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Research article

Inhibitory Effect of Water Soluble Fraction of *Monascus*-Fermented Rice on Lipid Accumulation in 3T3 L1 Adipocyte

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water soluble fraction;
cultivation conditions

Abstract

Excessive lipid accumulation in the body causes people to become overweight and obese, conditions that are associated with an amplified risk of serious diseases. The fungi of genus *Monascus* produce various secondary metabolites such as monacolins, citrinin and fungal pigments, which are water-insoluble and have inhibitory potency for the lipid accumulation in adipocytes. However, water-soluble adipogenesis inhibitors derived from *Monascus*-fermented products have not yet been reported. In this study, we investigated the inhibitory activity against intracellular lipid accumulation of water-soluble fractions of *Monascus*-fermented red mold rice (RMR) and red mold barley (RMB) on murine 3T3-L1 cells. Water soluble fractions of ten different *Monascus* strains were used and the inhibitory activity of their water-soluble fractions on lipid accumulation by differentiated 3T3-L1 cells was evaluated for 8 days using oil red staining. The water-soluble fraction from *Monascus pilosus* NBRC4507 fermented RMR cultivated at 30°C for 14 days was selected since it showed comparatively the lowest relative lipid accumulation (62±1.2%), which indicated the highest inhibitory activity of lipid accumulation in adipocytes. To study the presence of monacolin and citrinin in the water soluble fractions, thin-layer chromatography was done and the results showed that the water-soluble fractions tested were free from both monacolin and citrinin. Therefore, the present study strongly suggested that the water-soluble components, except for monacolin and citrinin, in the water soluble fraction obtained from *Monascus pilosus NBRC4507*-fermented rice can be used as functional food material to control overweight and obesity.

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1. Introduction

Adipogenesis is a differentiation process that includes lipid synthesis and lipid accumulation during differentiation of pre-adipocytes to mature adipocytes [1]. Excessive lipid accumulation in adipocytes eventually leads to obesity, which is a global health problem [2]. Obesity is a major risk factor for numerous pathological diseases such as diabetes, hypertension, and heart diseases [3]. Adipocyte hypertrophy caused by excessive lipid accumulate is closely associated with modulation of adipose mass and body weight gain [4]. Nowadays, adipogenesis modulation is generally accepted as having a crucial role in overcoming excess bodyweight and thus controlling or preventing obesity [2-4].

Solid-state fermentation by *Monascus* has a long tradition in East Asian countries, and the product is called *red yeast rice, red rice, angkak, red leaven, beni-koji, hong-zhu, hong-qu, zhitai, rotschimmelreis,* and *red mold rice* [5, 6]. The product has been consumed as a dietary staple and as a food additive in some Asian countries and it has been utilized as an enzyme source for making many fermented foods and folk medicine [6, 7]. In Okinawa, it has been used to make tofu which is a traditional fermented soybean food [8].

Monascus-fermented rice contains various secondary metabolites that can inhibit the adipogenesis process *in vivo* and *in vitro* [9-11]. Monacolin K, identical to lipophilic polyketide lovastatin, is the most famous secondary metabolite of genus *Monascus* [12]. Lovastatin is a strong inhibitor of a rate-limiting enzyme, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (EC 1.1.1.34), that is involved in cholesterol biosynthesis in the liver, and has been used as a cholesterol lowering prescription drug [13]. Recently, applications of *Monascus*-fermented rice have become more common as alternatives to statin therapies [14, 15]. In addition, *Monascus* spp. also produce nephrotoxic citrinin [16, 17], which is a potential threat to food sanitation [18] and shows moderate adipogenesis inhibitory activity [19]. However, there is no information on water-soluble lipid accumulation inhibitory agents from the *Monascus*-fermented products.

Barley extracts containing water soluble β-glucan have been reported to suppress total cholesterol and low-density lipoprotein (LDL)-cholesterol levels in the serum of hypercholesterolemic patients [20]. Fermentation of barley by lactic acid bacteria increases the contents of soluble fiber and β-glucan, and the same time decreases insoluble dietary fiber [21]. The water-soluble fiber from barley also decreases cholesterol levels in serum [22]. But there is no information about *Monascus*-fermented barley having a cholesterol lowering effect as the result of lipid accumulation inhibitory activity in adipocytes. Thus, steamed barley was also used for the determination of cultivation conditions as a solid-state substrate.

