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Determination of monacolin K and citrinin in red mold dioscorea and dried shrimp products using ultra high-performance liquid chromatography

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Abstract

In this study, red mold dioscorea (RMD) was utilized as a natural colorant to produce dried whiteleg shrimp (*Litopenaeus vannamei*). Whiteleg shrimp dyed with 50% diluted liquid RMD had a favorable appearance compared to control and undiluted liquid RMD. Dioscorea (*Dioscorea hispida* Dennst.) or bitter yam flour was used as a substrate in *Monascus purpureus* TISTR 3615 submerged fermentation to produce RMD. Dioscorea flour concentrations in a range of 10-50 g/L and various nitrogen sources at 4 g/L were investigated. The study showed that 10 g/L of diosorea flour and monosodium glutamate were chosen for further fermentation. Two major secondary metabolites in *Monascus* fermentation named monacolin K and citrinin, were determined in liquid RMD and dried shrimp products using UHPLC-DAD. Results showed that monacolin K were not detected in liquid RMD and all dried shrimp samples. Although, citrinin at a concentration of 2.06 mg/L was found in liquid RMD. Thus, RMD from the medium containing diosorea flour as a fermentation substrate can be used as colorant in dried shrimp production or other seafood products. However, the citrinin content in pigments remains an issue of concern.

1. Introduction

In the food industry, colorants play an important role in food processing in order to contribute to the sensory attributes of food. They not only improve acceptability and appeal but also tend to increase consumption (Dey and Nagababu, 2022). Synthetic colorants used in food industries have been reported as high health risks to consumers due to their carcinogenic or mutagenic activities (Mukherjee and Singh, 2011). Therefore, several attempts have been made to develop natural colorants, especially from pigments produced by microorganisms, since its eco-friendly, effective, and non-toxic (Sinha *et al*., 2017) to replace nitrate and nitrite salts in coloring and flavoring of food products (Ferysiuk and Wojciak, 2020). *Monascus purpureus* is a well-known edible fungus that has been used in the fermentation of starchy substrates for a long time in Asian countries (Zhu *et al*., 2019). *Monascus* strains can produce edible pigments of yellow to red colors depending on the fermentation process (Kim and Ku, 2018). Choices of carbon source affect pigment production (da Costa and Vendruscolo, 2017; Chen *et al*., 2021) while nitrogen sources are important to the composition of *Monascus* pigments (Widayanti *et al*., 2021). Different substances have been used in *Monascus* pigment production such as jackfruit seed, soybean flour, grape waste, sugarcane bagasse, durian seed, including dioscorea flour (Babitha *et al*., 2007; Silveira *et al*., 2008; Silveira *et al*., 2013; Handa *et al*., 2019; Srianta *et al*., 2020). Dioscorea or bitter yam root is regarded as a functional food, due to carrying many functional ingredients for the prevention of various diseases (Salehi *et al*., 2020). The root comprised approximately 75-84% of starch, 6-8% of crude protein and 1.2-1.8% of crude fiber (Oyeyinka *et al*., 2018). Therefore, dioscorea flour has been suggested as the substrate of choice for *Monascus* fermentation by Tseng *et al*. (2012) so called red mold dioscorea (RMD). A previous study reported that the RMD has a greater hypolipidemic and antiatherosclerotic effect than traditional red yeast rice and unfermented dioscorea in hamsters (Lee, Hung, Wang *et al*., 2007). Furthermore, research has also shown that RMD exerts a greater antidiabetic (Chen and Pan, 2015) and greater anti-hypertensive effect than traditional red mold rice in spontaneously hypertensive rats (Wu *et al*., 2009). Additionally, red pigment

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production in starchy substrate fermentation with *Monascus* spp. can produce a number of secondary metabolites such as monacolin K and citrinin (Lee *et al*., 2006). Monacolin K is a compound that inhibits cholesterol synthesis by blocking a key enzyme, hydroxymethylglutaryl-CoA (HMG-CoA) reductase (Yanli and Xiang, 2020). Therefore, the use of *Monascus* red pigments to color food products or food supplement possibly provides health benefits to consumers. However, citrinin which is a mycotoxin is also formed as byproduct during fermentation (Silva *et al*., 2021). Thus, the concentration for daily consumption is limited to not exceeding 200 ng/g (Nigovic *et al*., 2013). From our knowledge, the use of dioscorea flour as a substrate for red pigment production in submerged fermentation is still limited. The RMD was not widely used compared to red yeast rice although dioscorea was abundant in especially South East Asian and African countries. Thus, this study aimed to use dioscorea flour in submerged fermentation using *Monascus* spp. Then, use the RMD as colorant in dried shrimp production as well as evaluate monacolin K and citrinin concentration formed during the fermentation process.

