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A Novel Formulation Approach for Preparation of Nanoparticulate Red Mold Rice

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Monascus has been used for thousands of years. In China, *Monascus* has been widely used as a natural food-coloring agent for many kinds of foods. The metabolites of *Monascus* species, specifically, monacolin K, γ-aminobutyric acid, and dimerumic acid, have been proven to have cholesterol-lowering, blood pressure-lowering, and antioxidant effects. Nowadays, the public has recognized the importance of *Monascus* products for its many health benefits. The focus of this study is to explore the effects of nanoparticulate dispersion of red mold rice (RMR) after wet-milling technology treatment. An RMR nanoparticulate formulation was reproducibly obtained after milling in the presence of dispersing agent and water. Furthermore, the physical and chemical properties of these RMR particles were studied using electron microscopy, laser light scattering, pH meter, high-performance liquid chromatography (HPLC), and photometry. The results demonstrate that RMR (mean size = 20.15 μm), processed with wet-milling technology, forms an aqueous-based nanoparticle dispersion (mean particle size of less than 0.41 μm). In addition, HPLC analyses, performed on the secondary metabolites, demonstrated that monacolin K was reduced to 50−92% of its base level and citrinin was reduced to 48−74% of its base level. When testing for the levels of pH, the processed RMR had increased from a pH level of 4.47−4.82 to 5.56−6.4; also, pigment analysis showed that yellow and red pigments were reduced to 36 and 39% of its base level after the wet-milling process. Partial agglomeration has been observed in RMR dispersion when stored in refrigeration after 2 months. RMR can be formulated as a nanoparticulate dispersion without compromising its stability, but its secondary metabolite extraction rate was changed. Further experimentation will be needed to verify safety and functionality evaluations.

KEYWORDS: ***Monascu*; nanoparticulate; monacolin K; citrinin**

INTRODUCTION

Monascus, a traditional Chinese fermentation fungi, has been used in many kinds of foods for thousands of years (*1*, *2*). It is generally recognized for its health benefits because of the proven benefits of the secondary metabolites such as the monacolins (*3*−*5*), γ-aminobutyric acid (GABA), and dimerumic acid. One of the well-documented metabolites of *Monascus* is monacolin K, which has been identified for its cholesterol-lowering properties due to the competitive inhibitory effect on HMG-Co A reductase (*4*). In addition, the blood pressure-lowering effects of GABA (*6*, *7*) and the antioxidant effects of dimerumic acid (*8*) are also well-known. Although *Monascus* is capable of producing these bioactive compounds, there is a possibility of synthesizing citrinin, a hepatonephrotoxic mycotoxin, during fermentation (*9*). Red mold rice (RMR), the common fermented product of *Monascus*, has been recommended as a dietary supplement (*5*, *10*). As a healthy type food, the need to improve the absorption of monacolin K and reduce the effects of toxicity remains a key point for commercial development. Many approaches have been introduced and even applied for solving such problems; in fact, the application of nanotechnology, which reduces the particle size to nanometer scale, may be useful in this field (*11*). The unique characteristics of nanoparticles are their particle size effects and high surface reactivity, which offer broad applications in medicines and biomaterials (*12*). Pharmaceutically, the nanoparticles have been researched and developed for drug delivering, nanocoating, carrier, and numerous other applications (*13*, *14*). Using wet media-milling technology, a nanoparticle preparation of zinc−insulin was formulated and successfully improved the water solubility and bioavailability (*15*). However, there are few studies aimed at discussing the nanopaticulate process and its effects on biomaterials, especially in foods. In this study, we describe a physically stable nanoparticulate formulation of RMR processed by a high-energy wet-milling method. The nanoparticulate red mold rice (NRMR) dispersion appears to have different physical and chemical properties that can be measured by using electron microscopy, laser light scattering, pH meter, high-performance liquid chromatography (HPLC), and photometry.

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MATERIALS AND METHODS

Materials. RMR used in this study was obtained from a local traditional market in Taipei. The species of the rice used in this study was indica rice (*Oryza sativa* L. spp. *indica*). Yttria-stabilized zirconia grinding media were purchased from Toray Industries, Inc. (Chuo-ku, Tokyo, Japan). Monacolin K, citrinin, and sodium deoxycholate were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Arabic gum TCML and arabic gum no. 408 were purchased from Taiwan Gum Arabic Co. (Chung-Ho, Taipei county, Taiwan). The pluronic stabilizer F68 was purchased from BASF Pharma Solutions (Ludwigshafen, Germany). Dimethyl sulfoxide was purchased from Wako Pure Chemicals (Osaka, Japan).