The main goal of this study was to identify a novel water-soluble adipogenesis inhibitor from *Monascus*-fermented rice and barley products for application in the functional food industry. We investigated water soluble fraction of *Monascus*-fermented RMR and RMB, for its intracellular lipid accumulation inhibitory activity in adipocytes. Altogether, our results showed that *Monascus pilosus* NBRC4507 might be a suitable strain for the preparation of RMR that had water-soluble lipid accumulation inhibitory activity in the water extracts.

2. Materials and Methods

2.1 Chemicals and reagents

Dulbecco's modified eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from MP Biomedicals (Solon, OH, USA). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies Co. (Grand Island, NY, USA). Phosphate buffered salts (PBS) were purchased from Takara Bio Inc. (Shiga, Japan). Oil Red O, dexamethasone (DEX), dimethyl sulfoxide (DMSO),

insulin from bovine pancreas (INS), and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich (St. Louis, MO, USA). Lovastatin was obtained from TRC Inc. (Toronto, ON, Canada). Citrinin was obtained from LKT Laboratories Inc. (St. Paul, MN, USA). All the other chemicals used were of analytical and molecular grade.

2.2 Microorganisms and spore collection

Monascus strains used in the study were obtained from Biological Resource Center, NITE (NBRC, Chiba, Japan), Japan Collection of Microorganisms (JCM, Ibaraki, Japan), and stock cultures were isolated and maintained on potato dextrose agar (PDA) plates (pH 5.6).

The *Monascus* were cultured on PDA plates at room temperature (30°C) and spores of each strain were collected with sterilized 0.05% (w/v) Tween 80 solution (0.2 µm filtered). The spore suspensions were collected and centrifuged at 3,000 rpm for 10 min at room temperature. The precipitated spores were washed twice with sterilized distilled water, adjusted to a density of 5×10^4 spores/ml, and stored at 4°C until further use.

2.3 Preparation of *Monascus* fermented food products and extracts

A dry weight of 100 g of each polished rice and barley grain were soaked in distilled water at room temperature overnight. Excess water was drained off for 1 h and each soaked grain sample was autoclaved at 121°C for 20 min. After cooling, the collected fungal spores (5×10^4 spores/ml of each) were inoculated to sterilized rice and barley, and they were incubated at 30°C or 35°C for 7 days and 14 days. The collected RMR and RMB were stored at -20°C until further use.

Water-soluble fractions of RMR and RMB were extracted twice with 3 volumes of water by shaking at 200 rpm at 30°C overnight. The obtained water-soluble fractions of RMR and RMB were filtered using Whatman No. 5 filter papers, concentrated with a rotary evaporator in *vacuo*, and then freeze-dried to a constant weight. Solutions of RMR and RMB (1 mg/ml and 10 mg/ml) were prepared by dissolving the freeze-dried water-soluble fractions of RMR and RMB in 10X PBS. Under aseptic conditions, solutions of RMR and RMB in 10X PBS were filtered using 0.2 µm microfilters. Sterilized RMR and RMB in 10X PBS were used for further analysis of the lipid accumulation inhibitory assay in 3T3-L1 cells.

2.4 Lipid accumulation inhibitory assay in 3T3-L1 cells

Culturing and differentiation of 3T3-L1 cells were done as described by Roh and Jung [23] with slight modifications. 3T3-L1 preadipocytes were maintained in a basal medium (DMEM containing 10% (v/v) FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin) at 37°C in 5% CO_2. The cells were seeded into each well (2.5×10^4 cells/ml) of type-I collagen coated 48-well cell culture plate (Corning NC03548, Tokyo, Japan), and incubated at 37°C with 5% CO_2 for 48 h. The medium was changed at two-day intervals with continuous observation to achieve 100% confluence. At 100% confluence, the cells were stimulated with a differentiation induction medium (DMEM containing 10% (v/v) FBS, 1% penicillin/streptomycin, 0.5 mmol/l DEX, 1 µmol/l IBMX, and 5 µg/ml INS) for 2 days. Samples were prepared by mixing 100 µl from each RMR and RMB extract (1 mg/l and 10 mg/l) with the lipid accumulation medium (DMEM containing 10% (v/v) FBS, 1% penicillin/streptomycin, and 5 µg/ml INS) to a final volume of 1 ml and 1X PBS in lipid accumulation medium was used as the negative control. A volume of 1 µl from 10 mmol/l lovastatin in 100% ethanol mixed with 2 ml of lipid accumulation medium was used as the positive control. During differentiation, the cells were further cultivated for 8 days while changing the medium every two days.