2. Materials and methods

2.1 Reagents and chemicals

HPLC grade acetonitrile and methanol were purchased from Labscan (Bangkok, Thailand). HPLC grade formic acid was purchased from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q Plus system (Millipore, Inc., MA, USA). Authentic standards for monacolin K and citrinin were purchased from Fluka (Buchs, Switzerland). All the other chemicals were analytical grade. Culture media, such as yeast extract, potato dextrose agar (PDA), and peptone, were products of Himedia, India.

2.2 Microorganism

Monascus purpureus TISTR 3615 was obtained from Thailand Institute of Scientific and Technological Research. The strain was maintained on potato dextrose agar (PDA) slants at 4°C and subculture every 15 days. *Monascus purpureus* TISTR 3615 strain used in this study is shown in Figure 1.

Figure 1. *Monascus purpureus* TISTR 3615 strain used.

2.3 Preparation of substrate

Dried dioscorea chips (commercial products) were purchased from a local market in Pattani province, Thailand. The sample was dried in an oven at 60°C for 3 hrs. The dried dioscorea was ground and sieved through 120 mesh. Proximate composition and amylose content of dried dioscorea chips samples is shown in Table 1. Then, the powdered samples were vacuum sealed in plastic bags and kept at 4°C until used.

Table 1. Proximate composition of dried dioscorea chips samples (%).

Proximate analyses	Compositions [%]
Moisture	15.5±0.2
Total fat	0.3±0.0
Crude protein	2.6±0.0
Total carbohydrate (g)	80.3±0.2
Ash	1.2±0.0
Dietary fiber	1.8±0.1
Amylose (%)	30.1±0.1

2.4 Culture medium

The culture medium was prepared according to Srivastav *et al*. (2015) with slight modifications. Briefly, the culture medium had (g/L) 4 g MSG, 2.4 g KH_2PO_4, 2.4 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 10 mg $ZnSO_4 \cdot 7H_2O$, and 10 mg $FeSO_4 \cdot 7H_2O$. The medium pH was adjusted to 6 with 0.1 mol/L of HCl.

2.5 Effect of dioscorea flour concentration

Different concentrations of dioscorea flour for the production of RMD by *Monascus purpureus* TISTR 3615 were investigated. Briefly, 100 mL of the culture medium was prepared in a 250 mL Erlenmeyer flask and supplemented with dioscorea flour at concentrations of 10, 20, 30, 40, and 50 g/L. The culture medium was sterilized by autoclaving at 121°C for 15 mins. Then mycelial discs of 5 mm diameter were cut from the actively growing edges of *Monascus purpureus* grown on YM agar plates at 30°C for 6 days. Each flask of the culture medium was inoculated with 5 pieces of mycelial discs of *Monascus*. The fermentation broth from *M. purpureus* showed dark red color (Figure 2). Three replicates at each substrate concentration were incubated at 30°C for 4 days in a shaker at 180 rpm. Subsequently, the fermentation broth was centrifuged at 10,000 rpm for 5 mins for collecting supernatant. The extracellular *Monascus* red pigment content in the supernatant was analyzed by measuring the absorbance at 500 nm using a UV–Vis spectrophotometer (JENWAY, Model 6405). Distilled water was used as a blank. Red pigment yield was expressed as absorbance units (OD Unit/mL) multiplied by a dilution factor. The relative pigment

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yield at each concentration of dioscorea flour was also calculated. The concentration giving the highest absorbance units was considered 100%, therefore, the relative pigment yield of the other concentration was calculated based on the highest absorbance. The dioscorea flour concentration which gave the highest red pigment yield was chosen for further study.

