Optimization, Bio accessibility of Tricin and Anti-oxidative Activity of Extract from Black Bamboo Leaves

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ABSTRACT

Introduction: Bamboo and black bamboo leaves have long been used as folk remedies for the treatment of hypertension, cardiovascular disease in oriental regions. Many studies have shown that bamboo leaves extract and its main phenolic compounds have antioxidant activity before bio accessibility assessment. **Methods:** The optimized extraction methods for biological compound tricin by pressuriz ed liquid extraction (PLE) from black bamboo leaves were studied and tricin abundant black bamboo extracts were obtained. Subsequently, the digestive stability, bio accessibility and anti-oxidative activity (total phenolics, DPPH, ABTS) of tricin and tricin abundant extract were examined by *in vitro* simulated digestion system. **Results:** The optimized extraction methods for biological compound tricin by pressurized liquid extraction (PLE) from black bamboo leaves were obtained at 200°C, 50% ethanol, 20 min static time, and 425 µm particle size, achieving high extraction efficiency of 249 mg/100 g dry leaves. In simulated digestion process, tricin was unstable in duodenum and jejunum phases but recovery reached 100% after ileum phase and released to the maximum in ileum phase. Total phenolics of the extract was increased at the end of digestion. Moreover, ABTS assay showed the best anti-oxidative activity at the ileum phase with opposite result of DPPH assay. **Conclusion:** High temperature is beneficial to extract tricin from black bamboo leaves. Tricin was kept stable and the crude extract improved the antioxidant activity during gastrointestinal digestion. The information provided a scientific basis for further study pharmacological activity of tricin and the application of bamboo extract as an antioxidant additive.

Key words: Antioxidant, Bamboo leaves, Bioavailability, *In vitro* simulated digestion, Pressurized liquid extraction.

INTRODUCTION

One of the perceived health benefits derived from consumption of antioxidants is their putative ability to prevent various diseases such as cancer, diabetes, cardiovascular. Flavonoid, a large category of plant polyphenol secondary metabolites and biologically active non-nutrients, is one kind of antioxidants and widely distributed in herbs, fruits, vegetables, etc.[1] Numerous studies suggest that estrogen-like isoflavonoids present in soy foods promote cardiovascular, skeletal, and postmenopausal health.[2] Anthocyanidins from fruits have strong antioxidant protection towards lipid peroxidation.[3]

Tricin is one of the flavonoids (Figure 1), which can be discovered in plants such as wheat and bamboo, which can inhibit cyclooxygenase enzymes and interferes with intestinal carcinogenesis in mice,[4] interfering with the growth of human-derived malignant MDA-MB-468 breast cancer cells.[5] Good efficacy, lack of toxicity and reasonable bioavailability are three crucial prerequisites for the advancement of flavonoids into clinical development as chemopreventive agents. Tricin and bamboo extract had

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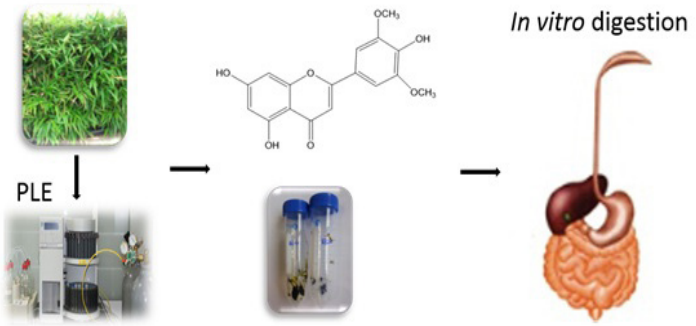

Graphical Abstract

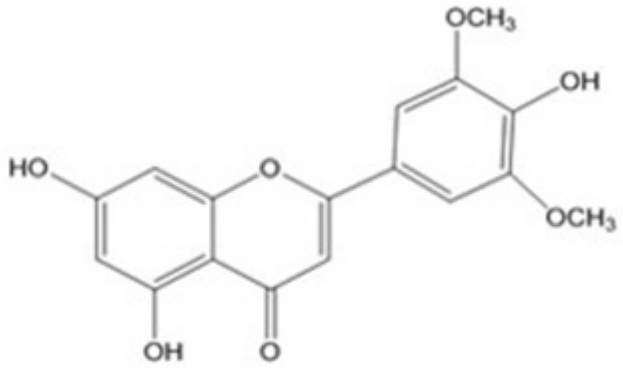

Figure 1: Chemical structure of tricin (5,7,4'-3',5'-dimethoxy-flavone)

been verified as agents without toxicity.[6] Jiao has developed the separation and purification method for getting pure tricin with efficiency from bamboo leaves.[7] However, efficiency extraction should also be consider for obtaining tricin extract, and the bioavailability should also be evaluated for the application as chemopreventative agents in functional food.

