



## IMPACT OF *IN VITRO* DIGESTION PHASES ON ANTIOXIDANT PROPERTIES OF MONASCAL WAXY CORN FROM 2-STEP FERMENTATION

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### Short communication



### ABSTRACT

The stability of monascal waxy corn (MWC) during *in vitro* digestion of MWC on bioaccessibility, using a standardized *in vitro* gastrointestinal digestion was focused on DPPH free radical scavenging ability, chelating ability on Fe<sup>2+</sup>, monacolins K and citrinin content of MWC fermented by *Monascus purpureus* TISTR 3090. The simulation of *in vitro* digestion was comprised of two stages: gastric and intestinal digestion. The results were found that, after gastric digestion, monacolins K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> of MWC of the sample were increased 1.35, 8.69, 33.33 and 13.47 %, respectively, suggesting a release of high potential antioxidants. After pancreatic digestion, however, monacolins K and citrinin contents and DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> between soluble and insoluble MWC were decreased between 14.69 and 76.92 % which these values were lost during treatment with pancreatin. It indicated that pancreatic digestion causes reduced antioxidant activity between soluble/insoluble fractions while the gastric digestion promoted more releasing the antioxidant activities.

**Keywords:** Stability; Monascal waxy corn; *In vitro* digestion

### INTRODUCTION

Monascal rice or angkak is one of functional foods which has been used in East Asian countries for centuries. Monascal rice is a dietary staple and is used to make rice wine, as a flavoring agent, and to preserve the flavor and color of fish and meat (Bunnoy *et al.*, 2015). *Monascus purpureus* is a red mold species, which produces the various hydrolyzing enzymes in order to convert the starchy substrates (white rice) into the secondary metabolites biosynthesis during the fermentation, i.e. the complexes formed between significant antioxidants and *Monascus* pigments (Kongbangkerd *et al.*, 2014). *Monascus* leads to the production of monacolins K and several antioxidants affecting DPPH free radical scavenging ability, inhibition of peroxidation, reducing power and chelating ability on Fe<sup>2+</sup> (Yang *et al.*, 2006). Moreover, monacolins K is a cholesterol-lowering agent. The mechanism of action monacolins K to block cholesterol production is an HMG-Co A (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibited competitively with respect to HMG-Co A (Saito *et al.*, 1991). According to this standpoint, *Monascus* pigments have been only determined through *in vitro* antioxidant activities and they showed high *Monascus* antioxidant activities from monascal rice products (Yang *et al.*, 2006). Besides, the antioxidant activities of monascal products after and/or during gastrointestinal digestion are doubted. Hence, the antioxidant activities of this product should be analyzed through mimicking *in vitro* gastrointestinal digestion. This model digestion benefit is a first approach to predict the *in vivo* antioxidant activities of substances (Emmons *et al.*, 1999; Zielinski and Kozłowska, 2000). Therefore, this research was aimed to determine monacolins K and citrinin contents and antioxidant capacities of MWC employing *in vitro* study.

### MATERIAL AND METHODS

#### Conventional fermentation method and 2-step fermentation of MWC

Conventional fermentation or step 1, 100 g of waxy corn seeds and 12.08 % of monosodium glutamate (MSG) (equivalent to 1.00 % N<sub>2</sub> content) were put into a flask 500 mL. The mixture was sterilized in an autoclave at 121 °C for 15 min

and then left until cool down. About 5 mL of 10<sup>6</sup> spores/mL spore suspension of *M. purpureus* obtained from actively growing slants in sterile water was inoculated into sterilized waxy corn and incubated at 25 °C for 12 days. Step 2 fermentation, MWC was then reinoculated with the same volume and spore suspension contents and continuously fermented with the same condition as the conventional method for another 12 days (Kraboun *et al.*, 2013). Then, the product was dried in an oven at 40 °C for 24 h. A fine powder (20 mesh) was obtained using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany). The sample was determined DPPH free radical scavenging ability, chelating ability on Fe<sup>2+</sup>, monacolins K and citrinin content.

