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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.11.002

Fate of polyphenols in pili (*Canarium ovatum* Engl.) pomace after *in vitro* simulated digestion

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ARTICLE INFO

Article history:
Received 6 Apr 2016
Received in revised form 27 Jul, 2nd revised form 4 Aug 2016
Accepted 14 Sep 2016
Available online 10 Nov 2016

Keywords:
Antioxidant
In vitro digestion
Phenolics
Bioavailability
Canarium ovatum
Pili

ABSTRACT

Objective: To evaluate the stability and bioavailability of polyphenols in pili (*Canarium ovatum* Engl.) pomace during simulated *in vitro* digestion.
Methods: Freeze-dried pili pomace was subjected to *in vitro* digestion simulating conditions in the stomach, small intestine and colon. Total polyphenols, anthocyanins, flavonoids and condensed tannins, and its antioxidant activity – 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azinobis-3-ethylbenzothiozoline-6-sulfonic acid, and ferric reducing antioxidant power were measured using standard spectrophotometric methods.
Results: *In vitro* digestion of pili pomace resulted in reduction of phenolic compounds. Condensed tannins and anthocyanins were released in the gastric and intestinal stages, while total polyphenols and flavonoids after fermentation simulating colonic conditions. Antioxidant values of the bioavailable fractions showed that more than 90% of activity was lost during simulated digestion.
Conclusions: Findings indicate that pili pomace is a promising functional ingredient for food and dietary supplements which can furnish potentially bioavailable phenolic antioxidants to the body.

1. Introduction

Pili (*Canarium ovatum* Engl.) is indigenous to the Philippines. Pili fruit pulp is rich in fat, and oil can be obtained by manual extraction using a mechanical pulp press. The exhausted paste referred hereinto as pomace, is the solid waste residue of such processing. Remnants consist of a mixture of peel and fibrous pulp. Typically, this by-product is turned as livestock feed and compost.

Enormous quantity of waste products, *i.e.* peels, seeds, stones and oil seed meals, are generated as a result of plant food processing. Aside from bioactive compounds such as phenolic compounds, discarded agricultural by-products are noted to contain sugars, minerals, organic acids and dietary fiber [1]. In fact, previous works have demonstrated that the amount of phytochemicals is greater in waste products compared to the edible portion [2]. Scientific work on the utilization of industrial residues from pili processing is scarce.

As opposed to the commonly used chemical extraction of dietary antioxidants, the potential physiological effects of bioactive substances from plant residues can be understood better if their stability during digestion is taken into account. Because *in vitro* methods are rapid, safe, and do not have ethical restrictions compared to *in vivo* techniques, they are extensively being used [3]. Moreover, it has been shown that *in vitro* bioavailability data correlated well with *in vivo* human and animal findings [4].

The bioavailability of the major classes of polyphenols in pili pomace has not been evaluated to date. Accordingly, an *in vitro* digestion process simulating the conditions in the stomach, small intestines and colon was utilized in the present study to determine the fate of these phenolic compounds upon consumption. The degree of antioxidant activity remaining in the digests was likewise measured.

2. Materials and methods

2.1. Raw materials

Collection of the crude defatted cake was carried out immediately after the oil extraction process. Pili pomace was

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Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

procured from a local pili pulp oil manufacturer located in Sorsogon City, Philippines. Samples were then packed into airtight polyethylene bags and shipped cold to the laboratory the next day. Test material was subjected to lyophilization and milled to a fine powder using a household grinder. Freeze-dried samples were stored in air-tight containers at −20 °C until use. Analytical grade solvents and chemicals were utilized in all experiments.

2.2. Extraction method

Lyophilized pili pomace was subjected to a slightly modified aqueous-organic extraction procedure detailed by Saura–Calixto *et al.* [5]. A 0.5 g sample weight was mixed with 20 mL acidic ethanol/water (50:50, v/v, pH 2) in capped centrifuge tubes. The mixture was shaken for an hour and then centrifuged at 2 500 r/min for 10 min. The solid residue was extracted a second time with 20 mL of acetone/water (70:30, v/v) under similar conditions. Both supernatants (ethanolic and acetonic) were combined and used for analysis.

2.3. Simulated in vitro digestion

An *in vitro* digestion procedure was performed using a previously published method by Trinidad *et al.* [6,7]. The amount of released phenolics, flavonoids, tannins and anthocyanins from pili pomace was assessed at different stages of digestion. Changes in antioxidant activities were likewise monitored.

