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Research Article

Assessment of Monacolin in the Fermented Products Using *Monascus purpureus* FTC5391

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Monacolins, as natural statins, form a class of fungal secondary metabolites and act as the specific inhibitors of HMG-CoA reductase. The interest in using the fermented products as the natural source of monacolins, instead of statin drugs, is increasing enormously with its increasing demand. In this study, the fermented products were produced by *Monascus purpureus* FTC5391 using submerged and solid state fermentations. Two commercial *Monascus*-fermented products were also evaluated for comparison. Improved methods of monacolins extraction and identification were developed for the assessment of monacolins in the fermented products. Methanol and ethanol were found to be the most favorable solvents for monacolins extraction due to their ability to extract higher amount of monacolin K and higher numbers of monacolin derivatives. Problem related to false-positive results during monacolins identification was solved by adding monacolin lactonization step in the assessment method. Using this improved method, monacolin derivatives were not detected in all *Monascus*-fermented products tested in this study, suggesting that their hypocholesterolemic effects may be due to other compounds other than monacolins.

1. Introduction

Cardiovascular disease, particularly coronary heart disease (CHD), is one of the main causes of mortality in developed countries [1, 2]. Atherosclerosis is the underlying disorder in the majority of patients presenting cardiovascular disease. Atherosclerosis is displayed when fat, cellular waste products, calcium, and other substances, particularly cholesterol, are deposited inside the artery, gradually reducing the inside diameter of the artery. Subsequently, this may cause blockage of large- and medium-sized arteries [1, 3]. Hence, the blood cholesterol level should be controlled to avoid atherosclerosis. Epidemiological studies have demonstrated a strong association between plasma cholesterol concentration and risk of CHD [4]. Statins (in particular, monacolin K, mevinolin, or lovastatin) form a class of molecules with a polyketide structure, a well-known inhibitor of HMG-CoA reductase. They are produced by secondary metabolism of several fungi such as *Aspergillus terreus*, *Penicillium citrinum,* and *Monascus* spp. [3]. At present, statins are widely used as hypercholesterolemia drugs for reduction of plasma cholesterol level [5].

Since, the great effect of food on health has been accepted, utilization of complementary and alternative therapies (natural treatments) for a variety of chronic diseases are increasing in demand. Red fermented rice or specifically, the *Monascus*-fermented product (MFP), is one of the popular remedies advertised as a therapy for hyperlipidemia.

Consumer spending on red yeast rice grew by about 80% from 2005 to 2008 in the United States, with the total sales of USD 20 million in 2008 [6, 7]. In Asia, particularly in China, Taiwan, Japan, and Indonesia, MFPs produced by *Monascus* spp. have been used as dietary supplements and remedy for decades [8, 9].

MFP contains at least 14 major chemical constituents which have chemical similarity to statin. It is believed that the cholesterol-lowering activity of MFP is mainly due to monacolin K (mevinolin or lovastatin). However, the effect of MFP on blood cholesterol could not be fully explained by the action of monacolin K alone, instead, it might be the combined effect of monacolins and other substances in MFP [10]. Since red fermented rice has not yet been approved as medicinal by the Food and Drug Administration (FDA), physicians may be limited in suggesting the guidelines of using this product by the patients. In this regard, generation of accurate information on the type and amount of monacolins present in MFP as a natural supplement is a complicated task.

The main objective of this study was to compare the quality and quantity of monacolins present in MFPs produced by *M. purpureus* FTC5391 with two commercial MFPs available in the market. The fermented products by *M. purpureus* FTC5391 were prepared using solid and submerged fermentation techniques with various types of substrate for enhancement of monacolins synthesis. To maximize the extraction of monacolins from the MFP, the extraction parameters such as type of solvent and temperature were optimized. Some important points for the improvement of methods for the detection of monacolins present in MFP were also identified in this study.

2. Materials and Methods

2.1. Materials. MK (mevinolin, lovastatin) standard, trifluoroacetic acid (TFA) analytical grade, corn steep liquor, and monosodium glutamate were purchased from Sigma (St. Louis, Mo, USA). Soybean, groundnut, coconut waste, gluten, pineapple waste, rice, and rice bran were purchased from local markets. The red yeast rice (RYR) capsule was obtained from Cosway (Malaysia), while Hypocol was purchased from Wearnes Biotech and Medicals (Malaysia). Yeast extract, malt extract, casamino acids, agar, standard Czapek medium, potato dextrose agar (PDA) and peptone were purchased from Difco (USA). Other chemicals, including methanol and acetonitrile (ACN), ethyl acetate, isopropanol, butanol (HPLC grade), and ethanol (analytical grade) were obtained from Merck (Germany).

2.2. Preparation of Monascus-Fermented Products. M. purpureus FTC5391 is a red-pigment-producing fungus isolated from fermented rice (angkak) and maintained at the culture collection in Malaysian Agricultural Research and Development Institute (MARDI). *M. purpureus* FTC5391 was maintained after routine inoculation on PDA and incubated at 30°C for 7 days, by storage at 4°C and refreshing monthly. For inoculum preparation, mycelia blocks (4 × 4 mm) of *M. purpureus* were transferred into 100 mL of YMP (3 g/L yeast extract; 3 g/L malt extract; 5 g/L peptone; 20 g/L glucose) in a 250 mL flask. The flasks were incubated in rotary orbital shaker at 30°C, agitated at 150 rpm for 4 days, and these cultures were used as a standard inoculum for all fermentations.

