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Original article

In natura tropical juices inhibit the *in vitro* carbonylation of bovine serum albumin

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Summary

Introduction – **Oxidative stress has been related with some pathologies, thanks to cellular homeostatic imbalance promoted by this condition. Therefore, current research has focused on finding tools for its prevention, like fruits consumption, thanks to their bioactive compounds. Here was evaluated antioxidant capacity of *in natura* tropical juices, by *in vitro* inhibition assay of bovine serum albumin carbonylation.** *Materials and methods* – **pH, soluble solids content (TSSC), titratable acidity and total flavonoids content (TFC) were determinated in four natural tropical juices from *Tamarindus indica, Passiflora edulis, Averrhoa carambola, Ananas comosus*, and *Carica papaya*. *In vitro* antioxidant capacity was evaluated through carbonyl index measurement from iron-oxidized BSA in presence of the juices by Dot-blot immunoassay.** *Results and discussion* – **Juices had an acid pH in a range of 3.0 and 5.5, while TSSC were found between 4.4 and 12°Brix. *T. indica* and *P. edulis* juices showed the greatest TSSC values with 12.0 ± 1.49 and 11.9 ± 1.11°Brix, respectively. Regarding TFCs, these were greater in juices with lower pH values. All juices showed an increasing antioxidant activity dependent of amount used, been greater in *P. edulis* and *T. indica*; this was attributed to a synergic effect of variables assayed (pH, TSSC and TFC).** *Conclusion* – **Natural juices tested have antioxidant properties by preventing iron-induced protein carbonylation and therefore could be used to attenuate the metabolic consequences of high oxidative stress.**

Keywords
antioxidants, flavonoids, fruits and protein carbonylation

Significance of this study

What is already known on this subject?

- Fruits have antioxidant properties due their content of secondary metabolites like flavonoids, thus are employed for diseases treatment involved in oxidative stress.

What are the new findings?

- Provide evidence of the protective antioxidant effects of natural juices on macromolecules such as proteins, adding scientific supports to its health effects.

What is the expected impact on horticulture?

- Promote the consumption of tropical fruits by supplying to horticulturists and consumers with scientific data on protective antioxidant effects of fruit juices.

Introduction

Oxidative stress (OS) is a complex biological condition, in which balance between production and elimination capacity of reactive oxygen species (ROS) in biological systems is altered. ROS formation occurs continuously in cells as a consequence of enzymatic reactions, including those involved in respiratory chain at the mitochondrial level (Hayashi and Cortopassi, 2015), inflammatory responses associated with macrophage-mediated microbial phagocytosis processes, and in prostaglandins synthesis, metabolism and biotransformation of drugs and xenobiotics through cytochrome P-450 complex enzymes (Veith and Moorthy, 2018). ROS can also be formed in non-enzymatic reactions between oxygen and organic compounds, and through exogenous sources such as exposure to X-rays, ozone, cigarette consumption, air pollutants, and industrial chemicals (Archibong *et al.*, 2018).

Currently OS is a worrying topic in biomedical research, given its relationship with etiology of a number of pathologies, including cancer, thanks to cellular homeostatic imbalance promoted by ROS in the main biomolecules (DNA, lipids and proteins) (Archibong *et al.*, 2018). ROS alters cell membranes stability, through reactive aldehydes formation, such as 4-hydroxynonenal (4-HNE), malondialdehyde (MDA) and acrolein (ACL), by a process known as lipid peroxidation. These aldehydes (4-HNE, MDA and ACL) also react with other biological molecules such as proteins and DNA, altering their structure irreversibly (Gasparovic *et al.*, 2018).

Proteins are responsible for most of the functional processes in cells, and also are direct target of ROS attack. Protein carbonylation is an irreversible and non-enzymatic post-translational modification, often used as a marker of oxidative stress (Weng *et al.*, 2017). Protein oxidative damage can be measured through the quantitation of carbonyl groups formation in side chains of their amino acid residues by spectrophotometric and immuno-enzymatic methodolo-

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gies. Within ROS that lead to protein oxidation are species like superoxide ($O_2^{\cdot-}$), hydroxyl (OH·), peroxyl ($RO_2^{\cdot}$), alkoxyl (RO·), hydroperoxyl ($HO_2^{\cdot}$), and species non-radicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$) (Hayashi and Cortopassi, 2015).

Carbonylated proteins (CPs) suffer alterations in their folding and favoring its backbone fragmentation, which results in loss of function and disruption of a variety of biochemical processes (Dalle-Donne *et al*., 2006). In fact, CPs have been related to primary or secondary pathophysiological mechanisms in age-related human diseases etiology, like atherosclerosis, ophthalmological and neurodegenerative diseases (Alzheimer, Parkinson's, Huntington's, tardive dyskinesia and epilepsy) (De Araújo *et al*., 2016).

In particular, a strong association between CPs and neurodegenerative diseases etiology like Alzheimer's disease (AD) have been established. According to Tramutola *et al*. (2017), brain of patients with AD is characterized by containing an elevate amount of CPs that include enzymes involved in the synthesis of ATP (pyruvate kinase, phosphoglucomutase-1, Eno1, triosa-phosphate isomerase, fructose bisphosphate aldolase A/C, ATP synthase, glyceraldehyde 3-phosphate dehydrogenase and malate dehydrogenase), whose inactivation besides is product of their oxidation. This situation could be a crucial event in ATP-dependent processes deterioration, commonly present in neurodegenerative diseases (Tramutola *et al*., 2017). On the other hand, high levels of amyloid peptide Aβ, a constituent of brain amyloid plaques, have also been associated with higher levels of protein, lipid and nucleic acid oxidation products in the hippocampus and cortex of patients with AD (Cheignon *et al*., 2018).

In vitro observations on cell cultures indicate that CPs are part to cancer factor risks due to numerous mechanisms independent to genotoxicity processes (Klaunig and Wang, 2018), and may play too an important role in diabetes, cardiovascular diseases and hypertension pathophysiology (Abbasian *et al*., 2018). Protein carbonylation is also associated with pathogenesis of a wide range of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, and systemic sclerosis, because these CPs are easier recognized by autoantibodies present in sera from patients with rheumatoid arthritis (Smallwood *et al*., 2018).

Due to the role of OS and protein carbonylation in prepathogenesis of chronic diseases, the scientific and medical communities have focused their efforts on search for tools for their prevention or attenuation, among which is the consumption of vegetables and fruits, thanks to their high content of bioactive compounds that include vitamins, polyphenols, carotenoids and complex carbohydrates, which together can act as antioxidant agents that inhibit ROS damage (Quan *et al*., 2018).

Based in the above described, the aim of this work was to evaluate the antioxidant capabilities of tropical *in natura* juices, by inhibiting the *in vitro* carbonylation of bovine serum albumin (BSA).

Methodology

Preparation of fruits juices

Fresh and ripe fruits, tamarind (*Tamarindus indica*), passion fruit (*Passiflora edulis*), papaya (*Carica papaya*), pineapple (*Ananas comosus*), and carambolo (*Averrhoa carambola*), were obtained from a local market (Cartagena, Colombia). Three units for each fruit were obtained as experimental elements; these were washed with deionized water, and from each of them a portion of 50 g of pulp was taken, which were individually processed with deionized water in a 1:1 ratio, using a commercial blender (Oster® BVLB07-ES 4655). Next, the fruit juices were centrifuged (3,000 rpm at 12 °C for 25 min), and the *in natura* juices obtained (supernatant) were storage at -20 °C until their use in antioxidant activity assays.

