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Characterization of Monascus Metabolites Isolated from Red Yeast Rice (*Monascus ruber*-fermented rice) and Their Proliferation Inhibitory Effects against Cancer Cells

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Qijun Wang [1], Changkeun Sung [2], Baojun Xu [3,*]

[1] College of Food and Bioengineering, South China University of Technology, Guangzhou 510640, China.

[2]Department of Food Science and Technology, Chungnam National University, Taejon 305-764, South Korea.

[3] Food Science and Technology Program, Beijing Normal University-Hong Kong Baptist University-United International College, Zhuhai 519085, China.

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ABSTRACT

The objective of current study was to characterize the secondary metabolites from red yeast rice and explore their anticancer activities. Solvent fractionation methods combined with open column chromatographic method were performed to isolate the major compounds from red yeast rice. High-resolution mass spectrum and ^{13}C and ^{1}H-NMR spectral techniques were further applied to elucidate the chemical structures of the isolated compounds. The isolated compounds were subjected to cancer cell proliferation inhibition analyzed by MTT method. Four isolated compounds were identified as lactone-form monacolin K, monacolin L, apigenin, and rubropunctatin based on the spectral analyses. The red pigment, rubropunctatin, exhibited strong anticancer effects against both SNU-1 cells and SK-OV-3 cells at the concentration of 10 μg/mL, its anticancer activities were close to or higher than the positive drugs. The IC_{50} values of monacolin L and rubropunctatin against cancer cells SNU-1 and SK-OV-3 were 9.8, 13.3, 28.9 and 8.2 μg/mL respectively.

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1. INTRODUCTION

Red yeast rice (RYR) produced by fermenting *Monascus* species on steamed rice is one paradigm of traditional foods consumed in Asia. Clinical observations clearly showed that functional RYR had the ability to lower blood lipid levels in humans (Heber, Yip, & Ashley, 1999), and these observations were partly due to the presence of cholesterol synthetase (HMG-CoA reductase) inhibitors. As cholesterol synthetase inhibitors, more than ten monacolin-related compounds (statins) were found in the metabolites of several fungal cultures. A couple of research work had been done on isolation and structure elucidation of monacolin compositions in the seventies and eighties of last century. On the other hand, more than 58 *Monascus* strains have been deposited in the American Type Culture Collection (ATCC, Bethesda, MD, U.S.A.) (Lin, Wang, Lee, & Su, 2008). The differences of *Monascus* strains and fermentation technology may conduce to the occurrence of different metabolites. In the past decade, some new pigments and metabolites have been isolated and characterized from RYR (Wild, Toth, & Humpf, 2002; 2003; Akihisa et al., 2004; Campoy, Rumbero, Martin, & Liras, 2006; Knecht, Cramer, & Humpf, 2006; Wu, Cheng, Yuan, Yech, & Chen, 2010; Zheng, Xin, Shi, & Guo, 2010; Cheng, Wu, Su, Chen, & Yuan, 2012; Hsu, Hsu, Liang, Liao, Kuo, & Pan, 2012; Zhu et al., 2012). In addition, RYR also contains several categories of bioactive compounds, such as unsaturated fatty acids, amino acids, phytosterols, isoflavones, and saponins. Consequently, the beneficial effects of RYR may derive not only from monacolins but also from some of other constituents.

Beyond cholesterol-lowering activities, the antibiotic activity (Cheng, Wu, Chen, Tseng, & Yuan, 2011), immunosuppressive activity (Martinkova et al., 1999), hypertension alleviating activity (Kohama, Matsumoto, Mimura, Tanabe, Inada, & Nakanishi, 1987), adipogenesis suppression activity (Jeon et al., 2004), bone formation promotion activity (Gutierrez, Mundy, Rossini, Garrett, Chen, & Mungy, 2006; Wong & Rabie, 2008), and anti-inflammatory activity (Cheng, Wu, Su, Chen, Yuan, 2012) of RYR have been found. Continuous discovery of new therapeutic activities of *Monascus* metabolites encourages more people to participate in a further study on this precious mold.

In recent years, there are emerging interests in analogs of monacolins use as anticancer agents based on preclinical evidence of their antiproliferative, pro-apoptosis, anti-invasive, and radiosensitizing properties (Dimitroulakos et al., 2001; Chan, Oza, & Siu, 2003; Hong, Seeram, Zhang, & Heber, 2008). In addition, the effect of *Monascus* pigment on tumor

promotion had been reported that *Monascus* pigment dose-dependent reduced the incidence of skin tumor formation (Yasukawa, Takahashi, Yamanouchi, & Takido, 1996). The pharmacological evidence also indicated that analogs of monacolins may inhibit colon cancer cell growth and thereby reduces the incidence of colon cancer (Agarwal et al., 2002; Ukomadu & Dutta, 2003; Lin, Song, & Pan, 2006). Hong, Seeram, Zhang, & Heber (2008) reported the anticancer effects of RYR on colon cancer cells. The anticancer effects were found in a pigment-rich fraction from RYR that showed anti-proliferation and pro-apoptotic activities (Martinkova et al., 1999). However, there is no exact evidence that which components are responsible the anticancer activity of RYR. Therefore, further studies have to be performed to discover the anticancer effect of active constituents from RYR. In order to illustrate its anticancer compositions and mechanisms, several monacolins compounds and pigments were isolated from RYR; the anticancer activities were further investigated by evaluating their anti-proliferation activity.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

