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Bioaccessibility of Phenolic Compounds of *Araucaria angustifolia* from Seed Water Extracts during *In Vitro* Simulated Gastrointestinal Conditions

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Abstract

Food by-products containing bioactive substances, such as phenolic compounds, have garnered attention due to the possibility to increase the value of what would otherwise be considered residue. The present work sought to evaluate the extraction of phenolic compounds and their bioaccessibility from pinhão "comum" (*Araucaria angustifolia* var. *angustifolia*) and pinhão "macaco" (*Araucaria angustifolia* var. *indehiscens*) cooking water extracts during *in vitro* simulated gastrointestinal conditions. Our findings indicate that changes occurred depending on the type of extract and the gastrointestinal step. Although both of the evaluated pinhão extracts displayed bioaccessible phenolic compounds, the gradual bioaccessibility decrease of pinhão "macaco" extract during *in vitro* simulated gastrointestinal condition steps, characterizes this extract as the one with the best functional property. The functional property is related to antioxidant properties which are able to generate protective effects against various diseases.

Keywords

Araucaria angustifolia, Pinhão Extract, Phenolic, *In Vitro* Bioaccessibility, Functional Properties

1. Introduction

Araucaria angustifolia is a tree which occupies large areas in south and southeast Brazil. Araucaria seeds which possess a resistant peel are called pinhão by Brazilians. The pinhão is usually consumed by boiling the seeds in water until they become soft, however, the peel and the cooking water are usually discarded as garbage [1]. Therefore, researchers emphasize that the sustainable use of pinhão can further assist in Brazil's conservation efforts that encourage the product's rational and moderate consumption [2]. Cooked pinhão seeds have established commercial value, while their peels have been used as adsorbent to remove ions from liquid effluents [1], the edible part of pinhão, known as almond, is considered a great alternative source of lectin [3], starch [2], and phenolic compounds [4]. Santos *et al.* [5] affirmed that pinhão seeds are rich in phenolic compounds. In a previous study by Koehnlein *et al.* [6], they stated that the cooking could promote the migration of phenolics compounds from the pinhão peel to the cooking water. However, to the best of our knowledge phenolic compounds from pinhão cooking water extract have not yet been studied.

Santos *et al.* [5] also highlighted that food by-products containing bioactive substances, such as phenolic compounds, have garnered attention due to the possibility of increasing value to food industry so called wastes. Considering the effectiveness of bioactive substances in the dietary system and health-promoting effects, the demand for these natural compounds is increasing. A countless number of studies have been published focusing on phenolics compounds of different foods, which exhibit a wide range of biological effects such as protective effects against cardiovascular diseases, neurodegenerative diseases, and cancer. However, no studies so far have taken into consideration the effects of the digestive process on phenolic compounds and bioaccessibility evaluations of pinhão cooking water extract.

Concerning these evaluations, some studies even used analytical curves of gallic acid to quantify the total phenolic compounds in a sample. Additionally, was also observed that many studies expressed the total phenolic content via the Folin-Ciocalteu method. Besides that Jara-Palacios *et al.* [7] defined the bioaccessibility as the amount of a food constituent that is released from a complex food matrix in the lumen of the gastrointestinal tract and could potentially be available for absorption into the body as well as promote biological actions. These authors also point that the first step to evaluate the possible effects of a compound is to determine its stability during gastrointestinal digestion. In this case, they also highlight that *in vitro* simulated gastrointestinal condition method is simple, affordable and one reproducible alternative to in vivo and clinical models. Another positive is that the *in vitro* method is exempt of any ethical considerations regarding the study, less expensive and suitable for screening a broad array of samples in different physiological conditions.

Aiming to evaluate *A. angustifolia*, a threatened species with particular so-

cio-cultural importance, often used as an economical alternative for local residents, we decided to evaluate two different varieties. Peralta *et al.* [8] cited that due to the endangered state of *A. angustifolia*, the cultivation of this species has received strong encouragement from governmental agencies related to environment and agriculture, and many efforts have been carried out in order to propagate and conserve it, such as pinhão "comum" and pinhão "macaco". The importance of pinhão "comum" is related to its greater frequency in rural properties, while pinhão "macaco", characterized by it's hard to remove peel and is widely consumed by monkeys, is an unexplored variety.