Physico-chemical parameters

Total soluble solids content (TSSC) in fruit juices was determined using a digital refractometer (ATAGO®, Digital Abbe Refractometer DR-A1) at 20 °C, and results were expressed as °Brix at 20 °C. Titratable acidity (TA) was calculated based on AOAC guidelines, for this, 5 mL of the *in natura* juices were taken, which were adjusted to a final volume of 50 mL with Milli Q water, and titrated with NaOH 0.1 N until reaching a pH of 8.1 ± 0.2 (Al-mentafji, 2016). Triplicates were prepared for the analysis, and the results were expressed as % ascorbic acid. pH was determined with a digital potentiometer (SI Analytics lab 860) at 25 °C. Finally, the maturity status (MS) of each fruit species evaluated was calculated through the relationship between TSSC and TA.

Quantification of totals flavonoids content

Total flavonoids contents *in natura* juices was measured thought an aluminium chloride ($AlCl_3$) colorimetric method described by Cimpoiu *et al.* (2011) and Said *et al.* (2018), with some modifications. Thus, 20 µL of $AlCl_3$ (5.0% w/v in ethanol 96% w/v), 20 µL of 1M sodium acetate and 950 µL of methanol (95% v/v) were added to 10 µL of each of the *in natura* juices. After incubation for 30 min at room temperature and dark, absorbance of the mixture was measured at 425 nm. Total flavonoids quantification was performed by the external standard method, using quercetin of known concentration, in a linear dynamic range between 100 and 800 mg L^{-1}. Five calibration curves of quercetin were constructed in two days of assaying, and their linearity, repeatability and reproducibility were evaluated by the correlation coefficient linear and the relative standard deviation of the slopes obtained (Cimpoiu *et al*., 2011; Said *et al*., 2018).

Inhibitory activity of protein carbonylation

Antioxidant activity of *in natura* juices was determined by the ability to inhibit the carbonylation of bovine serum albumin (BSA), promoted by a free radical generating system (10 mM $FeSO_4$ in 137 mM PBS). Thus, the oxidation generated on BSA was expressed as the carbonyl index (CI) in nmols of carbonyls mg^{-1} of proteins, which were calculated through the data obtained by combination of a spectrophotometric method with a Dot-blot immunoassay.

Antioxidant activity assay consisted of exposing 200 µL of BSA (1 mg mL^{-1}) to oxidant $FeSO_4$ in presence of increasing volumes of *in natura* fruits juice (5 µL, 10 µL, and 20 µL), during 2 h at 37 °C. As negative controls, BSA samples with 10 mM $FeSO_4$ in 137 mM PBS without *in natura* fruits juices were used, while as positive control, BSA samples with $FeSO_4$ but in presence of 8.2 mM acid ascorbic was employed. Likewise was used juice fruits in 137 mM PBS as reactive blank, for assessing their background on PVDF membranes. Three replicates were analyzed for BSA treatments with *in natura* juices, and two for the controls.

After incubation period, the level of carbonylation reached by BSA in each condition was determined with the help of an oxidized protein calibration curve. For curve, ini-

tially CI of a BSA stock solution (5 mg mL^{-1}) was determined through the alkaline method reported by Mesquita *et al.* (2014). Its value was considered as the basal CI in our conditions (non-oxidized BSA). Next, an aliquot of BSA standard solution was oxidized by incubation at 37 °C during 2 h with 10 mM $FeSO_4$ in 137 mM PBS. Oxidized BSA was precipitated with trichloroacetic acid, cleaned with acetone, resuspended in PBS and quantified by Bradford method (Bradford *et al.*, 1976). Next, stoichiometric combinations of oxidized BSA with non-oxidized BSA solutions were performed, to obtain working standard solutions with different CIs, which were used for the construction of calibration curves. Individual CIs for working standard solutions were determined by the alkaline method. A graphic representation of absorbance versus CI was built using Microsoft Excel v. 2019.

For Dot Blot assay, the methodology reported by Contreras-Puentes *et al.* (2019) was used. For this, samples (treatments and controls) and working standard solutions (oxidized BSA curve) were derivatized with DNPH probe (Contreras-Puentes *et al.*, 2019). Briefly, SDS was added to final concentration of 4% and heated for 2 min at 100 °C. Then, an aliquot 10 mM DNPH in HCl 2N was added to labelling carbonylated proteins for 10 min. After that, the reaction was stopped by addition of 85% glycerol/Tris-HCl 2M and 15% β-mercapto-ethanol freshly solution. 100 ng of treatments, controls and derivatized BSA oxidized standards were immediately spotted by quadruplicate onto PVDF membrane (Immun-Blot® PVDF Membranes for Protein Blotting, Cat. #10600023). PVDF membranes were blocked for 2 h at room temperature with 5% non-fat dry milk in phosphate-buffered saline (PBS). Later, blocked membranes were incubated with rabbit polyclonal anti-DNP antibody (Sigma Aldrich®) at 1:10,000 in PBS-milk 5%, for 2 h at room temperature with slow rocking, followed by incubation with peroxidase-linked anti-rabbit IgG antibody at 1:10,000 for 1 h at room temperature (Thermo Fisher Scientific). Chemiluminescence signals were developed using HRP Chemiluminescent Substrate IgG detection kit (Thermo Fisher Scientific) and captured in a ChemiDoc MP System (Bio-Rad) (Contreras-Puentes *et al.*, 2019). The intensity of each analyzed spot was obtained by optical densitometry using Image Lab 6.0.1 software (Bio-Rad). In order to maintain equal conditions during quantitative analysis, an area of 18 mm^2 was established for each protein spot and measured intensities were tabulated in a matrix table in Microsoft Excel 2019.

In vitro anti-oxidant effect of *in natura* juices was expressed as the carbonylation percentage inhibition of the BSA (CPI), which was calculated using Equation 1:

$$CPI(\%): \frac{CIs\ from\ negative\ control - CIs\ from\ treated\ samples}{CIs\ from\ negative\ control.} \times 100$$

Statistical analysis

Data obtained were expressed as the mean ± standard deviation, these were tabulated in a matrix table in a Microsoft Excel spreadsheet v. 2019. To establish the difference between *in natura* fruit juices and study parameters, an ANOVA analysis of variance was performed in GraphPad PRISM 8.01, followed by the Tukey test (5% significance). Likewise, a Pearson linear correlation analysis was performed between inhibition of *in vitro* carbonylation of BSA induced by fruit juices *vs.* variables such as pH, total soluble solids and total flavonoid content. In order to evaluate the joint correlation level of the independent variables *vs.* CPI, it was performed a general linear model, using the Statgraphics Centurion software 18.

