Byproduct from Triphala Extraction as Tannin and Rutin Sources for Production of Gallic Acid, Isoquercetin and Quercetin by Solid-State Fermentation

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Byproduct from Triphala extraction process (BTP) was studied as a substrate for gallic acid, isoquercetin and quercetin production by Aspergillus niger fermentation in this research. The results showed that BTP was a good source of tannin and rutin. Nevertheless, the activity of A. niger on BTP as a sole substrate was very low. Supplementing nitrogen sources was found to be a key to enhancing conversion of tannin to gallic acid, and rutin to isoquercetin or isoquercetin and quercetin. BTP with 0.75 % sodium nitrate was suggested to be an optimal supplemented nitrogen source for the production of gallic acid and isoquercetin in this research, which yielded the highest contents of 61.6±2.16 mg g⁻¹DS and 3.27±0.29 mg g⁻¹DS, respectively. In addition, the highest extraction yields of gallic acid, isoquercetin and quercetin were obtained by an ultrasound-assisted extraction using methanol as an extraction solvent as 12.24±2.12 mg g⁻¹DS which was around 0.5 time higher than the one without ultrasound-assisted extraction (8.84±1.12 mg g⁻¹DS).

Keywords: triphala, solid-state fermentation, gallic acid, isoquercetin, extraction

Introduction

Triphala is a well-known traditional herbal medicine in India and Thailand with formulation of Terminalia bellirica (Bahera), Terminalia chebula (Myrobalan) and Phyllanthus emblica (amla) in the ratio 1:1:1³². Its properties come from a combination of antioxidant agents in the formula, for example, gallic acid, ellagic acid, chebulic acid, tannin, and vitamin C¹, which have all been reported for their many medicinal properties including antimutagenic², anticancer⁴, stomach ulcer relieving⁵, rejuvenating⁶, and antibacterial and antiviral⁷,⁸ effects. Thus, Triphala has been used in products of pharmaceutical, food, and cosmetics industries. Hot water is usually used for Triphala extraction; however, 35 % tannin still remains in it⁹. Therefore, it was interesting to use it as a material for tannin hydrolysis by chemical¹⁰ and enzymatic reaction¹¹,¹² to produce gallic acid. Enzymatic hydrolysis by a fungal fermentation has been recommended to produce gallic acid because of high yield and low operating cost. Some studies have reported that Aspergillus oryzae, A. awamori, and A. niger produced tannase, which was more efficient in gallic acid production by solid-state fermentation (SSF) than other species¹³⁻¹⁵. Even though gallic acid is the primary constituent in the leftover Triphala, some rutin is still present in the byproduct from Triphala extraction process (BTP) because of its low water solubility¹⁶. It can be hydrolyzed into isoquercetin (quercetin-3-O-β-D-glucoside) by the ability of A. niger to produce α-L-rhamnosidase¹⁷,¹⁸, which is specific for releasing isoquercetin from rutin by derhamnosylation. A previous report¹⁹ showed that fermentation of BTP with A. niger increased both gallic acid isoquercetin content. This was interesting because isoquercetin is rarely found in nature and is difficult to isolate. It has been reported that it has higher pharmacological activity than rutin²⁰,²¹ to improve blood flow and brain function, thus benefiting Alzheimer treatment²². It also helps maintain proper levels of blood sugar and lipid by improving pancreatic islets functions²³. Since the advantages of high-performance thin-layer chromatography (HPTLC) are less solvent requirement and ability to simultaneously run multiple samples²⁴, it was used to separate bioactive compounds.

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Materials and methods

Chemicals

Gallic acid, rutin, quercetin, and isoquercetin (AR grade) purchased from Sigma-Aldrich were used for analytic standards. They were prepared at concentration of 1 g mL⁻¹ in methanol and used immediately. The stock solutions were diluted to 0.1 mg mL⁻¹ to use as the working standard.

Preparations of spore suspension and Triphala byproduct

*A. niger* ATCC 16888 was purchased from Micro-Biologics Inc., USA. It was inoculated to potato dextrose agar (PDA), and incubated for 3 days at temperature 30 °C. Its spores on the media surface were collected by adding sterilized water, and gently scraped to obtain a spore suspension. The concentration of suspension was determined by counting in a Neubauer chamber. It was adjusted to 1·10⁶ spores mL⁻¹ using sterilized-distilled water.