The ability of *M. purpureus* FTC5391 in producing MK was tested on solid (SSF) and in submerged fermentations (SmF) using different media, including: Hiroi or sucrose medium [11] (sucrose (100 g/L), yeast extract (3 g/L), casamino acids (5 g/L), $NaNO_3$ (2 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$ (0.01 g/L), and agar (15 g/L)); Hiroi-PDA (sucrose (100 g/L), yeast extract (3 g/L), casamino acid (5 g/L), $NaNO_3$ (2 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$(0.01 g/L), potato starch (4 g/L), dextrose (20 g/L), and agar (15 g/L)); Hajaj medium [12] (glucose (50 g/L), monosodium glutamate (12 g/L), KH_2PO_4 (2.5 g/L), K_2HPO_4 (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (1 g/L), KCl (0.5 g/L), $ZnSO_4 \cdot 7H_2O$ (0.001 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L) and $MnSO_4 \cdot 7H_2O$ (0.003 g/L)); glucose-glycerol-peptone (GGP) medium [13, 14] (glucose (30 g/L), glycerol (70 g/L), peptone (38 g/L), $MgSO_4 \cdot 2H_2O$ (1 g/L), and $NaNO_3$ (2 g/L)); glutenous medium (gluten (40 g/L), malt (9 g/L), glucose (30 g/L), $MnSO_4 \cdot 7H_2O$ (2 g/L), and $MgSO_4 \cdot 7H_2O$ (3 g/L)); Rice medium (rice (40 g/L), malt (9 g/L), glucose (30 g/L), $MnSO_4 \cdot 7H_2O$ (2 g/L) and $MgSO_4 \cdot 7H_2O$ (3 g/L)); and, glucose-peptone-corn steep liquor (GPC) medium [15, 16] (glucose (60 g/L), peptone (25 g/L), corn steep liquor (5 g/L) and NH_4Cl (5 g/L)).

SmFs were carried out in 1000 mL Erlenmeyer flasks containing 500 mL liquid medium. All media were autoclaved for 15 min at 121°C after adjusting the pH at 6.0. Glucose was autoclaved separately to avoid the caramelization process and then was added to the related media under sterile conditions. The flasks were inoculated with 10% (v/v) inoculum and incubated in a rotary orbital shaker at different temperatures (25–37°C) and different agitation rates (110–250 rpm) for 25 days. Samples (5 mL) were withdrawn at 48 h intervals for MK analysis.

SSFs were carried out using different solid substrates, including soybean, ground nut, grated coconut waste, gluten, pineapple waste, rice, and rice bran. The substrates were ground and mixed with 1% (w/v) acetic acid. The 500 mL beaker containing 200 g of substrate was sterilized for 15 min at 121°C. To start the fermentation, the beaker was inoculated with the standard inoculum (10% v/w) and incubated at different temperatures (25–37°C) until the color of media had completely changed to red, normally about 10 days. During the fermentation, the humidity was maintained at 50–60%.

2.3. Sample Preparation

2.3.1. Extraction Conditions. The following solvents were used for the extraction process: ethanol, methanol, ethyl acetate, ACN, isopropanol, ethyl acetate, and butanol. The extraction was conducted for 30 min, 1 h, 2 h, and 12 h at different temperatures (37–80°C).

Extraction of Monacolin Compounds. Slightly different methods for extraction of monacolins were applied to solid and

liquid samples of the prepared fermented products. Solid fermented product was dried and finely ground into powder. The sample (0.5 g) was mixed with 5 mL of ethanol/water solution (75 : 25) for 2 h at 60°C under agitation, followed by centrifugation for 10 min at 3000 ×g. Liquid fermented product was homogenized to break the mycelia cells. The homogenized sample (5 mL) was extracted with 5 mL of 95% ethanol for 2 h at 60°C under agitation and was subsequently centrifuged for 10 min at 3000 ×g. In both cases, the supernatant (1 mL) was concentrated and dried under vacuum and then was resolved in 1 mL ACN. The mixture was filtered through a membrane filter (0.45 μm) prior to HPLC analysis.

2.3.2. Preparation of the Standard Solution. Calibration curves were obtained using an MK standard. The purity of the standard was evaluated using LC/PDA. MK (10 mg) was dissolved in pure solvents aforementioned or ethanol in water (75 : 25 v/v) inside a 50 mL actinic volumetric flask. The solutions were diluted with solvents to obtain the standard solutions for the calibration curve in a concentration ranging from 0.2 to 20 ng/mL. Individual solutions were filtered through a 0.45 μm syringe membrane filter prior to injection (20 μL) into HPLC.

2.3.3. Lactonization. MK has two forms, lactone (MKL) and hydroxyl acid (MKA) forms. For lactonization of MK, 1 mL of the extracted supernatant was mixed with 0.1 N NaOH (1 mL) and kept for 2 h at 30°C. The mixture was concentrated and dried under vacuum, which was then resolved in 1 mL ACN. The solution was filtered through a 0.45 μm membrane syringe filter prior to analysis using HPLC.