Results and discussion

Physico-chemical parameters

In order to determine organoleptic and physiological quality attributes in fruits, physicochemical parameters (pH, TA, TSSC and MS) were evaluated. Results of these parameters for each fruit juice are summarized in the Supplemental Information, Table S1 and Figure 1. All *in natura* juices had an acid pH, in a range from 3.0 to 5.5. Significant differences were observed in pH values of *in natura* juices from each fruit assayed ($p < 0.05$). *T. indica* and *P. edulis* juices presented the lowest pH (3.1 ± 0.04 and 3.3 ± 0.10, respectively), while *C. papaya* and *A. carambola* were greater (5.4 ± 0.17 and 4.3 ± 0.17, respectively). This was consistent with their titratable acid values (1.9 ± 0.45, 4.5 ± 0.50 and 0.11 ± 0.03, 0.17 ± 0.05% ascorbic acid, respectively). A linear correlation analysis performed between the values of these two variables from *in natura* juices, showed an inverse and significant relationship, as was expected ($r = -0.6662$, $P = 0.0025$).

In the case of TSSC, we found a range between 4.4 and 12 °Brix in *natura* fruit juices. Greater values were observed in *T. indica* and *P. edulis* juices (12.0 ± 1.49 and 11.9 ± 1.11 °Brix, respectively) and the lowest in *A. carambola* and *C. papaya* (5.2 ± 0.59 and 5.3 ± 0.008 °Brix, respectively). Pearson correlation analysis performed between TSSC and the juices' pH showed an inverse and significant relationship ($r = -0.7279$, $P = 0.0006$), because many of the TSSC present in fruits correspond to organic acids and carbohydrates as fructose and sucrose. Regarding the MS, values obtained for the different *in natura* juices were very variable, even among biological replicas of the same fruit; this behavior is due to the effect exerted by environmental origin factors, such as temperature and relative humidity, as well as the harvesting time and fruit handling (Arrazola, 2015).

Total flavonoid content

Total flavonoid content (TFC) was determined in order to establish a relationship between the antioxidant activity of *in natura* juices and the concentration of these bioactive compounds; because many researchers have reported a positive correlation between the total content of these secondary metabolites, and the antioxidant properties shown by different extracts of plants and fruits in biological models (Said *et al.*, 2018; Wei *et al.*, 2019).

Quercetin curves showed good linearity in the stipulated concentrations range (100 to 800 mg L^{-1}) ($R^2 > 0.99$), with RSDs <21.7%. When comparing the slope from curved constructed in two days of assay, no statistical differences were found ($p = 0.7888$). Details of these evaluated parameters are summarized in Supplemental Information (Tables S2, S3; Figures S1 and S2).

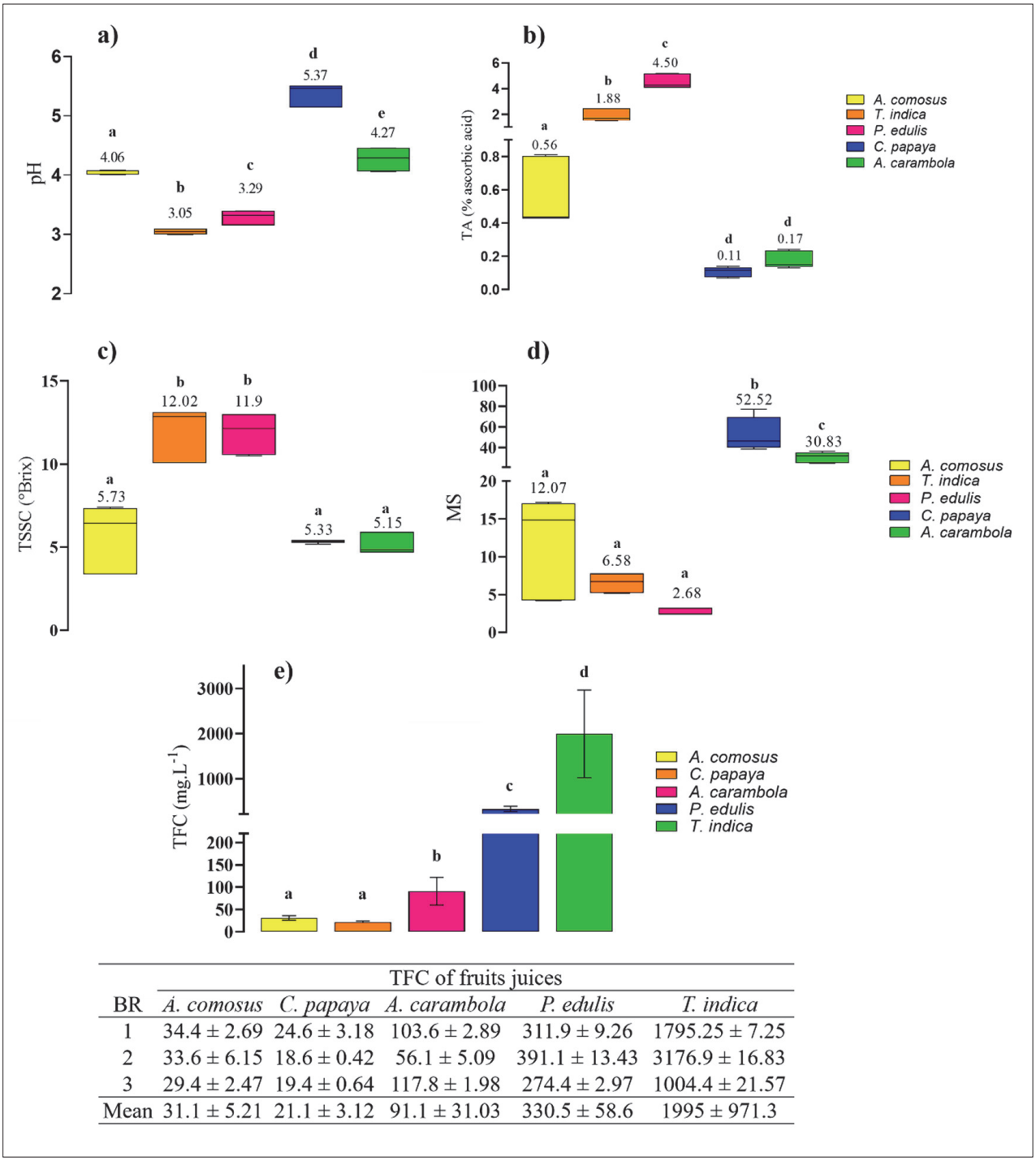
Experimental results showed that the TFC was greater in juices with a greater acid character, such as *T. indica* and *P. edulis* (1995.0 ± 971.3 and 330.5 ± 58.6 mg L^{-1}, respectively), while the *in natura* juices of *C. papaya* and *A. comosus* were those that presented lower TFC (21.1 ± 3.12 and 31.1 ± 5.21 mg L^{-1}, respectively). TFC data from *in natura* juices are shown in Figure 1e. A linear correlation analysis performed between TFC and pH from *in natura* juices, showed an inverse and significant relationship, as was expected ($r = -0.5938$, $P < 0.0001$).

Flavonoids are a group of secondary metabolites that play an important biological role in plants, accumulating mainly in flowers and fruits, due their importance in processes of pollination and protection of biotic and abiotic fac-

tors; these also act as UV filters, as molecules signaling, antimicrobial and temperature modulators (acclimatizers) in cold and warm weather (Hernández-Rodríguez *et al*., 2019). Therefore, biosynthesis of flavonoids in plants will depend to a great extent on external environmental factors and the intrinsic characteristics of each organism, being different even among individuals of the same species, which explains the variability of TFC found in the *in natura* juices assayed.