#### Study on the antioxidant stability of *Monascus* pigment from MWC via *in vitro* digestion

*In vitro* digestion of MWC, the technique of Miller *et al.* (1981), modified to our requirements, was followed. It was comprised of two stages: gastric and intestinal digestion. Shortly before use, 0.4 g of pepsin was dissolved in 2.5 mL of 0.1 M HCl. For intestinal digestion, 0.1 g of pancreatin and 0.625 g of bile salts were dissolved in 25 mL of 0.1 M NaHCO<sub>3</sub>. One gram of MWC with a final volume of 10 mL of milli-Q water was put together. The pH of the mixture was adjusted to 2 with HCl 6 N and a pepsin solution was added at a proportion of 0.05 g of pepsin/g of sample. The mixture was incubated at 37°C in a shaking water bath at 110 oscillations/min for 2 h for the gastric digestion. Then, the pH of the mixture was adjusted to pH 6 with 1 M NaHCO<sub>3</sub> dropwise. For the intestinal digestion, the digest and 2.50 mL of pancreatin + bile salt mixture was added. The pH was then adjusted to pH 7.5 with 1 M NaHCO<sub>3</sub>, and samples were incubated at 37°C at 110 oscillations/min for 2 h. After gastrointestinal digestion, the digestive enzymes were inactivated by heat treatment for 4 min at 100°C in a polyethyleneglycol bath. The samples were then cooled by immersion in an ice bath and centrifuged at 3200 x g for 60 min at 4°C (CS-6R centrifuge, Beckman) to separate soluble and non-soluble fractions. Monacolins K and citrinin contents and DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> were determined both of the samples after the gastric and intestinal digestion.

### DPPH radical scavenging activity

The scavenging activity (H/e-transferring ability) against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was measured spectrophotometrically by following Velazquez *et al.* (2003). Each 1-20 mg/mL of aliquot of 40  $\mu$ L appropriately diluted extracts mixed with 200  $\mu$ L of 0.02 mM DPPH solution and methanol 4 mL. Samples were kept for 15 min at 25 °C and the absorbance was measured at 517 nm. The absorbance of a blank sample containing the same amount of solvent was also measured. The extent of decolourisation is calculated as a percentage reduction of absorbance, and this is determined as a function of concentration and calculated relative to the 0.1-0.01 mM of equivalent Trolox concentration. The radical scavenging activity is expressed in mmol of equivalent Trolox per gram of sample (mmol Trolox /mL) with interpolation to 50% inhibition (IC<sub>50</sub>).

### Chelating ability on ferrous ions

Chelating ability on ferrous ions was evaluated spectrophotometrically by a slightly modified method of Kuo *et al.* (2004). Three hundred  $\mu$ L of 2 mM FeSO<sub>4</sub>.H<sub>2</sub>O were mixed with 1-20 mg/mL of each aliquot of 500  $\mu$ L test samples before addition of 600  $\mu$ L of 5 mM ferrozine. After the incubation at 25 °C for 10 min, 5 mL of ethanol was added and the absorbance was measured at 562 nm. IC<sub>50</sub> values (mg extract/mL) are the effective concentration at which ferrous ions was chelated by 50 % by interpolation from linear regression analysis.

### Monacolin K analysis

An 0.5 g sample was extracted with 25 mL of 70% ethanol at 50°C for 2 h, followed by filtration through a 0.2  $\mu$ m membrane (Chayawat *et al.*, 2009) and the extract was analysed by HPLC. The HPLC system consisted of Shimadzu LC-10AT VP Liquid Chromatograph, a FCV-10AL VP pump, an LDC Analytical SpectroMonitor 3100 detector set at 238 nm and an LDC Analytical CI-4100 integrator. A chromatography column Ascentis C18, 5 $\mu$ m, 250x4.6 mm was connected to a 20  $\mu$ L loop injector. An isocratic mobile phase of acetonitrile:water in the ratio of 65:35 (by vol.) was used. The flow rate and temperature were 1.0 mL/min and 28°C, respectively (Friedrich, *et al.*, 1995). Monacolin K dissolved in 70% ethanol was used as a standard.