Bamboo flavonoids extract has anti-oxidative activities and had been authorized as antioxidant additives in China.[7] However, lacking of the evaluation data for bio accessibility, bioavailability generally describes its utilization following transport across the brush border and basolateral membranes.[8] Whereas bio accessibility is defined as the amount of an ingested compound that is available for absorption in the gut after digestion.[9] It's meaningful to research on the digestion of the active components and bamboo extract to give important information for the industry application.

There are two evaluation methods for the behavior of bioactive compounds during digestion process: *in vivo* and *in vitro*. However, *in vivo* method is complex, expensive, lengthy and not easy to control variables, so *in vitro* method seems to be a suitable alternative to obtain proper simulation data.[10] In this research, tricin extraction conditions were optimized by pressurized liquid extraction (PLE) method according to previous study,[11] following the stability and bio accessibility of tricin and extract were evaluated, and antioxidative activities of digestion were also researched by *in vitro* simulated digestion system.

MATERIALS AND METHODS

Chemicals and plant materials

All HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). The organic solvents used for extraction were purchased from Daejung (Gyonggi, Korea). 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, Folin–Ciocalteu's phenol reagent, catechin and potassium persulfate were purchased from Sigma–Aldrich Chemicals (Saint Louis, MO, USA). Fresh leaves (500 g) of *P. nigra* Munro were harvested on September 12, 2010, in Gangnueng City (Gangwon-do, Korea), and a voucher specimen (No. BBL-0003) was deposited at the Herbarium of KIST, Korea. The leaves were air-dried in the shade and kept at room temperature until use. Alpha-amylase, pepsin, pancreatin, pancreatic lipase, and bile extract were purchased from Sigma-Aldrich for simulated digestion.

Extraction Optimization and quantification of tricin by PLE

PLE was carried out using a fully automated ASE200 system (Dionex, Sunnyvale, CA, USA). The optimization method was same with last study.[11] Briefly, sample size, temperature, static time, and solvent ethanol in water, four variables were optimized for PLE method by univariate method, and the HPLC was used as detection method with the same conditions with the previous study.[11]

The isolated tricin from last study was used as the standard for preparing standard solutions. HPLC-grade methanol was added to tricin to produce a stock standard of 2 mg/mL. Various standard solutions were prepared from the stock solution by dilution with methanol. The concentration

of tricin from 0.0036 to 0.48 mg/mL and injection of 10 μL for the standard curve establishment. The limits of detection (LOD) and limits of quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations. LOD and LOQ were defined as signal-to-noise ratios equal to 2 or 3 and 10, respectively.

All extracts from PLE were injected for HPLC analysis. The tricin peak identification in extracts was based on comparison of its retention time and ultraviolet (UV) spectra with that of the standard. The concentration of tricin in each sample was quantified by comparing its response with the standard curves.

To determine the stability of the tricin during high temperature of the PLE, a series of experiments were performed using pure isolated compound. Briefly, tricin (1.8 μg/mL) was evaporated and placed inside the extraction cell, where it was submitted to the heating process. In order to ensure that the solvent was not purged in the extraction cell, the extraction was aborted manually after preheating for 30 min at 200°C. The content of tricin after PLE heating was measured on HPLC analysis compared to before heating.