Inhibitory protein carbonylation activity of *in natura* juices

Antioxidant capability of the *in natura* juices was determined through the inhibition of BSA carbonylation in a pro-oxidant medium (10 mM $FeSO_4$ in 137 mM PBS). Initially, an oxidized BSA curve was built; since reproducibility of the BSA oxidation model with ferrous sulfate was demonstrated in previously published works (Contreras-Puentes *et al*., 2019; Rodríguez-Cavallo *et al*., 2018), it was not necessary to



	TFC of fruits juices				
BR	*A. comosus*	*C. papaya*	*A. carambola*	*P. edulis*	*T. indica*
1	34.4 ± 2.69	24.6 ± 3.18	103.6 ± 2.89	311.9 ± 9.26	1795.25 ± 7.25
2	33.6 ± 6.15	18.6 ± 0.42	56.1 ± 5.09	391.1 ± 13.43	3176.9 ± 16.83
3	29.4 ± 2.47	19.4 ± 0.64	117.8 ± 1.98	274.4 ± 2.97	1004.4 ± 21.57
Mean	31.1 ± 5.21	21.1 ± 3.12	91.1 ± 31.03	330.5 ± 58.6	1995 ± 971.3

Figure 1. Behavior of physicochemical parameters and total flavonoids contents of the *in natura* fruit juices assayed. Different letters on the bars indicate significant statistical differences ($p < 0.05$). All values are reported as mean ± standard deviation ($n = 3$). In the lower part of panel e), TFC quantified in the biological replicas of each fruit are detailed. TA: titratable acidity, TSSC: total soluble solids content, MS: maturity status, TFC: total flavonoids contents, BR: biologic replicas.