2.4. Detection of Monacolins. The HPLC system (Waters 2695 Alliance, Waters Inc., USA) was equipped with an online degasser, and an autosampler was used for detection of monacolin. The raw data were detected by 2996 PDA, acquired and processed by a Waters Millennium32/Empower software, chromatographic workstation loaded on an IBM computer. The column of Waters Symmetry C18 (150 mm × 3.9 mm i.d., 5 μm) was used as a stationary phase. A linear gradient of concentrated ACN (eluent A) and 0.1% TFA (eluent B) was used as the mobile phase at a flow rate of 0.9 mL/min. The amount of eluent A was increased from 5 to 75% in the first 15 min, kept at 75% for 5 min, increased to 95%, and then reduced to 5% in another 10 min. The PDA was used at absorbance ranging from 210 to 350 nm. The column temperature was set at 28°C, and the injection volume was 20 μL.

The Thermo-Finnigan LCQ Classic was employed to analyze separation via liquid chromatography, coupled with electrospray ionization (ESI) quadrupole ion-trap mass spectrometry. The chromatographic conditions of LC/PDA/MS were modified from the conditions of LC/PDA. The separations by LC/PDA/MS were performed using an arrow-bore reversed-phase Zorbax SB-C18 HPLC column (2.1 mm × 100 mm i.d., 5 μm, Agilent Scientific, Calif, USA) with a gradient elution consisting of ACN (eluent A) and aqueous 0.2% acetic acid (eluent B) at a flow rate of 300 μL/min. The gradient pattern was similar to that used for LC/PDA analysis. The HPLC elution after PDA was analyzed by the mass spectrometer (MS) using electrospray ionization (ESI). All analyses were performed using an ESI interface with the following settings: positive ionization mode; the capillary temperature was set at 250°C; spray voltage was set at 4.5 kV; capillary voltage was set at 6 V; sheath gas (N_2) flow was fixed at 30 AU; auxiliary gas (N_2) flow was fixed at 10 AU.

3. Results and Discussion

3.1. Chromatographic Peaks Identification. In the preliminary study, hypocholesterolemic activity in the fermented products using *M. purpureus* FTC5391 was analysed *in vivo* via animal testing. Hypocholesterolemic activity was detected in all MFPs tested, and it was assumed due to the existence of monacolins (unpublished data). In this study, the existence of monacolins in similar MFPs was evaluated using LC/PDA/MS.

Basically, different derivatives of monacolins have similar structures, and consequently, similar UV spectra. In this study, different concentrations of MKA and MKL standards were prepared and analyzed via LC/PDA/MS. Figure 1 shows the chromatogram and UV spectrum of the MK standard. The retention time (RT) of MKL and MKA were observed at 21 min and 17 min, respectively. The UV spectrum of MKL and MKA showed a pattern with three λ_{max} at 232, 239, and 248 nm. The chromatograms of Hypocol (Figure 2) and RYR (Figure 3) showed 12 and 3 extra peaks, respectively, with a similar UV spectrum pattern of MK. These extra peaks may be related to other derivatives of monacolin due to existence of similarities in their structures (common polyketide portion, a hydroxy-hexahydro-naphthalene ring system, where different side chains are attached).

The chromatogram results of all MFPs produced using *M. purpureus* FTC5391 by two different methods of fermentation (SSF and SmF) with various substrates showed that there was only one peak with the same RT as MK standard, while its UV spectrum was different. The UV spectrum of this peak showed the λ_{max} at 228 nm and that was not similar with UV spectrum of mountain-like peak at λ_{max} of 232, 239, and 248 nm (Figure 4). This means that all MFPs using *M. purpureus* FTC5391, tested in this study, did not contain monacolin derivatives. The UV spectrum peak at RT of 11.917 min was similar to yellow pigment spectrum [8] with λ_{max} pattern at 237, 280, and 332 nm (Figure 4).

LC/MS is one of the informative methods for the detection and identification of the bioactive molecules. Recently, rp-HPLC has been widely applied for the determination of secondary metabolites in MFP such as pigments, monacolins, GABA, and several other components [8, 9, 17–19]. Improvement of the technical performance of chromatography permits efficient separation of similar molecules. Adjustment and selection of the mobile phase is one of most important stages of analyses in the rp-HPLC. Although a large variety of organic solvents can be used in reversed phase chromatography, in practice only a few are routinely employed. Acetonitrile and methanol are the most