BTP (N mass fraction = 0.32) was collected from the Institute of Thai Traditional Medicine after the hot-water extraction process to use as a solid substrate. To control the properties of BTP, BTP used in this research was collected from 10 extraction batches. Each batch was washed and dried at 60 °C for 1 day. All batches were then mixed and ground by hammer mill to obtain particle size of 600 microns for the SSF study. Initial tannin and rutin content in BTP substrate were also analyzed by gravimetric method and HPTLC, respectively.

Solid-state fermentation (SSF)

Solid substrate was prepared for SSF by mixing BTP with different types and concentrations of nitrogen sources, including yeast extract (N mass fraction = 0.96), sodium nitrate (NaNO₃, N mass fraction = 0.16), and ammonium sulfate (NH₄)₂SO₄, N mass fraction = 0.21), as shown in Table 1. The portion of solid substrate determined the initial moisture content by drying in hot-air oven at 105 °C for 3 h. The moisture of 30 g of BTP was then adjusted to 55 % (wet basis) with an amount of sterilized distilled water calculated by Eq. (1), and mixed with *A. niger* 5·10⁵ spores per g of dry substrate. The solid substrate was placed in 125-mL Erlenmeyer flasks. The flasks were plugged with cotton stoppers, and incubated at 30 °C for 5 days. They were then dried at 60 °C for 24 h, and milled into a powder for analysis.

\[
W_3 = \frac{55}{100} (W_1 - W_2) \tag{1}
\]

where,

- \( W_3 \) = weight of the dry substrate,
- \( W_1 \) = initial moisture content of substrate,
- \( W_2 \) = amount of sterilized distilled water needed to adjust the moisture content of substrate to 55 %.

Extraction of gallic acid, quercetin, and isoquercetin

Control sample (C) was used in the extraction study. The fermented samples were extracted by maceration with and without sonication using water, ethanol, or methanol as extraction solvents. The ratio of sample and solvent was 1:10 (w/v). They were extracted for 5 days at 30 °C to obtain the crude extract solution. In the ultrasound-assisted extraction experiments, the ultrasonication was performed at 40 kHz for 60 min once a day. The solution obtained from extraction was filtered and

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**Table 1 – Details of sample codes used for this study**

| Sample | Description |
|--------|-------------|
| C      | Control (BPT (SSF) without nitrogen added) |
| GA     | Standard gallic acid |
| ISO    | Standard isoquercetin |
| RU     | Standard rutin |
| QU     | Standard quercetin |
| YE050  | Fermented BPT supplemented with 0.50 % yeast extract |
| YE075  | Fermented BPT supplemented with 0.75 % yeast extract |
| YE100  | Fermented BPT supplemented with 1.00 % yeast extract |
| YE125  | Fermented BPT supplemented with 1.25 % yeast extract |
| YE150  | Fermented BPT supplemented with 1.50 % yeast extract |
| SN050  | Fermented BPT supplemented with 0.50 % sodium nitrate |
| SN075  | Fermented BPT supplemented with 0.75 % sodium nitrate |
| SN100  | Fermented BPT supplemented with 1.00 % sodium nitrate |
| SN125  | Fermented BPT supplemented with 1.25 % sodium nitrate |
| SN150  | Fermented BPT supplemented with 1.50 % sodium nitrate |
| AM050  | Fermented BPT supplemented with 0.50 % ammonium sulfate |
| AM075  | Fermented BPT supplemented with 0.75 % ammonium sulfate |
| AM100  | Fermented BPT supplemented with 1.00 % ammonium sulfate |
| AM125  | Fermented BPT supplemented with 1.25 % ammonium sulfate |
| AM150  | Fermented BPT supplemented with 1.50 % ammonium sulfate |
evaporated to obtain a dry crude extract for calculating the extraction yield using Eq. (2). The dry crude extracts were then collected and used for determining the quercetin, isoquercetin, and gallic acid content. The experiments were performed in triplicate.

\[
\text{Crude extraction yield} = \frac{\text{Dry weight of extract}}{\text{Total dry weight of substrate}} \tag{2}
\]