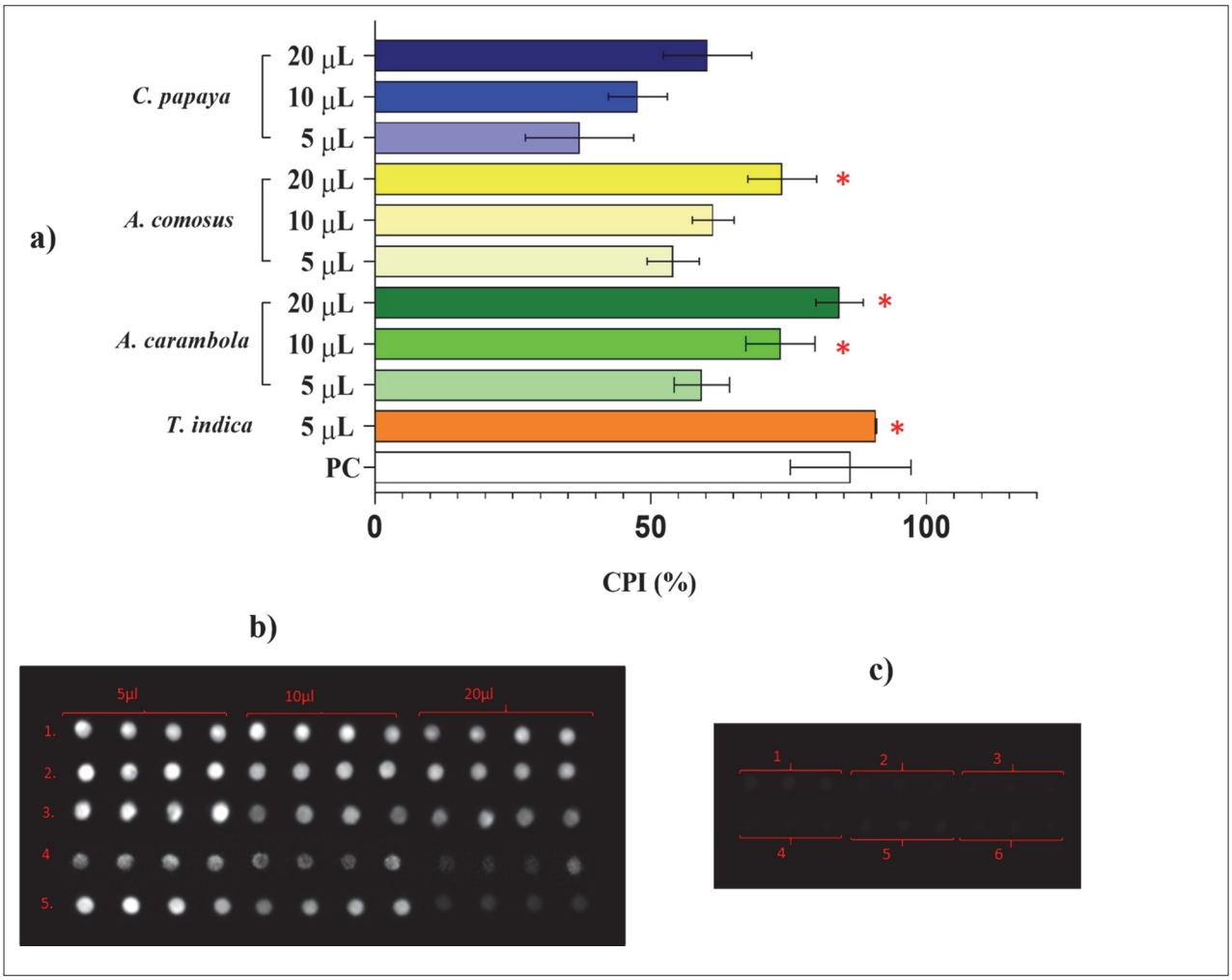


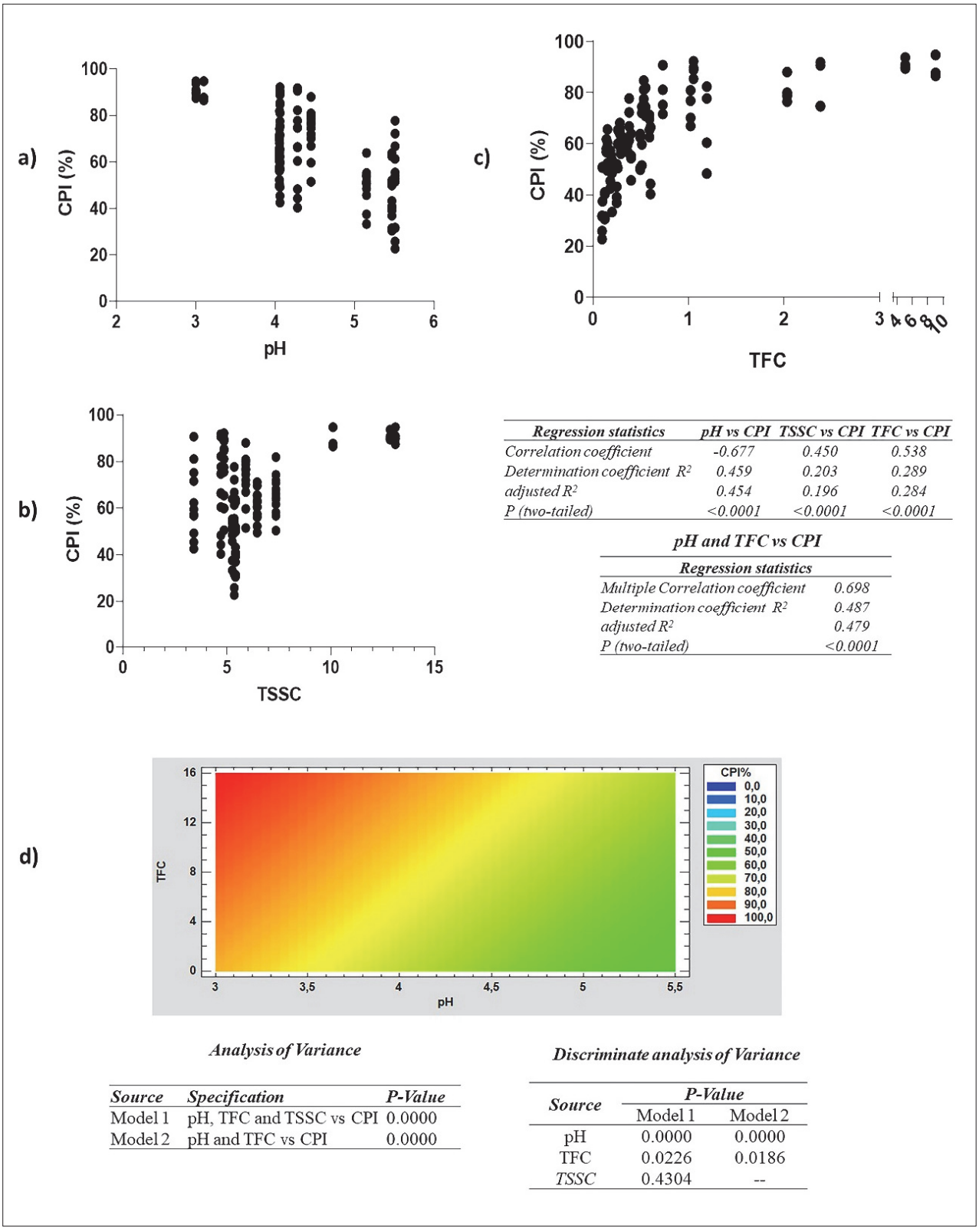
Figure 2. Carbonylation percentage inhibition of the BSA (CPI) induced by *in natura* fruit juices assayed. a) Results are shown as the percentage mean ± SD ($n = 3$). Asterisks (*) on the bars identify the CPIs induced by the *in natura* juices that were not statistically different from the positive control (PC) ($p > 0.05$). CPI were calculated using the Equation 1. It was not possible to calculate the CPIs induced by *T. indica* (10 and 20 µL) and by *P. edulis* at all the volumes assayed, because the CIs from BSA exposed to these juices were lower than the carbonylation value shown by the basal BSA in our assay conditions (5.0 nmol of carbonyl mg^{-1} protein). b) Spots' intensities from treated samples (BSA oxidized with 10 mM $FeSO_4$ in 137 mM PBS, in presence of 5 µL, 10 µL, and 20 µL of the assayed *in natura* juices). Numbers on the sheets indicate oxidized BSA samples in presence of natural juices from 1) *C. papaya*, 2) *A. comosus*, 3) *A. carambola*, 4) *P. edulis*, and 5) *T. indica*. c) Spots' intensities from *in natura* juices assayed (20 µL) and reactive blanks (PBS). Numbers on the sheets indicate juice intensities from: 1) *Tamarindus indica*, 2) *Carica papaya*, 3) *Ananas comosus*, 4) *Averrhoa carambola*, 5) *Passiflora edulis*, and 6) PBS.

re-evaluate this parameter, so that only curve linearity and repeatability were determined in a single day of assay. The behavior of the curve obtained for dot-blots assay are shown in Supplemental Figure S3 and Supplemental Table S4. Curve showed linearity in the range of CIs between 5 and 65 nmol of carbonyl mg^{-1} of protein ($R^2 > 0.99$), and spots' intensities from a same CIs presented RSDs lower than 5.9.

CIs from samples (BSA oxidized in the presence of *in natura* juices) and controls were determined using the dot-blots shown in Figure 2b, and the oxidized BSA curve equation (Supplemental Figure S3a). Blanks of juices in PBS did not show significant background in assays conditions (see Figure 2c) and CIs obtained for samples are listed in Supplemental Table S5. As can be seen, the negative control showed an average carbonylation of 499 ± 0.49 nmol of carbonyls mg^{-1} of protein, while samples oxidized in presence of natural juices assayed were lesser. These data show that *in natura* juices induced a protective effect of BSA oxidation, presenting CIs significantly lower than those of the negative control ($P < 0.05$) (Supplemental Table S5). *In vitro* antioxidant capacity from *in natura* juices was expressed as CPI, calculated using Equation 1.

All the *in natura* juices showed an increasing antioxidant activity (inhibition of BSA carbonylation), dependent of amount of juice used, being greater in all cases at 20 µL (see Figure 2a, and Supplemental Table S5). This antioxidant activity was even significantly superior to that shown by the positive control (81.2 ± 4.37%) in samples treated with *P. edulis* and *T. indica* to all three volumes assayed, and statistically equal in samples treated with 10 and 20 µL of *A. carambola* (73.5 ± 6.28 and 84.2 ± 4.31%, respectively) and 20 µL of *A. comosus* (73.9 ± 6.26%) (see Figure 2a and Supplemental Table S5).

P. edulis and *T. indica* juices were the ones with the greater antioxidant effect. In these treated samples, only the CIs from BSA exposed to 5 µL of the *T. indica* juice could be quantified;



Regression statistics	*pH vs CPI*	*TSSC vs CPI*	*TFC vs CPI*
Correlation coefficient	*-0.677*	*0.450*	*0.538*
Determination coefficient R^2	*0.459*	*0.203*	*0.289*
adjusted R^2	*0.454*	*0.196*	*0.284*
P (two-tailed)	*<0.0001*	*<0.0001*	*<0.0001*

pH and TFC vs CPI

Regression statistics	
Multiple Correlation coefficient	*0.698*
Determination coefficient R^2	*0.487*
adjusted R^2	*0.479*
P (two-tailed)	*<0.0001*

Analysis of Variance

Source	*Specification*	*P-Value*
Model 1	pH, TFC and TSSC vs CPI	0.0000
Model 2	pH and TFC vs CPI	0.0000

Discriminate analysis of Variance

Source	*P-Value*	
	Model 1	Model 2
pH	0.0000	0.0000
TFC	0.0226	0.0186
TSSC	0.4304	--

Figure 3. Multiple linear correlation analysis performed between CPI *vs*. a) pH, b) TSSC, and c) TFC. Parameters used to evaluate the variables' relation level were the correlation and determination coefficients. d) In these parts the response surface contours estimated by the generalized linear model are shown; in which correlation between independent variables from *in natura* juices such as pH and TFC on CPI can be observed (Red contours representative of values of CPI between 90 and 100% were reached in juices with pH lower than 3.5 and TFC greater than 8 mg L^{-1}). In turn, p values from analysis of variance obtained in the two models; model 1: comparison of independent variables (pH, TFC and TSSC) *vs*. CPI; model 2: independent variables (pH and TFC) *vs*. CPI. Due to adjusted coefficient of determination value from model 2 (pH and TFC *vs*. CPI) (R^2 = 0.479) was greater than the individual ones, we were able to attribute the antioxidant effect of natural juices to the synergic effect of these independent variables evaluated. TFC: total flavonoids contents, TSSC: total soluble solids content, CPI: carbonylation percentage inhibition of the BSA.

the others were below to the carbonylation value shown by the basal BSA under our assay conditions (5.0 nmol of carbonyl mg^{-1} of protein). This could indicate that volumes of 10 and 20 µL of *T. indica* juices and all assayed volumes of *P. edulis*, in addition to having a clearly marked antioxidant effect on BSA, could also be promoting the reduction of this protein.