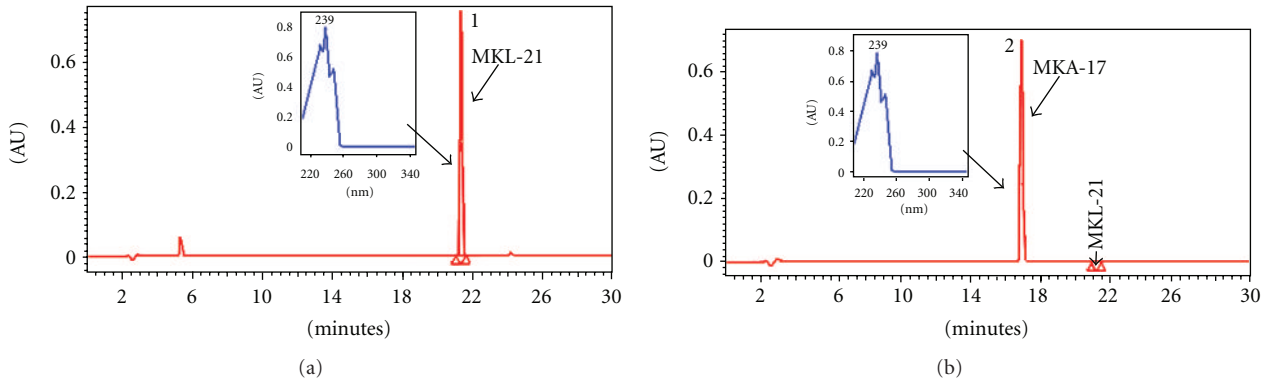

Figure 1: HPLC chromatogram profile with UV spectra of monacolin K standard. (a) Monacolin K lactone form (MKL) chromatogram, and (b) monacolin K acid form (MKA) chromatogram.

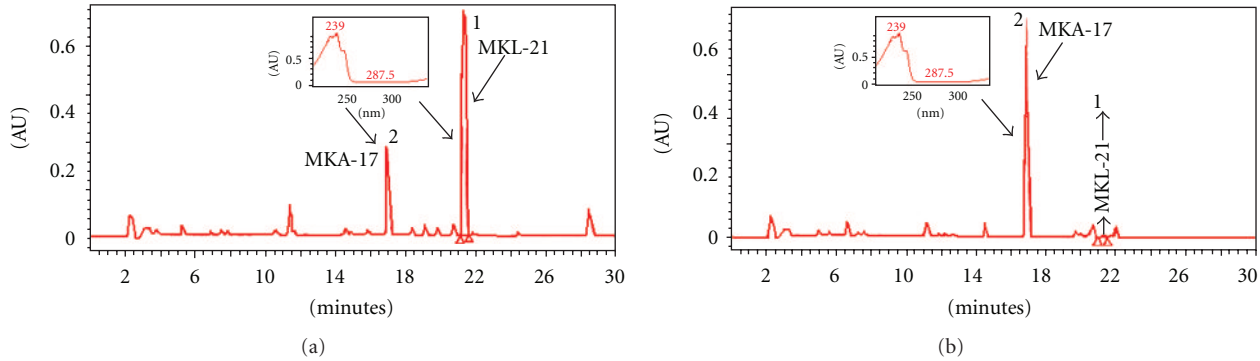

Figure 2: HPLC chromatogram of the supplement-fermented *Monascus purpureus* Hypocol. (a) Hypocol HPLC chromatogram profile before lactonization and (b) after lactonization.

commonly used solvents in this case, since both have low viscosity (even when mixed with aqueous solutions) and are UV transparent. Reverse phase separations are most often performed at low pH values, generally between pH 2 to 4. The low pH results in good solubility of the sample components and ion suppression, not only of acidic groups on the sample molecules, but also of residual silanol groups on the silica matrix. Acids such as trifluoroacetic acid, heptafluorobutyric acid, and orthophosphoric acid in the concentration range of 0.05–0.1% would be suitable in this type of separation. Hence, trifluoroacetic acid (0.1%) and concentrated acetonitrile were used in this study as the mobile phase.

3.2. Confirmation of Monacolin K Identification

3.2.1. Lactonization. Monacolin molecules exist in two forms of lactone and hydroxyl acid [15]. Lactonization or alkalization of monacolins was performed by mixing the sample with NaOH before applying to LC/PDA system. Alkalization of MK standard caused a shift in the RRT from 21 min to 17 min without changing the UV spectrum, indicating that both peaks have similar molecular structures (Figure 1), where it was in agreement with the previous report [20]. Comparison between two chromatograms of pre- and postalkalized Hypocol, RYR, and MFP FTC5391 extracts (Figures 2, 3 and 4, resp.) revealed that some peaks disappeared, while some new peaks appeared. In addition, after alkalization some peak areas increased. However, the similarity of UV spectra of the missed peaks and those with the increased peak areas was high. Although the typical chromatogram for MFP using *M. purpureus* FTC5391 extract before and after lactonization did not change (Figure 4), the comparison between Hypocol and RYR chromatograms and their UV spectra before and after lactonization showed some differences (Figures 2 and 3). The RYR chromatogram (Figure 3) showed a large peak in the RT of 21 min before lactonization, this peak shifted to 17 min after lactonization with similar spectrum of MKL. After the shifted of large peak, a small peak at the RT of 21 min appeared with different UV spectrum of MKL. This peak showed a similar UV spectrum with the peak for MFP using *M. purpureus* FTC5391 extract at RT of 21 min. In addition, the Hypocol chromatogram demonstrated a small peak in the RT of 17 min that grew up after lactonization. These results suggested that there were two different peaks belonging to different compounds, where the small peak was masked by the large peak in the same RT. However, lactonization shifted the MKL peak and helped to reveal the concealed peak.

