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# The effect of *in vitro* digestion on the antioxidant activity of fruit extracts (*Carica papaya*, *Artocarpus heterophillus* and *Annona marcgravii*)

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ABSTRACT

Fruit-derived antioxidant compounds that are involved in the prevention of many degenerative diseases have been investigated in several studies. To improve the knowledge of the antioxidant compound absorption process, this study evaluated the antioxidant activity (AA) of *Carica papaya* (papaya), *Artocarpus heterophillus* (jackfruit) and *Annona marcgravii* (araticum) extracts that were not digested and digested at physiological pH using enzyme solutions (*in vitro* gastrointestinal digestion). The antioxidant activity was measured by such methods as Trolox equivalent antioxidant capacity and Oxygen radical absorbance capacity (ORAC). Total phenol content (TPC) and flavonoid content (FC) were also evaluated. Decreased TPC levels were observed in araticum and papaya extracts, whereas increased TPC levels were detected in jackfruit. Increased FC levels and decreased AA was observed in papaya extract. However, the decreased AA was not associated with the degradation of the analyzed compounds. Although ORAC is among the best analytical chemical methods, it is necessary to evaluate AA by other methods, such as antioxidant cellular or *in vivo* analysis. Moreover, the AA of other fruits (araticum and jackfruit) increased after being digested. The increased AA of the digested jackfruit and araticum extracts can be associated with the release of phenolic compounds after the digestion.



## 1. Introduction

The World Health Organization (2003) indicates that a daily consumption of at least 400 g of fruits and vegetables (equivalent to five daily servings) decreases the risk of cardiovascular diseases, chronic diseases, and other diseases.

In addition to being healthy, fruits contain substances that can prevent and delay the development of some diseases. These substances include polyphenols, which have antioxidant activity (AA) (Senger, Schwanke, & Gottlieb, 2011; Van den Ende, Peshev, & De Gara, 2011). The beneficial effects attributed to compounds present in foods provided the foundation for several studies that were initially conducted using chemical extracts. However, these results may not reflect physiological activities.

*Carica papaya* (papaya) which is one of the most widely consumed fruits worldwide that has antioxidant properties (Simirgiotis, Caligari, & Schmeda-Hirschmann, 2009), and *Artocarpus heterophillus* (jackfruit), which is exotic to Brazilian popular culture but whose global consumption has not been studied, exhibit important features such as biological activity and AA (Jagtap & Bapat, 2010) and *Annona marcgravii* (araticum). However, the detailed mechanisms of these activities have not yet been determined. Other fruits that belong to the same family have shown significant AA (Roesler, Malta, Carrasco, & Pastore, 2006).

Recent research has elucidated the functional properties of total phenol content (TPC), flavonoid content (FC), anthocyanins and AA after *in vitro* gastrointestinal digestion of vegetables and fruits and their extracts (Bouayed, Hoffmann, & Bohn, 2011; Faller, Fialho, & Liu, 2012; Sancho & Pastore, 2012).

As described in the literature, the complexity of the extract influences the release of certain compounds and their specific chemical structures (de Oliveira & Bastos, 2011; Rechner et al., 2002). It is known that polyphenol molecules are associated with carbohydrates, acids, amino acids and other molecules and that the digestion process can cause the release of these associated compounds leading to changes in AA. For this reason, it is necessary to evaluate the influence exerted by these bound compounds. Several studies have compared the content of bioactive compounds and AA

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in undigested and digested extracts (Bouayed et al., 2011; Faller et al., 2012; Giménez, Moreno, López-Caballero, Montero, & Gómez-Guillén, 2013; Tavares et al., 2012).

To advance this knowledge, the present study assessed the levels of TPC, FC and AA of the extracts of the abovementioned exotic fruits that have not been widely studied (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004) prior to and after digestion.

