

Modified Riceberry rice extract suppresses melanogenesis-associated cell differentiation through tyrosinase-mediated MITF downregulation on B16 cells and *in vivo* zebrafish embryos

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Abstract

Background and purpose: Excessive melanin production caused by overactive tyrosinase (TYR) enzyme results in several dermatological problems. The TYR inhibitor, derived from metabolite changes during fermentation, has been well recognized for pigmentation control.

Experimental approach: This study is interested in alternative anti-melanogenic agents from bio-modified Riceberry rice through fermentation. Modified Riceberry rice extract (MRB) was evaluated for its cytotoxicity, melanin content, melanin excretion, and TYR activity in B16 cells. TYR and their melanogenesis-related molecules such as TYR-related proteins-1 and -2, and microphthalmia-associated transcription factor (MITF) were determined. The anti-melanogenic activity and toxicity were also tested using the embryonic zebrafish model. Furthermore, comprehensive genotoxicity testing was verified by cytokinesis-block micronucleus cytome assay.

Findings/Results: The study found that non-cytotoxic concentrations of MRB at 20 and 40 mg/mL inhibited melanogenesis and melanin excretion by interfering B16 cell morphology. Cellular TYR enzymatic activity was also suppressed in the treated cells. The mRNA transcription and protein expression levels of TYR and MITF decreased by dose-dependent and time-dependent manners with MRB treatment. In the animal model, MRB was found to be safe and potent for melanogenesis-related TYR inhibition in embryonic zebrafish at 20 and 30 mg/mL. The toxicity of effective doses of MRB showed no genotoxicity and mutagenicity.

Conclusion and implications: This study suggests that MRB has anti-melanogenesis potential through TYR and its-related protein inhibitions. MRB is also safe for applications and maybe a promising anti-melanogenic agent for hyperpigmentation control.

Keywords: Fermentation; Melanogenesis; MITF; Riceberry rice; Tyrosinase.

INTRODUCTION

Melanin is a dark biological pigment produced by a complicated process referred to as melanogenesis. Melanogenesis is regulated by the cooperation of three enzymes including tyrosinase (TYR); TYR-related protein 1 (TRP-1); and TRP-2 (1). All these enzymes are controlled by the activity of upstream protein microphthalmia-associated transcription factor (MITF), which regulates melanogenesis,

melanin excretion, and melanocyte differentiation (2,3). Accumulation of these melanogenic proteins causes several hyperpigmentation disorders including freckles, actinic damage, solar lentigo, and melasma (4). Improvement of these dermatological problems can positively affect the patients' emotional well-being and social lives (5).

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Anti-melanogenic agents have consequently been used for hyperpigmentation control, such as hydroquinone, tranexamic acid, phenylthiourea, and kojic acid. The long-term application of these agents has been reported to result in several side effects (6). Therefore, this study was interested in exploring a novel alternative anti-melanogenic agent obtained from natural sources.

Several phytophenolic compounds have previously shown anti-melanogenesis effects by inhibiting TYR enzyme (7-9). They are also potent antioxidants which have a role in MITF modulation-associated hyperpigmentation control (10). The present study investigated Riceberry rice, a purple-black colored Thai rice due to the presence of high antioxidants and phenolic compounds which act like TYR inhibitors (11). Overpowering rice seeds through germination and fermentation processes has been claimed to promote both the quantity and quality of valuable phenolic compounds for anti-melanogenesis, including ferulic acid, gallic acid, p-coumaric acid, 4-hydroxybenzoic acid, vanillic acid, chlorogenic acid, and syringic acid (12,13). In this study, biomodification of Riceberry rice by germination and fermentation with *Saccharomyces cerevisiae* was conducted. The modified Riceberry rice (MRB) product was developed with the aim of creating a novel, effective, and safe anti-melanogenic agent for hyperpigmentation control.

MATERIALS AND METHODS

Biomodification of Riceberry rice

Riceberry rice was obtained from Payao province, northern Thailand. The biomodification of the rice sample was performed based on the researchers' previous study with some modifications (14). The rice samples were soaked in reverse osmosis water at pH 7.2-7.4 for 60 min. Next, 100 g of soaked rice was germinated in a polyethylene tray (22 × 22 × 5 cm) under the dark condition at room temperature, with moisture controlled for 6 h. The rice sample was lyophilized to eliminate moisture content and stop any germination activity. The rice sample was then pulverized using a centrifugal mill before sieving through a 345 µm stainless-steel sieve.