Trifluoroacetic acid (TFA), propidium iodide (PI), 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyletrazolium bromide (MTT), 10-hydoxyl-camptothecin (HCPT), *cis*-diammineplatinum (Cis-platinum) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A). Taxol was purchased from Xi'an Tiancheng Drug and Bioengineering Co. (Xi'an, China). Column chromatographic material silica gel (silica gel 60, particle size 0.063-0.200 mm) was purchased from Merck Co. Ltd. (Germany), Sephadex LH-20 was purchased from Amersham Biosciences, Sweden). Cell culture medium RPMI 1640 and fetal bovine serum (FBS) were purchased from GIBCO (Carlsbad, CA, U.S.A). Penicillin-streptomycin and Trypsin-Versene Mixture were purchased from BioWhittaker (Walkersville Inc., MD, U.S.A). Sterilized cell culture materials were purchased from Beckton Dickinson Labware (NJ, U.S.A). Polyvinylidene difluoride (PVDF) syringe filters with pore size 0.2 μm were purchased from National Scientific Company (Duluth, GA, U.S.A). HPLC grade water and cell culture water was prepared with a Compact Ultra-pure water system (Compact Co. Ltd., Iowa, U.S.A). Chromatographic grade solvents were purchased from Duksan (Dusan Pure Chemical Co. Ltd., Ansan, South Korea). Deuterated solvents used for structural elucidation were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A).

2.2. Monascus strain and solid culture

The strain of *Monascus* employed in the production of functional RYR (producing monacolin K) at Dbio Co. Inc., Korea was isolated from a RYR sample from China. The strain was classified as *Monascus ruber* and proved to be 100% similarity with the type strain of AS3.549 *M. ruber* (Type Culture Collection of Chinese Academy of Science) (Wang, 2003). The solid-state fermentation on steamed rice was carried out in plastic mushroom bottle at 32°C for 15 days, solid medium consisted of 100 g rice, 14 g soybean powder, 2 g sucrose, and 1 g yeast extract according to our previous publication (Xu, Wang, Jia, & Sung, 2005).

2.3. HPLC analysis of secondary metabolites from red yeast rice

The HPLC system was System Gold ® (Beckman, U.S.A.) equipped with 128 solvent module and 168 photo-diode array (PDA) detector and a power supplier, a 7725 autosampler and an on-line degassing instrument. ODS column (250 × 4.6 mm, 5 μm) from Sphenomenex was used. Gold™ nouveau chromatography station was used for system control, data collection, and analysis.

The identification of monacolins was carried out by HPLC, using a Phenomenex reverse phase column. The flow rate was set at 0.8 mL/min. Monacolin K exhibits UV absorption (monacolin K: $^{MeOH}_{max}$ = 237 nm), so the detection was monitored at 237 nm, UV photodiode array (PDA) detection range was set from 200 to 600 nm. The injection volume was 5 μL, and run time was 40 min. The mobile phase consisted of acetonitrile–water contained 0.05% TFA (62:38, v/v) in this study.

2.4. Extraction and separation of monacolins and pigments from solid cultures

The dried RYR solid cultures (1 kg) were soaked in 5 L of 60% acetonitrile aqueous solution for 48 hr at room temperature and then extracted for 30 min under ultrasonication for three times. The extract was concentrated under reduced pressure. The resultant pellet was dissolved in 1 L H_2O and the insoluble materials were removed by filtration. The filtrate was extracted with petroleum ether. The water layer was extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness under reduced pressure. The residue was subjected to silica gel (63-200 mesh) column chromatography; the column was eluted with a gradient solvent system of dichloromethane-ethyl acetate. The crude crystals were found in the fraction eluted by dichloromethane-ethyl acetate (6:4), the compound was recrystallized from aqueous acetone, colorless needle-like crystals (compound **1**) was obtained. The fraction

eluted by dichloromethane-ethyl acetate (7:3) was subjected to silica gel column chromatography, the column was eluted with a gradient solvent system of *n*-hexane-acetone (9:1 and 8:2), the crude crystals were obtained from the fraction eluted by *n*-hexane-acetone (9:1), the crystals (compound **2**) were purified by solvents; the precipitation was found in the fraction eluted by *n*-hexane-acetone (8:2), the crystals (compound **3**) were obtained by recrystallization from acetone aqueous solution. The red pigments contained a solution infraction 4 from the first column chromatography was further gone through Sephadex HL-20 column chromatography; finally, a purified red pigment was yielded (compound **4**).