2.3.1. Gastric phase (stomach)

Freeze-dried pili pomace (20 g) was homogenized in a blender with 80 mL deionized water. The homogenate was acidified to pH 2.0 using 6 mol/L HCl before enzymatic digestion with 3.2 mL pepsin-HCl solution (8 g pepsin in 50 mL 0.1 mol/L HCl). The mixture was incubated at 37 °C in a shaking water bath for 3 h.

2.3.2. Intestinal phase (small intestine)

An aliquot of pepsin digest (20 g) was collected. The pH of the digest was raised to 7.5 with 0.50 mol/L KOH. A 5 mL pancreatin-bile solution (1 g pancreatin and 6.25 g bile extract in 250 mL of 0.1 mol/L $NaHCO_3$) was added. The mixture was transferred to a dialysis tubing (Spectrapor 1, 23 mm width × 14.6 mm diameter, 6 000–8 000 nominal molecular weight cut-off). The tube was immersed in 0.50 mol/L $NaHCO_3$ solution. Dialyzates were collected after 3, 6, 9 and 12 h incubation at 37 °C.

2.3.3. Colonic fermentation (colon)

In vitro fermentation procedure described by Trinidad *et al.* [6,7] was used. A 0.5 g of the pancreatin-bile digest was added into a 100-mL serum bottle containing 40 mL fermentation medium (mixture of 2 L deionized H_2O, 1 L $NaHCO_3$ buffer solution, 1 L macromineral solution and 5 mL 0.1% resazurin) and 2 mL reducing solution (mixture of 1.25 g cysteine-HCl, 50 pellets KOH in 100 mL deionized H_2O and 1.25 g Na_2S in 100 mL deionized H_2O). Bottle was flushed with carbon dioxide until colorless, then capped with rubber stopper and crimped metal seal. The solution was maintained at 4 °C overnight. The following day, bottle was warmed at 37 °C in a water bath 1–2 h before inoculation. Fresh feces obtained from a healthy adult donor were homogenized with 400 mL collection media (distilled water, fermentation medium and reducing solution, 15:5:2, v/v/v). The serum bottle was filled with 10 mL of appropriately diluted faecal slurry (1:15) using a syringe and kept at 37 °C. At the end of the 24 h incubation period, serum bottle was uncapped, then mixed with 1 mL $CuSO_4$ solution (10 g/L). The fermented digest was then filtered (0.22 μm Millipore) into storage containers.

Aliquots taken from each stage were stored frozen until analyses. Samples were thereafter thawed and centrifuged at 2 500 r/min for 10 min before use. Supernatants were assayed for total phenolics, total flavonoids, condensed tannins, total monomeric anthocyanins and antioxidant activity as described below.

2.4. Total phenolic content

Total phenolics were measured according to the Folin–Ciocalteu method. For this assay, 1.5 mL Folin–Ciocalteu reagent (1:10, v/v with distilled water) was mixed with 0.2 mL of the sample and incubated for 5 min. A 1.5 mL $NaCO_3$ solution (60 g/L) was added and reacted for 90 min. Absorbance was taken at 725 nm. Total phenolic content was expressed as mg gallic acid equivalents per 100 g dry basis, calculated from a prepared gallic acid standard curve [8].

2.5. Total flavonoids

Flavonoid contents were determined using the aluminum chloride colorimetric assay [9]. The sample (1 mL) was transferred into a 10-mL volumetric flask. This was followed by the addition of 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$. Exactly after 5 min, 0.3 mL of 10% $AlCl_3$ was added. The mixture was held for another minute before adding 2 mL of 1 mol/L NaOH. Final volume was adjusted to 10 mL with distilled water. Absorbance was recorded at 510 nm. Catechin was used as reference for the calculation of total flavonoids. Total flavonoid content was expressed as mg of catechin equivalents per 100 g dry basis.

2.6. Total monomeric anthocyanins

Total anthocyanin concentration was quantified by the pH differential method [10]. In brief, two dilutions were prepared separately on each sample using two buffer systems (0.025 mol/L potassium chloride – pH 1.0 and 0.4 mol/L sodium acetate – pH 4.5) based on a predefined dilution factor. Sample aliquot and buffer solution were mixed thoroughly. The mixture was then kept at room temperature for 15 min. Absorbance was taken at 510 nm and 700 nm against distilled water blank. Total monomeric anthocyanin content was expressed as mg cyanidin-3-glucoside (MW = 449.2 and extinction coefficient (e) = 26.900) per 100 g dry weight.