**Determination of total tannin content**

The method was modified from Makkar *et al.*

The extracts were prepared by adding 200 mg of BTP in 10-mL mixture of acetone and water (7:3) in a tube and centrifuged with Allegra X-15R (Beckman Coulter, USA) at 10,000 rpm at 4 °C for 10 min. The process was repeated until 110 mL of supernatant was collected and stabilized by keeping in ice at 0 °C for 4 h before analysis. The extracts were divided into 65-mL and 45-mL portions. The first portion (65 mL) was diluted with distilled water by 1:1 (v/v) before adding 6.5 g of polyvinylpyrrolidone (PVP) and stirring for 15 min at 4 °C. The supernatant was collected and centrifugation at 10,000 rpm at 4 °C for 10 min. 20 mL aliquots were taken from the PVP-treated portion and 10 mL aliquots from the untreated PVP (45 mL) portion. The aliquots were transferred into separate aluminum weighing dishes, and oven-dried at 100 °C until constant weight. The difference in weight of the two portions represented tannin weight and was reported as percent tannins on dry weight basis. This was done in triplicate for data analysis.

**High-performance thin-layer chromatography (HPTLC) analysis**

HPTLC was used to determine gallic acid and flavonoids, including rutin, quercetin, and isoquercetin. Four mobile phases were preliminarily tested for this study, including the mixtures of 1) formic acid: water: methyl ethyl ketone: ethyl acetate (10:30:50 v/v/v/v), 2) formic acid: water: ethyl acetate (9:9:82 v/v/v), 3) formic acid: water: ethyl acetate (1:1:6 v/v/v/v/v/v), and 4) methanol: formic acid: ethyl acetate: toluene (1:4:15:15 v/v/v/v/v/v). A twin trough glass tank (20 × 10 cm) was pre-saturated with mobile phase at room temperature for 60 min before placing HPTLC sample plates to perform linear ascending. The chromatogram height was 80 mm with 20–25 min developing time. A CAMAG TLC 3 scanner with winCATS software was used for densitometric scanning at 254 and 366 nm. The scanning speed was 20 mm s⁻¹ with 6.00 × 0.45 mm slit dimension. The retention factors (Rf) of rutin, quercetin, isoquercetin, and gallic acid were 0.08, 0.15, 0.40, and 0.55, respectively. The scanning was done in triplicate for data analysis.

A linearity study of HPTLC analysis was performed. Five concentration levels of individually prepared standard solution in methanol (20, 40, 60, 80, and 100 μg mL⁻¹) were used to determine the linearity. The solutions were spotted on a HPTLC plate with calibration range of 100–500 ng per spot. The calibration graphs were plotted as the peak area versus the standard concentration. The R² of the standards of gallic acid, isoquercetin, rutin, and quercetin standards were 0.996, 0.991, 0.994, and 0.993, respectively.

**Results and discussions**

**Solid-state fermentation of Triphala waste by Aspergillus niger**

The chromatograms of constituents in the fermented BTP were visualized under different wavelength. The chromatogram of isoquercetin of BTP was difficult to detect at UV illumination at 254 nm, but it was more visible at 366 nm and derivatizing with 10 % H₂SO₄ helped obtain a clearer band for gallic acid.

The concentration of gallic acid in the fermented BTP calculated from the band intensities is plotted in Fig. 1, together with the contents of tannin as a substrate for gallic acid production of A. niger. The results showed that supplementing nitrogen sources affected the fungal tannin utilization. The highest conversion of tannin to gallic acid was obtained in the SSF of BTP supplemented with 0.75 % nitrate (SN075). This was indicated by the highest reduction of tannin from the initial content of 133.8 mg g⁻¹ DS (156.4 mg g⁻¹ DS · (100–0.75) %) – 21.0 mg g⁻¹ DS, and the largest gallic acid production of 61.6±2.16 mg g⁻¹ DS. The effect of supplementing yeast extract and ammonium sulfate were
quite similar, especially in the experiments denoted with YE050 and AM050; YE125 and AM125; YE150 and AM150, despite the fact that nitrogen in yeast extract was in organic form, and that in ammonium sulfate was in inorganic form.