Riceberry powder was then prepared for fermentation by adding distilled water into 50 g of rice powder to adjust the humidity to 60%. The slurry was autoclaved for 15 min at 121 °C and then cooled to room temperature. Next, 10 mL of 1×10^8 CFU/mL *Saccharomyces cerevisiae*, provided by Innovation Center for Holistic Health, Nutraceuticals, and Cosmeceuticals, Faculty of Pharmacy, Chiang Mai University was added and incubated in a shaking incubator at 27-30 °C for 24 h. The enzymatic activity of the fermented broth in each condition was parallel determined.

Riceberry rice extraction

The Riceberry sample (100 g) was extracted by adding 200 mL of 80% ethanol solution and shaking at 120 rpm for 3 h under dark condition. The supernatant was harvested and dried by a rotary evaporator (Buchi rotavapor R-200, Switzerland) at 40 °C, 200 mm/Hg followed by dehydration with a lyophilizer (Thermo Supermodulyo-230, USA). Dried stock samples were kept in -20 °C before using in the experiment.

Cell culture

B16 melanoma cells (ATCC®, Number CRL-6475™) and V79 Chinese hamster lung fibroblast (ECACC, Number 86041102). The cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum, 10 U/mL of penicillin, 10 mg/mL of streptomycin in a 37 °C, 5% CO₂, and 95% humidified atmosphere.

Cell viability

The cytotoxicity of MRB was determined on B16 cells using a modified method of MTT assay (14). Briefly, 3×10^4 of B16 cells were treated with the MRB extract at 10 to 40 mg/mL for 24 h. Treated cells were incubated with 1.2 mM MTT solution for 4 h. Formazan crystals were solubilized by absolute DMSO and determined at 530 nm. The results are expressed as a percentage of cell viability compared with the control (100% cell viability).

Melanin content and melanin excretion

Intracellular and excreted melanin pigments of B16 cells were determined as described

previously (14). Briefly, 1.0×10^5 of B16 cells were treated with the MRB extract dissolved in phenol red-free DMEM at indicated concentrations for 72 h. Excreted melanin pigments were determined in the culture medium at 405 nm. Intracellular melanin pigments were disrupted from the pellet with 1 N NaOH solution at 80 °C for 4 h and then measured at 405 nm. Ten μ M hydroquinone and 40 mg/mL metabolite were used as a positive control and vehicle control, respectively. The results are expressed as a percentage of melanin content or melanin secretion compared with the untreated control (100%).

Morphological appearances

Melanin-containing B16 cells and their morphological appearances were observed using the Fontana-Masson staining which was modified from a previous study (15). Briefly, treated B16 cells were fixed in absolute methanol and rehydrated with dechlorinated water. Staining was performed using a Fontana-Masson Stain Kit (Bio-Optica, Italy) according to the manufacturer's instructions. The cells were then observed under a light microscope at 100 \times magnification to count the melanin-containing cells out of a total of 1,000 of B16 cells. The morphological appearances of differentiated B16 cells were scored in total 100 differentiated B16 cells as a low grade, a dendritic shape with a bipolar spindle, the cell size of 1-50 μ m with pigmentation; and high grade, a multipolar spindle with or without tip branches, cell size larger than 51 μ m.

Cellular tyrosinase activity

TYR enzymatic activity in B16 cells was determined using the previous method (14). Briefly, 1.0×10^5 of B16 cells were treated with the MRB extract at indicated concentrations for 72 h. The cells were solubilized with PBS, pH 7.1 containing 1% TritonTM X-100, and clarified the lysate by centrifugation at 15,000 g for 15 min. The supernatant was incubated with 2 mM levodopa and L-3,4-dihydroxyphenylalanine (L-DOPA) at 37 °C for 90 min before measuring the dopachrome product at wavelength 492 nm. Ten μ M hydroquinone and 40 mg/mL metabolite were

used as a positive control and vehicle control, respectively.