2.7. Condensed tannins

Condensed tannin content was analyzed using the vanillin–HCl assay [11] with a modification. A 1 mL volume sample was added to 2.5 mL of 1% (w/v) vanillin in ethanol and 2.5 mL of 9 mol/L HCl in ethanol. The reacting mixture was incubated at 30 °C for 20 min. Absorbance was detected at 500 nm. A standard plot was constructed using a series of known catechin solutions. The level of condensed tannins was reported as mg of catechin equivalents per 100 g dry basis.

2.8. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was based on the procedure reported by Shams Ardekani *et al.* [12] with minor modification. FRAP reagent consisted of 5 mL of 10 mmol/L 2,4,6-tripyridyl-*S*-triazine solution, 5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 50 mL of 300 mmol/L acetate buffer (pH 3.6). This solution was warmed at 37 °C before use. Sample extract (0.1 mL) was reacted with 3 mL of FRAP reagent at 37 °C for 10 min. Absorbance at 593 nm was then measured.

2.9. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH procedure described by Morales *et al.* [13] was slightly modified. A freshly prepared 0.1 mmol/L DPPH ethanolic solution (2 mL) was added to 1 mL aliquot of sample extract. The absorbance of the mixture was read at 515 nm after a 30 min standing period at room temperature.

2.10. 2,2′-Azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) assay

The ABTS method was conducted in accordance to Chew *et al.* [14]. ABTS radical cation ($ABTS^{\bullet+}$) solution was prepared by combining equal quantities of 7 mmol/L ABTS stock solution with 2.5 mmol/L $K_2S_2O_8$. The mixture was allowed to react in the dark at room temperature for 12–16 h prior to use. The radical solution was diluted with 70% ethanol to give an absorbance measurement of 0.70 (± 0.02) at 734 nm. A 10 mL of diluted $ABTS^{\bullet+}$ working solution was mixed with 0.1 mL of sample and incubated at ambient temperature for 6 min. Absorbance was determined at 734 nm.

Antioxidant activities obtained by the above mentioned methods were estimated from a standard curve of Trolox. Data were reported as μmol Trolox equivalent/g of dry weight. Losses in antioxidant activity were computed as the difference between the level of antioxidant activity determined in the non-digested and digested extracts.

2.11. Bioavailability calculations

The extent of release of the phenolic compounds from the solid pili pomace matrix was estimated at each stage of digestion. The calculation of bioavailability was based on the ratio of the amount present in the supernatants of digested and non-digested samples, expressed as percentage.

2.12. Scanning electron microscopy

The insoluble residues that remained after centrifugation of the digesta were freeze-dried and examined under scanning electron microscope (S-3000N, Hitachi). Specimens were mounted on scanning electron microscope stubs with double-sided adhesive tape and photographed.

2.13. Statistical analysis

Experimental data were reported as mean ± SEM for three determinations. For multiple comparisons, results were subjected to ANOVA. Significant differences between means were analyzed by Tukey's honest significant difference test at $P < 0.05$. Statistical analysis was carried out using SPSS software (version 20.0).

3. Results

The percent bioavailable fractions of total phenolics, flavonoids, monomeric anthocyanins, and condensed tannins are depicted in Figure 1. There were substantial changes in quantity of these phenolic compounds following digestion. The amounts determined in the digests were significantly lower than the original concentration found in the undigested sample ($P < 0.05$).

Anthocyanin level in the gastric digest was almost twice higher than its undigested counterpart. This increase was found to be statistically significant ($P < 0.05$). After intestinal digestion, significant lower total anthocyanin content was observed