2.5 MTT assay

Cell viability of 3T3-L1 cells was determined colorimetrically using MTT assay as described in Roh and Jung [23] with slight modifications. Cells were cultivated in a 96-well plate. After incubation at 37ºC in 5% CO_2 for 24 h, 10 µl of 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetraoliumbromide) was added to the wells and incubated for 24 h under the same conditions. The supernatant was removed carefully, 100 ml of DMSO was added and mixed, and the absorbance was read at 570 and 660 nm. All the tests were done in triplicate.

2.6 Oil Red O staining

Accumulated lipids in 3T3-L1 cells were examined as described in Roh and Jung [23]. After cell differentiation for 8 days, the culture medium was removed, and the cells were fixed with 10% (v/v) formaldehyde for 10 min at room temperature. The fixed cells were washed twice with PBS, and then replaced with 60% (v/v) isopropanol for 1 min at room temperature. After removing isopropanol, the accumulated lipids in the cells were stained with Oil red solution (1.8 mg/ml in isopropanol) for 20 min at room temperature. The stained Oil red colorant was extracted with 100% isopropanol, and then the extracted colorant was measured at 510 nm with the microplate reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA).

2.7 Thin-layer chromatography (TLC) analysis

To evaluate contamination of lipophilic compounds in the water extracts, an artificial model which consisted of steamed rice to which synthetic lovastatin and citrinin had been added. Both water and methanol extracts from the constructed model were prepared and were concentrated and freeze-dried. TLC analysis for both extracts was carried out with silica gel plates (Art. 5721, DC-Fertig platen Kieselgel 60, Merck, Darmstadt, Germany). For lovastatin detection, concentrates of both extracts were spotted on TLC plates and developed in a solvent system of dichloromethane: ethyl acetate (7:3 v/v). The mobility of these spots on TLC plates was visualized as a brown color spot by exposure to iodine vapor [24]. For detection of citrinin, both fractions were developed in a solvent system of acetone: ethyl acetate: water (5:5:2 v/v/v) and were visualized as yellow-green color spots with a UV transilluminator (312 nm) [25].

2.8 Statistical analysis

The results obtained from this study were presented as means of triplicate with standard deviations (±SD). Statistically significant differences were determined by one-way analysis of variance (ANOVA) using GraphPad Prism ver. 5 (La Jolla, CA, USA). One-way ANOVA, Tukey`s test was used as post-hoc test. Values of $P < 0.05$ were considered as statistically significance.

3. Results and Discussion

3.1 Effect of the water-soluble fraction of RMR on the lipid accumulation in 3T3-L1 adipocytes

Monascus-fermented cereal grains produce some secondary metabolites that possess adipogenesis inhibitory activity including inhibition of lipid accumulation [13, 19, 26]. Contamination due to some secondary metabolites, such as lipophilic polyketides, and citrinin, are prohibited under the

Food Sanitation Law in many countries [18, 27]. In addition, lovastatin is considered undesirable in the food industry because statins possess severe side effects even though they have similar structural and medicinal properties to monacolin K [27]. Therefore, this study was conducted to check whether the water-soluble fractions prepared from *Monascus*-fermented cereals in solid-state fermentation had an inhibitory activity on lipid accumulation in adipocytes.

In order to examine the inhibitory effect of the water-soluble fractions of RMR, from preliminary studies, 10 *Monascus* strains were selected. Those selected strains were further examined for their relative lipid accumulation inhibitory activity (Figure 1). Compared to the positive control lovastatin (5 μmol/l), the water-soluble fraction of NBRC4507-RMR cultivated at 30°C for 14 days showed significantly less lipid accumulation percentage (61.86±3.6%) at a final concentration of 1.0 mg/ml. This implied that the water-soluble fraction of RMR of *M. pilosus* NBRC4507 cultivated at 30°C for 14 days could inhibit lipid accumulation in adipocytes at a comparatively high level. These results suggested that *M. pilosus* NBRC4507 might be used as a suitable strain for the production of the inhibitory activity against lipid accumulation in the water-soluble fraction (Figure 1).