Melanogenic proteins expression

The expressions of melanogenic proteins were determined using the western blotting analysis which was modified from a previous study (16). Briefly, 40 μ g of protein lysate obtained from treated B16 cells was electrophoresed and transferred to polyvinylidene difluoride membrane (Millipore, Germany). The membrane was treated individually with primary antibody including rabbit polyclonal anti-MITF, 1:1000 (ab 20663, Abcam, UK); rabbit polyclonal anti-TYR, 1:1000 (ab 180753, Abcam, UK); mouse monoclonal anti-TRP-1, 1:100 (ab 3312, Abcam, UK); rabbit polyclonal anti-TRP-2, 1:100 (ab 74073, Abcam, UK), and rabbit polyclonal anti- β -actin, 1:1,000 (ab 8227, Abcam, UK) at room temperature for 1 h with agitation before washing with 1 \times tris-buffered saline and Tween[®] 20 (TBST), pH 7.2 for three times. The membrane was then treated with secondary antibody including polyclonal goat anti-rabbit IgG-horseradish peroxidase(HRP), 1:1,000 (P0448, Dako, Denmark) and polyclonal goat anti-mouse IgG-HRP, 1:1,000 (P0447, Dako, Denmark) at room temperature for 1 h with gentle agitation. Then, washed the membrane with 1 \times TBST, pH 7.2 for 5 min three times, and incubated with Luminata Forte western HRP substrate (Merck, Germany) for 10 min. The proteins were then visualized using a gel documentation analyzer (ImageQuant LAS 500, UK) and analyzed the densitometry by Image J software.

Quantitative polymerase chain reaction

Cellular RNA was extracted using RNAiso Plus (TaKaRa Bio, Japan). Extracted RNA was reversed transcribed to cDNA (PrimeScript RT Master Mix, Takara Bio, Japan). Quantitative polymerase chain reaction (q-PCR) was carried out using the primers as MITF forward 5'-CTGATGGACGATGCCCTCTC-3' and reverse 5'-AGCCAGCAGGTCAGATCCTA-3'; TYR forward 5'-GGCCAGCTTTCA-GGCAGAGGT-3' and reverse 5'-TGGTGCT-TCATGGGCAAATC-3'; TRP-1 forward 5'-ATGAAGCCAACCAGCCTCTC-3' and

reverse 5'-TGTGACTTTGTGGTGGCCTT-3'; TRP-2 forward 5'-GGGAAGAA-CGAG-TGTGACGTG-3' and reverse 5'-GAAAA-GCCAGCAACCCCAAG-3'; β -actin forward 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGAT-TTC-3'. The reaction was cycled in total volume 20 μ L for 30 cycles for 30 s at 94 °C, 60 s at 60 °C, and 60 s at 72 °C. Ten μ L of the reaction mixture was analyzed by electrophoresis on ethidium bromide pre-stained 1.5% agarose gels. The intensity of the bands was measured by a UV transilluminator.

Zebrafish maintain and embryo collection

All animal studies were approved by the Faculty of Science, Mahidol University Animal Care and Use Committee (MUSC62-003-467). Thirty zebrafish were kept (2-3 female/1 male density) in 60 L of glass tank at 27 ± 3 °C, pH 7.0 ± 1.0 , and re-filtered circulation of water with 14/10-h light/dark cycle. Zebrafish were fed three times a day with fleck food supplemented with live brine shrimps (*Artemia salina*). The harvesting of zebrafish embryos was operated by a natural spawning technique that was modified from a previous study (17). In the experiments, all embryos were treated with MRB at 6-h post-fertilization (hpf).

Toxicity assessment

A non-invasive technique for the investigation of toxicity on embryonic zebrafish was performed as described previously (18). Briefly, 5 embryos were treated with the MRB at indicated concentrations and incubated at room temperature until 48-hpf. The mortality and heart beating rate were observed under a stereomicroscope. Then, zebrafish embryos were fixed and prepared for histological section. The histology was observed using hematoxylin and eosin (H&E) staining.

Melanin content of zebrafish embryo

Melanin content in zebrafish embryos was determined by a modified method, previously published (19). Briefly, 100 synchronized embryos were treated with the MRB extract at non-toxic concentrations in a 6-well plate until 48-hpf. The embryos were lysed with 1 N

NaOH at 80 °C for 1 h. Melanin content was determined at 490 nm. In this study, 0.1 mM phenylthiourea was used as a positive control.