## 2. Materials and methods

### 2.1. Samples and extract preparation

About 10 kg (two units) of jackfruit (*A. heterophillus* L. cv J3, fruit with hard flesh) and 1.5 kg (three units) of papaya (*C. papaya*, cv. Sunrise, Solo group) were acquired in the wholesale fruit vegetable and flower market (CEASA) of Campinas city, in the state of São Paulo, Brazil, and 1 kg (four units) of araticum (*A. marcgravii*) was harvested and acquired in the Saputá nursery in Campina do Monte Alegre, located in the state of São Paulo, Brazil. The samples were acquired at the maturity level that was required for consumption based on the softness, color and specific aspects of each fruit. The samples were collected in April 2012. After harvest, the fruits were immediately taken to the laboratory where they were sanitized, fractionated (separating pulp, seed and peels) and then weighed (500 g of jackfruit, 860 g of papaya and 300 g of araticum) and were lyophilized. The peel and seed were discarded and only the pulp used for the extract preparation. This crude extract of fruits was prepared in the following manner: 10 g of each freeze-dried fruit (FDF) was macerated and dissolved in 100 mL of the ethanol 70° GL. The mixture was homogenized using an Ultra Turrax (Polytron, MR-2100) for three minutes, sonicated for 30 min in an ice bath, agitated in a 38 °C water bath for 60 min at 150 rpm, filtered (Whatman N°1), evaporated to 40 mL, aliquoted and the material was frozen at −80 °C for subsequent analysis. Crude extracts were prepared in triplicate.

### 2.2. Methods

#### 2.2.1. Total phenolic content

The determination of TPC was carried out according to the method of Folin Ciocalteau described by Roesler et al. (2006). Briefly, the crude extract prepared above was diluted at a concentration of 6 mg/mL in methanol, sonicated (Ultrasonic cleaner, Unique, São Paulo, Brazil) for two hours and filtered through 0.22 μm filters (Millipore, Merck). The reaction occurred by mixing 0.5 mL extract, 2.5 mL Folin Ciocalteau (10%) and 2.0 mL sodium carbonate (7.5%). The mixture was incubated for 5 min at 48 °C, and the absorbance at 760 nm was measured using a spectrophotometer (Beckman Coulter tm, USA D.U. 640). A calibration curve was constructed with standard gallic acid (10–100 μg/mL), and the results were expressed as mg gallic acid equivalent (GAE) per 100 g FDF.

#### 2.2.2. Flavonoid content

FC was measured by a colorimetric assay developed by Zhishen, Mengcheng, and Jianming (1999). Extracts crude of jackfruit, papaya and araticum were diluted at a concentration of 20 mg/mL in water distilled. 1.27 mL of diluted crude extract and 5 mL of distilled water were homogenized and 0.3 mL 5% $NaNO_2$ was added. After 5 min, 0.3 mL 10% $AlCl_3$ was added. After 6 min, 2 mL 1 M NaOH was added to the mixture. Immediately, the reaction flask was diluted with the addition of 2.4 mL $H_2O$ and thoroughly mixed. The absorbance of the mixture was compared to the water blank and measured at 510 nm. A calibration curve was generated with standard catechin (10–175 μg/mL), and FC was expressed as mg catechin equivalent (CE) per 100 g FDF.

#### 2.2.3. Antioxidant activity

*2.2.3.1. Trolox equivalent antioxidant capacity (TEAC).* Antioxidant activity was measured by the TEAC method described by Le, Chiu, and Ng (2007). Briefly, the radical cation ABTS′ (2,2′-azinobis 3-ethylbenzothiazoline 6-sulfonate, Sigma) was prepared 12–16 h prior to the assay. Radical production occurred by mixing an aqueous solution of 7 mM ABTS′ in 140 mM potassium persulfate. The radical was diluted with water until the absorbance reached a value of 0.7000–0.02 ± 734 nm. The extracts were analyzed at a concentration of 3 mg/mL, 9 mg/mL and 11.96 mg/mL (papaya, araticum and jackfruit respectively) dissolved in water. The assay was performed with a spectrophotometer and microplate reader adding 50 mL sample/Trolox and 250 mL ABTS′. A calibration curve was constructed with standard Trolox (10–250 μg/mL), and the results were expressed as μmol Trolox equivalents (TE) per 100 g FDF.

