The vitamin C, BC content, and AOX was minor in the Non-HHP fruit-smoothie; while 500 MPa treatments showed greater increase of the content of BC and AOX. Regarding to anti-inflammatory effect, it was observed that HHP fruit-smoothies caused a modulating effect in various inflammation markers; at 500 MPa for 45 s generated the modulation in the expression of NF-κB, TNF-α, IL-8, COX-1 and COX-2 markers. Therefore, it was possible to obtain valuable information about the effect of HHP on fruit-smoothie BC and its potential anti-inflammatory effect.

Introduction

The consumption of beverages from fruits and vegetable milks has become popular among people looking for a healthy diet. Fruit smoothies are a popular and convenient way of consuming fruit, they contain a large amount of fruit rich in nutrients and Bioactive Compounds (BC) responsible for health effects [1,2]. The term “smoothie” is given to a blended fruit drink characterized by a pulpy consistency, containing one or more fruits, yogurt, cow’s milk or, vegetable milk [3-5]. Fruits such as Mango (Mangifera indica) and Jackfruit (Artocarpus heterophyllus) are tropical fruits, which have proven to be a good source of vitamin C and BC as Phenolic Compounds (PC), and carotenoids (CC) mainly [6-8]. Other variants such as the addition of vegetable milk facilitate the consumption of the lactose intolerant population [9], and the contribution of flavonoids such as γ-tocotrienol, and δ-tocopherol (homologue of vitamin E) that come from the grain of rice. The BC act as antioxidants and offer some protection against oxidative stress-related diseases and in the state of inflammation [10,11]. Concerning human health, chronic inflammation is considered to be a critical factor in many chronic degenerative human diseases [12]. Several authors have reported the ability to modulate the expression of the inflammatory markers and reducing the expression of interleukins by different BC of mango [10,13,14], and jackfruit [15-17, 11]. High Hydrostatic Pressure (HHP) is a non-thermal technology that has shown positive effects on nutritional compounds and sensory characteristics, in different fruit-products, specifically in beverages [18]. HHP allows the inactivation of microorganisms and enzymes [19-22], a range of 580 MPa is recommended by the FDA for commercially products (FDA, 2012). Furthermore, HHP opens a possibility for functional foods by improving the extraction and maintaining of BC [9, 21, 22]. In this sense, the aim of this study was to evaluate the effect of HHP on BC in a fruit-based drink (mango, jackfruit, and rice drink), its Antioxidant Capacity (AOX) and anti-inflammatory properties using an HT-29 cell model.

Materials and Methods

Chemicals

All solvents, standards, salts, and acids were purchased from Sigma Chemical Co. (St. Louis, MO), J. T. Baker (Mexico City) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human colon cancer cell line HT-29 was obtained from the American Collection of Type Cultures (ATCC HTB-38TM) and was provided by the Laboratory of Functional Foods and Nutraceuticals (Instituto Tecnológico de Durango, Mexico).

Fruit-smoothie preparation

Jackfruit and Mango (“Ataulfo”) were acquired from a local market located in Tepic Nayarit, Mexico, in consumption maturity and the commercial rice beverage (Nature’s heart terrafertil®), with a capacity to 135 L and 600 MPa. A 2x2 factorial design was used, where the factors were pressure (500 and 600 MPa) and time (45 and 90 s). A non-pressurized sample (Non-HHP) was included as fruit-smoothie control. After obtaining the smoothies with HHP treatment and control samples were freeze-dried (LABCONCO, Freezone, USA), ground at 6 °C until HHP treatments were applied.

High hydrostatic pressure processing

All the treatments were performed in an industrial HHP (Verfruco de Mexico S de RL de CV Company), located in Urupau, Michoacán, Mexico. The fruit smoothies were subjected to treatments using an HHP equipment (Hiperbaric Wave Model 6000/135 NC), with a capacity to 135 L and 600 MPa. A 2x2 factorial design was used, where the factors were pressure (500 and 600 MPa) and time (45 and 90 s). A non-pressurized sample (Non-HHP) was included as fruit-smoothie control. After obtaining the smoothies with HHP treatment and control samples were freeze-dried (LABCONCO, Freezone, USA), ground at 6 °C until HHP treatments were applied.
Bioactive compounds analysis

Preparation of organic extracts for evaluation of total soluble polyphenols content and antioxidative capacity: The organic extract was prepared starting from dried samples (0.5 g) with methanol-water (50:50 v/v, 50 mL/g of sample, 60 min) followed by an extraction with acetone-water (70:30 v/v, 50 mL/g of sample, 60 min). After each extraction step, samples were centrifuged (3000 g for 15 min at 25 °C), and extracts were collected.