Figure 2. The fermentation broth from *Monascus purpereus* using dioscorea flour as a substrate showed dark red color.

2.6 Effect of nitrogen sources

The five different nitrogen sources including monosodium glutamate (MSG), peptone, yeast extract, $(NH_4)_2SO_3$ and NH_4NO_3 were studied in the production of RMD, at a fixed 4 g/L concentration. The culture medium with an initial pH of 6.0 was prepared and inoculated with 5 pieces of *Monascus purpureus* mycelial discs. After incubation at 30°C for 4 days in a shaker, the RMD pigments produced were measured at 500 nm. The nitrogen source that gave the highest red pigment content was chosen for further study.

2.7 Application of RMD red pigments in dried shrimp production

The liquid pigments of RMD obtained from the optimum fermentation broth containing dioscorea flour (10 g/L) and MSG (4 g/L) of *Monascus purpureus* TISTR 3615 were used as a colorant in dried white leg shrimp (*Litopenaeus vannamei*) production. Three types of dried shrimp products were prepared: (A) natural dried shrimp product (control), (B) dried shrimp using 50% dilution RMD liquid pigments, and (C) dried shrimp using undiluted RMD liquid pigments. The whiteleg shrimp were cleaned with tap water before soaking in the RMD liquid pigments for 10 mins, then boiled for 5 mins. The liquid colorant was drained and the boiled shrimp was cooled down for 20 mins before drying at 60°C for 10 hrs.

2.8 Dried shrimp extraction

Dried shrimp samples were extracted to determine monacolin K and citrinin contents, as these are secondary metabolites from the RMD fermentation. Powdered samples (0.1 g) of dried shrimp were accurately weighed into a 15 mL tube and 2.5 mL of 75% ethanol was added. The mixture was sonicated for 15 mins and centrifuged at 5000 rpm for 10 mins. The residue was re-extracted twice. The supernatants were combined in a 10 mL volumetric flask and then made up to the mark with 75% ethanol. The sample solution was then filtered through a 0.22 µm PTFE filter before analysis using UHPLC-DAD.

2.9 Determination of monacolin K and citrinin using UHPLC-DAD

Ultra high-performance liquid chromatography with a diode array detector (UHPLC-DAD) was used to determine monacolin K and citrinin in samples, according to Avula *et al*. (2014) with slight modifications. Agilent series 1290 ultra-high performance liquid chromatograph–Diode array detector (UHPLC–DAD) was used. Separation was done in an Agilent Zorbax SB-C18 RRHD (2.1×100 mm, 1.8 µm) column. The column temperature was set at 35°C. The mobile phase (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid was used at a flow rate of 0.35 mL/min. Analysis was performed using the gradient elution: 65% A to 35% B then 35% A to 65% B within 15 mins and in the next 3 mins to 100% B. Each run was followed by 5 mins wash with 100% B and 5 mins with 65% A and 35% B for an equilibration period. The precision of the UHPLC-DAD method was evaluated. Reproducibility was evaluated from five replicates (n = 5) of a standard solution for three different days (inter-day). Variations are expressed as relative standard deviation (%RSD).

3. Results and discussion

Red mold dioscorea (RMD) produces a mixture of pigments ranging from orange to red in color, at least six colors have been identified and are well-known (Kim and Ku, 2018). The absorbances at 410 and 470 nm corresponded to yellow and orange colors. The maximum absorbance was observed at 500 nm representing red color, so the RMD in this study had dominantly red pigments. The results agree with previous showing that *Monascus* red pigments are obtained from starchy substrates (Srianta *et al*., 2017). Thus, the peak at wavelength 500 nm was used for measuring red pigment in RMD throughout this study.