Formulation of NRMR Dispersion. A NRMR formulation was prepared using a wet-milling process and different dispersing agents to provide efficient particle size reduction and maximum stability. In the end, a physically stable NRMR product was produced. NRMR dispersions were prepared as follows: Crude RMR was ground into flour by utilizing a two-step process. The proper amount of RMR (10 g) was added into a bean grinder and ground for 30 s. Powder collected from the first step (30 g) was ground again using a cyclone sample mill until no obvious solid particles could be observed. Dried RMR powder was weighted (20 g) and stirred gently for 30 min with aqueous solutions (200 mL) of arabic gums, 0.5% F68/0.05% sodium deoxycholate, 0.1% F68/0.01% sodium deoxycholate, and distilled water, respectively. The crude RMR slurry was added to a roller mill jar containing zirconia grinding media (600 g) and placed on the mill NM0010 (Fu Chun Shin Co., Tainan, Taiwan). The wet-milling process was achieved by using 0.65 mm grinding media for 1 h and 0.2 mm grinding media for another 3 h. Processing was performed at 3000 rpm, and the power output of the mill was 80 W. The NRMR dispersion was collected for particle size determination and secondary metabolite analysis. After they were processed, the dispersions were harvested and used for the studies described below.

Particle Size Determination. The particle size was estimated by dynamic laser scattering and electron microscopy. Dynamic light scattering measurements were performed using the Zetasizer-3000HS (Malvern Instruments, Worcestershire, United Kingdom) for studying sizes and shapes of nanoparticulates in liquids (*16*). Prior to use, the instrument was calibrated using standard microspheres (0.199 ± 0.006 μm) and samples were assayed after being diluted with deionized distilled water, filtered with 3 μm filter paper, and then ultrasonicated for 20 min. Light scattering measurements were verified using electron microscopy. For transmission electron microscope (TEM) analysis, a diluted sample was visualized using a Hitachi H-7100 (Hitachi Co., Tokyo, Japan). For scanning electron microscope (SEM) analysis, samples were diluted with deionized−distilled water and an aliquot was dried, sputter-coated, and visualized using the TOPCON ABT-150S (Topcon Technologies Inc., Pleasanton, CA). In particle size analysis, there was insufficient evidence to estimate average sizes of RMR and NRMR particles by SEM or TEM images. The mean particle sizes can be estimated by dynamic laser scattering measurement (*16*).

HPLC Analysis. For chemical stability analysis, the secondary metabolite of the NRMR formulations was assayed using a modification of previously described methods. The NRMR dispersion was freeze-dried, and 1 g of dried NRMR samples was extracted with 10 mL of ethanol at 65 °C for 1.5 h (*17*). The extracts (10% w/v) were further filtered with 0.45 μm filter and analyzed by HPLC. HPLC was performed according to the method described previously (*18*) and carried out on an HPLC system PU2089 plus (Jasco Co., Tokyo, Japan). A Discovery C18 column, 25 cm × 4.6 mm i.d., 5 μm (Bellefonte, PA), was used as the analytical column. The mobile phase was formed using 45% water, 55% acetonitrile, and 0.5% triflouacetate at a flow rate of 1.0 mL/min. Monacolin K was detected using a UV detector UV2075 plus (Jasco Co.) set at 238 nm. For citrinin analysis, the fluorescence detector FL-1 (Rainin Co, Wobum, MA) was set with an excitation λ_{max} of 330 nm and an emission λ_{max} of 500 nm.

General Analysis. The pH value of RMR was acidic because of production of organic acid (*19*) by the *Monascus* species. The NRMR dispersion was assayed by pH meter model 6071 (Jenco Co., San Diego, CA) for determination of the pH value changes after the wet-milling process to see if the acid environment could be neutralized by the breakage of cell wall. The appearance of the aqueous-based NRMR was recorded during storage under 4 °C for 2 months. Red pigment and yellow pigment analyses of RMR were diluted and then measured by spectrophotometer U-2001 (Hitachi Instruments Inc.) at OD_{500} and OD_{400}, respectively (*20*).

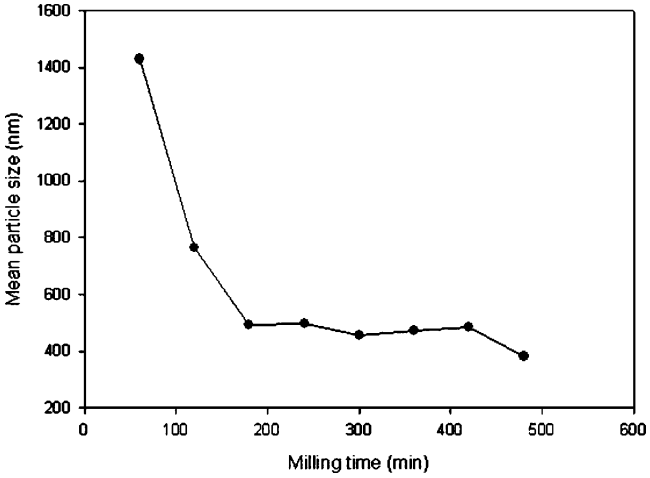

Figure 1. Particle size reduction/time curve for RMR dispersion processed using a high-energy wet milling technology for 8 h.