*2.2.3.2. Oxygen radical absorbance capacity (ORAC).* The ORAC was determined based on the method described by Ou, Huang, Hampsch-Woodill, Flanagan, and Deemer (2002). The experiment was performed using a fluorescence reader (New Star fluorometer, Labtechnologies BMG). The reading was performed every 1 min for 80 min with emission at 520 nm and excitation at 485 nm and the temperature controlled at 37 °C. For hydrophilic ORAC, 20 μL of each crude extract (papaya, araticum and jackfruit were diluted at a concentration of 6 mg/mL diluted in buffer) was added with 120 μL fluorescein (0.4 mg/mL) and 60 μL of the radical AAPH (2,2′-azobis 2-methylpropionamidine) dihydrochloride, Aldrich at a concentration of 108 mg/mL. The potassium phosphate buffer (pH 7.4, 75 mM) was used as the solvent. The area under the curve (AUC) was calculated using the following equation:

$$\mathrm{AUC} = 1 + \frac{f_2}{f_1} + \frac{f_3}{f_1} + \frac{f_4}{f_1} + \frac{f_n}{f_1}$$

where: $f_1$ = reading fluorescence at 1 min, $f_2$ = reading fluorescence at 2 min and $f_n$ = fluorescence reading at 80 min. The calibration curve was constructed with standard Trolox (25–700 μg/mL) and a blank without antioxidants, and the results were expressed as μmol TE per 100 g FDF.

#### 2.2.4. Gastrointestinal in vitro digestion

Based on the methodology described by Faller et al. (2012), with a few modifications, crude extracts were prepared in triplicate followed by digestion *in vitro*. 1 mL of crude extracts from papaya (500 mg/mL), araticum (500 mg/mL) and jackfruit (502 mg/mL) were mixed in a saline solution containing 140 mM NaCl, 5 mM KCl and 150 mM BHT at a ratio of 1:4 v/v (sample/saline) to obtain a final volume of 7 mL. This was followed by agitation at 21 °C for 10 min. Afterward, the mixture was acidified to pH 2.0 with 0.1 M/1 M HCl and was added to a 0.125 mL solution containing pepsin (0.2 g in 5 mL 0.1 M HCl), and the samples were incubated at 37 °C with stirring for 1 h.

After step-wise gastric digestion, the intestinal digestion of the sample was commenced by raising the pH to 6.9 by the addition of 1 M $NaHCO_3$/0.1 M $NaHCO_3$. Then, 0.625 mL bile and pancreatic solution (containing 0.225 g bile extract and 0.0375 g pancreatin in a volume of 18.75 mL 0.1 M $NaHCO_3$) was added followed by incubation with shaking at 37 °C for 2 h. The final volume of the digested sample was adjusted to 7 mL by the addition of brine. The samples were filtered and analyzed again by the methods described in Sections 2.2.1, 2.2.2 and 2.2.3.

#### 2.2.5. Statistical analyses

The results are presented as the mean ± standard deviation of three replicates of each experiment. A $p$-value < 0.05 was used to indicate significant differences between the mean values

determined by analysis of variance (ANOVA) using Statistica 12.0 (StatSoft, Inc., 2013).

## 3. Results

The results are shown in Table 1.

### 3.1. Araticum

The araticum extract showed significant difference ($p < 0.05$) in the reduction of TPC with values of 215.7 ± 0.005 mg GAE/100 g FDF in the undigested sample and 178.2 ± 0.04 mg GAE/100 g FDF in the digested sample. FC showed no significant difference ($p > 0.05$) for the undigested and digested samples with values of 405 ± 0.003 and 399.1 ± 0.03 CE mg/100 g FDF, respectively.

A significant increase ($p < 0.05$) in the AA was observed according to the results from the TEAC and ORAC assays. The values obtained for TEAC were 142.6 ± 0.006 and 1647.69 ± 0.008 μmol TE/100 g FDF for the undigested and digested extracts, respectively. The ORAC value for the undigested araticum extract was 9618.38 ± 3.17 μmol TE/100 g FDF and 32,611 ± 1.13 μmol TE/100 g FDF for the digested extract.