3.1 Effect of dioscorea flour concentration on red mold dioscorea red pigments production

The effects of the concentration of dioscorea flour on RMD production by *Monascus* strain were evaluated. As shown in Figure 3, dioscorea flour at 10 g/L gave the highest production of red pigments with the value of 4.51±0.21 OD unit/mL. Regarding pigment production,

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an increase in the concentration of dioscorea flour resulted in a decrease in red pigment yield. The dioscorea flour at 20, 30, 40 and 50 g/L yielded 3.48±0.18, 2.47±0.10, 1.80±0.16 and 1.91±0.22 OD unit/mL, respectively (Figure 3A). The percentage of relative pigment yields of those dioscorea flour concentrations compared to 10 g/L of dioscorea flour were then 77.1±3.9, 54.7±2.2, 39.9±3.4 and 42.3±4.9, respectively (Figure 3B). The results showed that 10 g/L of dioscorea flour was an optimum concentration for RMD production. While, a previous report showed that the optimum concentration of sweet potato was 3.3% (Srivastav *et al*., 2015). Tseng *et al*. (2012) showed that *Monascus purpureus* NTU 568 produced high amounts of pigments and monacolin K and polysaccharides when dioscorea was used as the substrate. Normally, glucose and its oligosaccharides were adequate carbon sources for both fungal growth and bio-pigment synthesis (Agboyibor *et al*., 2019). It is also well recognized that the concentration of glucose at 18 g/L is near optimal (Mukherjee and Singh, 2011; da Costa and Vendruscolo, 2017), while a high (50 g/L) concentration of glucose can reduce fungal growth rates, giving low pigment synthesis and ethanol production. The dioscorea flour used in this study contained 80.3±0.2% carbohydrates

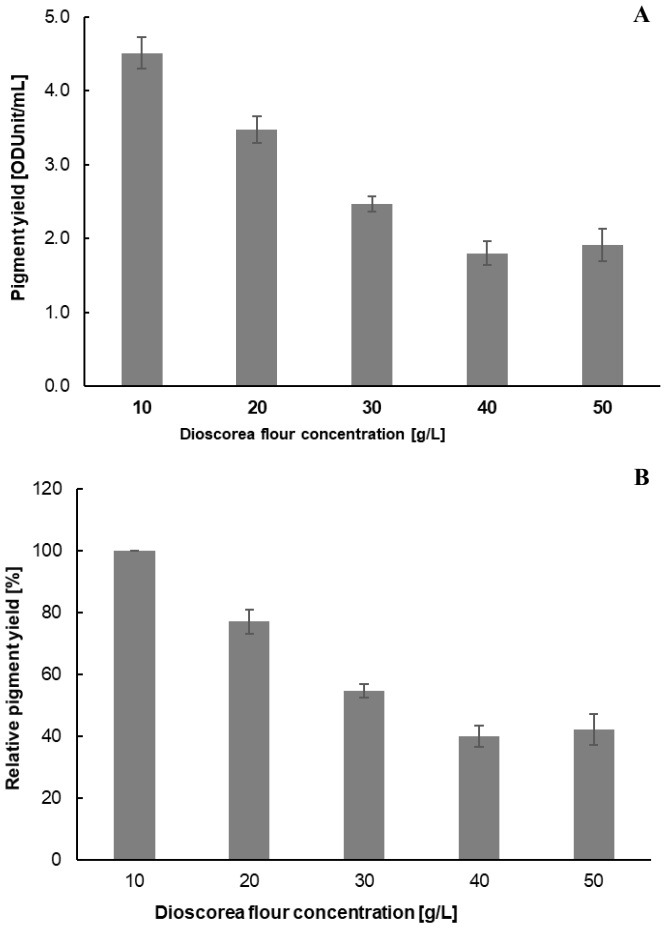

Figure 3. Effects of dioscorea flour concentration on RMD production by *Monascus purpureus* TISTR 3105 incubated at 30°C for 4 days in a shaker at 180 rpm. Values are expressed as mean±SD. (A) pigment yield, (B) relative pigment yield.

(Table 1) and 30.1±0.1% amylose. The high content of carbohydrates and amylose could be a source of released glucose due to amylolytic enzymes produced by a fungal strain that gave high yielding of red pigment.

