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Reduction of mycotoxin citrinin in Monascal Broken Rice (MBR) via exogenous tannic acid supplementation and chemometric analysis

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Abstract. *Phanumong P, Srisuvor N, Sarawong C, Norajit K, Wattanakul J, Kumtabtim U, Nuclear P, Kraboun K. 2024. Reduction of mycotoxin citrinin in Monascal Broken Rice (MBR) via exogenous tannic acid supplementation and chemometric analysis. Asian J Agric 8: 153-160.* Addressing the presence of mycotoxin citrinin in Monascal Broken Rice (MBR) is crucial. Incorporating Exogenous Tannic Acid (ETA) into *Monascus* substrate effectively diminished citrinin accumulation in angkak. This research explored the impact of 10-20 mg/mL ETA supplementation on reducing citrinin levels in MBR and altering its pigments, glucosamine, monacolin K, fatty acids, and antioxidant properties. The relationship among these factors in MBR was examined using Principal Component Analysis (PCA). ETA supplementation markedly decreased citrinin levels in MBR by over 73.26% compared to the control group while enhancing pigments, glucosamine, monacolin K, fatty acid content, as well as catalase (CAT), superoxide dismutase (SOD), superoxide anion ($\cdot O^{-2}$), and hydroxyl free radical (HO•) scavenging capabilities. The *M. purpureus* generated higher contents of unsaturated fatty acids in ETA treatments, such as linoleic acid (C18:2) at 30-32%, followed by oleic acid (C18:1) ranging from 23 to 24%, compared to the control (no ETA), 25.09% for C18:2 and 20.23% for C18:1. The association among oleic acid, linoleic acid, linolenic acid, and citrinin content was evident through PCA loading plots and Pearson's correlation coefficient. ETA supplementation is a viable approach to reduce citrinin concentration in MBR. Applying multivariate analysis, PCA, and Pearson's correlation coefficient provided a comprehensive understanding of the interrelation among various factors in MBR after ETA treatment.

Keywords: Angkak, exogenous tannic acid, monascal broken rice, *Monascus purpureus*, mycotoxin citrinin

INTRODUCTION

Monascus purpureus is responsible for the production of angkak, which is commonly referred to as monascal rice. This fermented food has a long-standing history in Chinese culinary traditions. The *M. purpureus* ferments a variety of cereals, such as rice, soybean, adlay, sweet corn, and waxy corn, for 3 weeks to yield angkak (Guo et al. 2019; Yang et al. 2019). It had extensive applications across various industries, including its use as a supplement for reducing Low-Density Lipoprotein (LDL), as food colorants, and as fabric dyes in Southeast Asian countries, particularly China, Japan, and others (Kraboun et al. 2019). The *M. purpureus* releases several hydrolytic enzymes during angkak fermentation, including amylase, protease, glucoamylase, maltase, pectinase, α-galactosidase, and ribonuclease. The cereal substrates are hydrolyzed into primary metabolite substrates, specifically acids and reducing sugars, through hydrolysis. The compounds are subsequently utilized to produce secondary metabolites, such as antioxidants and pigments, as documented by Kraboun et al. (2019, 2023) and Pechyen et al. (2024). The *M. purpureus* produces a range of secondary metabolites such as pigments, citrinin, monacolin K, γ-aminobutyric acid (GABA), dimerumic acid (DMA), and flavonoids (Kraboun et al. 2019). In angkak fermentation, the ideal conditions result in the production of high levels of antioxidants, citrinin (a mycotoxin), and pigments. Citrinin is a toxic substance that can lead to harmful effects on the kidneys and liver in mammals (Kraboun et al. 2023). Hence, to tackle this phenomenon, it may be necessary to enhance the levels of antioxidants and pigments and decrease the citrinin content during angkak fermentation. Controlling external factors such as carbon sources, oxygen levels, and pH during the growth of *Monascus* can effectively slow the pigments and antioxidants production (Pechyen et al. 2024). A study conducted by Yang et al. (2015) showed that the addition of antioxidants to *Monascus* substrate for angkak production could lead to an imbalance in oxidative stress conditions or Reactive Oxygen Species (ROS). This imbalance results in an increase in pigmentation and a decrease in citrinin content, as observed by Hu et al. (2017). Research has indicated that the inclusion of antioxidants during *Monascus* fermentation may help mitigate oxidative stress and enhance the overall process (Wei et al. 2022).

Tannic Acid (TA), a derivative of a hydrolyzable polyphenol with gallic acid, is identified by the United

States Food and Drug Administration (FDA or US FDA) as Generally Recognized As Safe (GRAS) (Hu et al. 2017). TA is classified as a secondary metabolite and is generally found in the peel and flesh of various fruits and vegetables. TA hinders malignant cholangiocyte growth, reduces ROS, controls lipid peroxidation, and regulates hydrogen peroxide levels (Lee et al. 2018). Similarly, Pechyen et al. (2024) noted that the addition of 20 mg/mL Exogenous Tannic Acid (ETA) reduced biogenic amine and citrinin levels in angkak after fermentation by 1.70 and 2.40-fold, respectively, compared to the control (no ETA supplementation). Consequently, exploring the impact of ETA supplementation on lowering citrinin levels and assessing the secondary metabolites in Monascal Broken Rice (MBR) should be investigated. This aspect has been unexplored in angkak-related research using broken rice as *Monascus* substrate.