Tyrosinase activity of zebrafish embryo

TYR activity in zebrafish embryos was determined according to the previous study (18) with some modification. Briefly, 100 synchronized embryos were treated with the MRB extract at non-toxic concentrations in a 6-well plate until 48-hpf. The embryos were lysed with 1 \times radioimmunoprecipitation assay buffer (RIPA) lysis buffer and centrifuged to obtain the supernatant. Then, the supernatant was incubated with 2 mM L-DOPA at 37 °C for 90 min before measuring the dopachrome product at wavelength 492 nm. In this study, 0.1 mM phenylthiourea was used as a positive control.

Cytokinesis-block micronucleus cytome assay

The genotoxicity of MRB extract was determined by cytokinesis-block micronucleus cytome assay, as described previously (20) with slight modification. In this study, V79 Chinese hamster lung fibroblasts were used and maintained as recommended in the standard protocol (OECD 476). Briefly, 2×10^5 cells/well V79 cells were treated with MRB extract at indicated concentrations and incubated at 37 °C, 5% CO₂, for 3 h. The treated cells have then blocked the cytokinesis by treatment with 3 μ g/mL cytochalasin B (Sigma-Aldrich, USA) for 18 h. The cells were then harvested to the glass slides through cytocentrifugation and stained by Giemsa staining according to their manufacturer instructions. The slides were observed under a light microscope at 400 \times of magnification to determine the cytostatic effect and the effect on DNA damage.

Ames test

The mutagenicity was tested as described by Maron and Ames (21). *Salmonella typhimurium* strains, TA98 (ATCC[®], Number BAA-2720TM), and TA100 (ATCC[®], Number BAA-2720TM), were incubated with Oxoid nutrient broth number 2 for 11 h at 37 °C, 110 rpm. Then 100 μ L of the culture was incubated with 5 mL soft agar containing 0.5 mM histidine-biotin and MRB at various

concentrations. The mixtures were then poured into glucose minimal agar plates and incubated for 48 h at 37 °C. The histidine revertant colonies were then counted. In the study, 4-nitroquinoline N-oxide at 0.4 and 0.2 mg were used as positive controls for *Salmonella typhimurium* strains, TA98 and TA100, respectively. A positive result is given when the number of revertant colonies is significantly higher than the spontaneous reversion.

Statistical analysis

All results were presented at the mean \pm standard deviation (mean \pm SD). Paired student t-tests were run to compare differences through time. One-way analysis of variance (ANOVA) followed by Dunnett's post-hoc tests were used for multiple comparisons among more than two

groups. The significance was considered at $P < 0.05$. GraphPad Prism version 6 (GraphPad Software Inc., CA, USA) was used to perform all statistical analyses.

RESULTS

Modified Riceberry rice extract inhibits melanogenesis and melanin excretion-associated morphological changes in B16 cells

The effect of MRB extract on melanogenesis was demonstrated in B16 cells at non-cytotoxic concentrations of 20 and 40 mg/mL, obtained from MTT assay (data not shown). The results showed that MRB significantly reduced the relative melanin content ($P < 0.001$) when compared with the control (100%) to $74 \pm 2.52\%$ and $52 \pm 2.58\%$, respectively (Fig. 1A).