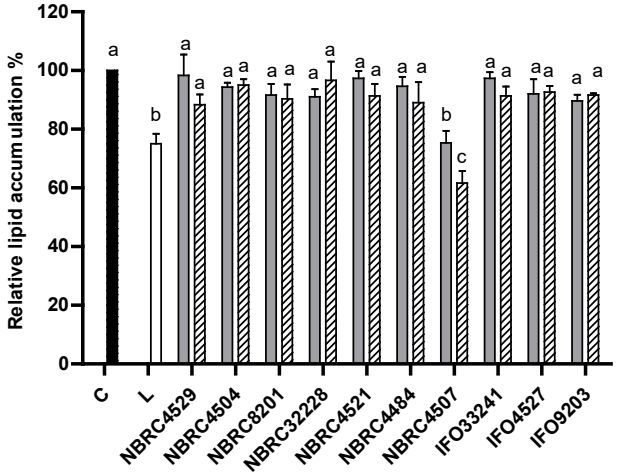

Figure 1. Effect of the water-extracts from RMR on the lipid accumulation in 3T3-L1 adipocytes cultivated at 30°C for 7 days and 14 days. Lovastatin (5 μmol/l) was used as the positive control (L). Closed black bar represents control (C); Open black bars represent lovastatin; Final concentration of the sample was 1.0 mg/ml. Ash bars represent 7 day cultivations and diagonal-line bars represent 14 day cultivations; Values represent means from triplicate±SD. The different letters indicate significant different at $P < 0.05$ (Tukey's test) between the control and treatment groups.

The cell viability was also examined in the water soluble fractions obtained from the above tested 10 strains of RMR in 3T3-L1 cells. The results in Figure 2 showed that the positive control (5 μmol/l lovastatin), negative control (buffer only), and all tested sample each at a final concentration of 1 mg/ml did not affect the viability of 3T3-L1 cells. This confirmed that the reduction of lipid accumulation by the water extract from RMR of the selected strain was not due to cell death, but due to a secondary metabolite produced during the production of RMR that could reduce the lipid accumulation. By considering these facts, NBRC4507 strain was selected for further study.

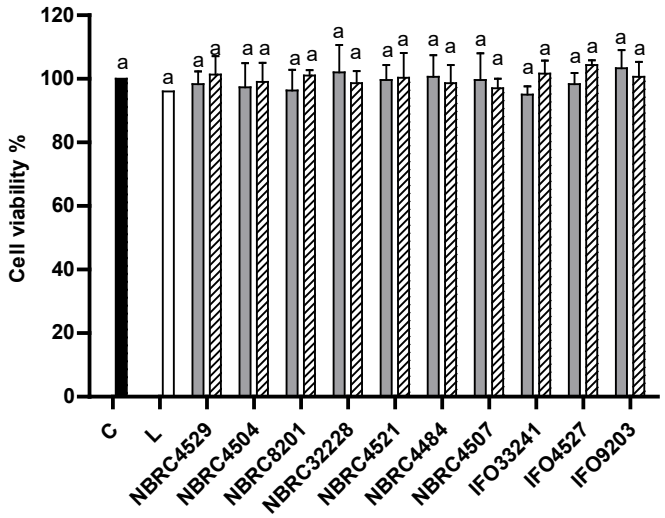

Figure 2. Effect of the water-extracts from RMR on cell viability in 3T3-L1 adipocytes cultivated at 30°C for 7 days and 14 days; Lovastatin (5 μmol/l) was used as the positive control (L). Closed black bar represents control (C); Open black bars represent lovastatin; Final concentration of the sample was 1.0 mg/ml. Ash bars represent 7 day cultivations and diagonal-line bars represent 14 day cultivations; Values represent means from triplicate ± SD. The same letter indicate non significance between the control and treatment groups.

3.2 Effect of the water-soluble fraction of NBRC4507-RMR and NBRC4507-RMB on the lipid accumulation in 3T3-L1 adipocytes

Optimization of the growth conditions for fungus *Monascus* is useful to increase the productivity of favorable secondary metabolites such as monacolin, and their pigments [28, 29]. Traditionally, steamed rice is widely used for the solid-state fermentation of *Monascus* in East Asian countries [8, 30, 31]. However, nowadays various raw materials in nature and some by-products except for rice are also used in solid-state fermentation technology to improve the productivity of beneficial secondary metabolites [32-34]. Barley is a major cereal crop and is used as a staple food in many countries [20]. Barley contains abundant water-soluble fiber compared with other cereals, and its water-soluble β-glucan contained in barley possesses the lowering effect of total cholesterol and LDL-cholesterol in plasma [20-22]. Hence, it was decided to study the inhibitory effects on lipid accumulation in adipocytes by the water-soluble fraction obtained from NBRC4507-RMB cultivated at 30°C and 35°C for 7 days and 14 days, and compared with those of NBRC4507-RMR cultivated under the same conditions.