In vitro digestion analysis

The extract (BB200) at optimized conditions was used for digestion evaluation. Simulated digestion was performed by the same method with previous studies of our center according to the method described by Garrett *et al.* with minor modifications.[12-14] In order to simulate the digestion from food to human intestine, six phases were designed to evaluate the stability and bio accessibility of extracts *in vitro* digestion system. Briefly, 100 mg of BB200 was homogenized in 12 mL of saline solution containing 120 mM NaCl, 5 mM KCl, and 6 mM $CaCl_2$ (pH 5.5). Then, 1000 units of α-amylase was added, and the pH was adjusted to 6.5. After the saline solution had been added to a volume of 12.5 mL, the samples were incubated at 37°C for 5 min in a shaking water bath (Lab companion, Jeio Tech, Korea) at 95 rpm for the simulated oral phase of digestion. To mimic the gastric phase of human digestion, the pH of the sample was acidified to 2.2 with HCl and 0.5 mL of a porcine pepsin solution (0.075 g/mL in 0.1 N HCl) was added. The samples were suspended in a saline solution to a volume of 15 mL and incubated at 37°C for 2 h in a shaking water bath at 95 rpm. To simulate the intestinal phase more accurately and specifically, that phase was separated into three intestinal parts (duodenum, jejunum, and ileum) and simulated sequentially. In the duodenum part, 250 mg of bile extract, 0.5 mL of pancreatic lipase (0.01 g/mL of 0.1 M sodium bicarbonate), and 0.5 mL of pancreatin (0.08 g/mL saline) were added and the pH was increased to 4.0 by adding 1 M sodium bicarbonate. Samples were incubated for 30 min at 37°C (final volume of 20 mL). In the jejunum part, the pH was adjusted to 5.5 and the samples were incubated for 90 min at 37°C (final volume of 22.5 mL). Finally, the pH was adjusted to 7.0 and the sample incubated for 300 min at 37°C (final volume of 25 mL) to mimic the ileum part. At the end of each digestion, digestate (100 μL) was collected and added to aqueous ethanol [1:1 (v/v)] to determine the bio accessibility. To measure the digestive stability of extracts, digestates of each intestinal phase were added to ethanol (25 mL) and extracted by sonication for 1 h. After the mixtures had been centrifuged at 12300 g for 10 min, their supernatants were collected and filtered through a 0.45 μm GHP filter (Smartpor) prior to HPLC analysis (same method with optimization part).

Measurement of in vitro anti-oxidative activity

Total phenolics analysis

Total phenolics content of the digestates from each digestion phase was determined with Folin-Ciocalteu colorimetric method with some modification. Briefly, digestates supernatants (10 μL) were mixed with 70 μL water and Folin-Ciocalteu's reagent 20 μL for 5 min, then sodium carbonate solution 100 μL (20%, v/v) was added. The mixture was stood for 30 min in the dark at room temperature then measured at 730 nm by Synergy HT-multimode microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The final result was expressed as milligram of catechin equivalent (CE) of 10 μL sample.

DPPH assay

The DPPH assay method was same with previous study.[11] A working DPPH solution (0.15 mg/mL) was prepared by dissolving 15 mg DPPH in 100 mL methanol. Five μL extracts from each digestion phase was seeded to 96-well microplate diluted by adding 45 μL methanol followed by 150 μL DPPH reagent and incubated at room temperature for 30 min. After incubation, the absorbance was measured at 515 nm using a Power wave XS microplate reader (Bio-Tek instruments, Winooski, VT). The DPPH scavenging effect was calculated as:

$$[(OD_{515control}-OD_{515sample})/OD_{515control}]\times 100\%$$

ABTS assay

The ABTS radical cation method was modified to evaluate the free radical scavenging effect of samples during the digestion phases.[15] The ABTS reagent was prepared by mixing 33 mg ABTS and 28.4 mg potassium persulfate in 900 mL water. The mixture was kept in the dark at room

Table 1: Regression data, LOD and LOQ for tricin from black bamboo leaves

Compound	Range (μg/ml)	Slope	Intercept	Coefficient of determination R^2	LOD (μg/ml)	LOQ (μg/ml)	Recovery	RSD(%)
Tricin	3.6-480	421.57	15.256	0.9997	0.54	9	115%	2.5

Values are the mean of three Calibration curves; slope and intercept refer to the regression equation, y=ax+b.

