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Impact of fermentation degree on phenolic compositions and bioactivities during the fermentation of guava leaves with *Monascus anka* and *Bacillus* sp.

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Chemical compounds studied in this article:
Gallic acid (Pubchem CID: 370)
Rutin (Pubchem CID: 5280805)
Isoquercitrin (Pubchem CID: 5280804)
Quercetin-3-O-β-D-xylopyranoside (Pubchem CID: 5320861)
Quercetin-3-O-α-L-arabinopyranoside (Pubchem CID: 5481224)
Avicularin (Pubchem CID: 5490064)
Quercetin (Pubchem CID: 5280343)
Quercitrin (Pubchem CID: 5280459)
Kaempferol (Pubchem CID: 5280863)

ABSTRACT

Guava leaves (GLs) were processed by solid-state co-fermentation with *Monascus anka* and *Bacillus* sp., and the impact of the fermentation degree on the phenolic composition and their bioactivities was investigated. Three representative HPLC fingerprints of phenolic compositions from GLs can be clearly distinguished for the different fermentation degrees. Four characteristic compounds, including gallic acid, quercetin-3-O-α-L-arabinopyranoside, quercetin, and kaempferol, that significantly responded to the changes of bioactivity in the fermented GLs were identified as the marker components by principal component analysis. High levels of the four marker components in the HPLC fingerprints are an indication that the fermented GLs are mature. In addition, the correlation analysis confirmed that the total phenolic, total flavonoid, and the quercetin and kaempferol contents strongly correlated with its bioactivities. These results can provide a theoretical basis to quickly evaluate and control the mature degree of fermented products, which will help to obtain high bioactive fermented products.

1. Introduction

Guava (*Psidium guajava* L.) belongs to the Myrtaceae family and is extensively cultivated throughout tropical and subtropical areas. The fruits of this plant are used as sources of functional beverages due to their pleasant taste and nutritional value. In addition to the fruits, the leaves also have a number of uses. In Brazil and China, guava leaves (GLs) have long been used as an edible folk herb or tea product to treat diarrhea and hyperglycemia (Gutiérrez, Mitchell, & Solis, 2008; Shao et al., 2012). Previous phytochemical research has confirmed that the bioactive components of GLs included phenolics, flavonoids, and polysaccharides. Among these, the phenolics and flavonoids are the main phenolic components and possess multi-directional biological activities such as antioxidant, hypoglycemic and hypertensive effects (Alshikh, de Camargo, & Shahidi, 2015; Ojewole, 2005; Vadivel & Biesalski, 2011).

Many researchers have recently verified that microbial fermentation can further enhance the release of phenolic components and the bioactivities of tea products, cereal food and agro-products. For example, solid-state fermentation was applied to enhance the antioxidant capacities of black bean, green tea, oat and plant matrices (Bei, Liu, Wang, Chen, & Wu, 2017; Cheng, Wu, Lin, & Liu, 2013; Dulf, Vodnar, & Socaciu, 2016; Hur, Lee, Kim, Choi, & Kim, 2014; Lee, Hung, & Chou, 2008; Liu, Costa et al., 2017; Liu, Wen et al., 2017; Liu, Zhang et al., 2017). Fermentation is used because microorganisms produce many enzymes that can break the linkages between phenolics and other substituents in molecular conjugations to release their phenolic components (Dulf et al., 2016). In our previous work, solid state co-fermentation with *Monascus anka* and *Bacillus* sp. could further enhance the bio-activity by releasing of phenolics contents from GLs. *Monascus anka* can also produce some natural functional bioactive compounds, such as γ-aminobutyric acid, Monacolin K, and pigments. However,

Abbreviations: GL, guava leaves; DM, dried mass; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt; Vc, ascorbic acid; TF, total flavonoids; TP, total phenolics; GIA, α-glucosidase inhibitory activity; PCA, principal component analysis; IFS, initial fermentation stage; MFS, mature fermentation stage; OFS, over-fermentation stage

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some reports also confirmed that, as the fermentation time increases, phenolics can continue to be hydrolyzed and produce bioactive compounds that lead to a loss of antioxidative activity (Othman, Roblain, Chammen, Thonart, & Hamdi, 2009). Dulf et al. (2016) found that the antioxidative capacity of food products was significantly reduced after overly long fermentation. Zhang et al. (2017) studied the dynamic changes in the biochemical composition and antioxidant activity of tartary buckwheat leaves during fermentation by *Aspergillus niger*. Their results revealed that the phenolic composition and antioxidant activity of the tartary buckwheat leaves were also divided into three stages that changed their concentrations during fermentation: increasing at the early stage of fermentation, declining at the medium stage, and changing slightly during the last stage. Consequently, the fermentation degree has an important effect on the phenolic compositions of fermented functional products and their bioactivities. Very little literature focuses on how to quickly evaluate the mature degree of fermented products. In recent years, HPLC fingerprints combined with chemometric analysis have been shown to have the potential to serve as a sensitive and quick method to differentiate the tea leaves or herbs from different seasons, regions or manufacturing steps (Alaerts et al., 2012; Wang et al., 2011; Yudthavorasit, Wongravee, & Leepipatpiboon, 2014). Therefore, HPLC fingerprinting technology was first introduced to evaluate the effect of fermentation degree on the biochemical composition of GLs.