Under the light of these facts, the present work aims to determine the total phenolic compounds content and its bioaccessibility from pinhão "comum" (*Araucaria angustifolia* var. *angustifolia*) and pinhão "macaco" (*Araucaria angustifolia* var. *indehiscens*) cooking water extracts during *in vitro* simulated gastrointestinal conditions.

2. Material and Methods

2.1. Material

The experiment was carried out with mature seeds locally regarded as pinhão "comum" (*Araucaria angustifolia* var. *angustifolia*), and pinhão "macaco" (*Araucaria angustifolia* var. *indehiscens*) harvested in 2018 in the state of Santa Catarina (Brazil) (Figure 1). The Folin-Ciocalteu phenol reagent and gallic acid used in the tests were purchased from Sigma Aldrich (St. Louis, USA).

During the *in vitro* simulated gastrointestinal conditions evaluation the following enzymes were used: α-amylase (28.75 U/mg protein); pepsin from porcine gastric mucosa (400 U/mg protein); pancreatin from porcine pancreas (digestive power − 8 × USP specifications); bovine bile salts. The aforementioned reagents were purchased from Sigma Aldrich. All chemicals used were of analytical grade.

(a) (b)

Figure 1. (a) Pinhão "comum" (*Araucaria angustifolia* var. *angustifolia*) and (b) pinhão "macaco" (*Araucaria angustifolia* var. *indehiscens*) seeds.

2.2. Preparation of Pinhão Extracts

The extract from each one variety of pinhão was obtained as described in Figure 2. Briefly, two extracts were prepared, one from pinhão "comum" seeds and another one from pinhão "macaco" seeds, which were previously washed in water and drained. Each one of these pinhão seeds varieties were cooked separately in triplicate with distilled water for 45 minutes, using a 1:100 seeds:water mass ratio, via pressure cooker (Clock®, São Bernardo do Campo, Brazil). This ratio was calculated from assays based on pinhão traditional cooking process. After the cooking, the seeds were drained, and the three extracts batches were pooled by turbulence vortex (Biomixer VTX-F, São Paulo, Brazil), in order to have a homogeneous sample. Both pinhão extracts were filtered with filter paper (12.5 cm diameter and 25 μm pore size); cooled, and maintained at a temperature of 4˚C ± 1˚C until the under *in vitro* simulated gastrointestinal conditions.

2.3. *In Vitro* Simulated Gastrointestinal Conditions

In vitro simulated gastrointestinal conditions steps were carried out in triplicate, simulating the typical predominant conditions in the human mouth, esophagus-stomach, duodenum and ileum, sequentially (Figure 3), as proposed by Verruck *et al.* [9]. Firstly, 2 mL of pinhão "comum" and pinhão "macaco" extracts were added into sterile flasks and exposed to the aforementioned gastrointestinal conditions. During *in vitro* tests pH adjustments in all samples were performed with 0.1 mol/L of $NaHCO_3$ or 0.1 mol/L HCl. The temperature was maintained at 37˚C ± 1˚C, and the intensity of peristaltic movements in each part of the human digestive system was assembled by using a water bath (Dist DI950M, Florianópolis, Brazil). The enzyme solutions were prepared right before use and filter-sterilized using a 0.22 mm-membrane filter (MF-Millipore, Billerica MA, USA).After sterilization, all solutions were kept in an ice bath until use in the simulated gastrointestinal conditions. As in the natural digestion, the