Additional MK standard solution in MFP using *M. purpureus* FTC5391 extract was employed to confirm the existence of MK. Figure 5 shows that the addition of MK standard reproduced the peak at RT of 21 min in the MFP

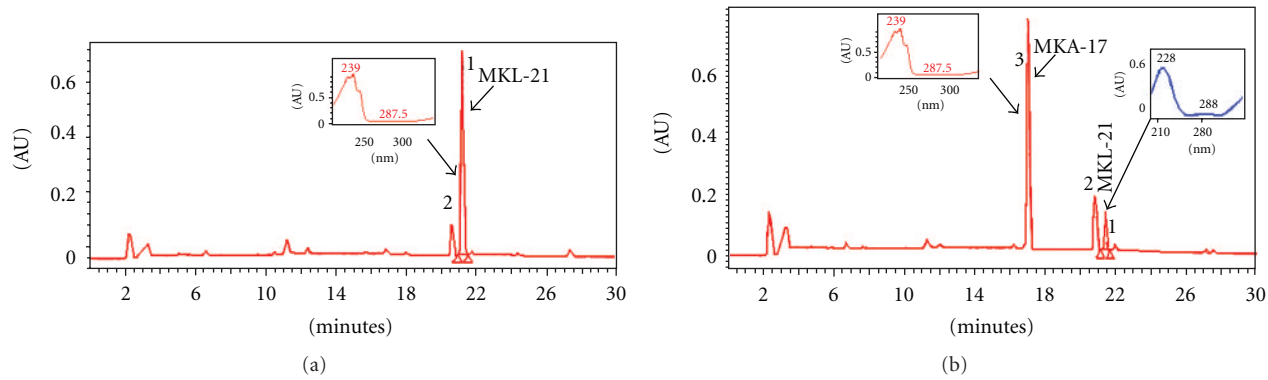

Figure 3: HPLC chromatogram with UV spectra of the supplement-fermented *Monascus purpureus* RYR (commercial product). (a) RYR HPLC chromatogram profile with UV spectrum before lactonization and (b) after lactonization. MKL: monacolin K lactone form, MKA: monacolin K acid form.

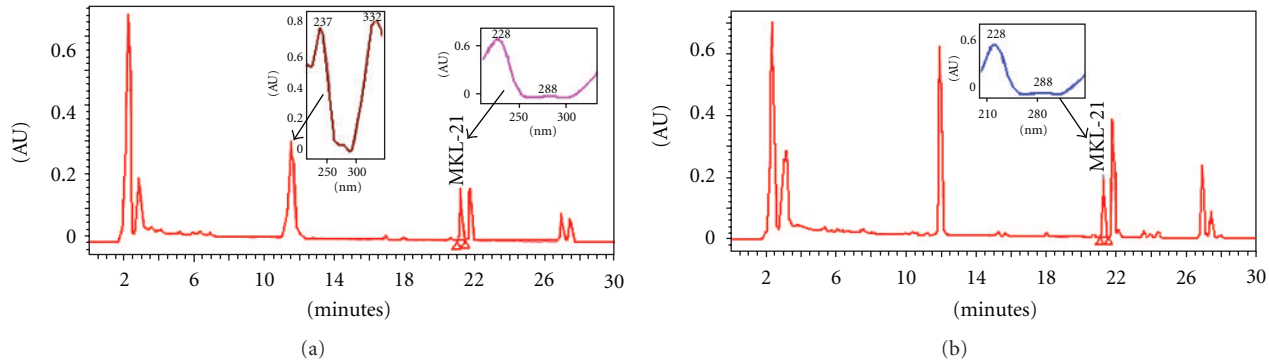

Figure 4: A typical HPLC chromatogram profile with UV spectra of MFP using *Monascus purpureus* (FTC5391). (a) Chromatogram before lactonization and the UV spectrum of 11.92 RT peak related to yellow pigment and (b) chromatogram after lactonization. The MFP was produced by submerged fermentation using Hiroi-PDA (sucrose (100 g/L), yeast extract (3 g/L), casamino acid (5 g/L), $NaNO_3$ (2 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$ (0.01 g/L), potato starch (4 g/L), dextrose (20 g/L), and agar (15 g/L)) MKL: monacolin K lactone form, MKA: monacolin K acid form.

chromatogram. Moreover, there was a significant difference in comparison between the UV spectra related to peaks at RT of 21 min in pre- and postmixing of the MK standard solution with MFP extract. The premixing UV spectrum associated with the peak at RT of 21 min showed λ_{max} at 228 nm, while the postmixing UV spectrum transformed to the MK spectrum shape with three λ_{max} at 232, 239, and 248 nm. The chromatogram of MFP using *M. purpureus* FTC5391 extract blended with MK standard solution after lactonization showed the appearance of a peak at RT 17, where the peak area at RT 21 decreased and its UV spectrum also changed. These results doubly confirmed the existence of two different coincided peaks in the same RT, those concealed each other. Therefore, it was concluded that all MFPs produced using FTC5391 *M. purpureus* FTC5391, tested in this study, did not contain MK.

It is interesting to note that the conversion of monacolins from the lactone form to acid form is a special clue for the development of simple and rapid method for monacolins detection via LC/PDA/MS. In fact, RT evaluation alone is not enough for the identification of bioactive compounds by the LC/PDA method. Thus, this method should be utilized with the examination of other special properties of the target molecule simultaneously. In these cases, where there are two or more compounds with the same RT in the sample, the peaks cover each other and the compound with the higher amount may dominate and mask the other molecules. For a case where the small peaks were related to monacolin, a step of lactonization of monacolins must be added in the detection procedure of LC/PDA/MS to enable identification.