To obtain gallic acid from BTP, the production of fungal tannase was required to hydrolyze tannins in BTP. Nitrogen sources are essential not only for a fungal growth, but also for promoting fungal enzyme activity, depending on their species because nitrogen is a precursor for nucleic acid and protein syntheses in fungal cells. Nitrate assimilation process is effective in which nitrogen is consumed by nitrate and nitrite reductases, and then converted to ammonia, which further reacts with glutamic acid to produce amino acids and glutamine. Nitrate reductase is also active in producing NAD+ (Nicotinamide Adenine Dinucleotide) needed for cell respiration. Thus, it promotes the growth of A. niger and results in an increase in tannase production. Beniwal et al. found that 0.2% (w/v) sodium nitrate was suitable for tannase and gallic acid production of A. heteromorphus MTCC8818. On the other hand, 0.3% (w/v) diammonium hydrogen phosphate, (NH₄)₂HPO₄ and 1% (w/v) potassium nitrate, KNO₃, were suitable nitrogen sources for promoting tannase and gallic acid production of P. atramentosum and A. niger ATCC 16620, respectively. Nevertheless, the imbalance of the C/N ratio in substrate caused osmotic stress, which suppressed enzyme activities.

The concentrations of isoquercetin and quercetin calculated from the band intensity of BTP after fermenting for 5 days with different nitrogen supplement sources were plotted together with the remaining content of rutin, as shown in Fig. 2. The

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Fig. 1 – Remaining tannin and released gallic acid contents in the BTP fermented with different supplementary nitrogen. Initial tannin content in BTP byproduct was 156.4 ± 2.2 mg g⁻¹ DS.

Fig. 2 – Content of A = gallic acid, B = rutin, quercetin, isoquercetin in fermented BTP with nitrogen-supplemented conditions. Initial rutin content of BTP = 5.30±0.03 mg g⁻¹ DS.
production of isoquercetin and quercetin is related to the content of rutin in substrate due to ability of \textit{A. niger} to produce naringinase, a multi-enzyme consisting of \( \alpha \)-\( \text{l} \)-rhamnosidase and \( \beta \)-glucosidase. \( \alpha \)-\( \text{l} \)-rhamnosidase hydrolyzes rutin to isoqueretin, and \( \beta \)-glucosidase hydrolyzes isoqueretin to quercetin.\(^{44}\) Compared with the rutin content from BTP in the control sample (1.43\( \pm \)0.06 mg g\(^{-1}\) DS), the highest decrease was detected in the SN075 sample (0.59\( \pm \)0.08 mg g\(^{-1}\) DS), which was not significantly different from YE050, SN050, AM050, YE075, and SN125 samples. Of the aforementioned samples, the highest isoqueretin content (3.27\( \pm \)0.29 mg g\(^{-1}\) DS) was found together with the lowest quercetin content (0.21\( \pm \)0.12 mg g\(^{-1}\) DS) with the SN075 sample. When compared with control sample, it was 57.5 and 9.6 times higher on gallic acid, followed by isoqueretin content. This suggested that supplementing BTP with sodium nitrate 0.75 % (SN075) enhanced only the conversion of rutin to isoqueretin, but decreased that of isoqueretin to quercetin. In other words, using SN075 condition raised \( \alpha \)-\( \text{l} \)-rhamnosidase activity but reduced that of \( \beta \)-glucosidase. On the other hand, supplementing nitrogen sources as in the conditions of SN050, AM050, YE100, AM100, YE125, and SN125 samples provided highest quercetin content (\( \sim \) 2.03–2.37 mg g\(^{-1}\) DS) with the lowest isoqueretin content (\( \sim \) 0.27–0.43 mg g\(^{-1}\) DS). Largely different conversions of rutin to isoqueretin, and isoqueretin to quercetin were found with different concentrations and types of nitrogen supplement sources. Moreover, the obtained isoqueretin contents in experiments with high nitrogen sources (AM125, YE150, SN150 and AM150) were found to be 3.5, 4, 2.4, and 8.3 times greater than in the control sample, even when the reductions in rutin level as its precursor were not significantly different. This showed that the addition of high content of nitrogen sources enhanced the utilization of rutin in the isoqueretin production only, but adding lower nitrogen content enhanced both the consumption of rutin, and conversion of rutin to isoqueretin. At the same time, using high nitrogen supplement levels depressed the conversion of isoqueretin to quercetin, except in the AM150 sample. Obviously, the types and concentrations of nitrogen sources used affected the \( \beta \)-glucosidase production as well as the observed quercetin content.\(^{45,46}\)