2.5. Structural analyses of isolated compounds

^{13}C-NMR and ^{1}H-NMR spectra of isolated compounds were recorded in $CDCl_3$ with a Brucker NMR spectrometer (DRX 300-MHz, Japan). The high-resolution mass measurement was performed by a Mariner mass spectrometer (Perseptive Biosystem, U.S.A.), an electrospray ionization (ESI) probe was operated in the positive ion mode, the mobile phase was 100% methanol (0.1% acetate acid), and sample solvent was 100% methanol. The analysis was also introduced to MS detector by injecting 5 μL of the sample through an HPLC system consisting of a 1100 separations module (Hewlett-Packard, U.S.A.) connected to a Sphenomenex RP-C_{18} column (250 × 4.6 mm, 5 μm).

Compound 1. ^{1}H-NMR (CD_3OD, 300 MHz) δ 5.38 (1H, H-1), 1.92 (2H, H-2), 2.41 (1H, H-3), 1.09 (3H, d, *J* = 7.2 Hz, 3-CH_3), 5.53 (1H, H-4), 5.98 (1H, dd, *J* = 9.6 Hz, H-5), 5.79 (1H, dd, *J* = 9.6, 5.7 Hz, H-6), 2.39 (1H, H-7), 0.91 (3H, d, *J* = 7.5 Hz, 7-CH_3), 1.72 (1H, H-8), 2.25 (1H, 8α), 2.64 (1H, ddd, *J* = 17.7, 2.3, 1.4 Hz, 2′ eq), 2.71 (1H, dd, *J* = 17.74, 4.2 Hz, 2′ ax), 4.37 (1H, 3′), 1.66 (1H, 4′ ax), 1.99 (1H, 4′ eq), 4.63 (1H, 5′), 1.32 (1H, 6′), 1.39 (1H, 7′), 2.36 (1H, 2′′), 1.10 (3H, d, *J* = 6.9 Hz, 2′′-CH_3), 1.42 (1H, 3′′), 0.88 (3H, t, *J* = 7.2 Hz, 4′′). ^{13}C-NMR (75 MHz, CD_3OD) δ 177.26 (C-1′′), 172.37 (C-1′), 132.97 (C-5), 132.12 (C-4α), 129.36 (C-6), 128.56 (C-4), 77.07 (C-5′), 68.56 (C-1), 62.30 (C-3′), 41.83 (C-8α), 38.16 (C-2′), 37.53 (C-2′′), 36.98 (C-8), 35.65 (C-4′), 33.05 (C-6′), 32.64 (C-2), 30.95 (C-7), 27.79 (C-3), 26.96 (C-3′′), 22.39 (3-CH_3), 15.60 (2′′-CH_3), 13.10 (7-CH_3), 11.16 (C-4′′). ESI-MS (m/z): 427, $[M + Na]^+$(calculated for $[M + Na]^+$, $C_{24}H_{36}NaO_5$: 427).

Compound 2. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 5.92 (d, H-5), δ 5.72 (dd, H-6), δ 5.43 (H-4), δ 4.71 (H-5′), δ 4.40 (H-3′), δ 2.72 (dd, H-2′ax), δ 2.64 (ddd, H-2′eg), δ 2.33 (H-3), δ 2.31 (H-7), δ 2.05 (H-8a), δ 1.97 (H-4′eg), δ 1.82, δ 1.80 (H-4′ax), δ 1.58 (H-2), δ 1.47 (H-6′), δ 1.42 (H-7′), δ 1.38 (h-8), δ 1.18 (H-1), δ 0.98 (H-3-CH_3), δ 0.89 (H-7-CH_3). ^{13}C-NMR (125 MHz,

$CDCl_3$) δ 170.69 (C-1′), δ 132.82 (C-5), δ 131.85 (C-4α), δ 130.71 (C-6), δ 128.58 (C-4), δ 76.21 (C-5′), δ 63.05 (C-3′), δ 42.11 (C-8α), 38.87 (C-2′), 36.30 (C-8), 36.34 (C-4′), 33.40 (C-6′), 33.24 (C-2), 30.95 (C-7), 27.79 (C-3), 22.39 (3-CH_3), 13.10 (7-CH_3). ESI-MS (m/z): 324.27, $[M + Na -3H]^+$ (calculated for $[M + Na -3H]^+$, $C_{19}H_{25}NaO_3$: 324).

Compound 3. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 8.31 (1H, s, H-3), 7.37 (1H, d, *J* = 8.4 Hz, H-2', 6'), 6.81 (1H, d, *J* = 8.4 Hz, H-3', 5'), 6.39 (1H, s, H-8), 6.23 (1H, s, H-6). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 181.08 (C-4), 165.12 (C-7), 162.86 (C-2), 159.53 (C-5), 158.45 (C-9), 158.27 (C-4'), 131.0 (C-3', 5'), 123.16 (C-1'), 115.93 (C-2', 6'), 105.34 (C-10), 99.82 (C-6), 94.52 (C-8). ESI-MS (m/z): 271.24, $[M + H]^+$ (calculated for $[M + H]^+$, $C_{15}H_{10}O_5$: 271.24).