3.3. Identification of Monacolins by LC/PDA/MS. For further identification of the existence of monacolins in the samples and to complete the monacolins identification, all samples and standard solutions were traced by hyphenated instrumentation of LC-MS. The quadruple ion trap mass analyzer serves to store, fragment in, and select ions for detection. Therefore, the total ion current (TIC) chromatogram, mass, UV spectra, and selective ion chromatograms (SIC) for each peak were evaluated, one by one. The results confirmed the existence of MKL, MKA, MJA, MJ, MXA, MLA, MX, MKA, ML, P1, MMA, MM, and DMK in Hypocol; MKL, MM, and DMK in RYR.

The molecular ion chromatograms and mass spectra patterns for MKL and MKA STD by the LC/PDA/MS

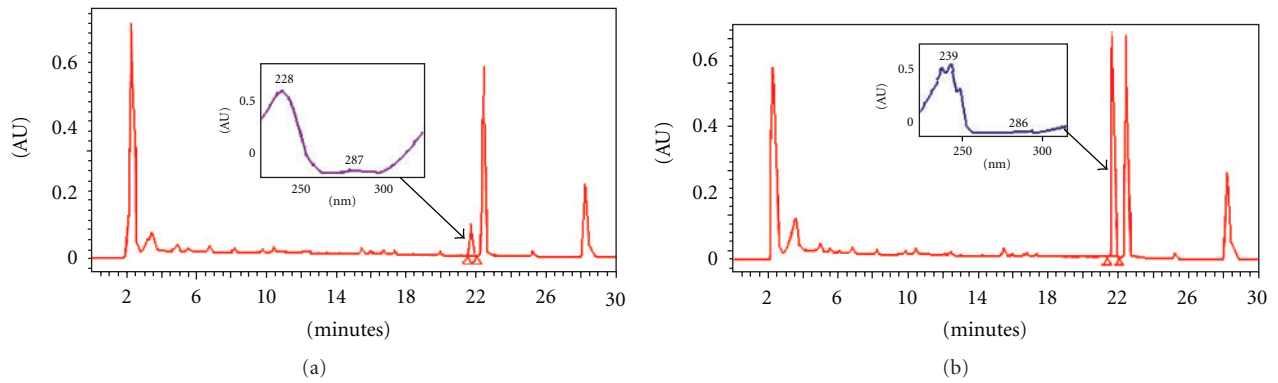

Figure 5: A typical HPLC chromatogram of t MFP using *Monascus purpureus* FTC5391 mixed with monacolin K as standard solution (STD). (a) HPLC chromatogram profile with UV spectrum before mixing with STD and (b) after mixing with STD. The MFP was produced by submerged fermentation using Hiroi-PDA (sucrose (100 g/L), yeast extract (3 g/L), casamino acid (5 g/L), $NaNO_3$ (2 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$ (0.01 g/L), potato starch (4 g/L), dextrose (20 g/L), and agar (15 g/L)).

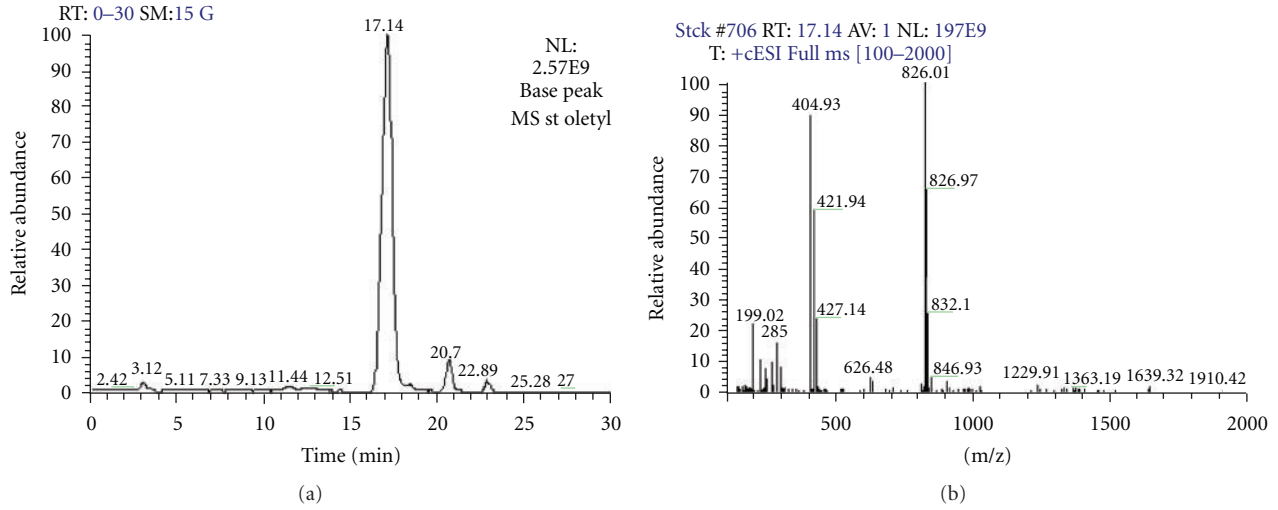

Figure 6: MS chromatogram profile of the MKL standard. (a) MS chromatogram, (b) MS spectrum.

showed the predominant peaks at RT around 15.82 min and 11.82 min; λ_{max} at 245 nm; mass spectrum 405 (M + 1) and 423 (M + 1), respectively. The LC/PDA/MS chromatograms of standards (MKL and MKA) in comparison to MFP using *M. purpureus* FTC5391, Hypocol, and RYR chromatograms revealed that the Hypocol included both MKL and MKA, whereas RYR included only MKL, and the MFP using *M. purpureus* FTC5391 tested had no monacolins, not even MK. Figures 6 and 7 show the differences between mass spectra of MK standard and MFP using *M. purpureus* FTC5391 compound with similar RT of MK.