Total soluble polyphenols content: Total Soluble Polyphenols (TSP) content was determined according to [23, 24]. Briefly, 250 μL of extract was mixed with 1000 μL of sodium carbonate solution (75 g/L), and 1250 μL of Folin-Ciocalteu reagent (100 mL/L) were combined in glass tubes and then mixed using a vortex. The solution was incubated at 50 °C in the dark for 15 min. Then, 270 μL of sample extracts or gallic acid were placed in the wells and the absorbance was measured at 750 nm using a multidetector microplate reader (Biotek, Synergy HT, Winooski VT, USA) with Gen5 software and the results were expressed as grams of gallic acid equivalents (g GAE/100 g of sample) using a gallic acid standard curve (0.0.2 mg/mL).

Total carotenoids content determination: Total Carotenoids (TC) were determined by spectrophotometric method according to the procedure of [25]. It was estimated in acetone-petroleum ether extracts. 0.5 g of lyophilized sample was used to make the extraction. The absorbance at 448 nm of the resultant sample was measured in spectrophotometer single cell holder (Jenway® model 6705, UK). β-Carotene was used as standard and results were expressed as mg of β-carotene equivalents per 100 g of sample (mg β-carotene/100 g of sample).

Total dietary fiber analysis: The beverages were analyzed in the Total Dietary Fiber (TDF) using the AOAC enzymatic-gravimetric method (method 991.42) modified [26], all data were expressed as g TDF/100 g of sample.

Antioxidant capacity (AOX)

The AOX was evaluated in the organic extract by three methods: A) 2, 2’-Azinobis (3-Ethylbenzothiazoline-6-Sulfonic (ABTS)) [27], B) 2, 2-Diphenyl-1-Pycrylhydrazyl (DPPH) [28], and C) Ferric Reducing Antioxidative Power Assay (FRAP). All data were expressed as Trolox Equivalents (mmol TE/100 g of sample). The absorbance was measured in a microplate reader (Biotek, Synergy HT®, Winooski VT, and EE.UU.) with the Gen5 software (Biotek®, Winooski, Vermont, USA).

Anti-inflammatory assay

The anti-inflammatory effect of the HHP fruit-smoothies was evaluated, using the techniques implemented by [29].

Cell culture: Human colon cancer HT-29 (colorectal adenocarcinoma) cell suspension of 1.2 x 106 was grown at preconfluence into 100 mm plates with the medium RPMI (Roswell Park Memorial Institute) medium supplemented with 10 % Fetal Bovine Serum (FBS), penicillin (50 U/mL) and streptomycin (50 μg/mL) for 72 h (5 % CO2 at 37 °C) in a humidified atmosphere. Cells at 70 % of confluence were used for assays, which were carried out between passages number 10-15.

Cell viability: Cell viability was assayed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) method. The cells were seeded into 96 well plate at a density of 9.6 x 103 cells/well. After the experimental treatments, cells were thoroughly washed with PBS (Phosphate-Buffered Saline) three times to avoid any interference of the oak with the MTT. Then, 0.1 mL of MTT reagent was added to the plate and was incubated for 4 h (5 % CO2 atmosphere). The MTT reduced by the viable cells to formazan product was dissolved in 0.2 mL of DMSO (Dimethyl sulfoxide solution) and absorbance was measured at a test (570 nm) and refer a wavelength (600 nm) using a microplate ELISA reader (ELX800®, Biotek® Instruments, Inc. Winooski, Vermont, USA). The percentage of mitochondrial enzyme activity was determined by the ratio of the mean absorbance for each treatment and control obtained.

Experimental treatments: Several concentrations (0.125, 0.5, 0.75 and 1.5 mg/mL) from the lyophilized samples of HHP and Non-HHP fruits smoothies were prepared in FSB-free DMEM (DuBecco’s Modified Eagle Medium). Subsequently, the treatments were applied and allowed to incubate for 24 h in a 5 % CO2 atmosphere. After the incubation period, the effect of pH and precipitation of the treatments on the possible cytotoxic effects generated by the changes in the physicochemical factors in the cellular microenvironment was evaluated. Only those concentrations that did not change the culture media conditions were selected for further experimentation. The final dose established for the experiments was that which did not induce changes in the physicochemical parameters of the culture and which did not show cytotoxicity (0.125 mg/mL).