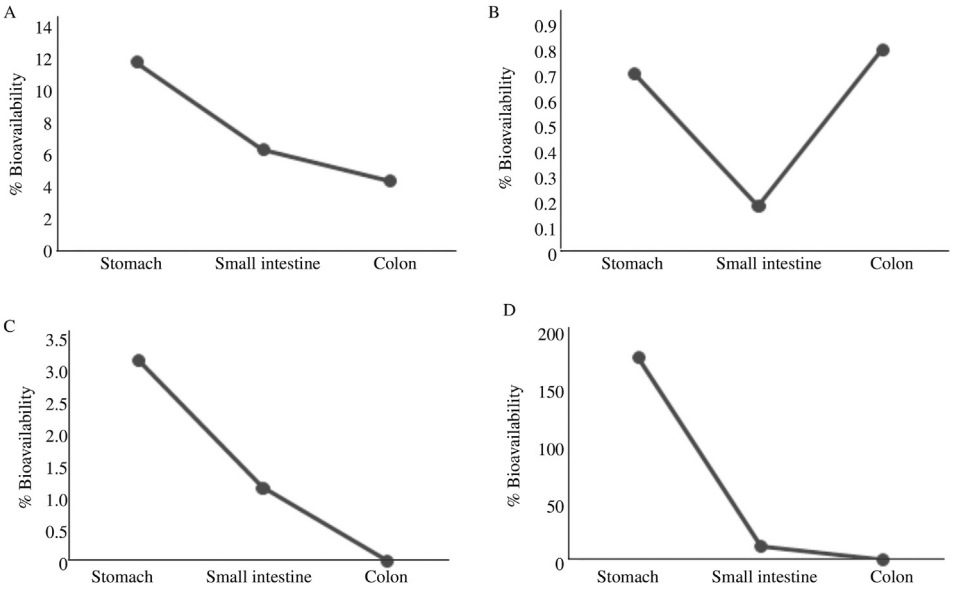

Figure 1. Recovery of (A) total phenolics; (B) total flavonoids; (C) condensed tannins; (D) total monomeric anthocyanins from pili pomace during *in vitro* digestion.

($P < 0.05$), while a further decline in total polyphenols, monomeric anthocyanins and condensed tannins was seen in the colon stage. Polyphenol decreased significantly after *in vitro* fermentation ($P < 0.05$). Both condensed tannins and anthocyanins were absent in the final digesta. However, it is interesting to note that fermentation significantly increased flavonoid bioavailability in the colon ($P < 0.05$).

Antioxidant activity was influenced by *in vitro* human digestion. All digestion products displayed significantly lower antioxidant activities than the non-digested sample (Figure 2, $P < 0.05$). As seen in Figure 2, ABTS and DPPH scavenging activities were more effective at basic pH conditions prevailing in the small intestine than in the acidic pH of the stomach. On the other hand, FRAP values were higher during gastric rather than intestinal phase.

Food is physically and chemically broken down as it travels along the gastrointestinal tract as a result of churning motions and hydrolytic enzymatic or chemical reactions. To examine the structural integrity of pili pomace particles as it underwent *in vitro* digestion, representative scanning electron microscope images of the digesta were captured at each stage. Figure 3 shows that granules were big and fully intact prior to digestion. As digestion proceeded, a gradual disruption in structure was seen. Hollow cavities became apparent and particle size decreased as well with *in vitro* digestion.

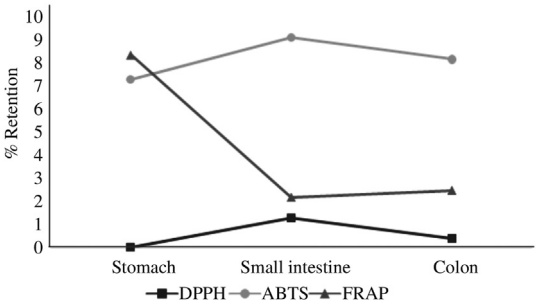

Figure 2. Remaining antioxidant activity in pili pomace during *in vitro* digestion.

4. Discussion

The simulated digestion presented in this study provided a glimpse of the changes in polyphenol content and antioxidant activity of pili pomace as it goes through the human gastrointestinal tract. Data obtained in this study support the apparent low bioavailability of polyphenols observed previously [15–17]. Results of the study revealed condensed tannins and anthocyanins were recovered only up to the gastric and duodenal stages, while flavonoids reached the colon. Recorded antioxidant values of the bioavailable fractions showed that more than 90% of activity was lost during simulated digestion. This observed reduction can be attributed to the susceptibility of these phenolic compounds to the effects of enzymes and pH in the gastric environment resulting to their decomposition. On the other hand, the strong acid and action of digestive enzymes effectively released the anthocyanins bound to the food matrix. Anthocyanins are able to survive under acidic gastric conditions due to the low pH in the stomach that favors the conversion of anthocyanins into its most stable flavylium cation form [18–21]. In relation to this, results of *in vivo* animal studies suggest that anthocyanins are directly absorbed from the stomach [15,22–25].