3.4. Effect of Fermentation Conditions on MK Production. Production of monacolins, secondary metabolites, is influenced by physicochemical factors of the fermentation conditions and is strain-dependent based on individual abilities of the microorganisms [21–25]. Therefore, manipulation of the fermentation conditions (temperature, aeration, nutritional factors, as well as fermentation method) is necessary to induce or enhance the ability of *M. purpureus* strains to secrete monacolins. Results from this study indicated that the use of two types of fermentation methods (SmF and SSF) with various medium formulations and fermentation conditions did not induce monacolins production by *M. purpureus* FTC5391.

Comparison between HPLC chromatogram profiles of Hypocol and RYR revealed that the UV spectra and the number of chromatogram peaks were not similar even though both extracts were derived from MFPs using *M. purpureus* FTC5391 (Figures 2 and 3). Hypocol chromatogram showed the existence of MKL, MKA, and several other peaks with MK-like UV spectra, while the RYR chromatogram revealed just the existence of MKL and a few numbers of peaks with MK-like UV spectra. Since the supplements tested in this study were produced by the different companies under different fermentation conditions using different strains of *M. purpureus*, some of the metabolites or all of them may not be existed in the products. This is in agreement with the results for the analysis of citrinin; and the lactone acid forms of MK in red mold rice [26].

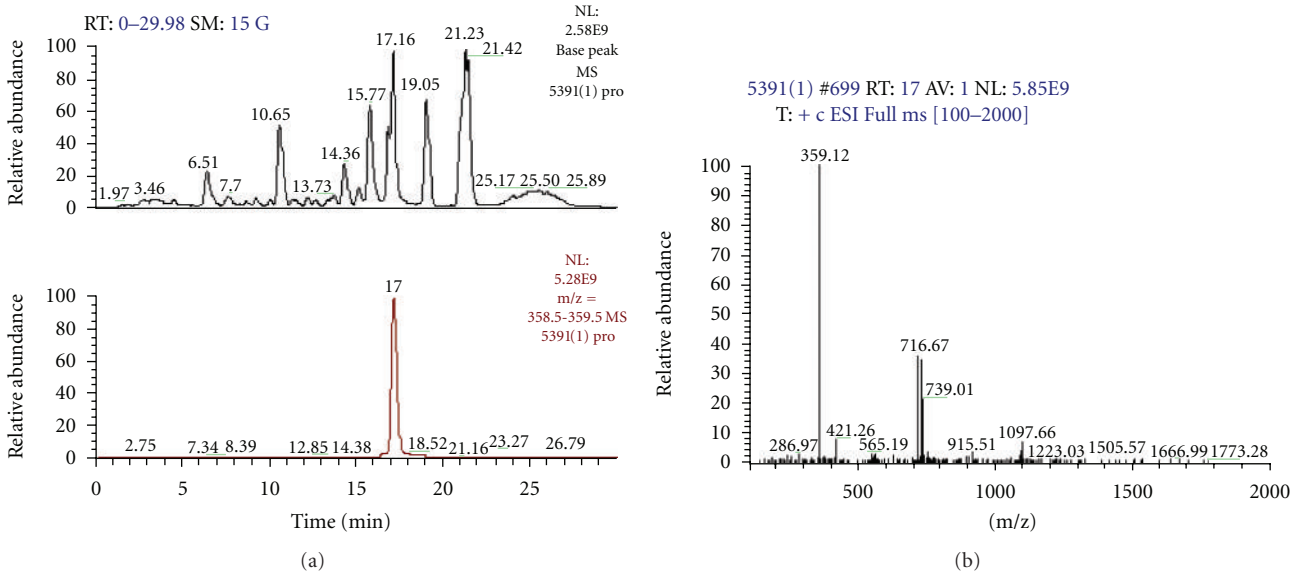

Figure 7: A typical MS chromatogram profile of MFP using *M. purpureus* FTC5391, which also highlighting the compound with similar RT of MKL standard. (a) MS chromatogram, (b) MS spectrum of compound with similar RT of MKL standard. The MFP was produced by submerged fermentation using Hiroi-PDA (sucrose (100 g/L), yeast extract (3 g/L), casamino acid (5 g/L), $NaNO_3$ (2 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$ (0.01 g/L), potato starch (4 g/L), dextrose (20 g/L), and agar (15 g/L)).