Western analysis: For the immunoblot analysis, pre-confluent cultures were washed with PBS IX and treated with medium alone (RPMI) and HHP and Non-HHP (0.125 mg/mL) fruits smoothies in DMEM as vehicle for 3 h at 37 °C. Cells were removed by a mixture of trypsin and verseno dissolved in phosphate buffer (pH 7.8), and then centrifuged at 3000 rpm for 5 min. The pellet was re-suspended in a phosphate buffer (pH 7.8) added with protease inhibitor (Bio-Rad), and then the cells were lysed on ice for a maximum speed using an Ultra-turrax cell (IKA T 10 basic). The suspension was centrifuged at 12,000 rpm, the supernatant was recovered to determine protein concentration and immunoblot analysis.

Protein concentration was determined using the Bio-Rad Bradford dye binding protein assay kit according to the manufacturer’s instructions. Fractions of cellular proteins were subjected to SDS-PAGE and elektrotransferred into nitrocellulose membrane for 1 h at 100 V. Membranes were immersed for three hours at 4 °C in 10 mL blocking solution which contained 5% (v/v) non-fat milk in PBS-T (20mM Tris/HCl-buffered saline/100 mM NaCl, 0.2%, 0.1% Tween 20) at pH 7.6. Membranes were washed three times with 10 mL PBS-T (20 mM Tris/HCl, 100 mM NaCl, 0.2% (v/v) Tween-20, pH 7.6), followed by incubation with the first antibody with an appropriate dilution in prepared 10 mL of PBS-T: for COX-1 and COX-2 (1:1000), for total NF-κB p65 (C-20) and IL-1 (1:200) and for TNF-α (1:800) for 2 h. After washing for three times of 10 mL PBS-T, membranes were incubated for 3 h with a secondary antibody, mouse anti-rabbit IgG or mouse anti-goat IgG, conjugated with alkaline phosphatase diluted 1:2000 for 1 h. Then, it was revealed using a chemiluminescence kit (ECL, GE Healthcare, USA). The bands were visualized using Hyperfilm ECL (Amersham Phamacia Biotech, USA). Densitometry was performed using ImageJ software (National Institutes of Health, USA). Results were expressed as g TDF/100 g of sample.

Statistical analysis

Experimental values are given as mean ± SD. Statistical significance was determined by one-way ANOVA (P < 0.05) and an analysis of Fisher’s LSD means to determine significant differences between all the HHP treatments and Non-pressurized sample. An analysis of correlation analysis (p<0.05) and the principal components analysis (PCA) was carried out (P<0.05) to correlate all the response variables analyzed for the different treatments applied. Statistical analyses were performed using Statistica 8.0 (StatSoft, Inc., Tulsa, OK).

Results and Discussion

Effect of HHP on vitamin C and BC content of fruit-smoothies

(Table 1) shows vitamin C and BC (TSP, TC and TDF) content in Non-HHP fruit-smoothie processed by High Hydrostatic Pressure. World J Food Nutr 2: 1012.
g). HHP treatments at different pressure levels and holding times did not cause an increase in the carotenoid extraction, but maintained same concentration as the Non-HHP smoothie. The vitamin C content was ranged from 14.35 to 17.73 mg AA/100 g of sample, being the highest content at 500 MPa for 45 s with significant difference (P<0.05) between samples.

Table 1: Effect of High Hydrostatic Pressure (HHP) on Total Soluble Polyphenols (TSP), vitamin C, Total Carotenoids (TC) and Total Dietary Fiber (TDF) content of a fruit-smoothie.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TSP (g GAE/100 g FW)</th>
<th>TC (mg F β-carotene/100 g FW)</th>
<th>Vitamin C (mg EAA/100 g FW)</th>
<th>TDF (g/100 g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-HHP</td>
<td>0.164±0.009 c</td>
<td>1.086±0.014 a</td>
<td>14.35±0.289 d</td>
<td>2.049±0.040 a</td>
</tr>
<tr>
<td>500 MPa-45 s</td>
<td>0.182±0.005 a</td>
<td>1.049±0.028 a</td>
<td>17.73±0.206 a</td>
<td>2.057±0.024 a</td>
</tr>
<tr>
<td>500 MPa-90 s</td>
<td>0.178±0.005 b</td>
<td>1.091±0.033 a</td>
<td>16.97±0.179 b</td>
<td>2.065±0.021 a</td>
</tr>
<tr>
<td>600 MPa-45 s</td>
<td>0.175±0.007 b</td>
<td>1.029±0.025 a</td>
<td>15.42±0.132 c</td>
<td>2.050±0.081 a</td>
</tr>
<tr>
<td>600 MPa-90 s</td>
<td>0.169±0.010 b</td>
<td>1.022±0.017 a</td>
<td>15.10±0.252 c</td>
<td>2.058±0.036 a</td>
</tr>
</tbody>
</table>