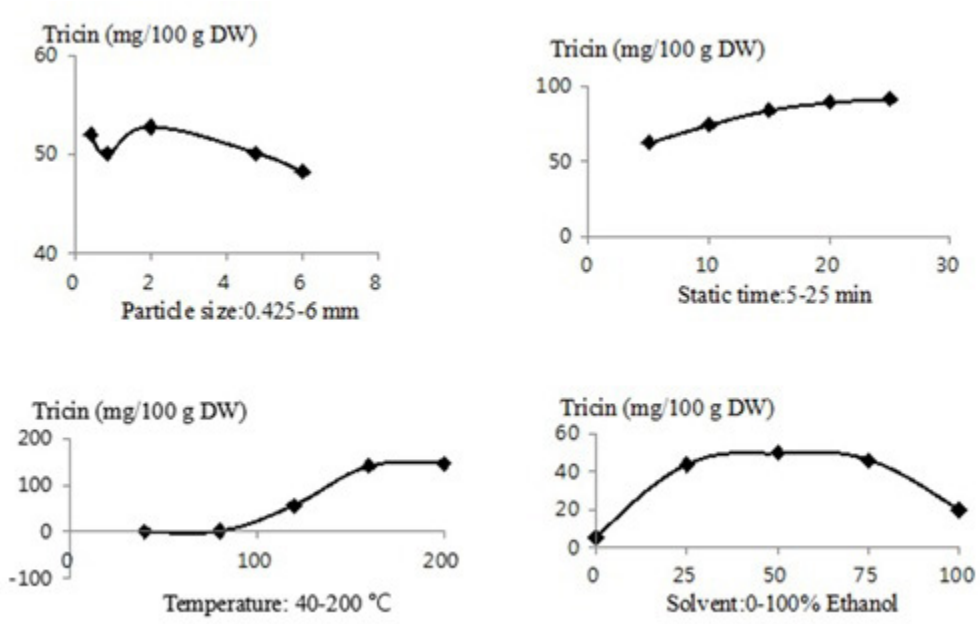

Figure 2: Effect of different extraction conditions on tricin extraction from black bamboo leaves

temperature for 16 h to allow for the completion of radical generation. To determine the scavenging activity, 5 μL extracts from each digestion phase was seeded to 96-well microplate and diluted by adding 45 μL water followed by 150 μL ABTS reagent and incubated at room temperature for 5 min. After incubation, the absorbance was measured at 734 nm using a Power wave XS micro plate reader (Bio-Tek instruments, Winooski, VT). The $ABTS^+$ scavenging effect was calculated as:

$$[(OD_{734control}-OD_{734sample})/OD_{734control}]\times 100\%$$

Data Analysis

The results are presented as the mean ± standard deviation of three replicates of each experiment.

RESULTS

The principal factors contributing to the efficiency of PLE are as follows: temperature, solvent, material state, static time, and number of steps.[11] Pressure normally has no effect on extraction results, default value 1,500 psi was used to enhance the PLE's efficiency by forcing the solvent into the matrix pores in the samples.

The standard curve of tricin was determined by HPLC analysis using pure isolated compound. Recovery of tricin (115%) was determined by pure tricin spikes in the black bamboo leaves extract from PLE; the higher recovery may be due to the interfering compounds from the extract, which may have increased the analysis errors for tricin in a higher peak area. Table 1 summarized the linear ranges and LOD (based on the signal-to-noise ratio of 3, S/N=3) and LOQ (based on the signal-to-noise ratio of 3, S/N=10). This shows that good linearity was observed in a concentration range of 0.0036 to 0.48 mg/mL for tricin with a correlation coefficient (r) above 0.9997. The LOD was 0.54 μg/mL and the LOQ was 0. 9 μg/mL for tricin; the relative standard deviation (RSD) value was 2.5% (Table 1).

The maximum tricin was obtained as 2.49 g/kg dry leaves and the optimized condition was verified as static time 20 min, extraction cycle 3, static time 5 min, temperature 200°C, and 50% EtOH (Figure 2).

To assess the stability and bio accessibility of tricin in extract during the simulated digestion, the aqueous extract of BB200 from each digestion step was submitted to

HPLC system.[11] To our knowledge, this is the first study addressing the stability and bio accessibility of tricin and extract for black bamboo leaves.

Generally, the bioavailability of a dietary compound is dependent upon its digestive stability, because the decomposition of compounds will lose the biological activities. The absorption of nutrition from the food should happen after the release of compounds from the food matrix. In here, the stability and bio accessibility of tricin in extract was evaluated during simulated digestion by measuring the amount of tricin after each digestion step. For the stability assay, the value of tricin was increased slightly during the initial digestion phase from food to stomach, following decreased markedly after Jejunum phase, and increased little much than initial after Ileum phase again (Figure 3).

There are several data in the literature regarding the polyphenol content in bamboo extracts, but there is no data regarding the polyphenols that are released after in vitro digestion and their effect on antioxidative activities.

The total phnolics content of extracts was quantified using the Folin-Ciocalteu reagent. Despite of digestion stage, the content of phenolics was increased for BB200 at the range 0.84-1.65 μg GAE/10 μL extracts (Figure 4). However, HPLC showed the same profile with original extract.