**Evaluation of maceration and ultrasound-assisted extraction with different solvents**

Yields of crude extracts obtained from the maceration and the ultrasound-maceration extractions of the fermented BTP are shown in Fig. 3. The maximum extraction rates, estimated from the slope of curve, were found in the first 24 h for all extraction experiments of 0.06\( \pm \)0.03, 0.09\( \pm \)0.04, 0.12\( \pm \)0.04, 0.24\( \pm \)0.07, 0.20\( \pm \)0.06, and 0.32\( \pm \)0.06 mg g\(^{-1}\) DS h\(^{-1}\) on WM, WUM, EM, EUM, MM, and MUM, respectively. This showed that ultrasonication increased the extraction rates by 1.5–2 times on water and ethanol extractions, but slightly on that of methanol. Regarding the extraction solvent in the maceration method, the highest extraction yield was found at 24 h with methanol as a solvent (MM, 8.84\( \pm \)1.12 mg g\(^{-1}\) DS), followed by that of ethanol (EM, 5.56\( \pm \)1.10 mg g\(^{-1}\) DS), and water (WM, 2.23\( \pm \)0.29 mg g\(^{-1}\) DS). The results clearly showed that the ultrasonication increased the extraction yield to the values of 12.24\( \pm \)2.12, 9.13\( \pm \)2.14, and 3.97\( \pm \)1.21 mg g\(^{-1}\) DS in methanol, ethanol, and water solvents, respectively. After maceration at 120 h, it was found that MM reached 1.7 and 3.3 times higher crude extraction compared to EM, followed by WM. For the ultrasound-maceration, the MUM achieved 1.2 and 4.9 times higher crude extraction compared to EM, followed by WM.

Most constituents in the fermented BTP had an affinity to methanol, the polarity (relative polarity 0.762) of which was between that of ethanol (relative polarity 0.654) and water (relative polarity 1.0)\(^{47}\). Moreover, ultrasonication significantly increased ethanol and methanol extraction yields (EUM, MUM), while it increased water extraction yield only slightly (WUM). The ultrasonication enhanced the extraction performance by generating cavitation bubbles in the suspension. Microjets produced by implosion of the cavitation bubbles improved the penetration of solvent into pores of BTP substrate, and increased the contacting surface area between BTP and solvent.\(^{48–50}\)

The content of target products, including quercetin, isoqueretin, and gallic acid in the fermented
BTP (without nitrogen supplement) quantified with HPTLC are shown in Fig. 4. The highest yields were obtained by methanol extraction with ultrasound-assisted maceration (MUM), and amounted to 0.028±0.001 mg g⁻¹ DS, 0.017±0.001 mg g⁻¹ DS, and 0.033±0.002 mg g⁻¹ DS for quercetin, isoquercetin, and gallic acid, respectively. All those components exhibited higher solubility in methanol than in ethanol and water, both for extractions with and without ultrasonic assistance. When compared among each other, the MM was 1.3, 0.9, 3.4 times higher than EM in quercetin, isoquercetin, and gallic acid content, respectively, and 67.8, 1.37, and 24.8 times higher than WM in quercetin, isoquercetin, and gallic acid content, respectively. For the ultrasound-maceration, the MUM was 1.1, 0.9, and 2.1 times higher than EUM in quercetin, isoquercetin, and gallic acid content, respectively. Even though methanol and ethanol maceration slightly differed in the quercetin and isoquercetin extraction, the superiority on gallic acid content in methanol made it a preferred solvent for the extraction in this study. The results contrast those of the works of Chebil et al.⁵¹ and Valentová et al.²¹, where higher solubility was achieved in water than in ethanol and methanol. Also, the finding that quercetin was more soluble in methanol than ethanol was inconsistent with the results of Idris et al.⁵² The results of this study imply that MUM technique can be applied to the BTP with supplemental nitrogen to enhance higher productivity of gallic acid and isoquercetin due to their better solubility in methanol, while sonication makes their extraction more efficient.

Conclusions

Tannin and rutin leftovers in the byproduct of Triphala extraction process were detected as possible substrates for gallic acid, isoquercetin, and quercetin production by solid-state fermentation with Aspergillus niger. However, the fungus required a supplemental nitrogen source to enhance the productions. The addition of 0.75 % sodium nitrate was found appropriate for production of gallic acid and isoquercetin, while supplementing 1.00 % sodium nitrate or 1.00 % yeast extract or 1.00 % ammonium sulfate enhanced quercetin production. Ultrasound-assisted maceration was found to be a technique that increased productivity of gallic acid and isoquercetin from the nitrogen-supplemented BTP substrate.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
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