3.5. Effect of Different Solvents on Monacolins Extraction Quality. Extraction of the monacolins is the first step required for detection and identification. Selection of appropriate solvent is important to maximize the efficiency of the extraction. MK is soluble in methanol, ethanol, acetone, chloroform and benzene but not in *n*-hexane and petroleum ether [15]. Moreover, ethyl acetate has been used for the extraction of MK [27]. The ability of several polar and nonpolar organic solvents (log *P* values: −0.76 to 0.8) to dissolve MK for the enhancement of the extraction of MK from Hypocol is shown in Table 1. The following sequence for solubility strength of solvents was achieved to dissolve the MK:

> Methanol > Ethanol > Isopropanol > Acetonitrile > Ethyl acetate > Butanol

The peaks pattern (MK-like-spectra) from the HPLC chromatograms was as follows (the numbers in parentheses indicate the numbers of peaks):

> Methanol (18) > Ethanol (17) > Acetonitrile (15) > Isopropanol (14) > Butanol (12) > Ethyl acetate(9)

Methanol and ethanol were identified as the preferred solvents for monacolins extraction due to their ability to extract the highest amount of MKL and the highest numbers of monacolin derivatives. In accordance with these results, Lee et al. [26] also reported that ethanol and methanol were the preferred extraction solvents for MKL, while ethanol was suggested as the best solvent to extract citrinin, MKL, and MKA. Methanolic and ethanolic Hypocol extracts included about 17 and 18 peaks, respectively, which were similar to the UV spectrum of MKL (mountain-like peak at λ_{max} of 232, 239, and 248 nm). The solvents that could dissolve the MK were in a range of semipolar to nonpolar. In spite of no clear correlation between log *P* values of organic solvents and the solubility of monacolins, results of this study indicated that the nonpolar solvents were the preferred solvents for monacolins extraction. The highest extraction efficiency was obtained by methanol (log *P* value of −0.76). Moreover, mixing a proper quantity of water to the water miscible solvents or powder (solid) samples increased the dissolving ability of the target compounds. The diluted solvent could diffuse better in the sample and, consequently, promoted the extraction of monacolins from the base material. Therefore, methanol, ethanol, and isopropanol, as water miscible solvents, were preferred for monacolins extraction from *Monascus*-fermented product prepared through SSF.

Table 1: The efficiency of different solvents on quantity of monacolin K and quality of monacolins.

Solvents	Log *P* value	Number of peaks with similar UV spectrum of MK	Amount of MK extracted (mg/mL)
Methanol	−0.76	18	0.046
Ethanol	−0.31	17	0.044
Acetonitril	−0.34	15	0.044
Isopropanol	0.05	14	0.024
Butanol	0.8	12	0.0072
Ethyl acetate	0.7	9	0.002

3.6. Effect of Physical Factors on Monacolins Extraction Quantity. One of the effective methods for improving the extraction of thermostable compounds is heating. Since MK is a thermostable molecule (mp = 157–159°C) [15], it was predictable that heating enhanced the amount of

MK extraction. The performance of MK extraction was also evaluated under different temperatures (37°C–80°C) for 30 min, 1 h, 2 h, and 12 h. The results showed that the extraction performance was increased up to 60°C for 2 h and decreased beyond this temperature. Extension of the extraction time beyond 2 h did not further improve the extraction performance of the target compound. In addition, agitation also significantly improved monacolins extraction. As a conclusion, the optimum conditions for MK extraction was a mixture of sample with ethanol and water (75 : 25) at 60°C for 2 h under shaking conditions for SSF and a mixture of concentrated ethanol with sample (1 : 1 v/v) for the SmF under shaking conditions at 60°C for 2 h.

4. Conclusions

Results from this study have demonstrated the uniqueness of HPLC chromatograms and UV spectrum profiles for the identification and quantification of monacolins in the fermented products of *M. purpureus*. Monacolins lactonization was found to be the important property of monacolins that can be used to solve the problem of false-positive results in monacolin identification and quantification using LC/PDA/MS. The methanolic extraction of MFP using *M. purpureus* FTC5391 pulled 18 compounds with a similar UV spectrum to MK was observed in this study, though the existence of 14 derivatives of monacolins has been reported to date. Using this improved technique, monacolin was not detected in all MFPs tested in this study (produced by *M. purpureus* FTC5391 using different fermentation techniques and substrates). The hypocholesterolemic activity of the MFPs using *M. purpureus* FTC5391 may be due to the present of other compounds aside from monacolins with hypocholesterolemic effects. Future study is necessary to identify the substance(s) that are responsible for this activity and the underlying molecular mechanisms involved.

Abbreviations

HMG-CoA: Hydroxymethyl glutaryl-coenzyme A
CHD: Coronary heart disease
TFA: Trifluoroacetic acid
PDA: Potato dextrose agar
ACN: Acetonitrile
MK: Monacolin K
MKL: Monacolin K lactone form
MKA: Monacolin K acid form
RYR: Red yeast rice
RRT: Relative retention time
RT: Retention time
MJA: Monacolin J acid form
MJ: Monacolin J lactone form
MXA: Monacolin X acid form
MX: Monacolin X lactone form
MLA: Monacolin L acid form
ML: Monacolin L lactone form
P1: Compactin
MMA: Monacolin M acid form
MM: Monacolin M
DMK: Dehydromonacolin K
STD: Standard
MFPs: *Monascus*-fermented products
SmF: Submerged fermentation
SSF: Solid state fermentations
MS: Mass spectrometer
LC/PDA/MS: Liquid chromatography/photodiode array/mass spectrometer.

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