Chemometrics is an effective method for representing omics data by illustrating dependent variable relationships graphically (Pollo et al. 2021). Chemometric analysis solves authentication problems as One-Class Classifiers (OCC). Common OCC tools include Soft Independent Modeling by Class Analogy (SIMCA) and Principal Component Analysis (PCA). Other OCC tools are Partial Least Squares (PLS) projection and PLS Discriminant Analysis (PLS-DA), which is a regression method (Rodionova et al. 2023). However, high-performance equipment is needed to obtain dependent variable data used for chemometric analysis, such as Ultra-High-Performance Liquid Chromatographic-Diode Array (UHPLC-DAD), Ion-Exchange Liquid Chromatography (IELC), and High-Performance Liquid Chromatography With Fluorescence Detection (HPLC-FD) (Chen et al. 2023). Kraboun et al. (2023) suggested that the recovery of angkak metabolites, such as organic acids, amino acids, monacolin K, pigments, and citrinin during *Monascus* fermentation could be used to determine angkak qualities. The relationship between the metabolites and fermentation time was studied using PCA. Gas Chromatography Time of Flight Mass Spectrometry (GC-TOF-MS), gas Chromatography-Flame Ionization Detection (GC-FID), and High-Performance Liquid Chromatography (HPLC) was used for secondary metabolite investigation in angkak for 30 days. Chemometrics is a suitable tool that explains the relationships among targeted-dependent variables during angkak fermentation.

This study aimed to determine the optimum ETA level to improve pigments, glucosamine, monacolin K, citrinin, fatty acid composition, antioxidant enzymes, and antioxidant activities in MBR. PCA and Pearson's correlation coefficient were utilized to examine the relationships between these targeted variables.

MATERIALS AND METHODS

Chemicals

Monacolin K, tannic acid, methyl alcohol, hydrochloric acid, *N*-acetyl glucosamine, citrinin, pyrogallol, EDTA, $FeCl_3$, deoxyribose, TCA, TBA, sodium hydroxide, and BHA were supplied by Sigma-Aldrich Co. (MO, USA). All substances were stored at -30°C until needed.

Microorganisms

The *M. purpureus* TISTR 3090 was cultivated on Potato Dextrose Agar (PDA) at 30°C for 14 days which was sourced from the Thailand Institute of Scientific and Technological Research (TISTR). Subsequently, *Monascus* mycelium was reinoculated into PDA slants and incubated under the same conditions or until spore concentration > 10^6 spores/mL before MBR fermentation. Next, for the purpose of determining *Monascus* spore concentration, 5 mL of distilled water was added to the PDA slants. Finally, a sterilized needle was used to disperse the *Monascus* spore in the water. An estimation of the spore concentration was made using a hemocytometer.

Materials

Broken rice was sourced from the Rung Charoen Market in Bangkok, Thailand.

Monascal Broken Rice (MBR)

The MBR preparation was following Kraboun et al. (2023) with slight adjustments. In a 500 mL flask, 50 g of broken rice was combined with 12 mL of ETA at concentrations of 10 and 20 mg/mL, except for the control using tap water. After autoclaving the mixture at 121°C for 15 min and allowing it to cool, a 5 mL suspension of *M. purpureus* spores > $1x10^6$ spores/mL (from PDA slants) was transferred into the sterilized samples. The samples were then placed in an incubator at 30°C for 20 days. The MBR was dried in a hot air oven at 55°C for 45 h, ground for 30 s to a fine powder using a Retsch ultra centrifugal mill, and then passed through a sieving machine (Haan, Germany) to achieve a 20-mesh consistency.

Monascus pigment intensity

The *Monascus* pigment intensity was assessed following the procedure detailed by Yongsmith et al. (2000) with slight adjustments. The extraction process involved mixing a 1 g sample with 100 mL of methyl alcohol and shaking using a shaker at 180 rpm for 1.5 h. The resulting mixture was filtered through Whatman No. 42 filter paper to collect the supernatant, which was then measured at 500 nm using a spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan) and compared to methyl alcohol used as the blank. The pigment concentration was determined using the formula below.

$$\text{Pigment intensity} = \frac{A_{500} \text{ X dilution factor X volume of methyl alcohol}}{\text{weight of sample (g)}}$$