### Citrinin analysis

Citrinin analysis was described by Lim *et al.* (2010). A 1 g sample was extracted with a solution (acetone : ethyl acetate = 1:1, v/v) at 65°C for 90 min under vigorous shaking. The supernatant was obtained by centrifugation at 1,600 x g for 10 min followed by filtration through a 0.45  $\mu$ m PTEE filter unit (National Scientific, Rockwood, TN). The citrinin was determined by HPLC using a chromatography column Ascentis C18 column (4.6 x 250 mm). The mobile phase consisted of methanol/acetonitrile/ 0.1% phosphoric acid (3:3:4, v:v:v) and the analysis was performed with a fluorescence detector set at excitation and emission wavelengths of 330 and 500 nm, respectively. The flow rate was 0.6 mL/min and the sample was spiked to confirm the presence of citrinin.

### Statistical analysis

All determinations were performed in triplicate and results were expressed as the mean  $\pm$  standard deviation calculated using spreadsheet software Microsoft Excel. This was carried out in a completely randomized experimental design (CRD) and the data were analysed by an analysis of variance ( $P \leq 0.05$ ) and means were separated by Duncan's multiple range test. The results were processed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for Windows.

## RESULTS AND DISCUSSION

### MWC after *in vitro* gastric digestion

This section plays a leading role in study of a simulated stomach digestion of MWC. Monacolin K and citrinin content, DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> of MWC and MWC through *in vitro* gastric digestion are given in Table 1. Gastric-simulated digestion of MWC with pepsin-HCl (pH 2.0) for 2 h had a significant effect on monacolin K and citrinin contents and both antioxidant activities of MWC.

The levels of monacolin K and citrinin in MWC after the stomach-treatment were slightly increased in the range of 1.35 – 8.69 %. The increased percentages after the *in vitro* gastric digestion were in the ascending order of monacolin K > citrinin. This indicated the high stability under *in vitro* stomach condition of monacolin K and citrinin. Our results on the stability of monacolin K and citrinin under the *in vitro* acidic condition are in agreement with those reported for raspberry (McDougall *et al.*, 2005) or pomegranate (Perez-Vicente *et al.*, 2002) anthocyanins. Furthermore, these results from this section and Perez-Vicente *et al.* (2002) reported a small increase in the antioxidants after *in vitro* stomach digestion. However, this was a disagreement with Bermudez-Soto *et al.* (2007), which showed that chokeberry juice after gastric-simulated digestion affected by a decrease in causing any of the major phenolic compounds.

DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> after the digestive simulation were increased 33.33 % and 13.47 %, respectively, which were mainly due to the fact that antioxidant activities of MWC were enhanced by gastrointestinal pH during gastric digestive processes (Baublis *et al.*, 2000). Rufian-Henares and Delgado-Andrade (2009) reported that, after the digestion process, the overall antioxidant activity statistically increased up to four times. Liyana-Pathirana and Shahidi (2005) found that, after a simulated gastric conditions, the antioxidant activity of flour measured by the DPPH method increased from 1.85 to 12.30 mmol trolox/kg of sample. Baublis *et al.* (2000) demonstrated that gastrointestinal pH condition caused a dramatic increase in antioxidant activities of aqueous extracts, suggesting that the acid condition caused alterations in antioxidant activities and compositions as well as concentration of water-soluble antioxidants. These studies stated that acid hydrolysis influenced monacolin K level by causing a release of monacolin K from MWC. Therefore, the digestive process indicated an influence on the enhancement of monacolin K and higher antioxidant activities.