As shown in Figure 3, when compared to the positive control lovastatin, the water-soluble fraction of NBRC4507-RMR cultivated at 30°C for 14 days showed the lowest relative lipid accumulation (62±1.2%) at a final concentration of 1.0 mg/ml. Moreover, no significant difference in lipid accumulation was seen among the treated groups, NBRC4507-RMR cultivated at 35°C for 7 days and 14 days. Furthermore, it was found that those lipid accumulation levels were higher than that of lovastatin control (Figure 3). These results revealed that the most effective lipid accumulation inhibitory activity for the NBRC4507-RMR was at 30°C for 14 days.

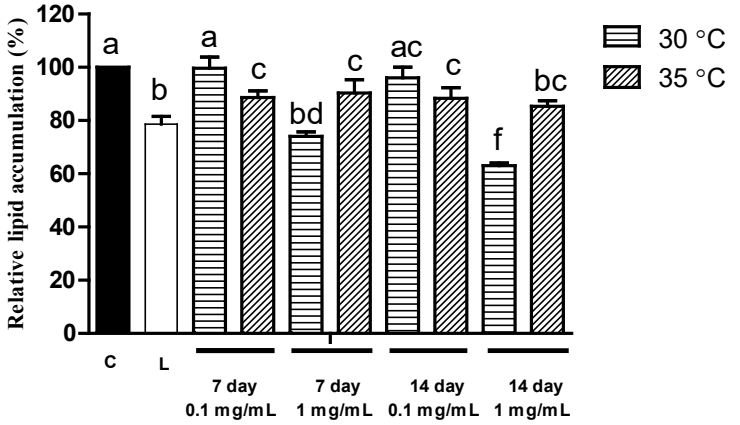

Figure 3. Effect of the water-extracts from RMR of NBRC4507 cultivated at 30°C and 35°C for 7 and 14 days on the lipid accumulation in 3T3-L1 adipocytes. Lovastatin (5 μmol/l) was used as the positive control (L). Closed black bar represents control (C); Open black bar represents lovastatin (L); Values represent means from triplicate±SD. The different letters indicate significant different at $P < 0.05$ (Tukey's test) among treatment groups.

On the other hand, the water-soluble fraction of NBRC4507-RMB, which had been cultivated at 30°C and 35°C for 7 and 14 days, showed less reduction in the lipid accumulation in the treated groups, but those inhibitory levels were not significantly different when compared with that of the lovastatin control and the negative control that was treated with the buffer only (Figure 4). When compared with all the treatment conditions, the highest reduction of lipid accumulation was observed when the 3T3L-1 cells were treated with the water-soluble fraction of NBRC4507-RMB cultivated at 35°C for 14 days at a final concentration of 1.0 mg/ml (Figure 4). However, no significant difference was observed compared to the positive control lovastatin.

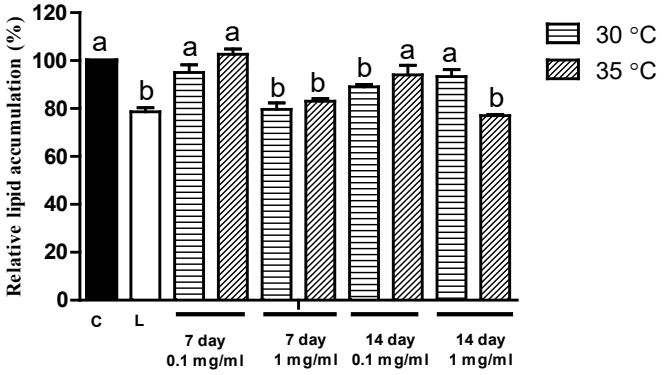

Figure 4. Effect of the water-extracts from RMB of NBRC4507 cultivated at 30°C and 35°C for 7 and 14 days on the lipid accumulation in 3T3-L1 adipocytes. Lovastatin (5 μmol/l) was used as the positive control (L). Closed black bar represent control (C); Open black bar represent lovastatin (L); Values represent means from triplicates±SD. The different letters indicate significant at $P < 0.05$ (Tukey's test) among treatment groups.