Compound 4. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 7.87 (1H, s, H-4), 6.89 (1H, s, H-5), 6.61 (1H, dq, *J* = 7.5 Hz, H-12), 6.15 (1H, s, H-1), 6.05 (1H, dd, *J* = 15.6, 1.2 Hz, H-11), 2.94 (4H, m, H-16, 17), 1.96 (3H, d, H-13), 1.71 (3H, s, H-10), 1.62 (2H, m, H-15), 1.32 (2H, m, H-18), 0.88 (3H, t, *J* = 6.6 Hz, H-19). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 197.6 (C-14), 191.0 (C-9), 171.9 (C-7), 169.4 (C-8a), 156.6 (C-3), 153.0 (C-1), 141.9 (C-6), 136.6 (C-11), 122.6 (C-12), 116.5 (C-4a), 113.4 (C-5a), 109.8 (C-5), 10.4.4 (C-4), 85.9 (C-9a), 41.8 (C-15), 31.6 (C-16), 28.5 (C-13), 23.6 (C-17), 22.7 (C-18), 18.9 (C-10), 14.2 (C-19). ESI-MS (m/z): 354, $[M + H]^+$ (calculated for $[M + H]^+$, $C_{21}H_{22}O_5$: 354).

2.6. Cell lines and cell culture

The human ovary adenocarcinoma cell line SK-OV-3 and human stomach adenocarcinoma cell line SNU-1 were purchased from the Korea Cell Line Bank (Seoul, Korea) and maintained in RPMI 1640 medium containing phenol red, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/mL) in 5% CO_2, 95% air, at 37°C.

2.7. Preparation of test sample solutions

Cytotoxic screening was done with a fast-growth cell line SNU-1, according to evaluation criteria of NCI (IC_{50} value of compound less than 10 μg/mL was considered as potential anticancer agent), 100 μg/mL of working solutions (final concentration was 10 μg/mL in MTT assay) were made by diluting 1 mg/mL of stocking solution. For plotting cell viability curve and measuring IC_{50} value, the samples which cell viability less than 50% in the preliminary screening were selected out (IC_{50} less than 10 μg/mL), gradient concentration solutions were further made by diluting 1 ml stocking solution with PBS buffer.

2.8. Cancer cell proliferation inhibitory assay

The anti-proliferation determination was performed using MTT assay (Carmichael, Degraff, Gazdar, Minna, & Mitchell, 1987) with minor modifications (Xu & Chang, 2012). Briefly, cells were seeded into 96-wells culture plates at seeding density of 1×10^4 SNU-1 cells per well, 1.5×10^4 SK-OV-3 cells per well in 180 μL RPMI 1640 medium. The cells were cultured in an atmosphere of 95% air and 5% carbon dioxide at 37°C and 90% humidity for 24 hr before sample-treatment. Subsequently, cells were exposed to samples for 48 hr. After the samples treatment, 20 μL MTT (5 mg/mL) was co-cultured with cells for 4 hr. Suspension cells (SNU-1) were centrifuged at 1760 rpm for 10 min, then the supernatant was removed, the formation of yellow formazan (product of the reduction of tetrazolium by viable cells) was dissolved in 150 μL DMSO, then gently shaken for 10-15 min under dark. Absorbance at 540 nm was measured by ELISA microplate reader (Molecular devices Emax, Sunnyvale, CA, U.S.A). Software Softmax Pro 4.6 was used for data processing. Cytotoxicity was evaluated by IC_{50} values (inhibitory concentration values, i.e. drug concentration required to inhibit viability by 50%) and each assay was done in triplicate wells. Cell viability curve against control was created by software SigmaPlot 7.0.

2.9. Statistical analysis

Data were expressed as mean ± standard deviation. The inhibitory concentration 50% (IC_{50}) was calculated from the concentration effect regression line. In each case, an appropriate range of 4–5 concentrations was used.

3. RESULTS AND DISCUSSION

3.1. Typical HPLC chromatogram analysis of solid cultures

The analysis of secondary metabolic compositions of the solid culture was achieved by separating the components of acetonitrile extract from solid state cultivation of *M. rubber* on steamed rice. Several monacolins were isolated from the extract of red yeast rice in sufficiently pure form for identification. The other compounds were presumed to be monacolin-related compounds based on HPLC and UV-PDA profiles.

Reversed-phase HPLC system was carried out for separation of monacolins. After HPLC separation, eluting substances were monitored with a photodiode array detector. A typical