Food Composition Analysis. The food composition analysis of RMR was performed according to the standard procedures of AOAC to determine the water, ash, lipid, protein, and carbohydrate contents (*21*). The water content was determined by drying the RMR in a 105 °C oven to constant weight. To analyze the ash content, RMR was preheated 250 °C for 2 h and heated at 550 °C for 16 h, and the ash content was calculated. The Soxhlet method was used for determination of the RMR lipid content. For protein content examination, RMR was treated with the Kjeldahl method to obtain the total nitrogen content and the nitrogen factor of rice (5.95) was used to calculate the crude protein content of RMR. Finally, the carbohydrate content of RMR was calculated by eliminating the total moisture, crude protein, crude lipid, and ash.

RESULTS

NRMRs were generated using wet-milling technology. As described in the Materials and Methods, 20 g of RMR powder with 200 mL of double-distilled water was mixed and then ball-milled without dispersive agents. The impaction and the shearing forces generated during milling were necessary in order to achieve the desired results. In **Figure 1**, the particle size reduction profile of the RMR powder is shown. The graph shows that within 3 h of processing, the mean particle size of the powder is reduced from 20 μm to 500 nm. Further processing yielded homogeneous particle preparation with a mean particle size of about 410 μm.

Using SEM, as shown in **Figure 2**, with 700× magnification, the larger unmilled RMR powder became smooth after 4 h of processing. Moreover, under 10000× magnification, the smooth surface area revealed the contours of NRMR structures. Using TEM, the homogeneity and the effectiveness of the wet-milling process were readily evident. As shown in **Figure 3**, with 4000× magnification, the milled NRMR is a highly dispersed nanoparticulate preparation. In addition, under 10000× magnification, nanoparticulates, which ranged from 300 to 500 nm in size, were clearly observed and demonstrated the ideal homogeneous and dispersive properties. SEM and TEM photos further support the particle size data from laser scattering measurement. After 1 week, unmilled RMR precipitated, but milled RMR still retained its dispersive condition (**Figure 4**).

