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Full Length Article

Effects of *In Vitro* Gastrointestinal Digestion on Phenolic Content and Antioxidant Capacity of Lotus Seeds N-Butanol Extract

Xiujuan Huang[1,2†], Jiamiao Hu[1†], Zhenyu Wang[1†], Shenghan Ge[1], Yilin Lin[1], Huiting Lin[1], Shaoxiao Zeng[1*] and Shaoling Lin[1*]

[1]*College of Food Science, Fujian Agriculture and Forestry University, Fuzhou, 350002, P.R. China*
[2]*Fujian Saifu Food Inspection Co. Ltd., Fuzhou, 350011, P.R. China*
[*]Correspondence author: zsxfst@163.com
[†]Contribute equally to this article

Abstract

This study investigated the changes in phenolic content and antioxidant capacity of lotus seeds n-butanol extract after *in vitro* gastrointestinal digestion. The results demonstrated that most of identified phenolics compounds remained stable in gastric digestion phase but declined after intestinal digestion. Moreover, the increases in antioxidant capacity (total antioxidant capacity; DPPH & hydroxyl radical scavenging capabilities) of n-butanol extract was observed after intestinal phase. This may attribute to newly formed digestive products with stronger anti-oxidant properties after *in vitro* digestion. In conclusion, lotus seeds may be developed as valuable dietary source of polyphenols with antioxidant potential. © 2019 Friends Science Publishers

Keywords: Lotus seeds; *In vitro* gastrointestinal digestion; Phenolic compounds; Antioxidants

Introduction

Lotus seeds, also known as lotus nuts, are considered as a functional food due to its richness in nutrients including resistant starch, polysaccharide, alkaloids and polyphenols (Zhang *et al.,* 2015). Particularly, as the naturally occurring secondary metabolites, polyphenols in lotus seeds attracted much interest due to their potential health functionality (Limwachiranon *et al.,* 2018). Previously it was also demonstrated that polyphenol-rich lotus seeds n-butanol extract (LBE) exhibited inhibitory effects on pre-adipocyte differentiation *in vitro* and suppressed diet-induced obesity (DIO) mice (Wang *et al.,* 2019).

Since the benefits of bioactive compounds are deeply affected by their absorption in digestive tract, therefore, bio-accessibility studies are important for studying the biological activities of phytochemicals (Luzardo-Ocampo *et al.,* 2017). Up-to-date, determining the bio-accessibility *in vivo* is still complicated and expensive (Juhasz *et al.,* 2011). In contrast, *in vitro* gastrointestinal digestion become widely adopted to measure the stability of phytochemicals under simulated gastrointestinal conditions. Indeed, a number of studies have highlighted that the bio-accessibility results obtained from *in vitro* assays were well correlated with *in vivo* studies (Egger *et al.,* 2018). Notably, *in vitro* methods may not only determine the stability of polyphenols upon gastrointestinal digestion, but also can measure the effects of gastrointestinal conditions on their antioxidant activity (Ryan and Prescott, 2010).

In this study, the stability of polyphenols previously identified in LBE was investigated, which has shown the anti-obesity potential in DIO mice. Furthermore, the effects of *in vitro* digestion on the antioxidant properties of this phenolic-rich extracts was also evaluated.

Materials and Methods

Reagents

The lotus (*Nelumbo nucifera* Gaertn.) seeds were provided by a local agriculture company (Fujian Lutian Co., Ltd.). The thermostable α-amylase (40000 U/g), papain (80000 U/g), Folin-Ciocalteu reagent, and phenolic standards were purchased from Solarbio (Beijing, China). The pepsin, trypsase, and bile salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform, ethyl acetate, n-butanol, and sodium bicarbonate were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Total antioxidant capacity assay kit, DPPH assay kit, and hydroxyl free radical assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Extraction of Phenolic-rich Compounds

The phenolics were extracted from lotus seeds as described (Lin *et al.,* 2018). In brief, the lotus seeds were extracted

using papain, α-amylase and ethanol (50%). The liquid-liquid partitioning was subsequently adopted to further extract phenolic-rich compounds. The obtained n-butanol extract was evaporated in vacuum and freeze-dried.