Two radical scavenging analysis methods were used to evaluate the anti-oxidative activities of extract during digestive system. For ABTS assay, the scavenging ability from 42% increased to 82% for extract (Figure 5). At the last phase, the antioxidant scavenging ability reached the highest. But for DPPH assay, the antioxidant activity was decreased largely between food phase (26%) and ileum phase (-0.89%) for stability and liberation analysis (Figure 6).

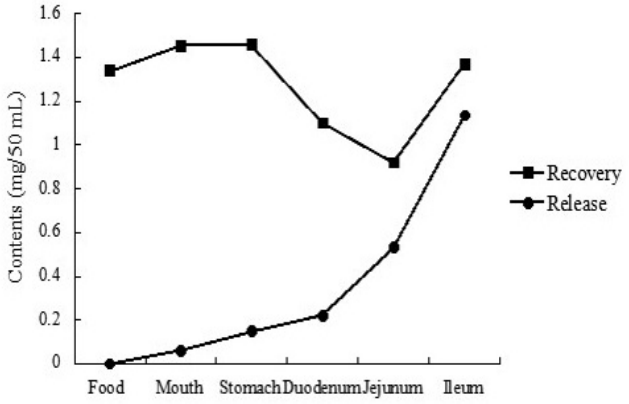

Figure 3: Digestive stability and release of tricin during simulated digestion

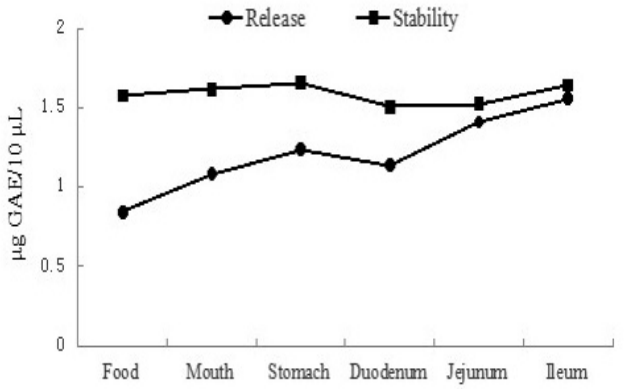

Figure 4: Total phenolics content in the extracts obtained during simulated digestion

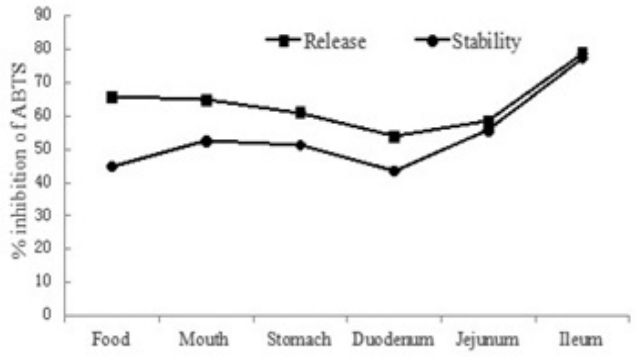

Figure 5: ABTS radical scavenging capacity of extracts obtained during simulated digestion

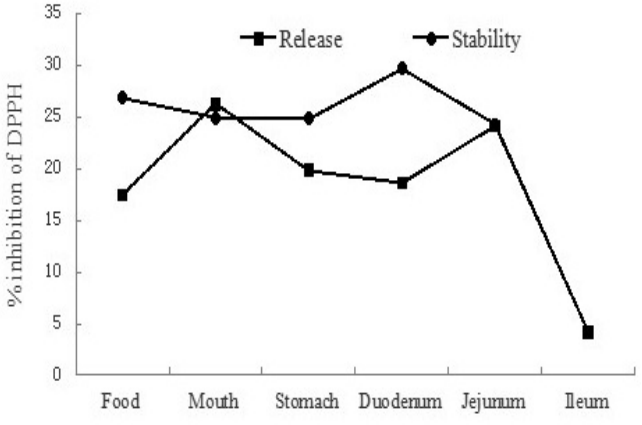

Figure 6: DPPH radical scavenging capacity of extracts obtained during simulated digestion