Taken together, the water extracts of NBRC4507-RMR showed a better reduction in lipid accumulation than the water extracts of NBRC4507-RMB. Therefore, these results suggested that rice is a better culture substrate for the production of the water-soluble lipid inhibitory components by *Monascus* in the solid-state fermentation. Also, it was found that cultivation of NBRC4507 at 30°C for 14 days was the best condition for the production of water-soluble lipid inhibitory components.

To further confirm these results, Oil Red O staining in 3T3- L1 was carried out (Figure 5). The cell pictures of Oil Red O stained 3T3-L1 cells treated with water extracts of NBRC4507 cultivated under different conditions are shown in Figure 5. The cell pictures of Oil Red O stained in 3T3-L1 cells revealed that the lipid accumulation in adipocytes treated with water extracts of NBRC4507-RMR cultivated at 30°C for 14 days were at a significantly lower level (Figure 5; 30°C for 14 days top panel) than that of other treated groups and also the positive control 5 µmol/l of lovastatin.

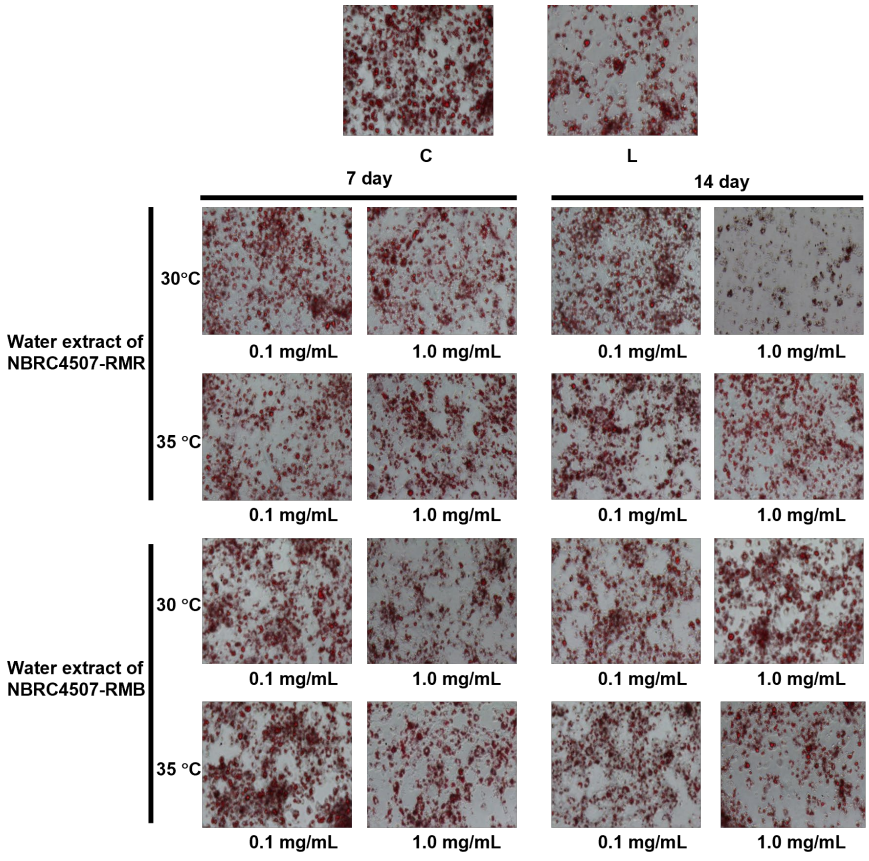

Figure. 5. Intracellular lipids stained with Oil Red O. Cells were treated with 0.1 mg/ml and 1.0 mg/ml of water extracts of RMR and RMB of NBRC4507. 1X PBS was used as the negative control (C), and lovastatin (5 µmol/l) was used as the positive control (L).

3.3 Confirmation of absence of lovastatin and citrinin in water soluble fraction of RMR by TLC

To confirm whether the water soluble fraction has been contaminated with lovastatin and citrinin, TLC analyses were carried out for both water and methanol soluble fractions obtained from the unfermented steamed rice model to which artificial lovastatin and citrinin had been deliberately added (Figure 6). Lovastatin was not observed in the water soluble fraction obtained from the unfermented rice prepared as the artificial model, while positive spots of lovastatin were observed only in the methanol-soluble fraction on the TLC plate (Figure 6A). In contrast, positive spots of citrinin were observed in both water and methanol extracts from the unfermented rice model (Figure 6B). These TLC results suggest that lovastatin had not escape into the water soluble fraction at least.