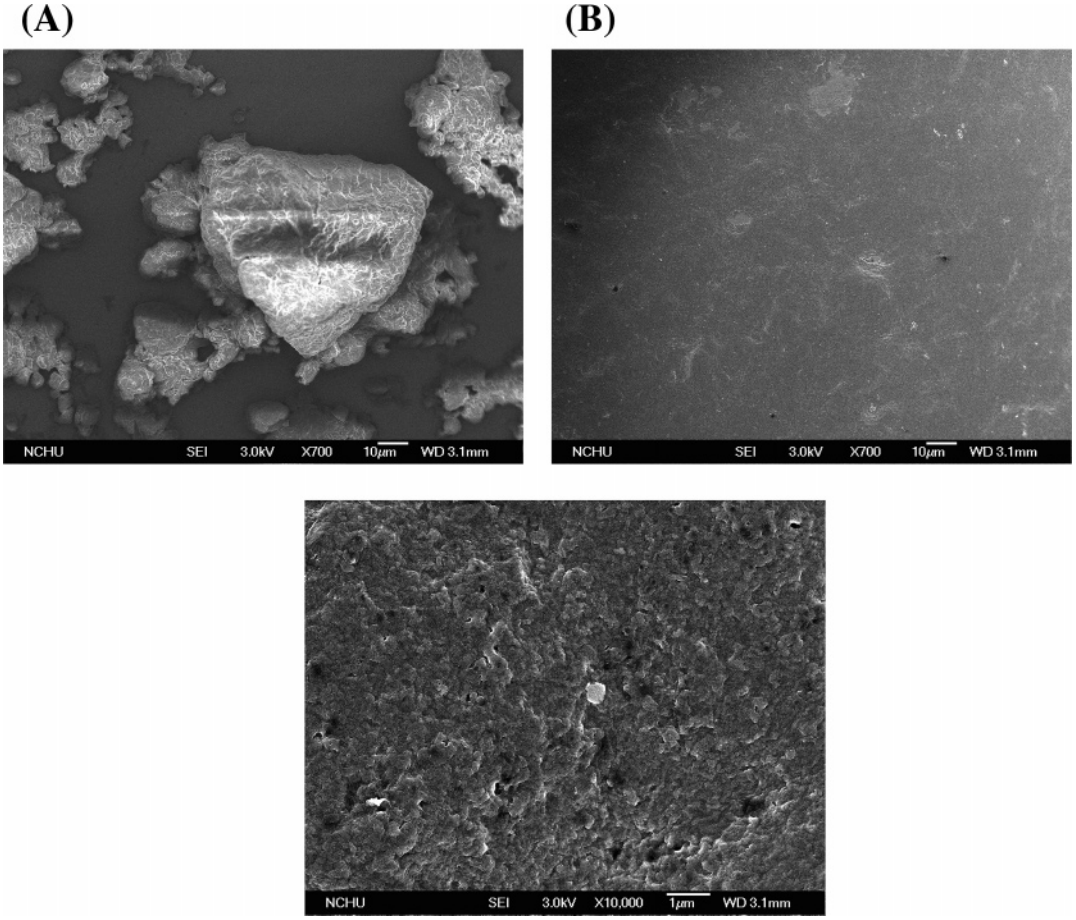

Figure 2. Micrographs compare (**A**) unmilled RMR powder and (**B**) milled RMR nanoparticulate using scanning electron microscopy at a 700× magnification. The measurement bar = 10 μm. (**C**) Milled RMR nanoparticulate using scanning electron microscopy at a 10000× magnification. The measurement bar = 1 μm.

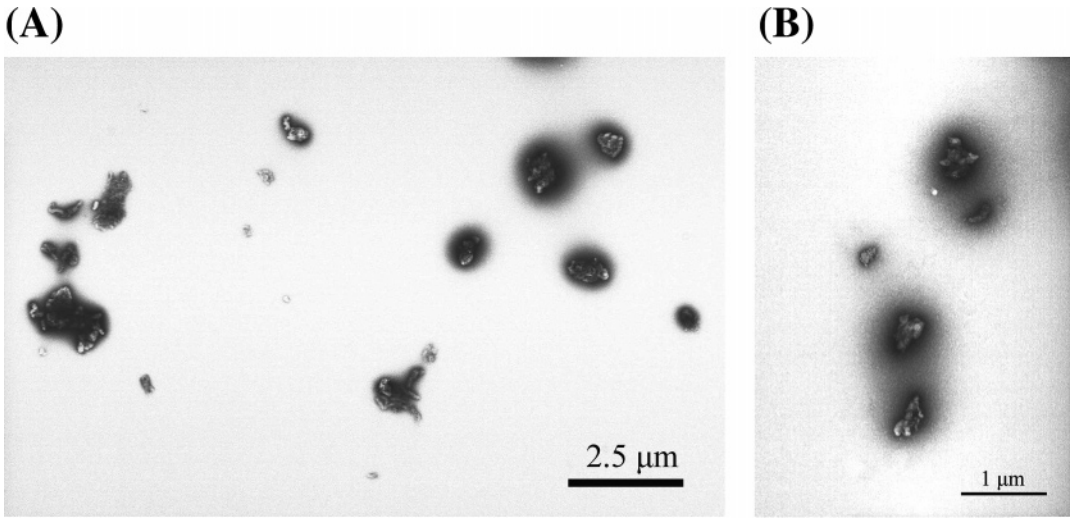

Figure 3. Transmission electron micrographs of (**A**) NRMR at a 4000× magnification. The measurement bar = 2.5 μm. (**B**) NRMR at a 10000× magnification. The measurement bar = 1 μm.

It is interesting to note that the impaction and the shearing forces generated during milling were necessary to achieve the desired results. Moreover, the size and concentration of the grinding media were important. As shown in **Figure 5**, mixing the RMR solution with a different size of milling media in the wet-milling process can cause different results. The results were based on the size report of dynamic laser scattering measurement, and the average size of nanoparticualtes can be calculated by these scattered intensity fluctuations on the time scale. When mixed with 0.65 mm grinding media, particle size reduction did not generate nanopartculates; the average size was larger than 1 μm. In the other milling processes, the average size of 75% RMR particle, milled with 0.2 mm grinding media, was about 521 nm and is not under ideal homogeneous conditions because 25% of the product's particle size was larger than 1 μm. The last milling process utilized a two-step process, which