HPLC profiles of the monacolins in red yeast rice was shown in **Fig. 1**. Nine peaks with full baseline separation were achieved. Their retention time and UV absorption maxima of peaks were peak 1, 6.8 min, UV 198, 237, 260 nm; peak 2, 7.1 min, 198, 237, 312, 415, 528 nm; peak 3, 9.5 min, UV 198, 237 nm; peak 4, 12.4 min, UV 231, 237, 246 nm; peak 5, 14.5 min, UV 237, 388 nm; peak 6, 16.2 min, UV 237 nm; peak 7, 18.4 min, UV 237 nm; peak 8, 21.9 min, UV 231, 237, 246 nm; peak 9, 24.5 min, UV 237, 388 nm; respectively. Peak 1, 3, 4, 6, 7 and 8 contained 237 nm UV absorption peak, so they were perhaps monacolin-related compounds; peak 2 contained 415 nm and 528 nm visible area absorption peak, meanwhile red visible spot (*Rf* value was 0.44) can be observed on TLC plate developed in cyclohexane-chloroform- isopropanol = 6:3:1 (v/v/v), so it was one kind of red pigment composition; peak 5 and 9 contained 237 nm UV area absorption peak and 388 nm visible area absorption peak. Meanwhile, yellow visible clear spot (*Rf* value was 0.71) can be observed on TLC plate developed by the same condition, so it was one kind of yellow pigment composition. In addition, the compounds of peak 5 and 9 may contain some basic structural unit of monacolins, due to the existence of a UV absorption peak at 237 nm; peak 4 and 8 contained 231 nm, 237 nm, 246 nm UV absorption peaks. The HPLC retention time of peak 4 was identical with that of alkaline hydrolyzed substance (acid-form monacolin K) of lactone form monacolin K standard, HPLC retention time of peak 8 was identical with that of standard (lactone form monacolin K), so the compounds of peak 4 and 8 were one pair of convertible structures between acid form and lactone form, and relationship and HPLC characteristics of them have been reported in our previous paper (Moon, Wang, Xu, & Sung, 2001). It is confirmed in the further study of this report. Among them, peak 8 (compound I) and peak 5 (compound II) were isolated from solid fermentation. Other two compounds (compound III and IV) were not found in typical HPLC chromatogram of solid fermentation of *M. ruber*, their spectral analyses in details will be further elucidated in the following section.

3.2. Identification of monascus metabolites from red yeast rice

Compound **1** was obtained as colorless needle-like crystal, its melting point was 165-166°C. The UV spectrum (in methanol) showed maxima peaks at 231, 237 and 246 nm. Its molecular formula $C_{24}H_{36}O_5$ was determined from the molecular ion peak and the pseudo molecular ion MH^+ peak at *m/z* 405 in the positive ESI-MS and its ^{13}C and DEPT-NMR spectral data. The molecular weight 404 was confirmed by elemental analysis (calculated: C 71.31, H 8.91, O

19.78%) and high-resolution mass spectrum. The ^{13}C-NMR spectrum (in CD_3OD) indicated the presence of 2 ester carbonyl carbons at δ 172.37 (C-1′′) and δ 177.26 (C-1′), 4 methyl carbons at δ 11.16 (C-4′′), δ 13.10 (7-CH_3), δ 15.60 (2′′-CH_3) and δ 22.39 (3-CH_3), and 4 olefinic carbons at δ 128.56 (C-4), δ 132.12 (C-4α), δ 129.36 (C-6) and δ 132.97 (C-5). The above-mentioned data were identical with those of reference (Endo, 1979), summed up all the data, the compound 1 was identified as **Monacolin K**, its chemical structure was elucidated in **Fig. 2**.

Compound **2** was obtained as a white amorphous powder, melting point: 146.8-147.8°C (in ethyl acetate). Its molecular formula $C_{19}H_{28}O_3$ was determined from its ^{13}C and DEPT-NMR spectral data. The ^{1}H-NMR spectrum showed proton signals at δ 5.92 (d, H-5) δ 5.72 (dd, H-6), δ 5.43 (H-4), δ 4.71 (H-5′), δ 4.40 (H-3′), δ 2.72 (dd, H-2′ax), δ 2.64 (ddd, H-2′eg), δ 2.33 (H-3), δ 2.31 (H-7), δ 2.05 (H-8a), δ 1.97 (H-4′eg), δ 1.80 (H-4′ax), δ 1.58 (H-2), δ 1.47 (H-6′), δ 1.42 (H-7′), δ 1.38 (h-8), δ 1.18 (H-1), δ 0.98 (H-3-CH_3), δ 0.89 (H-7-CH_3). The ^{13}C-NMR spectrum showed 19 carbons, in which there is a carboxylic at δ 170.69 (C-1′), 4 olefinic carbons at δ 128.58 (C-4), δ 131.85 (C-4α), δ 130.71 (C-6) and δ 132.82 (C-5) in low -field region, and other carbonic signals including two methyl, six methylene, and four methane carbons in the high-field region. All spectral data of compound 2 were identical with those of Monacolin L (Endo, Hasumi, & Nakamura, 1985). Therefore, the compound 2 from red yeast rice was **Monacolin L**, its chemical structure was elucidated in **Fig. 2**.

Compound **3** was obtained as a yellow amorphous powder, melting point: 288-290°C. Its molecular formula $C_{15}H_{10}O_5$ was determined from its ^{13}C and DEPT-NMR spectral data. The ^{1}H-NMR spectrum showed proton signals at δ 8.31 (1H, s, H-3), 7.37 (1H, d, *J* = 8.4 Hz, H-2', 6'), 6.81 (1H, d, *J* = 8.4 Hz, H-3', 5'), 6.39 (1H, s, H-8), 6.23 (1H, s, H-6). The ^{13}C-NMR spectrum showed 15 carbons, in which there is a carboxylic at δ 181.08 (C-4), and other aromatic carbon signals at 165.12 (C-7), 162.86 (C-2), 159.53 (C-5), 158.45 (C-9), 158.27 (C-4'), 131.0 (C-3', 5'), 123.16 (C-1'), 115.93 (C-2', 6'), 105.34 (C-10), 99.82 (C-6), 94.52 (C-8), respectively. All spectral data of compound 3 were identical with those of Apigenin (5,7,4'-trihydroxyl flavone) (Roh, Moon, & Zee, 2000). Therefore, compound 3 was identified as **Apigenin**, its chemical structure was elucidated in **Fig. 2**.