Under alkaline conditions in the intestines, anthocyanins are converted into labile chalcone pseudobase form, thereby promoting their rapid destruction [16,19,20,26]. Consequently, all other analyzed phenolic compounds showed greater instability in the intestinal phase as reflected by lower recovery values

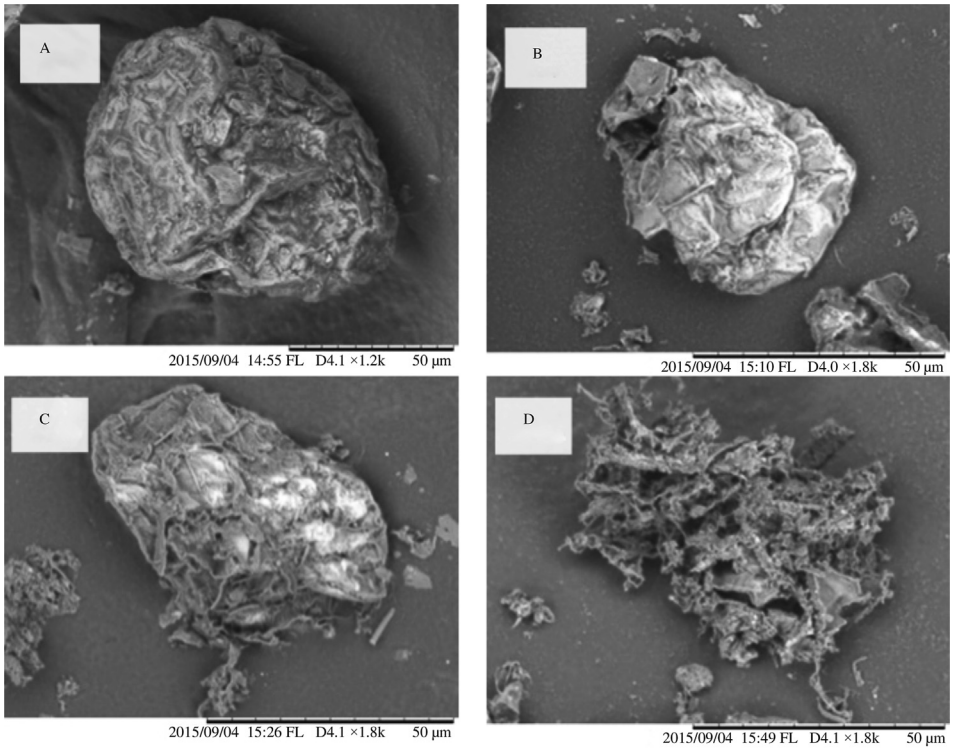

Figure 3. Scanning electron microscope morphology of pili pomace during *in vitro* digestion.
A: Initial raw material; B: Gastric stage; C: Intestinal stage; D: Colonic stage.

obtained compared to the gastric digesta. This finding further supports the aforementioned literature that phenolics are unstable at neutral or alkaline medium. As a result, majority of the ingested polyphenols in pili pomace are expected to exhibit poor intestinal absorption.

Previous studies by Bouayed *et al.* [4] and Soriano Sancho *et al.* [27] also reported that anthocyanins were not detected after small intestinal digestion. These observations add further support to the absence of anthocyanins in the colonic digest determined in this study. Meanwhile, only dimers and trimmers of condensed tannins or proanthocyanidins are absorbed through the small intestinal barrier. Unabsorbed tannin polymers that enter the colon are not inert but instead, they are degraded into low molecular weight aromatic acids. Moreover, it was shown that condensed tannins undergo many different chemical transformations in the gut, yielding soluble, non-tannin compounds [20,28,29]. This may explain why condensed tannins were also undetected in the colon.

The significant increase of flavonoids in the colonic conditions may be due to the partial release of bound flavonoids in pili pomace brought about by destruction of the entrapping matrix by the colonic microflora. These results are in agreement with Saura-Calixto and Díaz-Rubio who stated that polyphenols bound to dietary fiber are not bioavailable in the upper intestinal tract and that these complex polyphenols are absorbed and metabolized only after colonic bacterial fermentation [30]. This finding may have important implications on gut health. The presence of flavonoids and other types of phenolics can exert antioxidative actions, counteracting the harmful effects of free radicals in the colon [5,20,21,25]. A 24 h *in vitro* colonic fermentation was conducted in this study, but considering that colonic residence time can be as long as 72 h, further increase in flavonoids, as well as generation of more microbial metabolites are thus expected to occur at this stage.