3.2 Effect of nitrogen source on red mold dioscorea red pigments production

RMD production is influenced by nitrogen sources as nitrogen is essential for cell growth, and for the quantity and quality of the pigments (Chen *et al*., 2017). In this study, three organic nitrogen sources including; MSG, peptone, yeast extract, and two inorganic nitrogen sources which are ammonium sulfate and ammonium nitrate, were tested in red pigment production using dioscorea flour concentration at 10 g/L. In Figure 4A, the organic nitrogen sources MSG, peptone, and yeast extract were favorable choices for RMD production, while the inorganic nitrogen sources, ammonium sulfate and ammonium nitrate gave poorer results. MSG gave a good result on red pigment production followed by yeast extract and peptone corresponded to 4.80±0.19, 4.45±0.23 and 3.44±0.18 OD unit/mL, respectively. Red pigment yield from the medium in the presence of yeast extract and peptone related to MSG medium was 92.8±4.9 and 71.6±3.8, respectively (Figure 4B). The fermentation medium supplemented with ammonium sulfate and ammonium nitrate gave a pigment yield of 0.1-0.2 OD unit/mL, which their relative pigment yield was found in the range between 2.2 to 4.6% when compared to MSG. The results showed that an inorganic nitrogen source gave dramatically less red pigment production in the RMD with relative absorbance below 10% compared to organic N sources. The MSG was found to be the best nitrogen source for the RMD red pigments production. This result agrees with the report of Atalay *et al*. (2020) and Silbir and Goksungur (2019), in which monosodium glutamate was found to be optimal for red pigment production, followed by peptone, soybean meal and chitin powder, respectively. Additionally, the report of Silveira *et al*. (2013) found that 5 g/L MSG concentration was suitable for pigments production by *Monascus* strain using grape waste in submerged fermentation. However, the conflicting study by Mapari *et al*. (2010) considered ammonium chloride as the best for pigment production, followed by ammonium nitrate and then glutamate. Dioscorea has been reported to have 5-6% protein (Oyeyinka *et al*., 2018) which is important for cell growth. Results indicate that dioscorea flour is the substrate that requires a comparatively low concentration of MSG. This might be attributed to the nature of dioscorea flour used in this study which contains 2.6% crude protein; therefore, less nitrogen supplementation was required. In the present study, the fermentation broth was prepared with an initial

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pH of 6.0, which is in the range of suitable pH for red pigment production reported by Lee *et al*. (2002). A lower substrate pH promotes the biosynthesis of yellow pigments, whereas a higher pH favors red pigments (Yongsmith *et al*., 2000) and a pH range 5.5–8.5 has been found most suitable for red pigments production (Lee *et al*., 2001).

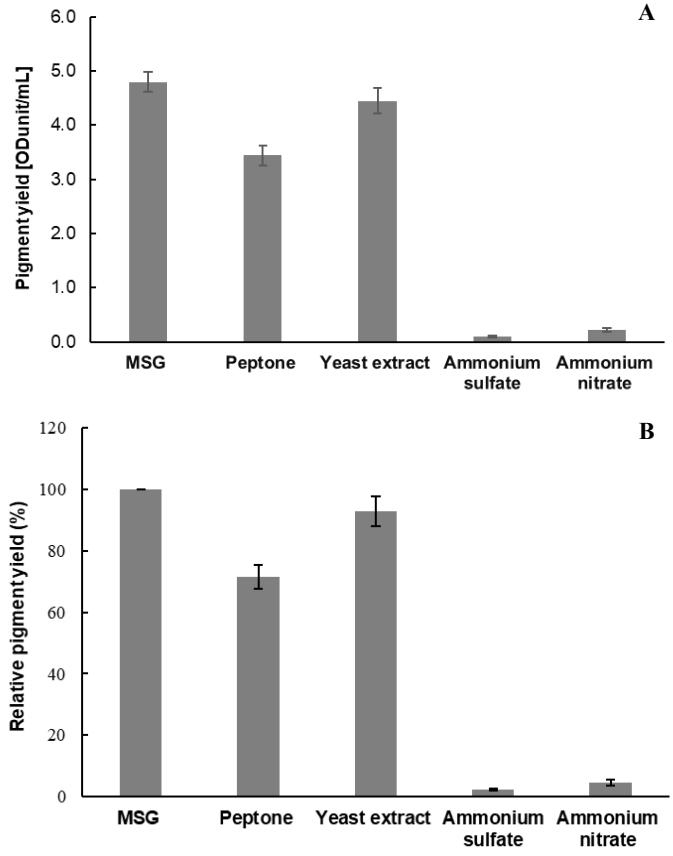

Figure 4. Effects of nitrogen source on RMD production by *Monascus purpureus* TISTR 3105 incubated at 30°C for 4 days in a shaker at 180 rpm. Values are expressed as mean±SD. (A) pigment yield, (B) relative pigment yield.