Compound **4** was obtained as a red amorphous powder, melting point: 152.5-153.5°C. Its molecular formula $C_{21}H_{22}O_5$ was determined from its ^{13}C and DEPT-NMR spectral data. The ^{1}H-NMR spectrum showed three methyl signals at δ 1.71 (3H, s, H-10), δ 1.96 (3H, d, H-13),

δ 0.88 (3H, t, *J* = 6.6 Hz, H-19), and four methylene signals at δ 2.94 (4H, m, H-16, 17), δ 1.62 (2H, m, H-15), δ 1.32 (2H, m, H-18). The ^{13}C-NMR spectrum showed 21 carbons, in which there are three carboxylic at δ 197.6 (C-14), δ 191.0 (C-2), δ 171.9 (C-4), and 10 olefinic carbons at δ 169.4 (C-8a), δ 156.6 (C-3), δ 153.0 (C-1), δ 141.9 (C-6), δ 136.6 (C-11), δ 122.6 (C-12), δ 116.5 (C-4a), δ 113.4 (C-5a), δ 109.8 (C-5), δ 86.2 (C-9a), three methyl carbonic signals at δ 28.5 (C-13), δ 18.9 (C-10), δ 14.2 (C-19), and four methylene carbons at δ 41.8 (C-15), δ 31.6 (C-16), δ 23.6 (C-17), δ 22.7 (C-18), respectively. All spectral data of compound 4 were identical with those of rubropunctatin (Martinkova et al., 1999). Therefore, compound 4 was identified as Rubropunctatin, and its chemical structure was elucidated in **Fig. 2**.

3.3. Anti-proliferation properties of the metabolites in red yeast rice

Anti-proliferation activities of isolated compounds (at the concentration of 10 μg/mL) from red yeast rice against gastric cancer cell line SNU-1 and SK-OV-3 were done by MTT assay. The percentage inhibitory rates of isolated compounds and positive anticancer drugs against SNU-1 and SK-OV-3 cell proliferation were listed in **Table 1**. The results showed that monacolin K, monacolin L, and rubropunctatin possessed strong anticancer activities against gastric cancer cells SNU-1, the inhibition rates were 93.1%, 57.4%, and 75.3%, respectively. These values were higher or close to that of the positive anticancer drugs (cis-platinum, taxol, and HCPT) at a concentration of 10 μg/mL. Rubropunctatin also exhibited strong inhibition activity against SK-OV-3; its inhibition rate (77.4%) was higher than that of positive anti-cancer drugs at a concentration of 10 μg/mL.

Dose-dependent inhibitory effect and IC_{50} value of the isolated compounds were further investigated using cell lines SNU-1 and SK-OV-3. After 48 hr exposing to the isolated compounds with various concentrations, the cell viability curves (**Fig. 3**) of SNU-1 and SK-OV-3 were created by software Sigma Plot 2001 at three parallel level treatments. The IC_{50} values of the isolated compounds were calculated from 3rd-degree polynomial fit by using software CurveExpert 1.3. The IC_{50} values of moma colin L against cancer cells SNU-1 and SK-OV-3 were 9.8 μg/mL and 28.9 μg/mL, respectively. The IC_{50} values of rubropunctatin against cancer cells SNU-1 and SK-OV-3 were 13.3 μg/mL and 8.2 μg/mL, respectively.

4. CONCLUSIONS

In summary, four compounds (Two statins, one flavonoid and one red pigment) were isolated from solid fermentation products (red yeast rice) of *M. ruber*. The four compounds were identified as monacolin K, monacolin L, apigenin, and rubropunctatin, respectively by mass spectrum and ^{13}C and ^{1}H-NMR spectral techniques. Cytotoxic assay indicated that monacolin L and rubropunctatin possessed potent anticancer activities against cancer cells SNU-1 and SK-OV-3. Therefore, as conventional cholesterol-lowering agents, red yeast rice, and its chemical compositions have a great potential to be developed into anticancer candidate agents.

Abbreviation used

RYR, red yeast rice; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyletrazolium bromide; NMR, Nuclear Magnetic Resonance; HCPT, 10-hydroxy-camptothecin; HPLC, High-Performance Liquid Chromatography.