DISCUSSION

Fifty percent ethanol give the best extraction efficiency, which can be explained that water is quite polar for tricin, as there is a free lipophilic aglycone accumulate on the surface of plant leaves, flowers, and other tissues;[16] at the same time, tricin comprises several hydroxyl groups, which hydrophilic and generally present higher solubilities in hydroalcoholic mixtures than in a pure alcoholic solvent.[17] The best extraction efficiency was obtained at 200°C, showing high temperature is suitable to improve both mass transfer and the penetration of solvent into the sample matrix;[18] tricin was stable in high temperature during 30 min (data not shown), which means that the increasing of tricin in extract was due to the tricin glycosides isomers decomposition.[19,20] Static time has no significant effect on tricin yield comparing to solvent and temperature; until 20 min, extraction yield was a little increasing then kept stable. As we known, decreased sample size will increase the surface area, resulting shorter diffusion paths for solvent-solution interactions, and thereby influencing extraction efficacy. In this study, however, we did not observe a large difference among different particle sizes for tricin extraction.

Tricin showed a good stability of to the gastric acidic medium following decreased and then increased little much than initial at the end of digestion. It is possible that the pancreatin digestion liberates compounds (macromolecules as proteins and fiber) are able to associate with tricin. However, when pH increased after Jejunum phase the tricin increased again which maybe contributed to the release of tricin from protein-aggregates.[21] The result was identical with other research data that tricin was stable in gastrointestinal tract.[5] For bio acessibility assay, tricin was not found in food stage because *O*–methylation exists in the structure which induces the bad solubility in aqueous. But released well from mouth to intestinal digestion process, we proposed that the enzyme and pH conditions were helpful to the decomposition of the extract matrix for releasing bioactive compounds.

Total phenolics increased during in vitro digestion were verified by many researches, garlic extracts and vegetable juices were found with increased total phenolics after simulated digestion.[22,23] One explanation for this is that various enzymes transform the phenolics into different structural forms, which were undetectable by the individual HPLC analysis. On the other hand, the physical properties of food matrix and digestion enzymes will affect the efficiency of digestion of nutritions, such as phenolics bonds with proteins and other biomolecules were gradually released during the hydrolysis process in the digestive system.[23,24] The current study shows that *in vitro* digestion increased the contents of total phenolics in black bamboo extract compared to the levels prior to digestion, which are more beneficial for human health.

The increase of ABTS scavenging ability of extract maybe due to the hydrolysis of bounded or/and esterified form phenolics in extracts, as the acidic conditions in the stomach and enzymatic are helpful to release the phenolics from its bounded and insoluble forms, such as release from polysaccharides, proteins and lignin.[25,26] Consequently, the antioxidant activity of extract was underestimated because the existence of insoluble antioxidants. The underestimated antioxidant activity, which were also found in vegetable juice, garlic and cereals.[22,23,26] Considering the decrease of DPPH radical scavenging ability, some explains maybe can be given. As DPPH radical is ethanol soluble, which can be used to evaluate the antioxidant capacity of lipophilic antioxidants. However, ABTS radical is water soluble, which can be used to measure the hydrophilic and lipophilic antioxidants. ABTS assay can give an appropriate evaluation, because the digestion products were water soluble components. We can get a conclusion that the type of solvent and polarity may give effect to the radical scavenging ability.[27]

CONCLUSION

This study optimized the tricin extraction conditions by PLE, and assessed the stability and bio accessibility of tricin and extract during *in vitro* digestion process. The optimized extract conditions (2.49 g/kg dry leaves) for tricin by PLE were confirmed as: 2 g sample, 200°C, 50% EtOH, 1,500 psi, 20 min for static time, and <425 μm particle size. Tricin and bamboo extract were kept stable (100%) and good bio accessibility in *in vitro* simulated digestion process with increased anti-oxidative activity of extract. Consequently, increased total phenolics and ABTS radical scavenging capacity for extract showing the "real" anti-oxidative efficacy was following digestion phases, which indicated that the quantity and quality of antioxidant compounds extracted by solvents may not reflect their bio availability. The study of *in vitro* simulated digestion of tricin and black bamboo extract could provide a scientific basis for the pharmacological activity of tricin and the application of bamboo extract as an antioxidant additive.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

ABBREVIATIONS

DAD: Diode array detector

SEM: Standard error of the mean

LOD: Limit of detection

LOQ: Limit of quantification

RSD: Relative standard deviation

Highlights of the Paper

- MExtraction methods for tricin from antioxidant additive-black bamboo leafs by PLE was optimized.
- Optimized extract and tricin were researched by *in vitro* simulated digestion system.
- Total phenolics of the extract was increased at the end of digestion.
- ABTS assay showed the best antioxidative activity at the ileum phase.

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