### 3.2. Jackfruit

TPC exhibited a significant increase ($p < 0.05$) in the undigested and digested extract 23.3 ± 0.004 and 33.9 ± 0.002 mg GAE/100 g FDF, respectively.

Analysis by the TEAC method indicated that the AA was significantly increased ($p < 0.05$) with 56 ± 0.005 and 318.4 ± 0.014 μmol TE/100 g FDF for the undigested and digested extracts, respectively. The ORAC values were also significantly increased ($p < 0.05$) with values of 2115.95 ± 1.7 and 3560 ± 1.5 μmol TE/100 g FDF in the undigested and digested extracts, respectively.

### 3.3. Papaya

The papaya extract exhibited a significant decrease ($p < 0.05$) in the TPC concentration: 79.5 ± 0.09–28.6 ± 0.03 mg GAE/100 g FDF after *in vitro* digestion. FC exhibited a significant increase ($p < 0.05$) after *in vitro* digestion with values of 15.9 ± 0.02 and 26.4 ± 0.004 mg CE/100 g FDF for the undigested and digested extracts, respectively.

The TEAC and ORAC assays did not indicate a significant difference ($p > 0.05$) between the digested and undigested extracts.

## 4. Discussion

In the present study, the effect of the *in vitro* digestion of polyphenols on the AA from araticum, jackfruit and papaya extracts was evaluated.

There are several data in the literature regarding the polyphenol content in fruit extracts, but there is a lack of data regarding the polyphenols that are released after *in vitro* digestion and their effect on AA.

The digestion process directly affects the composition of the extracts depending on the simulation of the physiological conditions and the sequence of events within the gastrointestinal tract. Thus, in an effort to address these factors, this study demonstrated that after *in vitro* digestion, the extracts of araticum and jackfruit significantly increased the AA ($p < 0.05$).

The TPC exhibited different results in the fruit extracts after *in vitro* digestion. The araticum and papaya extract exhibited significant reduction ($p < 0.05$) in the TPC, which was similar to what was reported by Bouayed et al. (2011) in the analysis of different apple varieties. As written by Friedman and Jurgens (2000), the reduction in TPC can be linked to the instability of phenolic compounds in high pH, as they speculated, larger molecules may be more stable, but when hydrolyzed to smaller molecules, such as gallic acid, were not stable at high pH.

The evaluation of jackfruit extract showed a significant increase ($p < 0.05$) in the TPC. This result agrees with what was demonstrated by Bhatt and Patel (2013), whereby the increase in the TPC was associated with the gradual release of polyphenols during the digestive process.

It was found that only the papaya extract exhibited a significant increase ($p < 0.05$) in flavonoid content after *in vitro* digestion. This same behavior was observed in a study of Ginkgo biloba leaves (Goh & Barlow, 2004).

As described for Heim, Tagliaferro, and Bobilya (2002), the conservation of dietary flavonoids after digestion can be related to the β-linkage between carbohydrate and aglycone, which cannot be hydrolyzed by the enzymes usually employed in simulated digestion model and subsequently fermented by bacteria in the colon. This same behavior was observed in extracts of jackfruit and araticum.

The AA of araticum and jackfruit extracts after *in vitro* digestion were significant ($p < 0.05$) by TEAC and ORAC methods.

The increase of AA in jackfruit extract could be directly related to the significant increase ($p < 0.05$) in TPC after *in vitro* digestion. This increase in AA was also observed by investigators who analyzed other matrices, such as juices (Ryan & Prescott, 2010) and clove and nutmeg extracts that also exhibited increased TPC and TEAC (Baker, Chohan, & Opara, 2013). In 2013, Bhatt and Patel reported increased TPC and TEAC, and they hypothesized that increasing the TPC-associated gradual release of polyphenols during the digestive process and changing their structural form would directly affect their chemical and functional properties.