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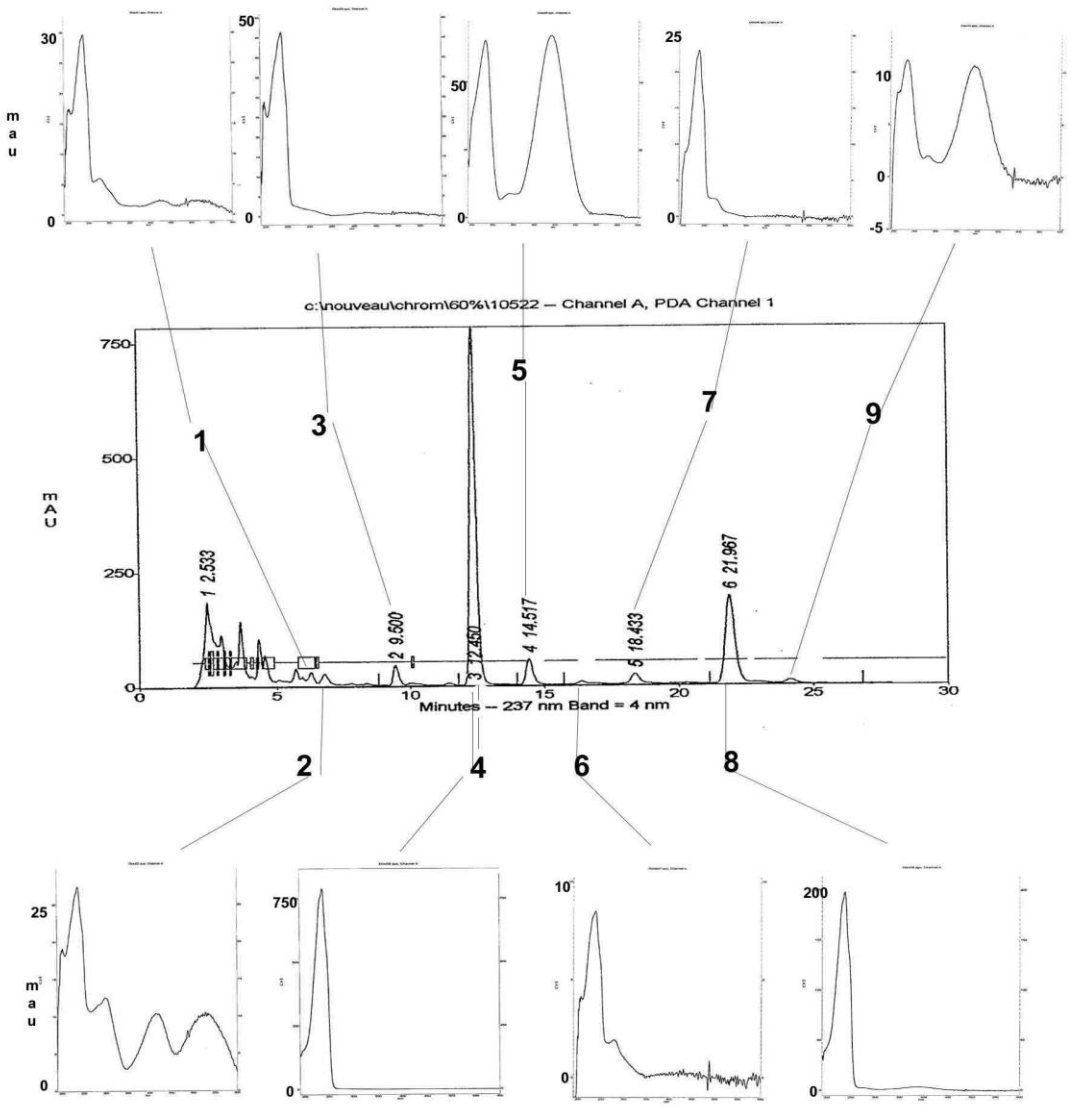


Fig.1. Typical chromatogram and spectrums (from 190 to 600 nm) of 9 typical peaks of red yeast rice by HPLC/UV/PDA.