Comparative analysis of CPIs from *in natura* juices reported in Supplemental Table S5, shown a relation between their antioxidant effect and parameters such as TFC, pH, and TSSC (Supplemental Table S1). In this sense, natural juices of *P. edulis* and *T. indica* induced the greater values of CPIs in the three volumes assayed (Figure 2a, Supplemental Table S5), and in turn presented a greater TFC, a more acid character, and a greater quantity of TSSC (Figure 1, Supplemental Table S1). While, *in natura* juice of *C. papaya* was one of those that induced the lowest antioxidant activity and congruently presented the lowest values of TFC, SSC, and the lowest acid character (pH = 5.4 ± 0.17).

Pearson's correlation coefficient was employed to calculating relationship between CPIs and the other independent variables (pH, TSSC and TFC), evaluating individual and multiples correlations, for it, the GraphPad prism 8.02 and Statgraphics Centurion 18 software were used, in order to analyze all the possible combinations of the evaluated variables and their joint effect on the CPIs. In these assays, data from samples treated with *P. edulis* and *T. indica* juices (10 and 20 µL) were not taken into account, because their CIs values could not be calculated, since they were below the lowest point of the BSA oxidized curve (Supplemental Tables S4 and S5).

Correlation analysis results between CPIs induced by *in natura* juices and their respective TFC, showed a positive and significant relation (r = 0.5382, P = 0.0001), indicating that flavonoids content is a crucial parameter in the antioxidant properties of *in natura* juices.

Many studies have reported the important role of flavonoids in fruits; these secondary metabolites have been attributed anti-inflammatory, antineoplastic, antibacterial, and antioxidant properties, either *in vitro* and *in vivo* models, both in plants and animals (Yang *et al*., 2019; Zheng *et al*., 2019). The mechanism whereby flavonoids (FV) promote an antioxidant effect at *in vitro* models has been widely described in the scientific literature; this is directly related to the chemical structure and glycosylation status of these compounds (Jabeen *et al*., 2018).

Main mechanism whereby FV exert antioxidant activity is through the elimination or inhibition of the free radical formation. They do this by donating H atoms or an electron from their hydroxyl groups to the free radical (R·), thus inhibiting the chain reaction, thanks to a stable semiquinone structure formation (Chen *et al*., 2018). FVs also have the ability to act as chelating agents for transition metal cations, including Fe (II), Fe (III) and Cu (II), which are related to the catalysis of the Fenton reaction, one of the important routes in the production of oxidative stress (Jabeen *et al*., 2018). In this sense, these antioxidant mechanisms from FVs described above, may be involved in one of the routes by which the *in natura* juices from fruits assayed inhibit the *in vitro* carbonylation of BSA.

On the other hand, correlation analysis between CPIs induced by *in natura* juices and their respective pH, showed a negative and significant relation (r = -0.6774, P = 0.0001), suggesting that acidic character from juices is a protective factor of the *in vitro* oxidation of the BSA, which are related to the greater concentration of organic acids (citric, malic, quinic, succinic and shikimic), which besides contributing to organoleptic characteristics have also been attributed antioxidant properties, being able to prevent lipid peroxidation and capture free radicals (Font *et al*., 2019).

In addition to described above, some authors have attributed indirect antioxidant properties to organic acids, because the antioxidant activity of flavonoids is hampered by pH and ionic strength of the medium. Some flavonoids, such as fisetin, exhibit a good antioxidant capability at acid pH, where the electron donation is promoted easier instead of H· and at the same time improve its radical elimination capacity (Farsad and Alizadeh, 2017).

Regarding the relation analysis between CPIs and the TSSC from juices, a positive correlation was observed, however this was the lowest of all, but even so it was significant (r = 0.4501, P = 0.0001).

When comparing adjusted determination coefficients obtained in correlation analysis, we were able to establish a hierarchy in the effect exercised by independent variables evaluated (pH, TSSC and TFC) on the CPI. This analysis showed the following increasing order: pH> TFC> TSSC (Figure 3). However, in order to evaluate the joint correlation level of the independent variables *vs*. CPI (%), it was performed a general linear model (GLM), using the Statgraphics Centurion software 18. Results obtained when correlating pH, TSSC and TFC *vs*. CPI (%) gave a p value <0.05, denoting the existence of a possible statistically significant serial correlation between CPI and the evaluated factors, with a confidence level of 95%. However, when significance level of each individual factor was compared, TSSC presented a p value that was not significant (0.4304), so it was removed from the model, re-performing the GLM, now correlating only the pH and TSSC *vs*. CPI (%). Due results obtained were significant (p < 0.05), we predicted with a confidence level of 95% that there was a correlation between assayed variables with the CPI (%).

Finally, R^2 adjusted values obtained in the linear correlation analysis between (CPI *vs*. pH) and (CPI *vs*. TFC) and the multiple linear correlation analysis of these two independent variables *vs*. CPI were compared. Since R^2 from combined variables (0.47867) was higher than the individual one (0.454232 and 0.283644 for the pH and TFC, respectively), we were able to attribute antioxidant effect of natural juices to the synergic effect of the independent variables evaluated (Figure 3).

Role of natural juices on prevention of diseases associated with oxidative stress

Protein carbonylation inhibition in biological systems is a desired effect, which could be useful to attenuate the consequences of high oxidative stress that resides in neurodegenerative, inflammatory, cardiovascular, and autoimmune diseases, among others. Results obtained in this work, show that the consumption of juices from natural fruits assayed may represent a good strategy for the prevention of these pathologies.

On the other hand, the model protein used in this study is the most abundant protein in the blood circulation. BSA transports hormones, fatty acids, and various compounds through the bloodstream, also helps maintain osmotic blood pressure, and can interact with a variety of ligands, including drugs (Lee and Wu, 2015). Therefore, the inhibitory capacity of the oxidation of this protein promoted by the evaluated natural juices, represents a protective factor to its functions.

Conforming as reported by Hyson, natural juices intake increases the antioxidant capacity in consumer's serum one hour after ingestion, and the effect can be maintained for several hours afterwards, depending on the volume, type of juice, subject characteristics, type of assay, and other unknown factors. In addition, bioavailability studies performed on active biomolecules such as flavonoids have shown that these are found in plasma and urine after consumption in natural fruit juices (Hyson, 2015).

Fruits like *T. indica* have shown multiple beneficial effects for health, in addition to the antioxidant properties exposed in this work. According to Kuru, *T. indica* has been used by various cultures for the treatment of abdominal pain, diarrhea and dysentery, shown in turn antibacterial, antimicrobial, antiparasitic, anti-inflammatory, antidiabetic, antimalarial, cardioprotective, hepatoprotective, anti-asthmatic, laxative and antihyperlipidemic properties. All this thanks to the bioactive compounds present in this plant such as catenin, procyanidin B2, epicatechin, tartaric acid, mucilage, pectin, arabinose, xylose, galactose, glucose, uronic acid and triterpene (Kuru, 2014).