Is important to note that the increase in AA could also be related to pH and enzymatic interactions that occur during *in vitro* digestion. Further investigation is required to differentiate the effects of

**Table 1**
Phenolic content, flavonoid content and antioxidant activity of araticum, papaya and jackfruit undigested and digested extracts.

| | Araticum | | Papaya | | Jackfruit | |
|---|---|---|---|---|---|---|
| | Undigested | Digested | Undigested | Digested | Undigested | Digested |
| Total phenol content[1] | 215.7 ± 9.5[a] | 178.2 ± 11[b] | 79.5 ± 0.13[a] | 28.6 ± 2.2[b] | 23.3 ± 3.5[a] | 33.9 ± 0.8[b] |
| Flavonoid content[2] | 405.0 ± 6.6[a] | 399.1 ± 44[a] | 15.9 ± 2.63[a] | 26.4 ± 3.7[b] | 33.0 ± 9.5[a] | 28.4 ± 1.2[a] |
| TEAC[3] | 142.6 ± 3.7[a] | 1647.7 ± 5.5[b] | 447.5 ± 18[a] | 383.35 ± 17[a] | 56 ± 2[a] | 318.4 ± 12[b] |
| ORAC[4] | 10038 ± 984[a] | 31165 ± 4113[b] | 3112 ± 866[a] | 2017 ± 393[a] | 2117 ± 388[a] | 3047 ± 455[b] |

Means followed by the same letter are not different according to ANOVA ($p < 0.05$).
[1] TPC is expressed as mg gallic acid equivalent in 100 g FDF.
[2] FC is expressed as mg catechin equivalent in 100 g FDF.
[3] TEAC is expressed in μmol Trolox equivalents per 100 g FDF.
[4] ORAC is expressed in μmol Trolox equivalents per 100 g FDF.

the pH environment and the digestion enzymes with regard to TPC (Baker et al., 2013).

It is known that the change in the composition of phenolics and flavonoids in an array can interfere with the increase or decrease of AA in extracts. In one of our experiments, we observed increased AA in the araticum extract, with a statistically significant reduction ($p < 0.05$) in TPC and no significant difference in FC ($p > 0,05$). Based on these results, we hypothesize that the matrix obtained from this extract contains other substances that were not analyzed in this study, including non-phenolic substances involved in increasing AA after the *in vitro* digestion process. These compounds, such as amino acids, peptides, were released during digestion or were changed, which could have affected the capture of radicals due to the increased AA of the extracts (Castro & Sato, 2014). Other associations can be made based on the loss of the volatile compounds, since it have antioxidant properties (Lee & Shibamoto, 2001).

The AA of papaya extract did not exhibit a significant difference ($p > 0.05$) between digested and undigested extracts. This was also reported in orange juice by Ryan and Prescott (2010) who suggested the possibility of the compounds being resistant to changes in pH and enzymatic hydrolysis. Furthermore, Bermúdez-Soto, Thomas-Barberán, and Garcia-Conesa (2007) demonstrated the possibility that structural transformation of polyphenols might not be detected by the same method of analysis.

Another relevant aspect to be considered is that the carotenoids of jackfruit and papaya, although they have not been assessed, may have antioxidant activity (Rivera-Pastrana, Yahia, & González-Aguilar, 2010; Swami, Thakor, Haldankar, & Kalse, 2012). In their study of the gastrointestinal digestion of papaya, Breithaupt, Bamedi, and Wirt (2002) determined that cholesterol esterase, and not pancreatic lipase, which was used in our study, was responsible for carotenoid hydrolysis.

Some studies suggest that in addition to the concentrations of phenols and flavonoids, the AA of chemical food extracts could be underestimated because the solvents used in the extraction could be less effective in the complete extraction of polyphenols (Bhatt & Patel, 2013; Goni, Serrano, & Saura-Calixto, 2006). Furthermore, other factors, such as maturity at harvest, environmental factors, processing and storage, can affect the polyphenol content of fruits (Manach et al., 2004).

## 5. Conclusions

To the best of our knowledge, this report describes the first time that TPC, FC and the AA of extracts from the afore-mentioned fruits have been evaluated after *in vitro* digestion. The results obtained in the present study indicate that different fruit extracts have different behaviors in tests of TPC, FC and AA after the *in vitro* digestion process. Further studies involving the relationship between polyphenols, AA and digestion under physiological conditions, including cell-based assays and *in vivo* studies, are warranted.

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