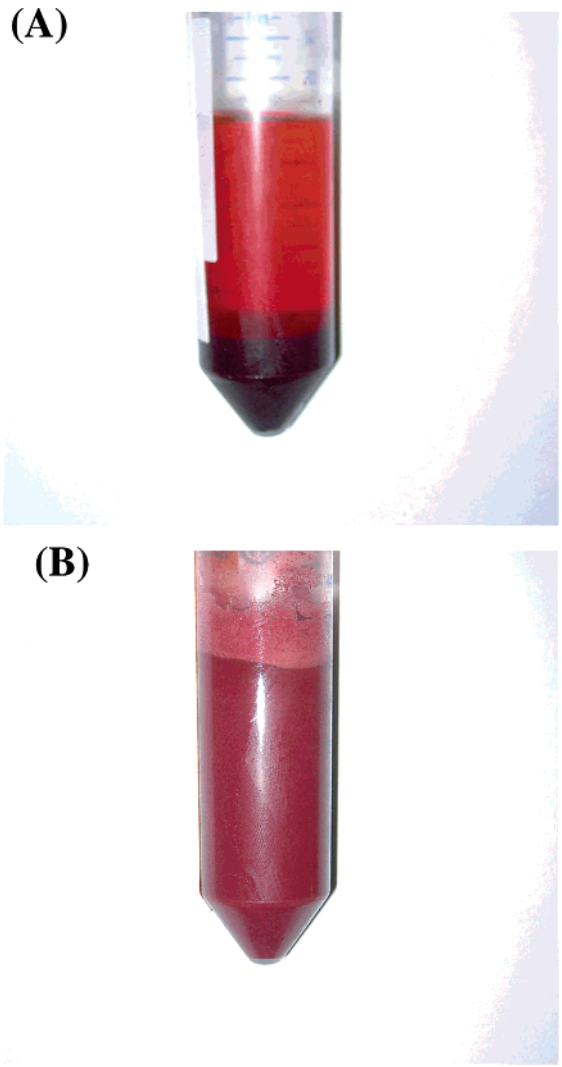

Figure 4. Graphs compare (**A**) unmilled RMR and (**B**) milled RMR stored at 4 °C for 1 week.

yielded a very homogeneous particle preparation with a mean particle size <410 nm. In addition, two kinds of stabilizer systems were selected. They are as follows: different concentration of pluronic F68 in combination with sodium deoxycholate and gum Arabic that has been widely used in food additives, such as chewing gum.

As shown in **Table 1**, despite using the 0.5% F68 with 0.05% sodium deoxycholate, the particles aggregated to about 1 μm; in addition, the other stablizers had similar nanoparticulate formulations and particle sizes ranging from 344.9 to 493.8 nm. However, when stored in a refrigerator at 4 °C for 2 months, agglomeration can obviously be found in stabilizers like 5% Arabic gums no. 408 and 0.1% F68 with 0.01% sodium deoxycholate. The particle sizes increased nearly 2-fold from 2 months ago. Much in contrast to the other stabilizers, RMR powder milled with distilled water had only partial agglomeration after 2 months. As demonstrated in **Figure 6**, the mean particle size of this preparation increased slightly during storage from an initial mean particle size of 410−480 nm because partial agglomeration was observed in the RMR dispersion when stored in refrigeration after 2 months.

The secondary metabolite concentrations of the unprocessed and processed RMR particle were studied using HPLC as shown in **Figures 7** and **8**. Comparison between unprocessed and processed RMR particles shows that these two kinds of