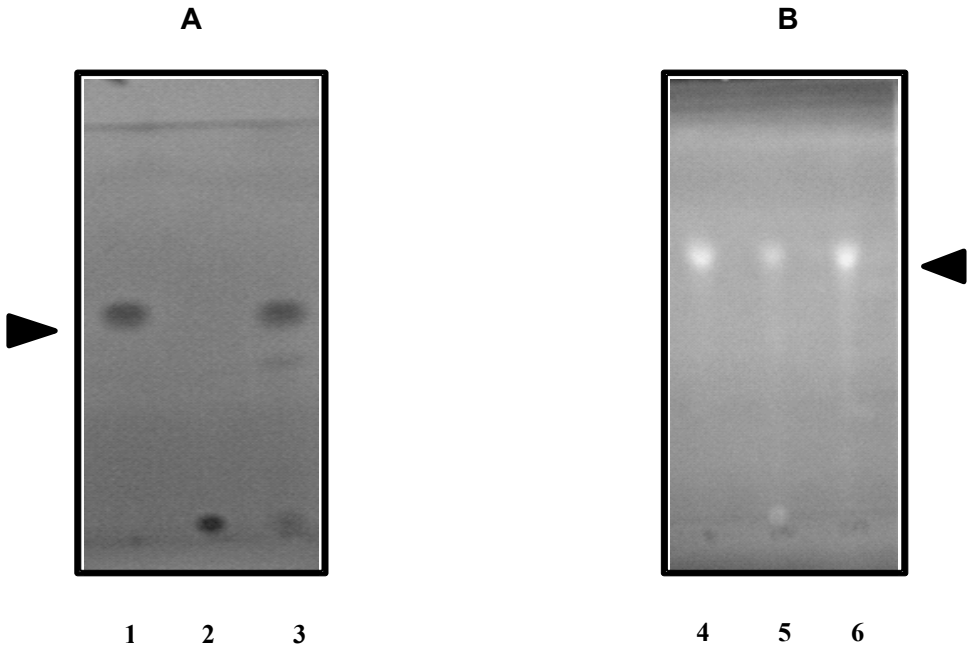

Figure 6. TLC profiles of lovastatin and citrinin for methanol and water-extracts from the rice artificial model. Lovastatin (A) and citrinin (B), Lane 1, 5 mmol/l lovastatin in methanol; Lane 4, 0.75 mmol/l citrinin in methanol; Lane 2 and 5, 10 mg/ml water-extracts; Lane 3 and 6, 10 mg/ml methanol-extracts

To assess lovastatin and citrinin contamination, the water-soluble fraction of NBRC4507-RMR cultivated at 30°C for 14 days was spotted on the TLC plate at a final concentration of 10 mg/ml (Figure 7). It was observed that the water extract of NBRC4507-RMR was free from lovastatin and citrinin even at a 10-fold higher concentration than the tested final concentration in the 3T3-L1 cell assay.