Pigment intensity: Unit/g dry weight

Monascus growth analysis

The level of *N*-acetyl glucosamine present in the mycelium cell wall is an indicator of *Monascus* growth derived from chitin broken down by acid. To extract *N*-acetyl glucosamine, a 2 g sample was immersed in 70 mL of 5.7 M hydrochloric acid and agitated at room temperature for 35 min. Following centrifugation at 3500 *g*

for 15 min and rinsing with distilled water, the supernatant was treated with 25 mL of 16 M hydrochloric acid at 30°C for 15 h before diluting with 50 mL of distilled water for chitin hydrolysis. This mixture underwent further hydrolysis in an autoclave at 115°C for 1.5 h. After adjusting the hydrolysate to pH 7.5 with 13 M and 0.05 M sodium hydroxide, 2 mL of acetylacetone reagent was combined with 1 mL of the hydrolysate, boiled for 60 min, cooled, and mixed with a reagent (10 mL of ethyl alcohol and 2 mL of Ehrlich reagent) for incubation at 75°C for 10 min. The absorbance at 530 nm was measured using a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Japan) against *N*-acetyl glucosamine as the standard (Arikan et al. 2020).

Monacolin K analysis

The procedure for analyzing monacolin K followed the methodology outlined by Ye et al. (2023). A 2 g sample aliquot was extracted using 60 mL of 85% ethyl alcohol at 60°C for 15 h in the absence of light and then passed through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter (CHM®, Ukraine). The analysis was performed by high-performance liquid chromatography (HPLC) using a system consisting of a Shimadzu LC-10AT VP Liquid Chromatograph, FCV-10AL VP Pump, LDC Analytical SpectroMonitor 3100 detector set at 240 nm, and an LDC Analytical CI-4100 integrator. A ZORBAX SB-C18 column (5 µm, 4.6 x 250 mm, Agilent Technologies, USA) was linked directly to a 20 µL loop injector. 1 µL volume of sample extract was injected. The mobile phase employed to elute was a consistent mixture of acetonitrile and water (65:35, v/v). The flow rate and temperature were maintained at 2.0 mL/min and 25°C, respectively. Monacolin K was used as the standard, dissolved in 80% ethyl alcohol. A calibration curve was prepared within a range of 2-10 mg/mL of monacolin K. The equation for the calibration curve is $y = 1.4748x + 28.937$, and the correlation coefficient is $R^2>0.999$. The calibration curve equation was determined to estimate the LOD and LOQ values, which were found to be 0.021 and 0.063 mg/mL, respectively. The reported recovery percentage for monacolin K yield was 98.2 ± 1.34%.

Examination of citrinin

The procedure outlined by Kraboun et al. (2023) was used to analyze citrinin. A 1 g sample was mixed with a solution of acetone and ethyl acetate in a 1:1 ratio (v/v) and shaken at 75°C for 130 min. After centrifugation at 2500 *g* for 15 min, the supernatant was obtained by filtration through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter (CHM®, Ukraine). The filtrate underwent analysis via an HPLC system with a ZORBAX SB-C18 chromatography column (5 µm, 4.6 x 250 mm, Agilent Technologies, USA). The mobile phase consisted of methyl alcohol, acetonitrile, and 0.2% phosphoric acid in a 4:4:5 ratio (v/v/v). A fluorescence detector was utilized with excitation and emission wavelengths set at 330 and 500 nm, respectively. 1 µL volume of sample extract was injected. The flow rate was kept constant at 1 mL/min, and the sample was supplemented with the citrinin standard (Sigma-Aldrich, USA) to ascertain the existence of citrinin, as the initial sample reported a concentration of 0.054 µg/mL. A calibration curve was generated using citrinin concentrations ranging from 2 to 10 µg/mL. The calibration curve equation and correlation coefficients were as follows: $y = 1.3681x + 38.648$ and $R^2 > 0.9992$. These calibration curve equations were derived to estimate the Limit of Detection (LOD) and Limit of Quantification (LOQ) as 0.044 and 0.085 µg/mL, respectively. A monacolin K yield recovery percentage of 97.5 ± 2.78% was recorded.

Validation methods for the analysis of monacolin K and citrinin

Linearity

Linearity is the ability of analytical research to produce test results that are directly proportional to the concentration of the analyte in the sample within a defined parameter range. An evaluation was conducted using working standards ranging from 2 to 10 mg/mL of monacolin K in the mobile phase, prepared by dilution in series. Similarly, a dilution of citrinin ranging from 2 to 10 µg/mL was included in the mobile phase. We conducted at least five injections of each concentration into The High-Performance Liquid Chromatography (HPLC) system (Davani et al. 2024). The assessment included the evaluation of the correlation coefficient (R^2), calibration curve equations, and linearity range.

The Limits of Detection (LOD) and Quantification (LOQ)

Through the statistical assessment of the signal deviation of monacolin K and citrinin standards, we have established the Limit of Detection (LOD) at 3×SE/m and the Limit of Quantification (LOQ) at 10×SE/m. In order to calculate the LOD value, the Standard Error (SE) obtained from the calibration line of the standard solutions was magnified by a factor of three and subsequently divided by the slope of the line. Analysis of the calibration line using regression provided a precise description of the standard error. In order to establish the LOQ value, the SE from the calibration line was multiplied by 10 and subsequently divided by the slope of the line (Davani et al. 2024).