In vitro Gastrointestinal Digestion

In vitro gastrointestinal digestion was carried out as reported (Lima *et al.*, 2019). In brief, a total of 200 mg extracts was dissolved in 50 mL of NaCl (0.9%), mixed with 160 mg of pepsin and then immediately adjusted pH to 2.0 using HCl (1 *M*). The mixture was kept in water bath (37°C) with 100 rpm shaking speed in dark. After gastric digestion for 2 h, the mixture was added with $NaHCO_3$ (0.5 *M*) immediately to adjust to pH 7.0 followed by the addition of 12 mL $NaHCO_3$ (0.1 *M*) containing trypsase (2 mg/mL) with bile salt (12 mg/mL). The mixture was also immediately conducted to incubate in water bath at 37°C with 100 rpm shaking speed. All digestive juices were concerned to 10 mL and kept frozen before further analysis.

Total Phenolics Content Analysis

The total phenolics content were measured using the Folin-Ciocalteu assay (Singleton *et al.*, 1999). Briefly, sample (50 μL) was diluted in 2.0 mL H_2O and mixed with undiluted Folin-Ciocalteu reagent (250 μL) for 1 min before addition of 20% (w/v) Na_2CO_3 (750 μL) and H_2O (1950 μL) followed by 2 h incubation. The absorbance at 765 nm was recorded and gallic acid (GA) was used as the reference standard.

HPLC Analysis

The chromatographic separation was carried out on Agilent 7890A (Agilent Technologies Co. Ltd., U.S.A.) equipped with an ODS C_{18} column (0.46 × 25 cm, 0.5 μm) and a flame-ionization detector (FID). The mobile phases and the gradient elution program were illustrated in Table 1 (Lin *et al.*, 2018). The injection volume was set as 10 μL with flow rate at 0.8 mL/min and column temperature at 30°C. The analytes were monitored at 200–590 nm.

Total Antioxidant Capacity (T-AOC) Assay (Colorimetric Method)

The total antioxidant capacity was examined by a colorimetric method as described (Wei *et al.*, 2010). The working solution of chromogenic agent was prepared according to manufacturer's protocol. Then, fresh prepared working solution was allowed to react with diluted samples at 37°C for 30 min. The absorbance was measured at 520 nm. The total antioxidant capacity was calculated using the following equation:

$$\text{Total antioxidant capacity (U/mL)} = \frac{As-Ac}{0.01\times 30\times V}\times n$$

Where As and Ac represent the absorbance of the sample and blank control; V (mL) is the sample volume; n is dilution factor.

DPPH Radical Scavenging Activity Assay

The scavenging ability of antioxidant substances toward DPPH radical was examined as described (Brand-Williams *et al.*, 1995). In brief, samples (150 μL) were added into 2850 μL DPPH working solution (DPPH stock solution diluted in methanol) and kept at room temperature for 30 min in the dark. The absorbance at 517 nm was recorded against a reagent blank. The antioxidant activity was calculated as percentage inhibition of DPPH free radical as below:

$$\text{DPPH radical-scavenging rate (\%)} = \left(\frac{Ac-As}{Ac}\right)\times 100\%$$

Where A_s is the absorbance of the sample, A_c is absorbance of DPPH standard solution. Results were expressed as mg GA equivalents.

Hydroxyl Radical Scavenging Activity Assay

Hydroxyl radical scavenging activity of the extracts was determined as reported (Lopes *et al.*, 1999). The reaction solutions (KH_2PO_4-Na_2HPO_4 (pH7.4, 50 m*M*), DMSO (200 μM), Fe (III)-EDTA (1:1), H_2O_2 (120 μM)) was prepared according to manufacturer's protocol. Sample (200 μL) was then mixed with reaction mixture and the reactions were carried out at 37°C for 10 min before stopped by the addition of 1.5 mL 4% phosphoric acid (v/v) followed by 0.5 mL 1% TBA (w/v). After boiling for 15 min, the absorption of control and tested tube was measured at 550 nm.