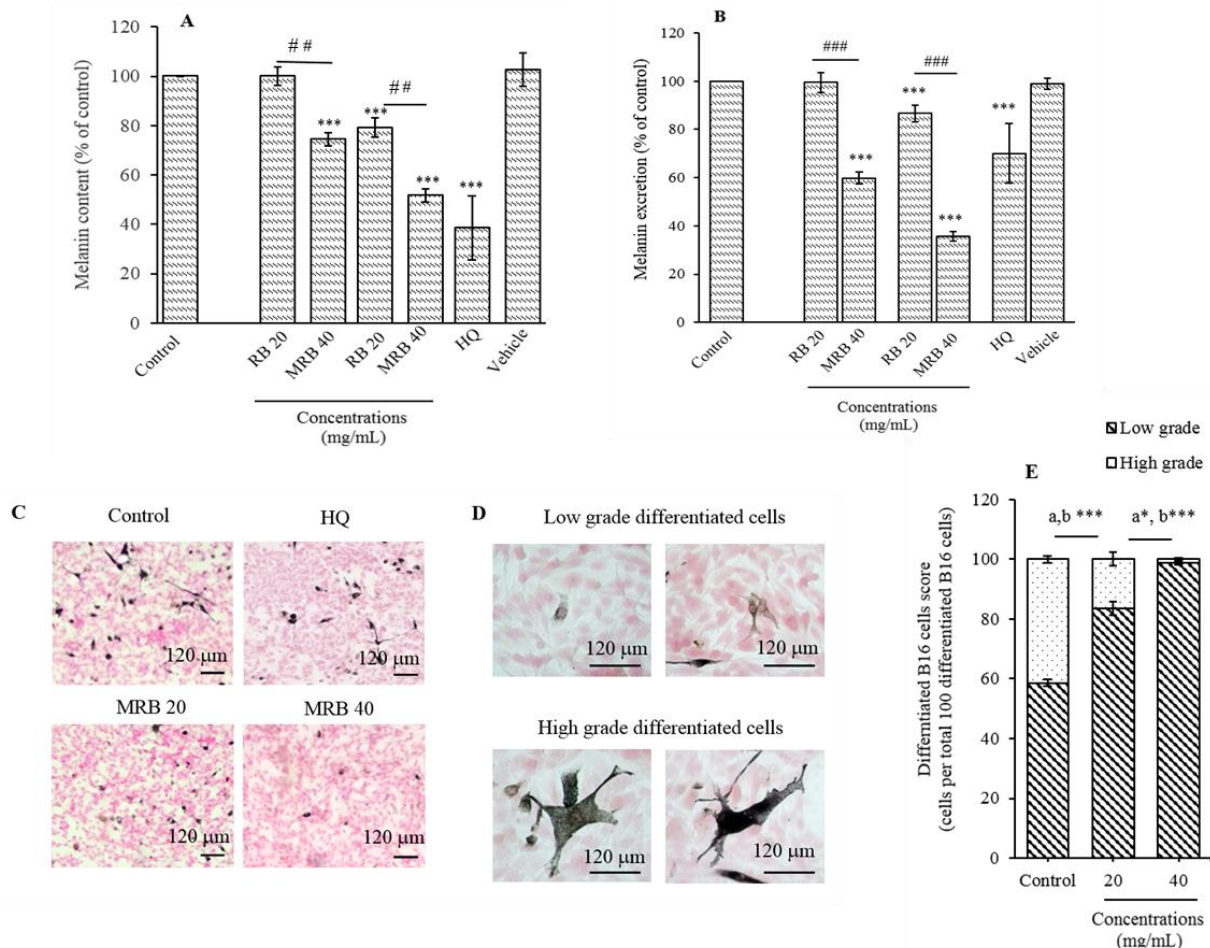


Fig. 1. Effect of MRB extract on melanogenesis. B16 cells were treated with rice extracts at 20 and 40 mg/mL for 72 h. (A) Melanin content and (B) melanin excretion (B) were obtained. (C) Morphology was determined by Fontana-Masson staining and observed under a light microscope (100 \times). (D) Differentiated cells were then scored into low and high grades (400 \times). (E) The results are expressed in a bar graph by mean \pm SD compared with the control. Ten mM HQ and 40 mg/mL metabolite were used as a positive control and vehicle control, respectively. *** $P < 0.001$ Indicates significant differences compared to the control group; ## $P < 0.01$ and ### $P < 0.001$ show differences between defined groups; and a, b compared to low grade and high grade differentiated B16 cells, respectively. MRB, Modified Riceberry rice; HQ, hydroquinone.

Meanwhile, Riceberry rice extract without bio-modification provided a significant reduction of relative melanin content in B16 cells at 40 mg/mL to $79 \pm 3.93\%$ in comparison with the control ($P < 0.001$). The melanin excretion was also determined in this study (Fig. 1B). Treatment of MRB at 20 and 40 mg/mL significantly reduced melanin excretion ($P < 0.001$) when compared with the control (100%) to $60 \pm 2.53\%$ and $36 \pm 1.98\%$, respectively. Meanwhile, B16 cells treated with the Riceberry rice extract without bio-modification provided a significant reduction of melanin excretion at 40 mg/mL to $87 \pm 3.54\%$ when compared with the control ($P < 0.001$). In the experiments, 40 mg/mL of the metabolite was used as a vehicle control that showed no significant inhibition of either melanin content or melanin excretion when compared with the control. Thus, the bio-modification of Riceberry rice efficiently enhanced the hyperpigmentation control effect. Cellular melanin biosynthesis and melanin excretion are determinants of melanocyte