Percentage of recovery

The percentage of recovery refers to the percentage of analyte that is successfully retrieved using the stated method. It facilitates the quantification of analyte losses, therefore demonstrating the precision of both the extraction and HPLC determination methods. Furthermore, the recovery was assessed by comparing the amount of total monacolin K or citrinin released by HPLC with their known amounts. The recovery percentage was reported as the mean value + standard deviation (Davani et al. 2024).

Profile of fatty acids

The acid-heating technique outlined by Huang et al. (2017) was employed to extract lipids. Initially, a 2 g sample was combined with 15 mL of 4.5 M hydrochloric acid, heated to 110°C for 4 min, cooled to 45°C for15 min, and mixed with 15 mL extraction solution (methyl alcohol and chloroform in a 1:1 ratio, v/v). The mixture was

vortexed for 1 min to facilitate extraction. Subsequently, centrifugation at 7,500 rpm for 15 min separated the sample into two phases, with the lower chloroform-dissolved phase retained. This chloroform phase was then transferred to a test tube containing 8 mL of 1% w/v sodium chloride, followed by centrifugation at 4,500 rpm for 12 min and evaporation for 30 min with nitrogen to yield the crude lipid.

Fatty acids were isolated through purification techniques and converted into Fatty Acid Methyl Esters (FAMEs) following the procedure of Kraboun et al. (2023). Subsequently, they were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS system comprised an Agilent 7890A GC linked to an Agilent 5975 C Mass Selective Detector (MSD). Separation was achieved using a quartz capillary column (DB-5ms, dimensions 30 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA). The injector was set at 300ºC, 1.0 μL, and splitless mode. The column temperature was programmed to initiate at 55°C for 5 min, then ramp up to 255°C at a rate of 7°C/min, and finally reach 310°C for 12 min at 13°C/min.

Sample preparation for antioxidant abilities

The MBR extract preparation followed the technique of Yang et al. (2015) with a minor adjustment. A 2 g sample was mixed with 200 mL of methyl alcohol, agitated at 170 rpm for 24 h, and filtrated through Whatman No. 4 filter paper. Subsequently, the filtrate was evaporated at 65°C until the solvent disappeared. The crude extract was then assessed for its antioxidant properties.

Scavenging abilities of superoxide anion ($\cdot O^{-2}$) and hydroxyl free radical (HO•)

The scavenging activity of the superoxide anion ($\cdot O^{-2}$) of MBR was determined using the pyrogallol autoxidation method of Yang et al. (2015). Briefly, 1 mL of 4 mg/mL MBR extract was added to 5 mL of Tris-HCl buffer (0.2 M, pH 7.5, 28°C), followed by 2 mL of pyrogallol (2 mM, 28°C), and finally mixed using a vortex mixer for 10 min. The reaction was halted by adding four drops of 13 M hydrochloric acid, and the absorbance was measured at 400 nm (UV-1700 PharmaSpec spectrophotometer, Shimadzu, Japan). The superoxide anion ($\cdot O^{-2}$) scavenging effects were quantified as inhibition percentages.

The assessment of HO• radical scavenging capability was conducted using the method of Kongbangkerd et al. (2014). To prepare for the analysis, ferric chloride ($FeCl_3$) and ascorbate were dissolved in de-oxygenated water. A solution composed of 150 μL of various substances including 4 mg/mL MBR extract, $FeCl_3$ (350 μM), EDTA (200 μM), hydrogen peroxide (2.5 mM), deoxyribose (5 mM), and L-ascorbic acid (250 mM) in potassium phosphate buffer, was incubated at 50°C for 2.5 h, then boiled for 35 min in a water bath. Subsequently, 2.5 mL of 25% v/v Trichloroacetic Acid (TCA) and 2-thiobarbituric acid (TBA) (2% v/v of TBA in 2M sodium hydroxide comprising 1% w/v of Butylated Hydroxyanisole (BHA)) was added. The absorbance at 540 nm was measured using a spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan) to determine the scavenging activity of HO• radicals, and expressed as percentage of inhibition.

Assessment of SOD and CAT antioxidant enzymes

The assessment of Superoxide Dismutase (SOD) was carried out using the Nitro Blue Tetrazolium (NBT) technique, as outlined by Kongbangkerd et al. (2014). A buffer solution was prepared by mixing 55 mM Tris (pH 8.5), 65 mM cacodylic acid (pH 8), 3 mM EDTA, and 11 mM hydrochloric acid. Subsequently, 20 μL of 4 mg/mL MBR extract was added to 5 mL of the buffer along with 200 μL of 3 mM pyrogallol, and the mixture was mixed using a vortex mixer. After a 4-min incubation period, the solution was analyzed at 417 nm using a spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan) to determine SOD activity, with 1 SOD unit defined as reducing pyrogallol auto-oxidation by 50%. Catalase (CAT) activity was assessed following the method of Misra et al. (2019), where 65 μL of 4 mg/mL extract was blended with 5 mL of distilled water and 2 mL of hydrogen peroxide before analysis at 260 nm using the same spectrophotometer.