Values means ± standard deviations of triplicate measurement. For different treatments, means in each column with different letters were significantly different (P < 0.05). FW-fresh weight.

Different assays show different results and particular mechanisms of action. To elucidate the AOX of smoothie samples, DPPH, ABTS, and FRAP assays were used. Non-significant differences were observed between Non-HHP and 600 MPa samples, to 45 s and 90 s, on the AOX by DPPH. However, HHP samples at 500 MPa showed a high AOX, being the highest AOX at 500 for 45 s (0.11±0.005 mmol TE/100 g of sample). The AOX measured by ABTS detected significant differences between Non-HHP and HHP samples, where the lowest AOX was observed in the Non-HHP sample (0.51±0.01 mmol TE/100 g of sample) and the highest was observed in 500 MPa for 45 s sample (0.65±0.007 mmol TE/100 g of sample). Contrary to DPPH and ABTS, AOX by FRAP showed non-significant differences (P>0.05) between Non-HHP and HHP fruits smoothies samples. This can be explained, because antioxidants may respond differently to different radical or oxidant source. Furthermore, an individual antioxidant may, in some cases, act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system [34,28].

Since fruits are rich in TC, they have high antioxidant activity and are a good source of nutrients, therefore, the use of HHP in fruits and beverages reduces the destruction of antioxidant components and favors their AOX [8]. In soy smoothies a slight increase of AOX by FRAP was observed of 2.9% at 550 MPa and 6.6% at 650 MPa and a slight increase by DPPH of 3.8% at 550 MPa, and 3.4% at 650 MPa [9, 4]. Reported higher antioxidant activities at lower operating pressures compared to higher pressures in fruit smoothies (450 vs. 650 MPa). HHP at 500 MPa for 10 min led to better retention of antioxidant activity with the ORAC value of 481.68 mmol TE/mL in mulberry juice [28]. The variability of the effects of HHP depends not only to the treatment conditions (pressure, temperature, time) but also the type of food (whole fruit, juice or puree) and food matrix (hydrophilic compounds, lipophilic, hydrophilic, etc.) [3,35,20]. Others, on the other hand, AOX by ABTS and DPPH methods was strongly correlated (P<0.005) with the content of vitamin C of HHP fruits smoothies. Vitamin C and TSP are correlated with major bioactive compounds that contribute to radical scavenging activity [9]. The fruit-smoothie that showed a higher AOX also contained a higher content of vitamin C and TSP, so that its AOX could be related by a possible synergistic effect of vitamin C with phytochemicals as polyphenols and carotenoids, these last one also contained in the fruit-smoothies.

Accumulation level of pro-inflammatory and anti-inflammatory proteins in vitro

HHP fruits-smoothies modulated the levels of pro-inflammatory and anti-inflammatory proteins in HT-29 cells (Figure 2).

HHP fruits smoothies differentially modulated the levels of pro-inflammatory proteins in HT-29 cells. All HHP fruits-smoothies decreased COX-1, COX-2, TNF-α, NF-κB and IL-8, so that its AOX could be related by a possible synergistic effect of vitamin C with phytochemicals as polyphenols and carotenoids, these last one also contained in the fruit-smoothies.