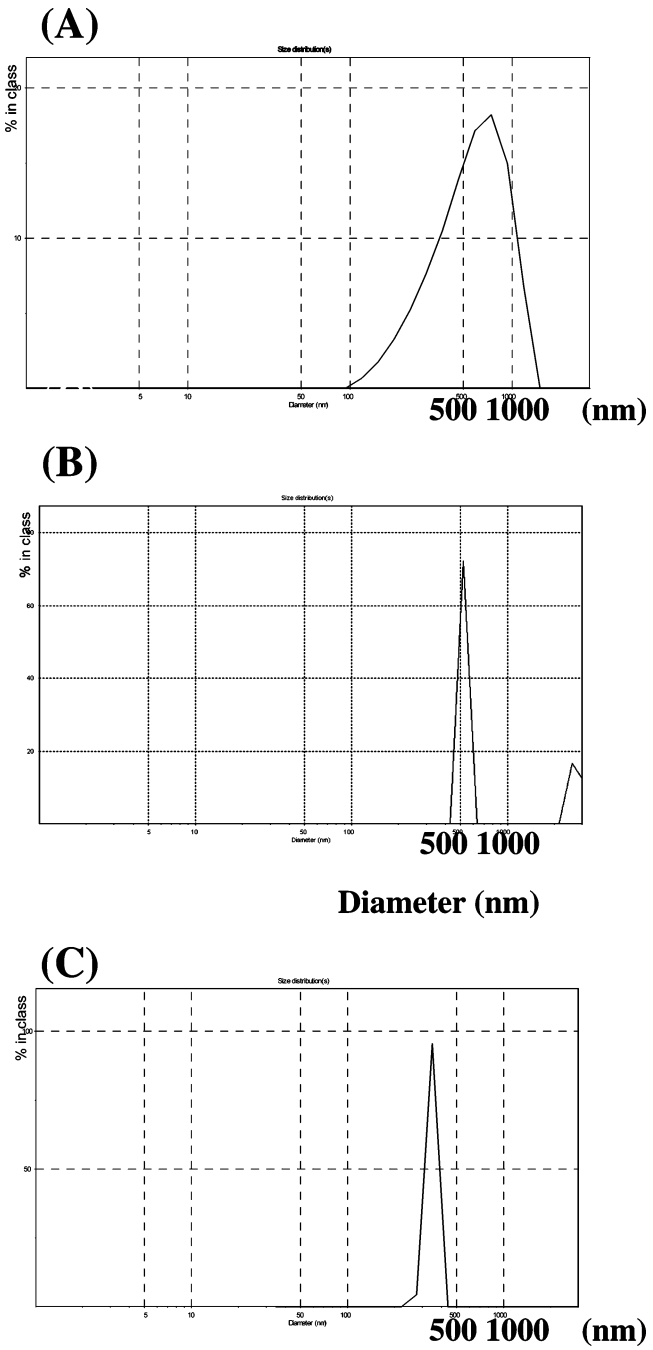

Figure 5. Effect of different grinding media size on the particle size distribution of NRMR. (**A**) Processed by 0.65 mm grinding media for 3 h; (**B**) processed by 0.2 mm grinding media for 3 h; and (**C**) two-step milling process processed by 0.65 mm grinding media for 1 h and then 0.2 mm grinding media for 3 h.

secondary metabolites have different reduction rates. The HPLC profiles of monacolin K, shown in **Figure 7**, reveal that its major peak with a retention time of 20.43 min was identified in this study. The extraction of monacolin K was reduced from 3942 mg/kg of unprocessed RMR to 3136 mg/kg of processed RMR, which is about a 20% reduction.

In **Figure 8**, the HPLC profiles of citrinin show that its major peak with a retention time of 6.66 min was identified. The extraction of monacolin K was reduced from 3815 to 1834 μg/kg (52% reduction). There is evidence that the milling process affects the chemical integrity of the secondary metabolites and generates different reduction rates. Furthermore, with the

Table 1. Comparison between Dispersive Agents with Average Particle Size and Dispersive Agents with HPLC Analysis[a]

dispersive agents	average particle size (nm) after milling process	average particle size (nm) after 2 months	HPLC analysis MK[b] (%)	HPLC analysis CT[c] (%)
distilled water	410.2	480.2	80 ± 4.5	48 ± 4.5
arabic gums TCML 5%	407.8	496.3	50 ± 2.5	56 ± 4.7
arabic gums no. 408 5%	344.9	628.7	71 ± 3.5	48 ± 5.5
0.1% F68/0.01% sodium deoxycholate	493.8	673.2	74 ± 1.6	53 ± 8.3
0.5% F68/0.05% sodium deoxycholate	944.2	986.4	92 ± 2.7	74 ± 5.5

[a] The values represent the mean ± SD ($n = 3$). [b] MK (%) = NRMR monacolin K concentration/RMR monacolin K concentration. [c] CT (%) = NRMR citrinin concentration/RMR citrinin concentration.

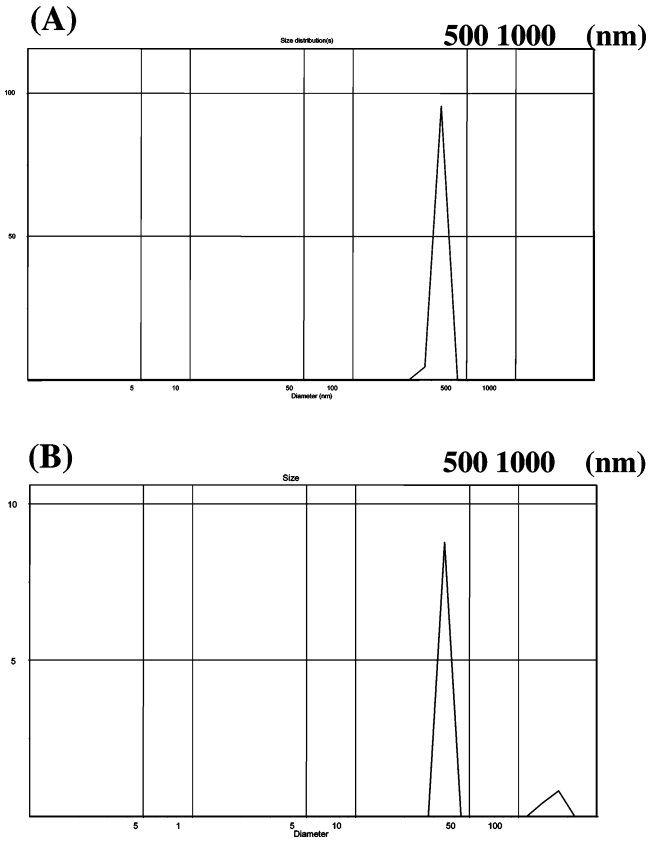

Figure 6. Particle size distribution profiles of nanopaticulate red mold rice using laser light diffraction: (**A**) measured immediately after milling (410 nm) and (**B**) measured after storage for 2 months (496.3 nm).

combination of other dispersive agents added to the secondary metabolite, 0.5% F68 with 0.05% sodium deoxycholate has the highest extraction rate of monacolin K but 5% Arabic gums no. 408 and distilled water have the lowest extraction rate of the mycotoxin citrinin.