Data analysis

All the experiments were conducted in triplicate, with results presented as mean ± standard deviation. Duncan's New Multiple Range Test (DMRT) was utilized to separate the means with significance at $p<0.05$. Statistical analyses, including ANOVA, Principal Component Analysis (PCA), and Pearson's correlation coefficient, were conducted with SPSS 26.0 software (SPSS Inc., USA). A linearity analysis was conducted using Microsoft Excel, 2019 (Microsoft, WA, USA) to determine the correlation coefficient (R^2), calibration curve equations, and linearity range.

RESULTS AND DISCUSSION

Effect of ETA supplementation on *Monascus* pigment intensity and glucosamine content

Correlations between *Monascus* pigment intensity and glucosamine levels exposed to ETA supplementation ranging from 0 to 20 mg/mL are shown in Table 1. The biosynthesis of *Monascus* pigments had an increasing trend with glucosamine production, proving that pigment production and the growth of *M. purpureus* had a positive relationship. This finding aligned with Kongbangkerd et al. (2014), who demonstrated the simultaneous production of glucosamine and pigments during Monascal Waxy Corn (MWC) fermentation; with increasing amounts of ETA, the pigment intensity and glucosamine levels in MBR showed an upward trend. Application of ETA (at levels of 10-20 mg/mL) during *Monascus* fermentation resulted in a substantial increase in pigmentation by 125.56% and glucosamine content by 39.62% compared to the control (without ETA). In comparison, Wei et al. (2022) observed that Exogenous Ascorbic Acid (EAA) used as a supplement for *Monascus* substrate exhibited lower pigmentation enhancement compared to ETA. The EAA at a concentration of 16 mg/L increased *Monascus* pigment intensity by only 29% compared to the control.

Supplementation of ETA played a crucial role in boosting both the growth of *Monascus* and its pigment production. Notably, broken rice substrate, rich in macronutrients, such as carbohydrates, proteins, and minerals, along with the presence of *M. purpureus*, a potent strain known for high pigment synthesis, acts as a favorable medium for pigment and antioxidant production. Therefore, factors such as ETA, broken rice substrate, fermentation conditions, and *Monascus* strain act synergistically to enhance *Monascus* pigment intensity and growth. Maintaining a fermentation temperature of 30°C and an initial moisture content of 30% in sterilized broken rice (data not shown) creates an optimal environment for angkak fermentation (Kongbangkerd et al. 2014; Kraboun et al. 2023).

Effect of ETA supplementation on changes in monacolin K and citrinin contents

Table 2 displays the levels of monacolin K and citrinin in MBR supplemented with 0-20 mg/L ETA. The addition of ETA resulted in higher monacolin K content in MBR compared to the control (no ETA), while citrinin content decreased. Specifically, at 10 and 20 mg/L ETA supplementation, monacolin K levels in MBR increased by 99.93% and 197.94%, respectively, compared to the control. Conversely, citrinin amounts in MBR were reduced by 74.82% and 73.26% at ETA concentrations of 10 and 20 mg/L, respectively, compared to the control. Wei et al. (2022) also observed that *Monascus* substrate added with EAA at 16 mg/L led to a 67.60% decrease in citrinin content, indicating the superior effectiveness of ETA in reducing citrinin accumulation in angkak compared to EAA. Huang et al. (2017) noted that incorporating exogenous genistein or rutin into the *Monascus* substrate decreased citrinin levels in angkak. However, the mechanism of ETA's citrinin reduction remains unclear. ETA was anticipated to improve the balance between Reactive Oxygen Species (ROS) and antioxidants, thus reducing oxidative stress and, subsequently, lowering citrinin accumulation.

The presence of citrinin in monascal rice products is a significant concern because this mycotoxin is directly generated by *Monascus* sp. Various countries have set allowable limits for citrinin content in angkak products. In Japan and the European Union (EU), the general citrinin content limit is <200 and 100 µg/kg, respectively. At the same time, the Chinese Food and Drug Administration (CFDA) mandates <20 µg/kg (Diaz et al. 2016). Fortunately, MBR supplemented with 10 and 20 mg/L ETA (5.32-5.65 µg/kg) did not exceed these regulations, except for the control group without ETA supplementation.

Impact of ETA addition on fatty acids

The fatty acid composition of MBR resulting from 0-20 mg/L ETA supplementation is shown in Table 3. The proportions of unsaturated fatty acids (USFAs) to Saturated Fatty Acids (SFAs) in MBR varied based on the added ETA levels. The presence of ETA influenced *Monascus* by enhancing the release of unsaturated fatty acids, particularly linoleic acid (C18:2), oleic acid (C18:1), palmitic acid (C16:0), and stearic acid (C18:0) compared to other fatty acids. The *M. purpureus* utilized ETA to produce higher levels of linoleic acid (C18:2) ranging from 30 to 32% and oleic acid (C18:1) ranging from 23 to 24%. In comparison, the control (no ETA) reported 25.09% for C18:2 and 20.23% for C18:1. This observation aligned with Wei et al. (2022), who noted higher USFA levels in *Monascus* broth supplemented with EAA during a 144-h fermentation period. Myristoleic acid (C14:1) was specifically identified in MBR treated with 10 and 20 mg/L ETA. The increased ratios of USFAs to SFAs in MBR due to ETA supplementation impacted cell membrane properties such as fluidity and permeability adjustments, balancing intracellular and extracellular pigments (Table 1). This finding was supported by Huang et al. (2017), while Hajjaj et al. (2000) suggested that the secretion of USFAs by *Aspergillus flavus* or *A. parasiticus* suppressed aflatoxin accumulation. Interestingly, higher ETA concentrations increased USFA accumulation (Table 3) while also reducing citrinin levels (Table 2) in MBR.