differentiation. The effect of MRB on melanin pigmentation and the cellular morphology of B16 cells were then determined by Fontana-Masson's staining. The results demonstrate that MRB extract at 20 and 40 mg/mL reduced the number of differentiated B16 cells and also affected B16 cell morphology when compared with the control (Fig. 1C). Additionally, the morphological appearances of differentiated B16 cells were scored into either low or high grade differentiated B16 cells (Fig. 1D). MRB shifted the differentiated B16 cells to the left by reducing the number of high grades differentiated B16 cells to 16.33 ± 2.31 and 1.33 ± 0.58 cells/100 differentiated B16 cells at 20 and 40 mg/mL, respectively (Fig. 1E). The reduction was statistically significant at $P < 0.01$ when compared with the untreated control (41.33 ± 1.16 cells/total 100 differentiated B16 cells). These results demonstrate that MRB affected melanogenesis and melanin excretion by interfering with cell differentiation.

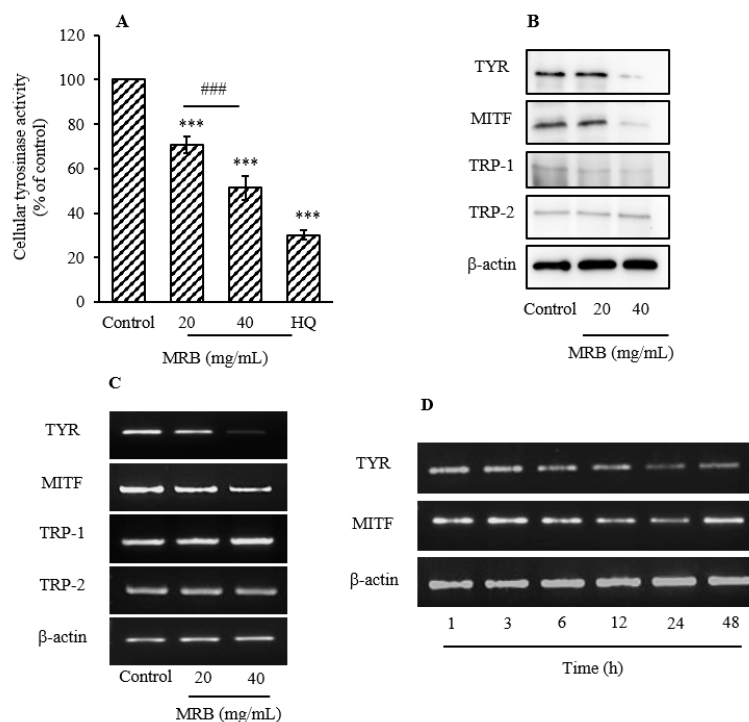


Fig. 2. Effect of MRB extract on melanogenesis-related protein and mRNA. B16 cells were treated with MRB extract at 20 and 40 mg/mL for 48 h. (A) Cellular TYR activity was determined by enzymatic assay. (B) Expression of melanogenic proteins including MITF, TYR, TRP-1, and TRP-2 were investigated by western blotting method. (C) The mRNA expressions were determined at 24 h of treatment. (D) The onset of MRB (40 mg/mL) effectiveness was determined at various durations of 1, 3, 6, 12, 24, and 48 h. Ten mM HQ was used as a positive control. $***P < 0.001$ Indicates significant differences compared to the control group; $###P < 0.001$ shows difference between defined groups. MRB, Modified Riceberry rice; HQ, hydroquinone. TYR, tyrosinase; MITF, microphthalmia-associated transcription factor; TRP, tyrosinase-related protein.

Modified Riceberry rice extract inhibits tyrosinase activity through the modulation of melanogenesis-related proteins expression in B16 cells

TYR and its related proteins are recognized to control melanogenesis. The mechanism of MRB on melanogenesis inhibition was then investigated by determining cellular TYR enzymatic activity of MRB treated B16 cells. Treatment of B16 cells with MRB extract showed a gradual suppression of cellular TYR activity in a dose-dependent manner (Fig. 2A). At concentrations of 20 and 40 mg/mL, MRB significantly suppressed TYR activity when compared with the control (100%) at $P < 0.001$ to $71 \pm 3.76\%$ and $51 \pm 5.33\%$, respectively. MRB also affected the protein expressions of TYR, MITF, and TRP-1 without altering TRP-2 (Fig. 2B). There are consistent with the mRNA level, only TYR and MITF were downregulated in MRB treated B16 cells (Fig. 2C). Then, the onset of MRB that affected melanin inhibition was determined in B16 cells treated with MRB at 40 mg/mL for 1, 3, 6, 12, 24, and 48 h (Fig. 2D). MRB exhibited an effect on TYR and MITF suppressions from 6 to 48 h and achieved the minimum at 24 h.