Scavenging activity was calculated as below:

$$\text{Hydroxyl radical-scavenging rate (\%)} = \left(\frac{Ac-As}{Ac}\right)\times 100\%$$

where As is the absorbance of the sample, Ac is the absorbance of the water presented sample.

Statistical Analysis

The results were expressed as means ± standard deviation (SD). The statistical calculation (Student's t-test or analysis of variance (ANOVA) followed by Tukey test) was performed using GraphPad Prism 5 with a significance level of $P < 0.05$.

Results

Changes in the Total Phenolic Contents (TPC) of LBE

The influence of *in vitro* digestion on TPC of LBE showed that in undigested samples, the TPC was 9.75 ± 0.24 mg/g. After 2 h simulated gastric digestion, the TPC did not change significantly (9.70 ± 0.13 mg/g), indicating that the

phenolics in extracts were stable to the acidic pH during the gastric digestion. Interestingly, the TPC of samples after the intestinal digestion phase showed an obvious decrease (8.57 ± 0.14 mg/g) ($P < 0.05$ compared to samples after gastric digestion, Table 1).

Table 1: The effect of *in vitro* digestion on free and bound phenolics of freeze-dried FLS

Samples	TPC (mg/g)
Undigested samples	9.75 ± 0.24[a]
Samples after Gastric digestion	9.70 ± 0.13[a]
Samples after Intestinal digestion	8.57 ± 0.14[b]

Different letters represent significant differences between group

Changes in Phenolic Compounds of LBE

HPLC analysis was further conducted to monitor the influence of *in vitro* digestion on individual phenolic compounds of n-butanol extract. The 11 phenolic compounds were identified by matching their retention times (Fig. 1A). In addition, the analysis of obtained chromatograms also revealed majority of the peaks showed no significant shifts only with slight area decreases between the gastric digested and undigested samples, supporting the findings that phenolics are stable under acidic conditions (Fig. 1B). But after intestinal digestion, the peak chromatography of phenolics changed significantly. For example, the peak chromatography of gentisic acid almost disappeared, and it is interesting to note the peak area and height of sinapic acid increased (Fig. 1C). This phenomenon may be due to that phenolic compounds undergo transformation and be catalyzed by enzymes when exposed to gastrointestinal conditions. In conclusion, lotus seeds n-butanol extract is rich in phenolics compounds, the content and structural forms of which may change upon *in vitro* gastrointestinal digestion. Apparently, there exist plenty of peaks and some phenolics not being identified by HPLC, which may need to be analyzed by other methods (*e.g.*, mass spectrometry) in the following research.