On the other hand, *A. comosus in natura* juice has shown antiproliferative properties in an *in vitro* study in which sorghum seeds were used (*Sorghum bicolor*) as a biological model, suggesting a high potential as an anticancer agent, attributed among its different bioactive compounds, to vitamins C and E (Chinedu *et al*., 2016).

Finally, quercetin, a flavonoid present in most of the *in natura* juices evaluated in the present study, in addition to exhibiting antioxidant, anti-inflammatory and cardiovascular diseases protective properties (Lesjak *et al*., 2018), has shown anti-tumor activity through the anti-proliferative effect mediated by mechanisms related to oxidative stress in a study conducted in LoVo cells from human colon cancer and MCF-7 breast cancer cells (Zhang *et al*., 2012).

Conclusions

Inhibitory capabilities of protein carbonylation to four *in natura* juices *(P. edulis*, *T. indica*, *A. carambola*, *A. comosus*, and *C. papaya*), were demonstrated through an *in vitro* assay. Acidity degree and total content of flavonoids were variables that exerted a direct and synergistic effect on the antioxidant capacity of the *in natura* juices. According to antioxidant capacity and total content of flavonoids of the *in natura* juices evaluated, it is possible to propose them as promising to attenuate the metabolic consequences of high oxidative stress for some human diseases, especially for *T. indica* and *P. edulis* juices.

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Conflict of interest declaration

None.

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Supplemental Information

Supplemental Information – Table S1. Physicochemical parameters of natural fruit juices.

Fruit juices	BR	pH	TA (%)	TSSC (°Brix)	RI
Ananas comosus	1	4.06 ± 0.03	0.81 ± 0.000	3.4 ± 0.000	4.2 ± 0.037
	2	4.05 ± 0.037	0.44 ± 0.007	6.4 ± 0.212	14.6 ± 0.247
	3	4.05 ± 0.035	0.43 ± 0.000	7.4 ± 0.071	17.2 ± 0.164
Tamarindus indica	1	3.09 ± 0.001	1.51 ± 0.021	10.1 ± 0.000	6.7 ± 0.095
	2	3.00 ± 0.001	1.69 ± 0.007	13.1 ± 0.000	7.8 ± 0.033
	3	3.04 ± 0.001	2.45 ± 0.007	12.9 ± 0.071	5.3 ± 0.014
Passiflora edulis	1	3.39 ± 0.001	5.14 ± 0.021	12.1 ± 0.071	2.4 ± 0.004
	2	3.15 ± 0.002	4.09 ± 0.014	13.0 ± 0.000	3.2 ± 0.011
	3	3.32 ± 0.001	4.29 ± 0.007	10.5 ± 0.069	2.5 ± 0.021
Carica papaya	1	5.47 ± 0.001	0.13 ± 0.014	5.4 ± 0.000	41.8 ± 4.546
	2	5.51 ± 0.001	0.08 ± 0.007	5.4 ± 0.067	71.7 ± 7.702
	3	5.15 ± 0.002	0.12 ± 0.015	5.2 ± 0.061	44.1 ± 4.599
Averrhoa carambola	1	4.45 ± 0.002	0.24 ± 0.007	5.9 ± 0.000	25.9 ± 0.756
	2	4.06 ± 0.002	0.15 ± 0.014	4.8 ± 0.071	32.1 ± 2.589
	3	4.28 ± 0.002	0.14 ± 0.006	4.7 ± 0.00	35.6 ± 1.826

BR: biological replicas, TA: titratable acidity, TSSC: total soluble solids content, RI: ripening index.
All values are reported as mean ± standard deviation.

Supplemental Information – Table S2. Data used to evaluate repeatability and reproducibility of quercetin curve.

Conc. (mg L^{-1})	Repeatability – day 1 (3 curves)				Repeatability – day 2 (2 curves)			Reproducibility	RSD
	Absorbance			Mean ± SD	Absorbance		Mean ± SD	Mean ± SD	
	R1	R2	R3		R1	R2			
100	0.038	0.053	0.048	0.046 ± 0.01	0.045	0.059	0.052 ± 0.01	0.049 ± 0.01	16.0
200	0.083	0.091	0.057	0.077 ± 0.02	0.072	0.103	0.088 ± 0.02	0.081 ± 0.02	21.7
400	0.174	0.201	0.166	0.180 ± 0.02	0.166	0.255	0.211 ± 0.06	0.192 ± 0.04	19.7
600	0.274	0.292	0.227	0.264 ± 0.03	0.252	0.326	0.289 ± 0.05	0.274 ± 0.04	13.9
800	0.328	0.394	0.295	0.339 ± 0.05	0.325	0.383	0.354 ± 0.04	0.345 ± 0.04	12.1

Conc.: concentration of standard quercetin solutions. 5 calibration curves of quercetin by spectrometric assay were used for the determination of total flavonoids contents.

Supplemental Information – Table S3. Parameters to evaluation of repeatability and reproducibility of quercetin calibration curves.

Parameter	Repeatability / Reproducibility curves		
	Day 1	Day 2	Mean ± SD
Slope (m)	0.0004 ± 0.0001	0.00045 ± 0.0001	0.00044 ± 0.0001
Intercept (b)	0.0003 ± 0.01	0.00245 ± 0.02	0.001134 ± 0.01
Correlation coefficient (R^2)	0.9917 ± 0.01	0.9806 ± 0.02	0.98724 ± 0.01

Supplemental Information – Table S4. Data used to evaluate repeatability of oxidized BSA curve.

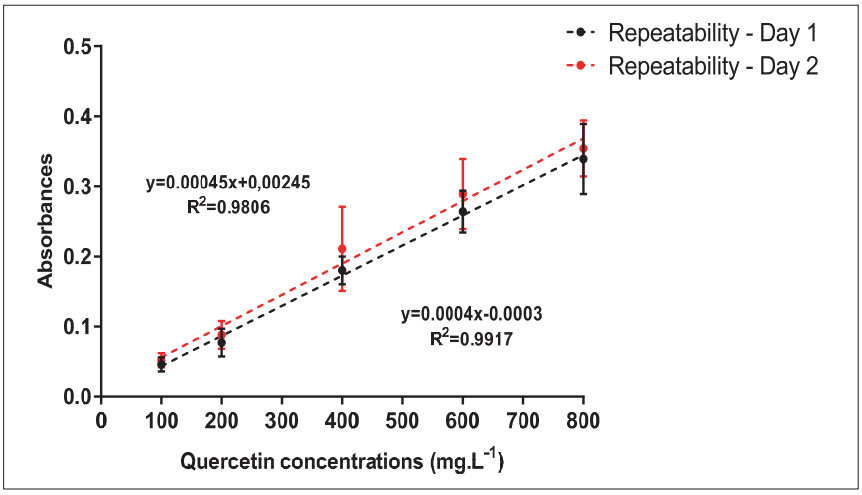
CIs	Replicas				Mean	RSD %
	R1	R2	R3	R4		
5	6.0	6.4	6.6	6.2	6.3 ± 0.21	3.4
25	8.2	8.1	8.2	8.1	8.2 ± 0.06	0.7
35	8.5	8.6	8.5	9.6	8.8 ± 0.45	5.1
45	10.9	9.8	9.2	10.1	10.0 ± 0.59	5.9
65	12.2	12.0	11.6	11.2	11.8 ± 0.39	3.3

CIs: carbonyls index of the oxidized BSA curve.