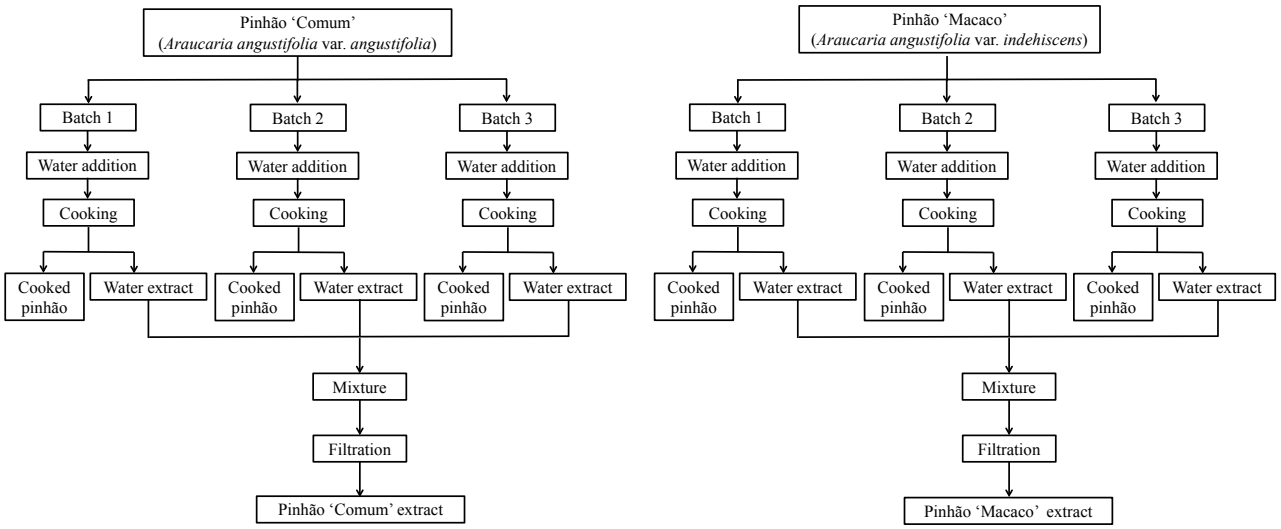

Figure 2. Diagram of the pinhão extracts preparation.