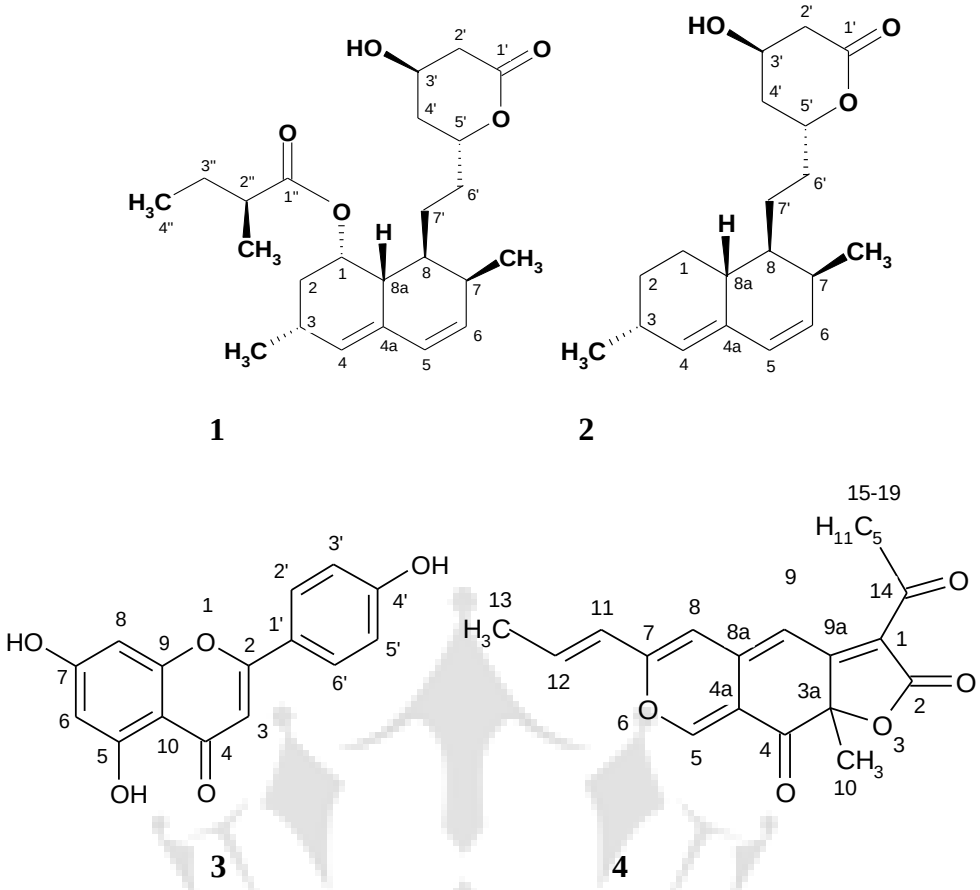
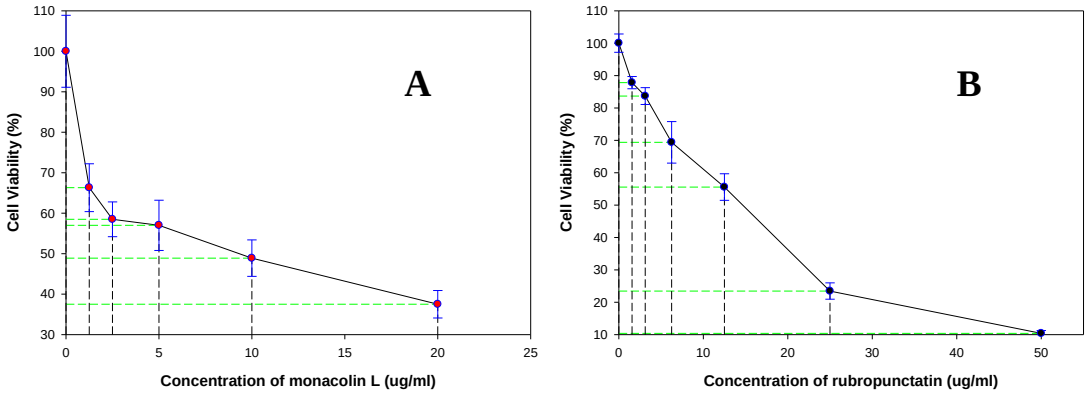


Fig. 2. Chemical structures of isolated compounds from red yeast rice. **1.** monacolin K; **2.** monacolin L; **3.** apigenin; **4.** rubropunctatin



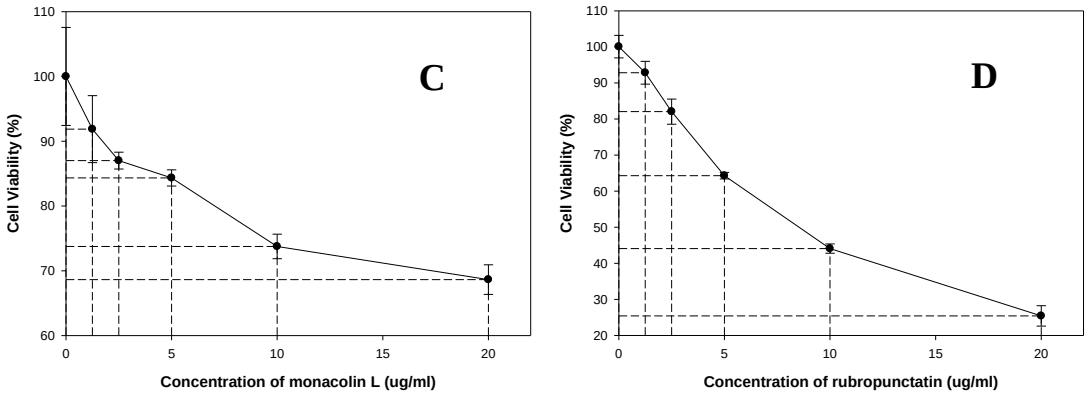


Fig. 3. Cell viability curves of cancer cells SNU-1 (A, B) and SK-OV-3 (C, D) after 48 hr exposing to the isolated compounds

Table 1. Effects of isolated compounds (10 µg/ml) on cancer cells proliferation *****

Compounds	Proliferation inhibitory rate (%)	
	SNU-1	SK-OV-3
Monacolin K	93.1	17.98
Monacolin L	57.4	18.68
Rubropunctatin	75.3	77.37
Cis-platinum	89.8	70.74
Taxol	88.2	56.29
HCPT	68.2	49.61

* Proliferation inhibiting rate of cancer cell line SNU-1 after 48 hr exposure to compounds.