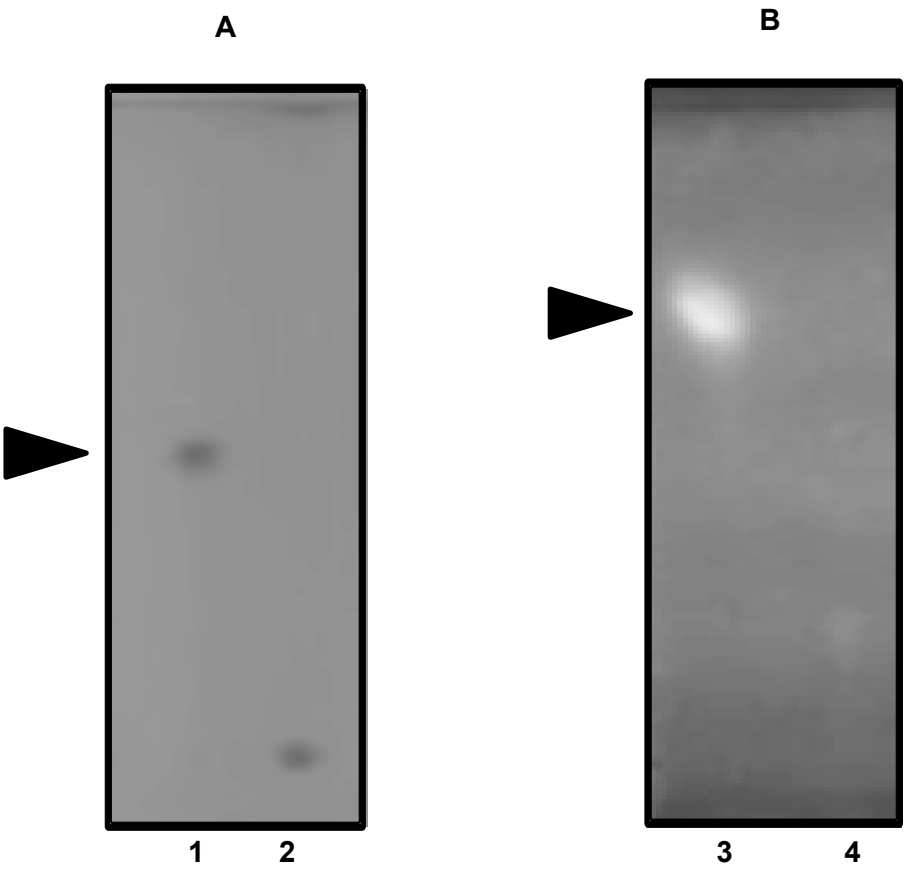

Figure 7. TLC confirmation of lovastatin and citrinin contamination for the water-extracts from RMR of *M. pilosus* NBRC4507 cultivated at 30°C for 14 day. Lovastatin (A) and citrinin (B), Lane 1, 5 mmol/l lovastatin in methanol; Lane 3, 0.75 mmol/l citrinin in methanol; Lane 2 and 4, 10 mg/ml of the water-extracts

According to Jeon *et al.* [10], hot water-extracts prepared from some *Monascus*-fermented products effectively inhibit proliferation in preadipocytes and lipid accumulation in adipocytes. Furthermore, Jeon *et al.* [10] and Lee *et al.* [11] reported that polyketide compounds are water-insoluble, but a part of those compounds including monacolin, citrinin, and pigments possessing the antiadipogenic effects can be extracted into the water-soluble fraction using hot water. However, the water-soluble fraction used in this study did not contain either lovastatin or citrinin. Although the inhibition mechanism against lipid accumulation in adipocytes by the water-soluble fraction remains unclear, the inhibitory effects observed in this study might be caused by new water-soluble components and not by lovastatin and citrinin.

Monacolin K, also known as the biological version of lovastatin, is produced by *Monascus* species. Monacolin K has a similar structure to polyketide and very low water-solubility [13, 31, 35]. Numerous studies have shown cholesterol lowering effects and lipid accumulation reduction in adipocytes using Monacolin K *in vivo* and *in vitro* studies [9-11]. This confirmed the absence of Monacolin K in the water-soluble fractions by TLC analysis using the unfermented model to which lovastatin had been added. These TLC results clearly showed that the water-soluble fraction used in

this study was free from lovastatin. Based on this evidence, the inhibitory effects on lipid accumulation in 3T3-L1 cells were attributed to water-soluble compounds in the fraction, and not due to the lovastatin.

Citrinin is a nephrotoxic mycotoxin produced by some fungi including *Monascus*, and it possesses cholesterol lowering effect in the plasma of rats [16, 17, 19]. However, when compared to other mycotoxins, contamination of food by citrinin is found to be relatively rare. As it is mentioned in the European Union regulation, the maximum allowed concentration of citrinin in food supplements based on fermented red yeast rice is established as 2000 µg/kg [36]. Moreover, according to Urraca *et al.* [37], the maximum allowed level of citrinin that can be found in red yeast fermented rice in China and Japan is 50 and 200 µg/kg, respectively. Citrinin is slightly soluble in water but is readily soluble in organic solvents [18]. In the TLC analysis, using the unfermented model, positive spots corresponding to citrinin were partially detected in the water-extracts, although it was detected in the methanol-extracts. Based on the TLC results, the water-soluble fractions obtained from NBRC4507-RMR were free from citrinin contamination. Hence, the water-soluble fraction obtained from NBRC4507-RMR should be safe to use in the food industry.

4. Conclusions

The present study revealed that the water-soluble fraction obtained from NBRC4507-RMR, cultivated at 30°C for 14 days can inhibit the lipid accumulation in 3T3-L1 cells at a level greater than or equal to that of lovastatin. TLC studies proved that the water-soluble fraction obtained from NBRC4507-RMR was free-from lovastatin and citrinin. Considering these results, NBRC4507-RMR can be used to develop new functional food materials that can be used to manage excess bodyweight and obesity.

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