**Table 1** Monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> in a digestive fraction after gastric digestion of MWC

Compounds and antioxidant activities	Raw material	Digested MWC	% Increase*
Monacolin K content (mg/kg dry weight) <sup>ns</sup>	79.52 $\pm$ 1.00	80.61 $\pm$ 9.00	1.35
Citrinin content ( $\mu$ g/kg dry weight) <sup>ns</sup>	10.50 $\pm$ 0.05	11.50 $\pm$ 1.28	8.69
DPPH (IC <sub>50</sub> mmol Trolox/mL) <sup>ns</sup>	0.03 $\pm$ 0.007	0.02 $\pm$ 0.004	33.33
Chelating ability on Fe <sup>2+</sup> (%)	82.20 $\pm$ 4.90 <sup>a</sup>	95.67 $\pm$ 9.03 <sup>b</sup>	13.47

\*Percentages of the total increase at the end of the digestion process. Different letters within the same row indicate statistical differences (one-way ANOVA and Duncan test,  $P \leq 0.05$ ). Values are mean  $\pm$  S.D of triplicate determinations

### Soluble and insoluble MWC after *in vitro* gastric and pancreatic digestions

Following simulation of gastric digestion, soluble and insoluble MWC were then incubated for 2 h digestion under conditions mimicking those of the duodenum. The results of monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> are shown in Table 2. Monacolin K and citrinin contents and DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> between soluble and insoluble MWC were lost between 14.69 and 76.92 % during treatment with pancreatin. Rufian-Henares and Delgado-Andrade (2009) confirmed that dietary antioxidants were highly sensitive to the mild alkaline conditions in the small intestine and suggested that, during digestion in the duodenum, a proportion of the compounds may be transformed into different

structural forms with different chemical properties. Moreover, light and O<sub>2</sub> are two important factors to consider as they can alter the structures and properties of phenolic compounds, due to oxidative degradation and polymerization reactions (Talcott and Howard, 1999). The antioxidant activities and monacolin K and citrinin contents in insoluble fraction from the MWC were less than those in soluble fraction. It is possible that acid and enzymatic hydrolysis influenced on the release of monacolin K and phenolic compounds in soluble fraction from MWC which this experiment was agreed with Ohta *et al.* (1994) reported an increase between the soluble antioxidants from food matrix by alkaline hydrolysis.

**Table 2** Monacolin K and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> in soluble and insoluble fractions after gastric and pancreatic digestion of MWC

Compounds and antioxidant activities	Raw material after the gastric digestion	Soluble MWC	Insoluble MWC	% Loss*
Monacolin K content (mg/kg dry weight)	79.52 ± 6.23 <sup>c</sup>	54.61 ± 6.00 <sup>b</sup>	13.23 ± 5.43 <sup>a</sup>	14.69
Citrinin content (ug/kg dry weight)	10.50 ± 0.01 <sup>b</sup>	5.50 ± 1.20 <sup>a</sup>	1.45 ± 0.00 <sup>a</sup>	64.54
DPPH(IC <sub>50</sub> mmol Trolox/mL)	0.03 ± 0.01 <sup>a</sup>	0.04 ± 0.00 <sup>a</sup>	0.09 ± 0.00 <sup>b</sup>	76.92
Chelating ability on Fe <sup>2+</sup> (%)	82.20 ± 3.00 <sup>c</sup>	65.56 ± 5.67 <sup>b</sup>	2.43 ± 0.41 <sup>a</sup>	17.29

\*% Loss indicates the lost values of raw material MWC after pancreatic digestion, Different letters within the same row indicate statistical differences (one-way ANOVA and Duncan test,  $P \leq 0.05$ ). Values are mean ± S.D of triplicate determinations

## CONCLUSION

For its stability after gastric digestion, the digestive process allowed an increase of 1.35 % of monacolin k content, 8.69 % of citrinin content and DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> of digested MWC increased in the range of 13.47 - 33.33 %. After pancreatic digestion, it has been demonstrated that monacolin k and citrinin contents, DPPH free radical scavenging ability and chelating ability on Fe<sup>2+</sup> decreased both soluble and insoluble MWC in the range of 14.69 - 76.92 %, indicating that the simulation of pancreatic digestion caused reduction of these antioxidant activities. However, this mimicking gastrointestinal digestion is a *in vitro* model only in order to know a trend for mechanism of action of *in vivo* human condition so that *in vitro* methods need to be further improved and validated with more *in vivo* animal and human studies (Bermudez-soto *et al.*, 2007).

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