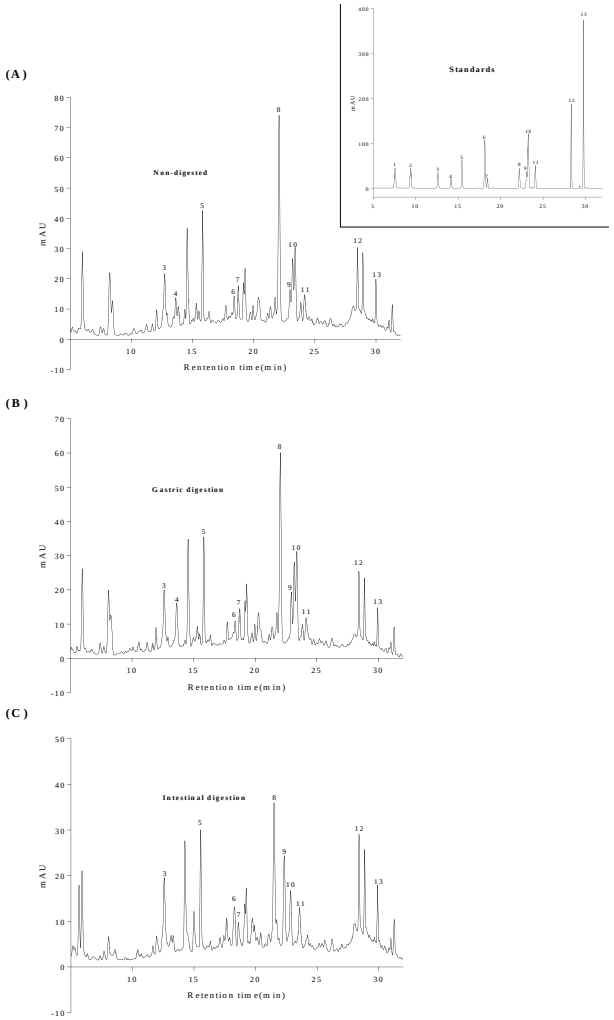

Fig. 1: Representative HPLC chromatograms of lotus seeds n-butanol extract **A**) before digestion; **B**) after gastric digestion; **C**) after gastrointestinal digestion. 1: gallic acid; 2: coumaric acid; 3: protocatechuic acid; 4: gentisic acid; 5: chlorogenic acid; 6: caffeic acid; 7: epicatechin; 8: ellagic acid; 9: sinapic acid; 10: ferulic acid; 11: naringin; 12: phloridzin; 13: cinnamic acid

Changes in Total Antioxidant Capacity (T-AOC) of LBE

To further explore the effects of *in vitro* digestion on the antioxidant capacity of LBE, the changes in the T-AOC of samples before and after digestion were evaluated. The T-AOC of the undigested n-butanol extract of lotus seeds was 4.07 U/mL (Fig. 2A). After *in vitro* gastric digestion at 37°C for 2 h, T-AOC increased to 5.28 U/mL. After intestinal digestion, a further significant increase was observed (6.20 U/mL).

Changes in DPPH Scavenging Activity of LBE

The DPPH scavenging antioxidant potential of undigested and digested samples of LBE was also analyzed. Changes in DPPH scavenging capability of LBE during the *in vitro* digestion had a similar increasing trend as T-AOC (Fig. 2B). Both gastric and intestinal digestion increased the hydroxyl scavenging activity of LBE significantly (67.10% and 72.22% *vs*. 61.51%, respectively, $P < 0.05$).

Changes in Hydroxyl Radical Scavenging Activity of LBE

The scavenging effects of n-butanol extract on ·OH was also determined (Fig. 2C). Compared with other two antioxidant index (T-AOC, DPPH), The hydroxyl radical scavenging capability of n-butanol extract showed minor changes during gastric digestion, while a significant increase ($P < 0.05$) in antioxidant capacity against hydroxyl radical was found after intestinal digestion. In conclusion, the phenolics compounds in fresh lotus seeds showed better scavenging capacity for hydroxy radical.

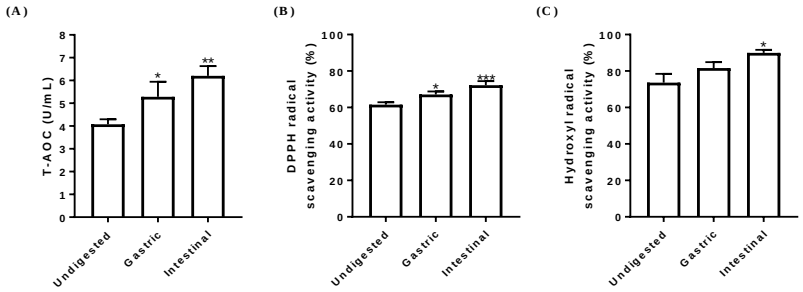

Fig. 2: Antioxidant activities of n-butanol extract obtained from lotus seeds after the simulated gastrointestinal digestion. * $P < 0.05$, ** $P < 0.01$. (**A**) Total antioxidant capacity, (**B**) DPPH scavenging activity, (**C**) Hydroxyl radical scavenging activity