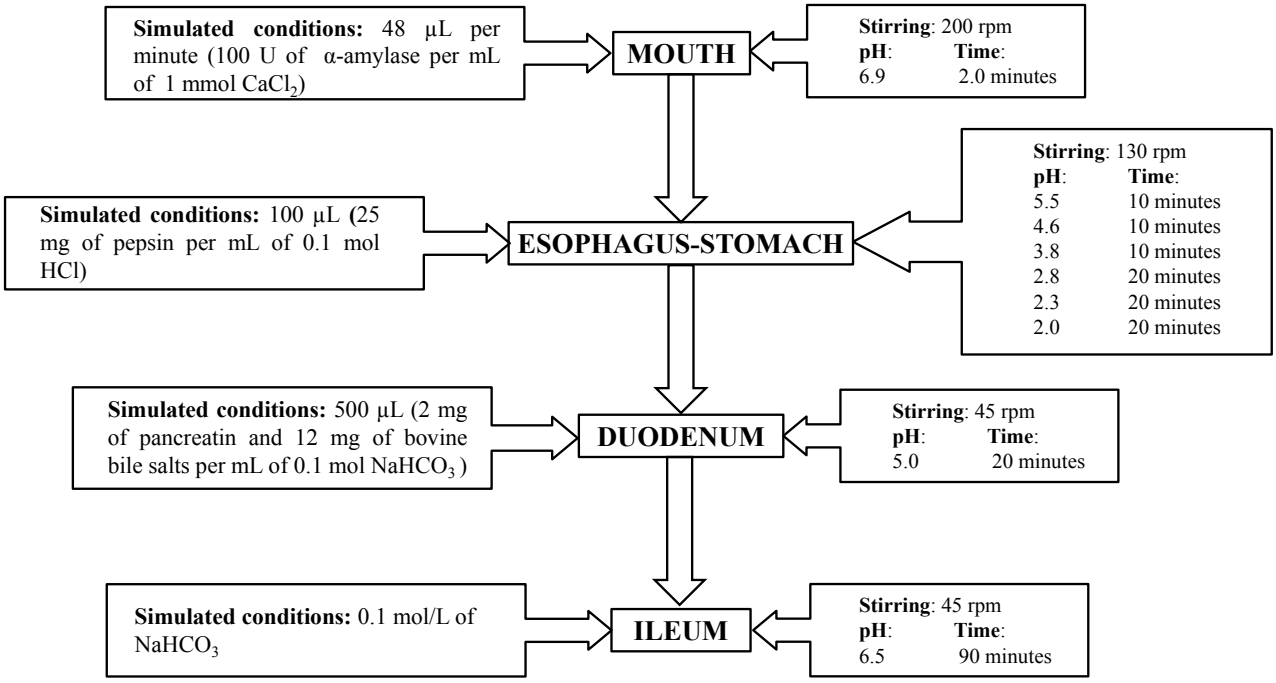

Figure 3. Protocol of pinhão extracts under *in vitro* simulated gastrointestinal conditions, by Verruck *et al.* (2015).

overall working volume increased during *in vitro* simulated gastrointestinal conditions steps, for this reason these volumes were correctly diluted before total phenolic content analyses.

2.4. Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay [10]. Pinhão extracts and aliquots of each *in vitro* simulated gastrointestinal conditions step were used for TPC analyses. In order to do this, 500 µL of pinhão extracts or samples from each step, and 2500 µL of 0.2 N Folin-Ciocalteu reagent were mixed in a vortex (Biomixer VTX-F, São Paulo, Brazil), and left still for 5 minutes. After, 2000 µL of sodium carbonate solution (7.5%) was added. This mixture was homogenized in vortex and kept in the dark for two hours. Absorbance was measured at 765 nm in a spectrophotometer (Hitachi U1800, Tokyo, Japan). Gallic acid was employed as a calibration standard and results were expressed as gallic acid equivalents (mg GAE/100 mL).

2.5. Bioaccessibility of Total Phenolic Compounds

To evaluate the effect of each step of the *in vitro* simulated gastrointestinal conditions (mouth, stomach, duodenum, and ileum) on the phenolic content, the bioaccessibility was calculated according to the Equation (1), as described by Juaníz *et al.* [11]:

$$\text{Bioaccessibility}(\%) = (\text{TPCA})/\text{TPCBX}\ 100 \quad (1)$$

where TPCA is the phenolic content (mg/g of pinhão "comum" or pinhão "macaco" extracts) quantified after simulated gastrointestinal conditions steps, and

TPCB is the phenolic content quantified before the simulated gastrointestinal conditions (initial) and expressed in the same unit.

2.6. Statistical Analysis

The data reported in all results were subjected to one-way analysis of variance (ANOVA), and Tukey test was employed to determine the significant differences ($P < 0.05$) between sample results, using the software STATISTICA 13.3 (TIBCO Software Inc., Palo Alto, CA).

3. Result and Discussion

The phenolic compounds contents (TPC) of pinhão "comum" and pinhão "macaco" extracts; as well as that of samples from each simulated gastrointestinal conditions steps are shown in **Figure 4**. Before digestion, pinhão "macaco" extract showed highest ($P < 0.05$) TPC (72.22 mg GAE/100 mL of extract) than pinhão "comum" extract (46.62 mg GAE/100 mL of extract). These results are in accordance to those found by Williams *et al.* [12], who verified that the type and bioactive compounds content could vary significantly between cultivars. Herranz *et al.* [13] cited that the phenolic compounds are usually bound to cell wall constituents such as polysaccharides and proteins, requiring the disruption of the cell wall, in order to allow for the cellular compartments to become bioaccessible. However, the phenolic bioaccessibility can be affected by some factors. Zheng *et al.* [14] highlighted that the phenolic content of foods do not reflect its bioaccessibility potential, which depends on many factors such as their release capacities from the food matrix and digestive stability. It is noteworthy to say that these points can be responsible for differences in the phenolic compounds behaviors of both pinhão extracts, before and after simulated gastrointestinal conditions steps. However, these behaviors are in accordance with those obtained recently by Herranz *et al.* [13], Jara-Palacios *et al.* [7], Qin *et al.* [15], and Zheng *et al.* [14] for onion and apple products, different extracts of white winemaking byproducts, dried fruits and seeds of *Rubusidaeus* L. and for Chinese hawthorn, respectively. It is possible to note that in our study, large changes in the TPC were found between digested and undigested samples, including between digestion steps.