When testing for the levels of pH, three different kinds of processed RMR sample had increased from pH levels of 4.58 to 6.4, 4.82 to 6.2, and 4.47 to 5.56; also, pigment analysis showed that yellow and red pigments were reduced to 36 and 39% of their base levels after the wet-milling process. In the RMR fermentation period, *Monascus* can produce organic acid (*19*, *22*, *23*) that makes the RMR more acidic; however, under a wet-milling process, the neutralization of NRMR dispersion may be due to the breakage of the cell wall. In food composition

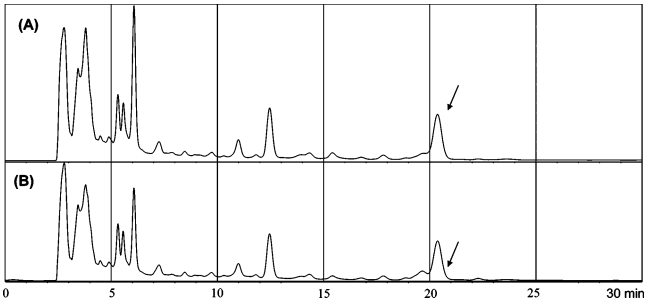

Figure 7. HPLC profile of monacolin K before (**A**) and after (**B**) the milling process at 4 °C for 4 h. The milling process brings about a significant change in the percent of sample having retention times of 20.43 min (100% in **A** to 80% in **B**).

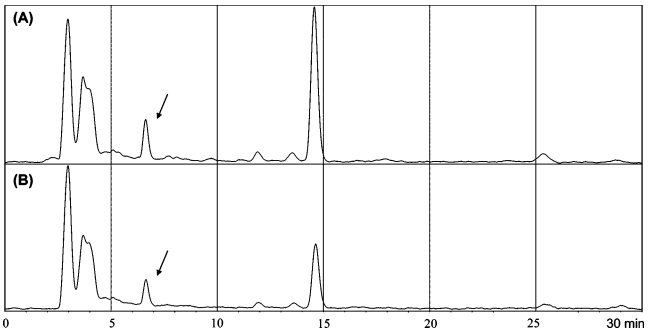

Figure 8. HPLC profile of citrinin extration before (**A**) and after (**B**) milling process at 4 °C for 4 h. The milling process brings about a significant change in the percent of sample having retention times of 6.66 min (100% in **A** to 48% in **B**).

Table 2. Food Composition of RMR

components	content (g/100 g)
moisture[a]	8.1
crude ash	0.78
crude fat	2.16
crude protein	8.42
crude carbohydrate	80.54

[a] Moisture was presented based on fresh RMR weight; others were presented on dry weight.

analysis, RMR was composed of 80.54% carbohydrate, 8.42% protein, 2.16% fat, 8.1% moisture, and 0.78% ash (**Table 2**).

DISCUSSION

In this study, we use wet-milling technology to grind RMR powder into a NRMR dispersion. This method has been applied successfully over the last few years (*13*, *24*). In drug therapy applications, many drugs like danazol, naproxen, etoposide, piposulfan, camptothecin, paclitaxel, and Zn−insulin are made into nanoparticulate dispersions (*25*−*27*). In fact, oral administration of drugs in the form of drug nanoparticles has been reported to have a full range of positive effects such as improving bioavailability, improving dose proportionality, reducing target organs irritation, reducing fed/fasted variability, and enhancing the absorption rate (*28*). RMR has been used for dietary supplements for its effects in lowering cholesterol levels. However, there are few studies focused on the discussion of the nanopaticulate process and its effects on healthy foods. RMR was chosen for this study for a number of reasons. In RMR production, citrinin (mycotoxin) that is produced from the fermentation process may have toxic effects on the human body at high concentrations (*9*).