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It is observed that there are two behavior patterns, the first (Factor 1) that explains the 65.15 % of the variability describes that as the AOX (ARTS, FRAP, and DPPH), the content of TSP and TNF-α marker decrease. Likewise, a decrease is observed in the biological markers IL-8, COX-2, COX-1, and NF-κB. Factor 2 with 24.43 % of the variance describes an increase in the content of carotenoids and fiber, also reveals low variations in AOX, TSP, vitamin C, and in the TNF-α marker. This behavior indicates that IL-8, NF-κB, COX-1, and COX-2 markers decrease could be associated with carotenoids and fiber content increment. HHP treatment at 600 MPa for 90 s represents a greater correlation with the decrease of the NF-κB, COX-1 and COX-2 markers, while at 600 MPa for 45 s treatment follows the trend according to (Factor 2), which appreciates a low variation concerning the AOX and content of TSP. The HHP treatment at 500 MPa for 90 s does not follow any of the trends indicated in the response variables, while treatment at 500 MPa for 45 s maintains a positive correlation with the (Factor 2), thus presenting higher concentrations of carotenoids and fiber. These values demonstrate that 500 MPa for 45 s, 600 MPa for 45 s, and 600 MPa for 90 s treatments are the most suitable for decreasing the concentrations of inflammation markers. About the results obtained, HHP extract of ginseng reduced mRNA levels of pro-inflammatory genes such as TNF-α and IL-6 in rats fed a high-fat diet, and it was attributed to the increase of total phenolic content polyphenols by HHP [2, 1]. Determined that the consumption of HHP extract of red ginseng in rats induced obesity generates a regulation of pro-inflammatory genes (TNF-α and IL-6).

The anti-inflammatory effect obtained is attributed to the BC (carotenoids, vitamin C, and phenolic compounds) present in the HHP fruit-smoothies since BC can act directly in the inflammatory signaling cascade, so that prevent the activation of different transcription factors as the factor nuclear kB (NF-kB), which triggers signaling cascades that releases cytokines and inflammation [36]. Several authors have reported that these compounds are capable to inhibit the expression of these markers [37,13] and numerous studies in different cell lines or animal models suggest a protective role of the synergetic effect of the bioactive compounds resulting beneficial biological functions, such as the inhibition of proliferation of human cancer cells is related to the anti-inflammatory effect [38, 39]. In this connection a correlation analysis (p <0.05) between the concentration of BC, AOX with the anti-inflammatory effect indicated that carotenoids and vitamin C influence repressed the expression of NF-κB and COX-2, as well as, FRAP and DPPH antioxidant activity influenced significantly (p <0.05) in suppressing the expression of IL-8, COX-2, and NF-κB. Regarding the TSP content in fruit smoothies and its relationship to the anti-inflammatory activity, HHP smoothies can have a significant impact on the inflammatory state and modulation of the expression gene of inflammatory markers TNF-α, TLR4, IL-6 [40].

The anti-inflammatory properties of vitamin C can be attributed to its ability to modulate the expression of the marker NF-kb and reducing the expression of interleukins [41]. The biological activity of mango can be attributed mainly to gallic acid, which is the major polyphenolic compound and which has been shown to reduce the expression of the biological markers responsible for inflammation [37]. On the other hand, some authors report that jackfruit carotenoids can be important for the prevention of several chronic degenerative diseases, such as cancer and inflammation [42]. Studies have also explored the anti-inflammatory role of jackfruit, phytochemical investigations of ethyl acetate extracts of jackfruit led to the isolation of the phenolic compounds artocarpesin [5,7,2',4'-tetrahydroxy-6-(3-methylbut-3-enyl) flavone] [43-46], which was further shown to possess potent anti-inflammatory effects in RAW264 [6]. Also, three phenolic compounds were characterized of jackfruit as artocarpesin [5,7,2',4'-tetrahydroxy-6-(3-methylbut-3-enyl) flavone], nororoticarpin [5,7,2',4'-tetrahydroxysteviol], and oroticarpin [trans-2,4,5'-tetrahydroxysteviolene] exhibited potent anti-inflammatory activity [7]. On the other hand, the rice drink could also be contributing to the polyphenol content and flavonoids such as ferulic acid, γ-tocotrienol and δ-tocopherol (homolog of vitamin E) [47-49].

Conclusions

HHP processing is an efficient treatment for the preservation of the BC present in a fruit-smoothie. Only a small number of studies have assessed the effect of HHP on mixtures of fruits, such as fruit smoothies, its biological effect on health, and the anti-inflammatory activity. This study demonstrated that high pressure conserves and/or increases BC, enhancing as consequence the AOX in fruit-smoothie beverages. The HHP fruits smoothie treatments were very effective in reducing oxidative stress with decreased COX-2, NF-κB, TNF-α, and IL-8 expression. Therefore, the high pressures are a promising technology for the conservation of nutritional properties in different food-matrix and open possibilities to functional foods, which when consumed may be generating beneficial effects for health.

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Conflict of Interest

The authors declare that they do not have any conflict of interest.

References