After mouth step, pinhão "comum" extract showed higher ($P < 0.05$) phenolic compounds (67.02 mg GAE/100 mL) bioaccessibility, which is 2.2 times greater (**Table 1**) than pinhão "macaco" extract (44.96 mg GAE/100 mL). That is to say that the amount of released phenolics from the pinhão "comum" extract showed higher ($P < 0.05$) content of released phenolics after mouth digestion compared with the initial pinhão extract. We believe such differences might arise due to major differences in the sample varieties.

It is probable that a portion of phenolic compounds might transform into structurally different forms with different chemical characteristics, resulting consequently in different bioaccessibility results. Overall, these different levels of

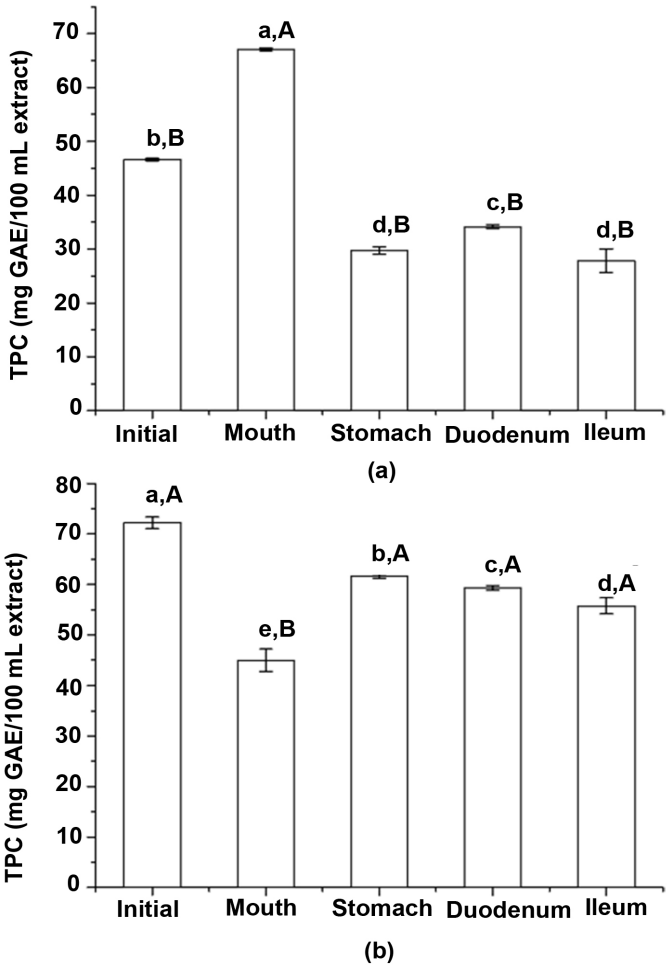

Figure 4. Total phenolic content (TPC) from (a), pinhão "comum" extract (initial) and (b) pinhão "macaco" extract (initial); and after each simulated gastrointestinal conditions step (mouth, stomach, duodenum, and ileum). [a-e]For the same sample, different lowercase letters denote significant differences ($P < 0.05$) between the gastrointestinal steps. [A-B]Different uppercase letters denote significant differences ($P < 0.05$), among the same step of the simulated gastrointestinal conditions.

Table 1. Total phenolic compounds (TPC) bioaccessibility results (median ± standard deviation) from pinhão "comum" extract and pinhão "macaco" extract after each step of the simulated gastrointestinal conditions.

Steps	Pinhão "comum" extract	Pinhão "macaco" extract
Initial	-	-
Mouth	$143.90^{aA} \pm 1.32$	$63.59^{dB} \pm 2.58$
Esophagus-Stomach	$63.25^{cB} \pm 1.66$	$86.18^{aA} \pm 1.98$
Duodenum	$73.55^{bB} \pm 0.67$	$82.39^{bA} \pm 0.66$
Ileum	$60.28^{cB} \pm 4.87$	$78.80^{cA} \pm 1.62$

Initial = is the phenolic content quantified before the simulated gastrointestinal conditions. [a-c]Within a column, different superscript lowercase letters denote significant differences ($P < 0.05$) among the different steps of the simulated gastrointestinal conditions for each sample. [A-B]Within a line, different superscript uppercase letters denote significant differences ($P < 0.05$) among the same step of the simulated of gastrointestinal conditions between samples.