At this point, it is noteworthy to mention that although an enormous amount of phenolic compounds was lost throughout the whole digestion process, it is likely that some of these complex compounds degraded into simpler forms or synthesized to create new products, while others may have undergone structural transformations, eventually escaping detection using methods described herein. It is also possible that the amount of phenolic metabolites present in the sample was below the detection limit of the methodology. Derivatives of breakdown and synthesis reactions of pili pomace polyphenols during *in vitro* digestion remain to be fully elucidated.

A number of literature have demonstrated the strong correlation between phenolic content and antioxidant activity. Thus, it can be speculated that the loss in antioxidant activity is due to the drop in polyphenol levels during digestion. However, it has been observed in the study that antioxidant activity was detected at all stages of digestion indicating that a portion of the original antioxidant activity survived the entire digestion process. Moreover, results suggest that antioxidant components in pili pomace have potential protective capabilities to combat free radicals formed throughout the digestive tract.

Besides concentration, antioxidant property of phenolics is pH-dependent. Current findings presented in this paper are in agreement with Bouayed *et al.* [4] who reported a similar trend using ABTS and FRAP methods to evaluate antioxidant activity in fresh apples at different phases of simulated digestion. pH modifications may lead to alterations in structure and conformation of phenolic compounds, consequently affecting antioxidant activity. It has been shown that antioxidant activity increased with higher pH values [4]. Thus, pH variations in the different compartments employed in the *in vitro* digestion procedure, as well as varied buffer systems used in the test solutions can impact antioxidant activity assessment. Accordingly, Bouayed *et al.* [4] and Guldiken *et al.* [16] hypothesized the suitability of FRAP assay performed in acetate buffer pH 3.6, for antioxidant measurements in the gastric digesta, while ABTS assay was carried out with phosphate buffer pH 7.4, for the intestinal digested fractions.

Recent research in our laboratory found that pili pomace contains 79% dietary fiber. This fiber which is predominantly insoluble, is highly resistant to digestive juices and enzymes, and thus can pass through the digestive tract unaltered. Together with dietary fiber, the presence of protein (5%) in pili pomace may suppress the digestive extraction of phenolic compounds. Phenolics bind strongly with macromolecules and limit their release during digestion [5,25]. The low bioavailability observed in this *in vitro* experiment could likewise be related to the incomplete hydrolysis of structural carbohydrates and protein that make up pili pomace, which in turn reduces the release of bound phenolic compounds. This assumption is further corroborated by results of the scanning electron microscopy analysis. Scanning electron microscope images revealed that although structural disintegration of pili pomace particles was evident during digestion, it was observed that some granules remained intact and did not rupture. Insoluble fiber, perhaps cellulose, which constitutes the structure of pili pomace remained essentially undamaged after digestion.

In summary, the decline in phenolic contents in pili pomace observed during digestion may be due to: (1) failure to release bound polyphenols that are chemically associated with macromolecules such as fiber and proteins; (2) instability of phenolics in the digestive environment, or; (3) anabolism and catabolism of new phenolic compounds or metabolites that are undetected using current available techniques of analysis.

Overall, findings demonstrated that *in vitro* digestion of pili pomace resulted in reduction of phenolic compounds and antioxidant activity in the gastrointestinal tract but flavonoids were more bioavailable in the colon. In addition, this research highlighted the advantage of the *in vitro* physiological approach over chemical procedures in the scientific evaluation of antioxidants. Further work is necessary to determine process variables, *i.e.*, particle size, chemical or enzymatic hydrolysis, heat treatment, that can enhance liberation of polyphenols from the cellular matrix of pili pomace, eventually leading to better absorption. This study provided initial evidence that pili pomace is a promising functional ingredient for food and dietary supplements which can furnish potentially bioavailable antioxidants to the body.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are grateful to the Department of Science and Technology–Science Education Institute (DOST–SEI) for providing scholarship to E. Arenas. This work was supported by a dissertation grant from the Student Research Support Fund (SRSF) of the DOST–SEI Accelerated Science and Technology Human

Resource Development Program. Technical expertise and assistance given by Dr. Rosario S. Sagum, Aida C. Mallillin, Amster Fei P. Baquiran, James David S. Alcantara, Queency H. Alcantara and Mark Ryan Q. Ibardaloza of the Food and Nutrition Research Institute (DOST-FNRI) is kindly acknowledged.

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