In addition, RMR is a kind of fermented rice, consisting of rice starch and intact *Monascus* cells. *Monascus* species belong to the group of ascomycetes with strong protective cell walls composed of chitin, β-glucan, and glycoprotein. Chitin, a homopolymer of N-acetyl-D-glucosamine (Glc-NAc) residues linked by β-1−4 bonds, is a kind of indigestible fiber in the intestine (*29*). Moreover, in our previous study, *Monascus* species can live in the acidic environment (*30*). The cell wall structure of *Monascus* is very durable and not easily broken down. Therefore, it is difficult for the matter inside to be released, much less absorbed, into the human body. In some patents, methods for producing sporoderm-broken *Ganoderma lucidum* spores claim to have medicinal effects on patients with immunological disorders, cancer, AIDS, hepatitis, diabetes, and cardiovascular diseases (*31*, *32*). Furthermore, the absorption rate of NRMR particles increases because the specific area increases after size reduction. In order to overcome these problems, milled technology, described above, is used for drug formulations to produce NRMR and to examine the effects and influences due to the wet-milling process. As shown in **Figures 1**−**4**, RMR powder was readily processed into a physically stable, nanoparticulate dispersion. As compared with the un-milling RMR particle size (20.15 μm), the NRMR particle size was reduced to 410 nm. If the particles are near spherical in shape, nanoparticles are further reduced in size from 20 μm to 400 nm and, thus, generate a 50-fold increase in the surface area to volume ratio. This increase in surface area can have a major impact on its absorption. Improving the dissolution rate of a poorly water-soluble compound generally correlates with faster absorption rates (*25*). Freeze-dried NRMR extraction at 37 °C for few seconds has nearly the same extraction efficiency in the secondary metabolite monacolin K as extraction at 65 °C for 1.5 h (data not shown). This suggests that an increase in the surface area to volume ratio increases the extraction efficiency at human body temperature.

A high-energy milling process will create a large amount of heat that may affect the monacolin K structure whose melting point is at 174.5 °C (*33*). In the milling process, the temperature condition is under control at 4 °C and the aqueous phase effectively dissipates the heat generated during processing. Under controlled temperatures, there are many factors that influence NRMR particle size like milling time, grinding media particle size, the composition of grinding media, and stabilizer. In regards to milling time, the time required to obtain drug nanocrystalline dispersions with unimodal distribution profiles and mean diameters <200 nm is 30−60 min in batch mode mill (*13*). However, in a noncrystalline drug such as peptide drug Zn−insulin, the milling time required to obtain the same mean diameters of <200 nm is more than 4 h (*15*). As shown in **Figure 1**, the graph indicates that within 1 h of processing, the mean particle size of the powder has reduced from 20 μm to the nanometer size range, but after 3 h, there is no more significant change observed in the particle size. This may be due to the size limitation of the grinding media. With a reduction in the size of grinding media in a media mill, the number of contact points is increased exponentially resulting in improved grinding and dispersing action. In **Figure 5**, the smaller the grinding media is, the smaller the NRMR particle size is. For smaller grinding media, the increase of the contact probability and impact force may cause the media sphere to fracture to pieces. To overcome this problem, we use yttria-stabilized tetragonal zirconia polycrystals (Y-TZP) as the grinding media. Among various kinds of grinding media, Y-TZP offers a combination of properties (high hardness, fracture toughness, spherical shape, and chemical stability), which make them very suitable for this study (*34*).

The stabilizer selections are used to promote the particle size reduction process, generate physically stable formulations, and prevent the high surface energy of nanometer-sized particles from agglomerating or aggregating. Too little stabilizer induces agglomeration or aggregation and too much stabilizer promotes Ostwald ripening (*13*). In this study, we select two kinds of stabilizer systems: pluronic F68 in combination with sodium deoxycholate and gum Arabic. Pluronic F68 is a block copolymer of ethylene oxide (hydrophilic) and propylene oxide (hydrophobic). When F68 is mixed with sodium deoxycholate (cosurfactant), the mixture is widely used in modifying drug nanoparticle surfaces to avoid agglomeration or aggregation after high-energy wet milling. Gum arabic (gum acacia), a kind of food additive, is composed of protein and high molecular weight polysaccharides that can be used as a thickener, stabilizer, and dispersion media (*11*). Unfortunately, these have problems such as aggregation and coagulation (**Table 1**). Surprisingly, as compared with other stabilizers, NRMR powder milled with distilled water has a similar particle size distribution (410 nm) and only partial agglomeration after 2 months (**Figure 6**). In HPLC analyses, its monacolin K extraction level was the second highest, maintained at 80% of its base level, while citrinin only constituted 48% extraction (the lowest). Moreover, as shown in food composition analysis (**Table 2**), RMR is a mixture of carbohydrate, protein, and fat that is entirely different from crystalline drugs. The data may imply that RMR powder itself may be used as a kind of stabilizer. The above data suggest that mixing RMR with distilled water to formulate NRMR dispersion provided an economical, simple, and physically stable product that can be used for further biological and food industrial uses.

It is concluded that the wet-milling technology can be used to formulate physically stable NRMR dispersions along with higher monacolin K extractions and lower citrinin extractions. This technology can be applied not only to poorly water-soluble drugs but also to functional foods. Further studies are being conducted to demonstrate the biological effect and safety evaluation of NRMR and to utilize this approach for other functional foods.

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