Effect of ETA supplementation on antioxidant abilities via scavenging activities of superoxide anion ($\cdot O^{-2}$) and hydroxyl free radical (HO•) and antioxidant enzyme abilities in terms of SOD and CAT

Superoxide anion radicals ($\cdot O^{-2}$) act as initial free radicals that stimulate the production of other important free radicals. In the process known as the Haber-Weiss reaction, $\cdot O^{-2}$ combines with H_2O_2 to create hydroxyl free radical (HO•), which is more reactive than $\cdot O^{-2}$. Additionally, $\cdot O^{-2}$ inhibits the activity of glutathione peroxidase (GPx) and catalase (CAT) (Lee et al. 2018), making $\cdot O^{-2}$ and HO• crucial indicators of oxidation levels in food items. Alterations in the scavenging capabilities of $\cdot O^{-2}$ and HO• by MBR with ETA supplementation are illustrated in Figure 1A.

Table 1. Pigment intensity and glucosamine level in MBR at 0-20 mg/mL ETA supplementation

Treatment	Pigment intensity (unit/g dry weight)	Glucosamine)mg/g dry weight(
Control (without ETA)	207.12+6.12[a]	32.38+1.21[a]
ETA 10 mg/mL	467.18+7.56[b]	45.21+2.98[b]
ETA 20 mg/mL	513.11+8.14[c]	55.88+3.44[c]

Note: Mean values with different letters in the same row are significantly different (p<0.05)

Table 2. Monacolin K and citrinin contents in MBR at 0-20 mg/mL ETA supplementation

Treatment	Monacolin K (mg/kg dry weight)	Citrinin (µg/g dry weight)
Control (without ETA)	30.13+2.12[a]	21.13+1.20[b]
ETA 10 mg/mL	60.24+3.22[b]	5.32+0.04[a]
ETA 20 mg/mL	89.77+2.43[c]	5.65+0.06[a]

Note: Mean values with different letters in the same row are significantly different (p<0.05)

Table 3. Fatty acid profiles in MBR at 0-20 mg/mL ETA supplementation

Category	Fatty acid	Relative amount)%(		
		Control (without ETA)	ETA 10 mg/mL	ETA 20 mg/mL
Unsaturated fatty acid)USFA(	Myristoleic acid (C14:1)	ND	0.50+0.01^{a}	0.53+0.01^{a}
	Palmitoleic acid (C16:1)	1.52+0.03^{b}	1.24+0.02^{a}	1.32+0.04^{ab}
	Oleic acid (C18:1)	20.23+1.11^{a}	23.13+2.15^{b}	24.22+3.12^{b}
	Linoleic acid (C18:2)	25.09+1.34^{a}	30.12+1.21^{b}	32.11+2.12^{b}
	Linolenic acid)C18:3(	0.45+0.01^{a}	0.56+0.04^{a}	0.87+0.01^{b}
	Eicosenoic acid (C20:1)	0.21+0.01^{a}	0.23+0.01^{a}	0.25+0.02^{a}
	Arachidonic acid (C20:4)	0.21+0.02^{a}	0.22+0.02^{a}	0.24+0.02^{a}
	Total UFA	47.71+1.67^{a}	56.00+0.45^{b}	59.58+2.12^{b}
Saturated fatty acid)SFA(	Myristic acid (C14:0)	0.56+0.02^{a}	0.59+0.02^{a}	0.55+0.02^{a}
	Palmitic acid (C16:0)	12.21+0.23^{c}	9.98+0.13^{b}	7.66+0.25^{a}
	Stearic acid (C18:0)	11.88+0.54^{b}	7.11+0.03^{a}	7.45+0.02^{a}
	Arachidic acid (C20:0)	0.39+0.02^{a}	0.43+0.01^{b}	0.38+0.02^{a}
	Behenic acid (C22:0)	3.23+0.12^{a}	3.45+0.03^{a}	3.44+0.05^{a}
	Lignoceric acid (C24:0)	0.56+0.01^{a}	0.58+0.02^{a}	0.55+0.01^{a}
	Total SFA	28.83+1.89^{b}	22.14+2.54^{a}	20.03+1.25^{a}
Ratio of USFA: SFA		1.65+0.02^{a}	2.53+0.03^{ab}	2.97+0.29^{b}