In this study, *Monascus* anka and *Bacillus* sp. were utilized to conduct the fermentation process on GLs, and the effect of the degree of fermentation on its antioxidant ability, *α*-glucosidase inhibitory activity, and chemical compositions were investigated. Importantly, HPLC fingerprinting combined with chemometric analytical methods were first applied to evaluate how mature the fermented products were. We expect that this study will provide a theoretical basis to increase the bioactivities of fermented products.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid, quercetin-3-O-*α*-L-arabinopyranoside, quercetin-3-O-*β*-D-xylopyranoside, rutin, isoquercitrin, quercitrin, avicularin, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH, > 99.7%), 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, > 99.7%), *α*-glucosidase (EC 3.2.1.20), *p*-Nitrophenyl-*α*-D-glucopyranoside, and Trolox were all purchased from Sigma-Aldrich (St. Louis, MO, USA). $FeCl_3$, Folin-Ciocalteu phenol reagent (> 99.8%), potassium persulfate ($K_2S_2O_8$, > 99.8%), and Na_2CO_3 were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). HPLC-grade formic acid and acetonitrile solvents were purchased from Fisher Scientific (Waltham, MA, USA). Guava leaves were provided by the Jiangmen Nanyue Guava Farmer Cooperatives (Jiangmen, Guangdong, China).

2.2. Solid-state co-fermentation (SSF)

Monascus anka GIM 3.592 was deposited in the publicly accessible culture collection GDMCC/GIMCC, Guangdong Culture Collection Centre of Microbiology, China. *Bacillus* sp. was provided from School of Biology and Biological Engineering, South China University of Technology. The *M. anka* GIM 3.592 inoculum contained 20 g L^{-1} glucose, 3 g L^{-1} yeast extract, 10 g L^{-1} peptone, 4 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl, and 0.01 g L^{-1} $FeSO_4{\cdot}7H_2O$. The *Bacillus* sp. inoculum contained 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl. *M. anka* was cultivated in a 250-mL Erlenmeyer flask containing 50 mL of media at 30 °C that was shaken at 200 rpm for 30 h. The *Bacillus* sp. was cultivated in a 250-mL Erlenmeyer flask containing 50 mL media at 35 °C that was shaken at 200 rpm for 20 h. The composition of the fermentation substrate media was GLs (25%, *w/w*, air-dried) and rice flour (15%, *w/w*). The mixture of the SSF media was steam-sterilized at 121 °C for 15 min before it was used. After being thoroughly mixed with the *Bacillus* sp. inoculum (5%, *v/w*) and the seed cultivation of *M. anka* GIM 3.592 (10%, *v/w*), the SSF media was incubated on a round screen (4-mesh, GB/T6003.1, China) at 28 °C for 20 days under 65% relative humidity. The substrates were turned over to re-disperse and release the heat that had been generated on the 11th day. During fermentation, approximately 1 g of sample was removed to carry out the chemical characterization. The sample was dried and stored at 20 °C in a drying cylinder with a silica gel indicator. All of the experiments were performed in triplicate.

2.3. Extraction of the phenolic components

A total of 0.4 g (dry mass, DM) of dried fermented GLs powder was extracted with 10 mL of 70% methanol by ultrasonic extraction (320 W, 40 °C) for 30 min. Then, the suspension was filtered by passage through filter paper (0.45 μm, Whatman, Maidstone, UK). All of the sample extracts were stored at −20 °C before analysis.

2.4. Total phenolic and flavonoid content

The total phenolic content (TPC) of all of the sample extracts was monitored as described with a minor modification (Wang, Wei, Tian, Shi, & Wu, 2016). Briefly, the reaction system (180 μL) containing 30 μL of Folin–Ciocalteu reagent, 150 μL of 20% Na_2CO_3 and 100 μL of the phenolic extracts or a standard solution of gallic acid was incubated at 35 °C for 30 min. The absorbance was measured using a SpectraMax Gemini microtiter plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 760 nm (A_{760}) with purified water as the blank. A standard curve was plotted using gallic acid (10–100 μg mL^{-1}) as a standard (R^2 = 0.9995). The phenolic contents were all expressed as mg of gallic acid equivalents (GAE) g^{-1} sample in a dry mass (DM). Samples were measured in triplicate. All of the reported values were presented as the mean values ± the standard deviation (SD). The total flavonoid content (TFC) was determined as previously described with a slight modification (Cai et al., 2011). Briefly, 100 μL of the extract was placed in a 2-mL Eppendorf tube. Seventy percent methanol solution was added to bring the volume of the solution to 500 μL. Then, 30 μL of 5% $NaNO_2$ solution was added. After standing at room temperature for 5 min, 30 μL of 10% $AlCl_3$ solution was added, and the mixture was left to react for an additional 6 min. Then, a total of 200 μL of 1 M NaOH was added, and the total volume was brought to 1 mL with 70% methanol. The solution was thoroughly mixed again and allowed to stand for 30 min at 35 °C. The absorbance was determined at 510 nm using the 70% methanol solution as a blank. The total flavonoid contents were expressed as mg rutin equivalents (RE) g^{-1} sample in dry mass. The calibration curves of rutin were prepared with a standard chemical concentration between 0.01 and 0.1 mg mL^{-1} (R^2 = 0.9997).

2.5. HPLC analysis

A Waters 2695 HPLC with a DAD detector (Waters 2998, USA) was used to analyze the phenolic compositions of the sample extracts. A Zorbax Eclipse Plus C18 column (250 mm × 4.6 mm, 5 μm, Waters) was used to separate the phenolic compounds. A non-linear gradient elution was carried out using 0.1% formic acid (solvent A) and acetonitrile (solvent B) at 0.8 mL min^{-1} with an increasing time course of solution B to 15% B during 0–5 min, 15–20% B during 5–10 min, 20–25% B during 10–20 min, 25–35% B during 20–30 min, 35–50% B during 30–40 min, 80% B during 40–44 min, and 15% B during 44–50 min. The column temperature was set at 30 °C. The detection of the biochemical component was conducted between 200 and 550 nm wavelengths.

2.6. Determination of the individual phenolic compounds

Nine of the main phenolic compounds were quantified by external calibration using the standard analytes with a combination of the