TPC bioaccessibility observed after mouth step, among both pinhão extracts, may be due to their different bioactive compositions. Celep *et al.* [16] concluded that significant increase in total phenolic content after mouth step could be related to the gradual release of phenols from proteins and other biomolecules, leading to the alteration in the chemical structure and functional properties. These authors also postulated that the increment in the total phenolic acid content might be due to the stirring or the enzyme activity during the mouth step. Concerning the pinhão "macaco" extract bioaccessibility decrease, this behavior is not consistent with TPC results usually found after mouth step. However, it is noteworthy to point that gallic acid also appears, although at a lower concentration.

The low pH and the pepsin action in the esophagus/stomach steps were expected to release some phenolic compounds bound to carbohydrates, rending the phenolic compounds more bioaccessible. In this step, for the pinhão "macaco" extract the TPC (61.60 mg GAE/100 mL) was higher ($P < 0.05$) than what was found for pinhão "comum" extract TPC (29.76 mg GAE/100 mL), indicating a higher increase in the bioaccessibility of pinhão "macaco" extract (86.18%) versus pinhão "comum" (63.25%). However, according to Qin *et al.* [15], the decrease of TPC and bioaccessibility observed for the pinhão "comum" extract might be related to the high number of phenolic compounds released previously during the mouth step. Comparing the TPC after the esophagus/stomach steps with the results found at the end of duodenum step, we hypothesize that phenolic compounds previously released are also responsible for the increase ($P < 0.05$) and decrease ($P < 0.05$) observed for the TPC and bioaccessibility of pinhão "comum" (54.13 mg GAE/100 mL and 73.55%) and pinhão "macaco" (59.31 mg GAE/100 mL and 82.39%) extracts, respectively.

Regarding phenolic compounds, after ileum step, gallic acid values decreased ($P < 0.05$) for both pinhão "comum" (27.80 mg GAE/100 mL) and pinhão "macaco" (55.78 mg GAE/100 mL) extracts. This follows previous studies by Tagliazucchi *et al.* [17] and Jara-Palacios *et al.* [7], who reported that the gallic acid was degraded under pancreatic conditions. This behavior is associated with a decrease ($P < 0.05$) in the phenolic compounds bioaccessibility, after ileum step, for both pinhão extracts (**Table 1**), when compared to the results found at the end of duodenum step.

After the ileum step, we observed that pinhão "macaco" extract showed a higher ($P < 0.05$) phenolic bioaccessibility (78.80%) than pinhão "comum" extract (60.28 %). Considering that not all the phenolic compounds ingested can be absorbed after stomach step, and that the pinhão "macaco" extract showed a gradual bioaccessibility decrease ($P < 0.05$) of these compounds after stomach, duodenum and ileum steps, this leads us conclude that pinhão "macaco" extract is a greater potential source of phenolic compounds that are able to reach the colon and could be metabolized by microbiota present there.

4. Conclusion

Changes in the total phenolic content of pinhão extracts were tested before and

after simulated gastrointestinal conditions, with pinhão "macaco" extract showing the highest concentration of phenolic compounds after *in vitro* digestion. Our findings also indicated that total phenolic compounds were affected by digestion steps regardless of the type of extract. This behavior can be related to structural transformation of phenolic compounds. We observed that the high amount of phenolic compounds released during the gastrointestinal condition steps is responsible for the decrease in bioaccessibility in the next step. Still, even though both pinhão extracts displayed bioaccessible phenolic compounds, the gradual bioaccessibility decrease of pinhão "macaco" extract during *in vitro* simulated gastrointestinal condition steps, has led us to conclude that this extract is the one with the best functional properties. The functional properties credited to phenolic compounds could exhibit a wide range of biological effects such as protective effects against various diseases due to their well stablished antioxidant properties.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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