Note: ND = Not Detected; mean values with different letters in the same row are significantly different (p<0.05)

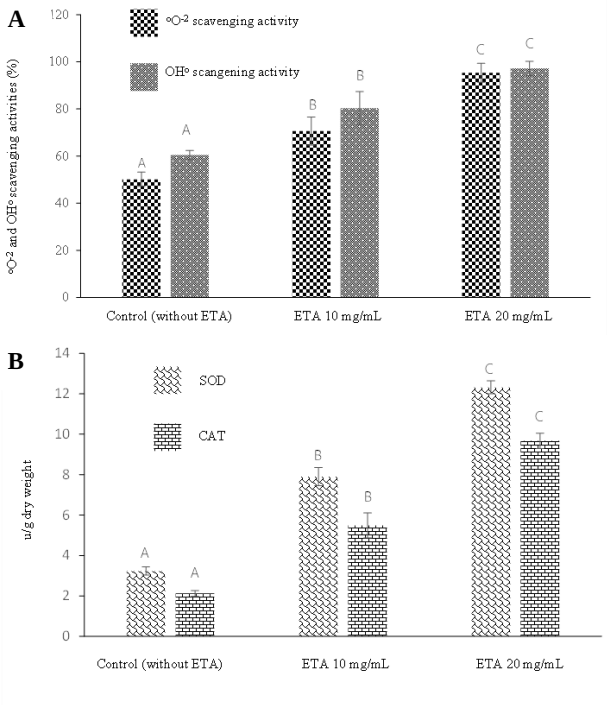

Figure 1. Superoxide anion (·O^{-2}) and hydroxyl free radical (HO•) scavenging activities (A) and SOD and CAT (B) in MBR from ETA at 0-20 mg/mL supplementation. Different capital letters indicate significant differences (p < 0.05) among treatments.

When comparing all ETA treatment groups, MBR displayed higher HO• scavenging efficacy than ·O^{-2} scavenging. The control (without ETA) exhibited relatively low scavenging rates of 50.16% for ·O^{-2} and 60.36% for HO•. This suggested that incorporating ETA improved the ability of MBR to scavenge ·O^{-2} and HO•. Compared to the control, adding 10 and 20 mg/L ETA increased the ·O^{-2} and HO• scavenging of MBR by nearly 20%. Hence, supplementing ETA in the *Monascus* substrate decreased the levels of ·O^{-2} and HO• radicals, with a reduction in ROS formation and oxidative stress, as indicated by Wei et al. (2022).

Superoxide Dismutase (SOD) can hinder ·O^{-2} radicals when faced with oxidative stress, which is crucial for safeguarding cellular components from the damage caused by ·O^{-2} radicals (Kraboun et al. 2022), while catalase (CAT) serves as a marker for the removal of H_2O_2 (Lee et al. 2018). Figure 1B shows the performances of SOD and CAT in MBR with ETA supplementation ranging from 0-20 mg/L. The trends in SOD and CAT activities in MBR were similar to the scavenging actions of ·O^{-2} and HO• displayed in Figure 1A. The most notable enhancements in the activities of SOD and CAT antioxidant enzymes in MBR were observed at 20 mg/L ETA supplementation, followed by 10 mg/L ETA supplementation, and lastly, the control (without ETA). Compared to the control, the introduction of 20 mg/L ETA led to a 3.81-fold increase in SOD activity and a 4.56-fold increase in CAT activity in MBR. In summary, incorporating ETA as a supplement in *Monascus* substrate enhanced the antioxidant capabilities of MBR, as evidenced by increased SOD and CAT activities, along with elevated ·O^{-2} and HO• scavenging abilities.

The relationship between pigments, glucosamine, monacolin K, citrinin, fatty acid composition, antioxidant enzymes, and antioxidant activities

In Figure 2, the loading plots of Principal Component Analysis (PCA) for various factors in MBR, such as pigment intensity, glucosamine, monacolin K, citrinin contents, fatty acid composition, antioxidant enzymes, and activities. PC1 and PC2 explained 100% of the total variance, with PC1 covering 78.78%. PC1 showed a strong association with multiple dependent variables, including

pigment intensity, glucosamine, monacolin K, antioxidant activities, and specific fatty acids. At the same time, PC2 explained 21.22% of the total variance and included compounds such as citrinin and various fatty acids. The correlation analysis in Table 4 indicated that palmitic acid significantly negatively correlated with SOD, CAT, monacolin K, and $\cdot O^{-2}$ and HO• scavenging activities. Citrinin negatively correlated with oleic acid, linoleic acid, and linolenic acid by -0.959, -0.956, and -0.689, respectively (p>0.05 and 0.01). Hence, USFAs such as oleic acid, linoleic acid, and linolenic acid may be used as an indirect index for citrinin synthesis and impact the amount of citrinin during *Monascus* fermentation (Table 2).