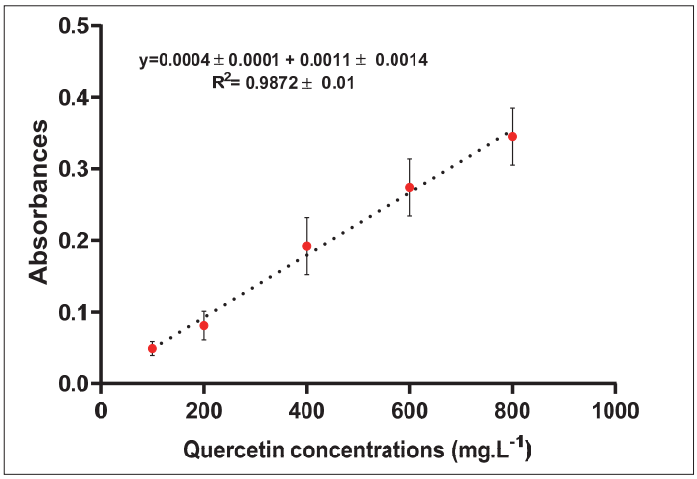
Supplemental Information – Table S5. Carbonyl index and antioxidant activity of the *in natura* juices.

Samples	BR	CIs (nmol of carbonyls mg^{-1} of protein)			CPI (%)		
		Volume assayed					
		5 µL	10 µL	20 µL	5 µL	10 µL	20 µL
C. papaya	1	320.2 ± 27.9	285.6 ± 33.3	216.3 ± 37.1	35.8 ± 5.6	42.8 ± 6.7	56.6 ± 7.4
	2	359.5 ± 22.4	232.4 ± 8.5	152.2 ± 35.1	28.0 ± 4.5	53.4 ± 1.7	69.5 ± 7.0
	3	262.0 ± 33.1	265.4 ± 46.2	225.5 ± 37.2	47.5 ± 6.6	46.8 ± 9.2	54.8 ± 7.5
A. comosus	1	256.4 ± 32.1	205.3 ± 13.1	101.6 ± 41.6	48.6 ± 6.4	58.9 ± 2.6	79.6 ± 8.3
	2	214.9 ± 36.8	202.8 ± 15.8	163.6 ± 19.5	56.9 ± 7.4	59.4 ± 3.2	67.2 ± 3.9
	3	216.0 ± 23.6	171.4 ± 9.6	126.2 ± 25.0	56.7 ± 4.7	65.7 ± 1.9	74.7 ± 5.0
A. carambola	1	177.7 ± 53.7	131.2 ± 31.7	96.2 ± 25.2	64.4 ± 10.8	73.7 ± 6.4	80.7 ± 5.0
	2	204.8 ± 30.9	101.2 ± 20.3	54.7 ± 14.1	59.0 ± 6.2	79.7 ± 4.1	89.0 ± 2.8
	3	227.7 ± 69.9	163.8 ± 78.3	85.0 ± 47.7	54.4 ± 14.0	67.2 ± 15.7	83.0 ± 9.5
T. indica	1	45.5 ± 22.8	-*	-	90.0 ± 4.4	-	-
	2	46.2 ± 15.1	-	-	90.7 ± 3.0	-	-
	3	45.2 ± 9.5	-	-	90.9 ± 1.9	-	-
PC			94.0 ± 21.8			81.2 ± 4.4	
NC			499.0 ± 0.49			0.0	

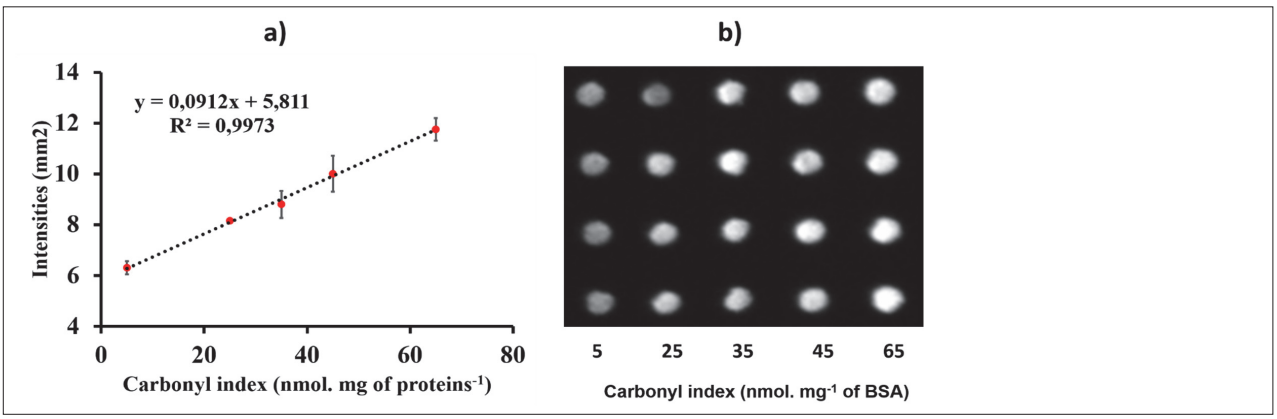
CIs: carbonyl index, CPI: carbonylation percentage inhibition of the BSA, PC: positive control (oxidized BSA samples with $FeSO_4$ in presence of acid ascorbic 8.2 mM) NC: negative control (oxidized BSA samples with $FeSO_4$ 10 mM in PBS 137 mM in the absence of *in natura* fruit juices). * Blanks in the table (-) from *T. indica* juices could not be quantified, because they were below the carbonylation value shown by the basal BSA under our assay conditions (5.0 nmol carbonyl mg^{-1} of protein).



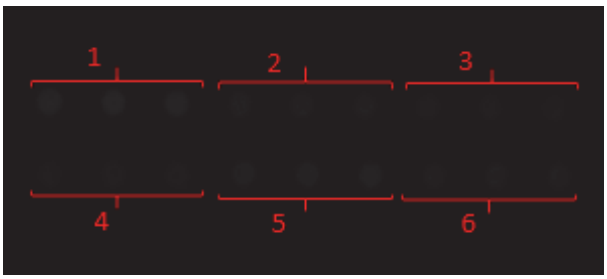
Supplemental Information – Figure S1. Repeatability of quercetin curves used for the determination of total flavonoids contents. The calibration curves were constructed in triplicate and duplicated on two different days.



Supplemental Information – Figure S2. Reproducibility and linearity of quercetin curve used for the determination of total flavonoids contents. Curve was elaborated by average absorbance of each quercetin concentration from 5 replicas.



Supplemental Information – Figure S3. Calibration curve of oxidized BSA for quantitation of carbonyl indexes by Dot-blot. a) Graphic representation of the oxidized BSA curve behavior. The intensities were normalized by dividing them by 100,000. b) Dot-blot of calibration curve, 100 µL derivatized BSA with DNPH at 1 ng µL^{-1} were spotted by quadruplicate to standard solutions employed PVDF membranes.



Supplemental Information – Figure S4. Spots' intensities from *in natura* juices assayed (20 µL) and reactive blanks (PBS). Numbers on the sheets indicate juices intensities from 1) *T. indica*, 2) *C. papaya*, 3) *A. comosus*, 4) *A. carambola*, 5) *P. edulis*, and 6) PBS.