Discussion

The previous studies found that n-butanol extract of lotus seeds is rich in polyphenols (Lin *et al.,* 2018), which may protect against obesity through the inhibition of adipogenesis, highlighting its potential as a functional food for the prevention of obesity (Wang *et al.,* 2019). Since understanding the changes of bioactive compounds in this extract (especially phenolic compounds) upon digestive juice treatment may provide better understanding about their possible metabolic fate and bioactivities, therefore, it was examined the stability of identified polyphenols throughout the different phases of *in vitro* gastrointestinal digestion.

In this study, obtained results suggested *in vitro* gastrointestinal digestion generally decreased the total phenolics content. These results are consistent with findings from previous reports (Gullon *et al.,* 2015; Garbetta *et al.,* 2018), that *in vitro* intestinal digestion led to losses of polyphenols while a number of phenolics appear to be stable after gastric digestion (Lima *et al.,* 2019). Several possible mechanisms have been proposed to explain this phenomenon such as bile acids-regulated bio-accessibility enhancement of polyphenols (Kida *et al.,* 2000; Yang *et al.,* 2018), and instability of phenolics in the mild alkaline pH values in the small intestine (Tagliazucchi *et al.,* 2010).

Since bioactive compounds have several antioxidant mechanisms, a good number of antioxidant assays have been developed (Alam *et al.,* 2013). Therefore, in present study three methods were adopted to evaluate the antioxidant activities. The results from these three methods demonstrated that antioxidant activities (total antioxidant capacities, DPPH scavenging capabilities and hydroxyl scavenging capabilities) of LBE were enhanced by *in vitro* digestion. The previous studies have also shown that gastrointestinal digestion may have positive effects on the antioxidant activities of polyphenol or anthocyanins-rich foods and beverages (Bermudezsoto *et al.,* 2007; Ryan and Prescott, 2010). Although the total phenolic content showed a declined trend, the *in vitro* intestinal digestion seems to have stronger effects on antioxidant activities. These results also suggested the antioxidant properties of LBE may not only be affected by the amount of phenolic compounds, but also be influenced by the physiologic properties of bioactivate compounds contained in the extract (*e.g.*, particular structures and functional groups of polyphenols). Indeed, studies have showed that polyphenols may undergo changes in structure and chemical composition when exposed to digestive juice (Burgos-Edwards *et al.,* 2018; Ovando-Martinez *et al.,* 2018), which led to the decrease in the total amount of phenolic compounds with increased antioxidant activities due to newly formed metabolites (Pavan *et al.,* 2014; Chen *et al.,* 2018). This observation had also been reported from a number of previous studies that gastrointestinal digestion, especially the intestinal step, enhances the anti-oxidant activities of phenolic compounds (Pavan *et al.,* 2014; Chen *et al.,* 2018). Indeed, previous research has shown higher pH values in intestinal digestion is an important factor resulting in the increase in hydroxyl radical scavenger activity (Tyrakowska *et al.,* 1999). This may explain that intestinal digestion has greater influences in elevating hydroxyl radical scavenging activity of n-butanol extract.

In addition, increasing evidence has suggested that dietary components with high nutritional value and antioxidant properties may provide health benefits for human beings. Taking it into consideration, the possible health benefits of lotus seeds consumption may, at least partially, derive from the antioxidant properties of their containing polyphenols.

Conclusion

Taken together, the present study demonstrated that LBE maintained strong antioxidant activity after a simulated gastrointestinal digestion. This highlighted lotus seeds may function as a valuable dietary source of polyphenols with antioxidant potential. However, further studies are needed to validate their health benefits and elucidate their metabolic fate *in vivo*.

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