3.3 Dried shrimp production

The colored liquid RMD from optimal submerged fermentation of dioscorea flour by *M. purpureus* TISTR 3615 was tested as colorant in dried white leg shrimp production. Subjectively, treating dried shrimp with 50% diluted liquid RMD gave preferable color when compared to commercial products. The color of dried shrimp became more intense after being treated with undiluted liquid RMD. All results are compared as shown in Figure 5. Thus, RMD can be used as an alternative colorant for dried shrimp and could be able to replace synthetic dyes. Moreover, RMD could also serve as colorant for other seafoods, as well as for other meat products and food supplementary.

Figure 5. Application of RMD red pigments in dried whiteleg shrimp (*L. vannamei*) production. (A) Control dried shrimp without liquid RMD treatment, (B) dried shrimp with 50% diluted liquid RMD treatment, and (C) dried shrimp treated with undiluted liquid RMD.

3.4 Determination of monacolin K and citrinin using UHPLC-DAD

The UHPLC-DAD method was tested for precision in an inter-day variation analysis, by using standard monacolin K at 5 and 50 mg/L and standard citrinin at 2.50 and 10 mg/L. The results in Table 2 showed RSD of 0.5% and 0.2% for monacolin K and citrinin, respectively, along with 90.1-92.6% monacolin K and 88.2-90.7% citrinin recovery for the extraction efficiency used in this study. Thus, the results indicate the effectiveness of the UHPLC-DAD and extraction with acceptable precision and accuracy. Monacolin K and citrinin in liquid RMD and in dried shrimp samples were identified by the retention time of the standard compounds. The retention times of citrinin and monacolin K standards were 4.461 and 14.224 mins, respectively. In the fermentation process; monacolin K and citrinin were formed as a by-product, monacolin K, normally gives benefits to human health, while citrinin is a compound which needs to be considered for the safety of consumption. In this study, monacolin K was not detected in liquid RMD, while citrinin was found in the liquid RMD at 2.06 mg/L. However, there are no monacolin K and citrinin detected in any dried shrimp samples. Citrinin was not detected in dried shrimp products although it was present in liquid RMD, because it might decompose during boiling in water and drying, as it is a heat-sensitive compound. The decomposition products of citrinin include temperature-dependent products called citrinin H1 and citrinin H2. Citrinin H1 is more toxic than the original citrinin, while citrinin H2 is less toxic (Kitabatake *et al*., 1991; Lee, Chen, Wang *et al*., 2007). Therefore, citrinin-free RMD would probably be preferred in the food industries, to ensure quality and

Table 2. Monacolin K and citrinin validation data using UHPLC-DAD.

Compounds	t_R (min)	Conc. (mg/L)	(%RSD) n = 5	% recovery	Linear range (mg/L)
Citrinin	4.461±0.003	5.00	0.32	88.2±0.1	2.50-1,000.00
		50.00	0.45	90.7±0.1	
Monacolin K	14.224±0.002	2.50	0.14	90.1±0.2	1.00-1,000.00
		10.00	0.22	92.6±0.1	

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safety. This study also confirmed that the citrinin in *Monascus* fermented products could be reduced after heat treatment. However, optimization of medium parameters and conditions affecting fermentation products by *Monascus* strain is required in order to gain beneficial products before it would be applied as food colorants.

4. Conclusion

The present study describes the conditions for the production of red pigment by *M. purpureus* TISTR 3615 in submerged fermentation using dioscoria flour as a substrate. High yields of red pigment were obtained in a medium containing 10 g/L dioscoria flour and 4 g/L MSG. Neither monacolin K nor citrinin were detected in the dried shrimp samples. Therefore, the red pigment produced in this study can be used as a substitute for nitrate and nitrite salts in food products and is considered an alternative value-added product of dioscoria.

Conflict of interest

The authors declare no conflict of interest.

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