Categorization among ETA treatments using Principal Component Analysis (PCA)

Figure 3 shows the discrimination of ETA treatments using PCA based on pigment intensity, glucosamine, monacolin K, citrinin contents, fatty acid composition, antioxidant enzymes, and antioxidant activities in MBR. PC1 and PC2 described 91.13% of the total variance. PC1 (containing 10 and 20 mg/mL ETA supplementation) and PC2 (the control, without ETA addition) explained 55.03 and 36.10% of the total variance, respectively. Results indicated that classifying ETA treatments may depend on higher amounts of dependent variables found in 10 and 20 mg/mL ETA supplementation than the control (Tables 1-3).

Table 4. Pearson's correlation coefficient among pigment intensity, glucosamine, monacolin K, citrinin contents superoxide anion ($\cdot O^{-2}$), hydroxyl free radical (HO•) scavenging activities, SOD and CAT activities, oleic acid, linoleic acid, linolenic acid, palmitic acid, and stearic acid

	Pigment intensity	Gluco-samine	Monacolin K	Citrinin	Superoxide anion ($\cdot O^{-2}$)	Hydroxyl free radicals (HO•)	SOD	CAT	Oleic acid	Linoleic acid	Linolenic acid	Palmitic acid
Glucosamine	0.946											
Monacolin K	0.929	0.999*										
Citrinin	-0.988	-0.883	-0.860									
Superoxide anion ($\cdot O^{-2}$)	0.905	0.994	0.998*	-0.827								
Hydroxyl free radicals (HO•)	0.945	1.000**	0.999*	-0.882	0.994							
SOD	0.933	0.999*	1.000**	-0.865	0.997*	0.999*						
CAT	0.902	0.993	0.998*	-0.823	1.000**	0.994	0.997*					
Oleic acid	0.992	0.979	0.969	-0.959	0.952	0.979	0.971	0.950				
Linoleic acid	0.990	0.982	0.971	-0.956	0.955	0.981	0.974	0.953	1.000**			
Linolenic acid	0.795	0.949	0.963	-0.689	0.977	0.949	0.960	0.979	0.866	0.871		
Palmitic acid	-0.923	-.998*	-1.000*	0.851	-.999*	-.998*	-1.000*	-0.999*	-0.964	-0.967	-0.967	
Stearic acid	-0.979	-0.861	-0.835	0.999*	-0.800	-0.859	-0.841	-0.796	-0.946	-0.942	-0.656	0.826

Note: *Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).

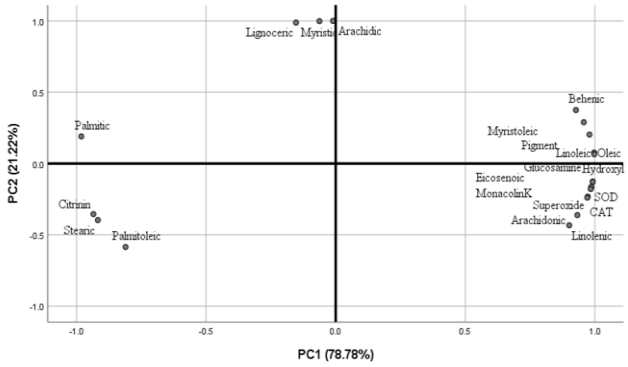

Figure 2. Loading plots of Principal Component Analysis (PCA) based on pigment intensity, glucosamine, monacolin K, citrinin contents, fatty acids, antioxidant enzymes, and antioxidant activities in MBR

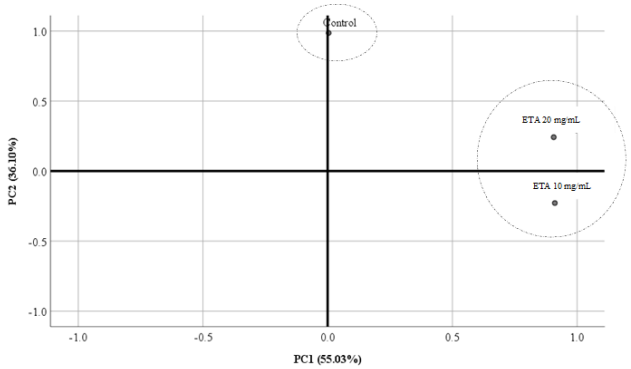

Figure 3. Principal Component Analysis (PCA) for ETA treatment discrimination based on pigment intensity, glucosamine, monacolin K, citrinin contents, fatty acids, antioxidant enzymes, and antioxidant activities in MBR

In conclusion, adding ETA led to a significant 73.26% reduction in citrinin accumulation in MBR compared to the control with no ETA. ETA addition increased the production of pigments, glucosamine, and monacolin K as well as $\cdot O^{-2}$ and (HO•) scavenging activities and SOD and CAT activities in MBR. To enhance the ratio of unsaturated fatty acids to saturated fatty acids, broken rice substrate should be supplemented with ETA concentration > 10 mg/L. PCA loading plots and Pearson's correlation coefficient suggested that oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) could be used as markers for citrinin. PCA analysis confirmed that MBR treated with 10 and 20 mg/L of ETA differed from the control due to the impact of the dependent variable content.

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