Modified Riceberry rice extract inhibits melanogenesis-related tyrosinase activity in embryonic zebrafish

The melanin inhibitory effect of MRB extract was also investigated in the embryonic zebrafish model. Zebrafish embryos at 6-hpf were exposed to various concentrations of MRB until 48-hpf. Treatment with the MRB extract at 10, 20, and 30 mg/mL showed no toxicity on zebrafish embryos by not altering the viability (Fig. 3A) and heart rate (Fig. 3B) when compared with the control group. The histopathological effects of MRB on the zebrafish embryos were observed at non-toxic concentrations and no histopathological changes were found to occur on the embryonic yolk sac, spinal cord, skeletal muscle, and pericardia when compared with the control group (Fig. 3C). Thus, non-toxic concentrations of MRB at 10 and 30 mg/mL were selected for further investigation.

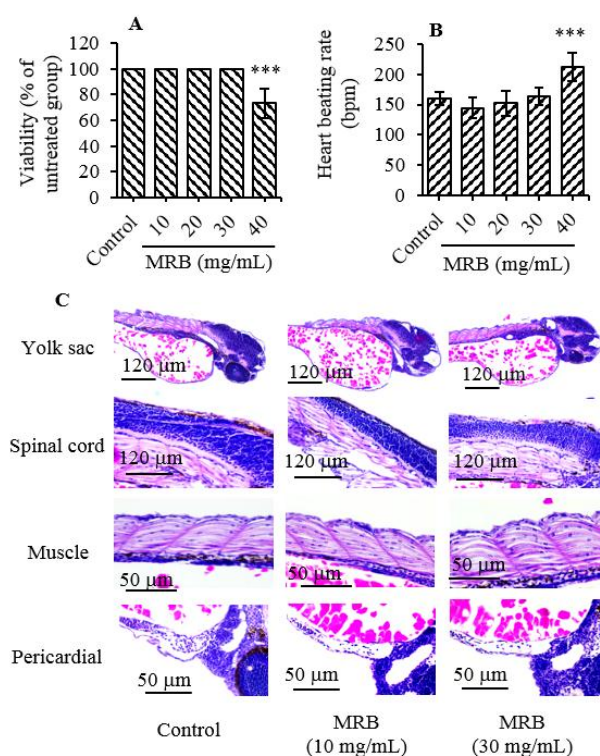


Fig. 3. Toxicity of MRB on zebrafish embryos. Six-h post fertilization of embryonic zebrafish were treated with MRB extract at 10, 20, 30, and 40 mg/mL until 48-h post fertilization. (A) The viability and (B) heart beating rate were observed under a stereomicroscope. The results are expressed in a bar graph as mean \pm SD. (C) Non-toxic concentrations of MRB at 10 and 30 mg/mL were further tested for histopathological toxicity. The histopathological appearance of the yolk sac (100 \times), spinal cord (400 \times), skeletal muscle (400 \times), and pericardial (400 \times) were observed by hematoxylin and eosin staining. *** $P < 0.001$ Indicates significant differences compared to the control group. MRB, Modified Riceberry rice.

MRB treatment at non-toxic concentrations of 10 and 30 mg/mL showed a gradual reduction of phenotypic-based pigmentation throughout zebrafish embryos (Fig. 4A). Additionally, the reducibility of pigmentation of MRB was characterized by melanophore shrinkage. The melanin content was then optically determined and showed reductions to $84 \pm 6.69\%$ and $71 \pm 1.46\%$, significant at $P = 0.01$ and $P < 0.001$, respectively when compared with the control (100%) (Fig. 4B). At similar concentrations, TYR activity was significantly suppressed at $P < 0.001$ when compared with the control to $79 \pm 10.40\%$ and $63 \pm 9.71\%$ (Fig. 4C). These results indicate that MRB suppressed melanogenesis in *in vivo* zebrafish embryos through TYR-associated melanophore differentiation.

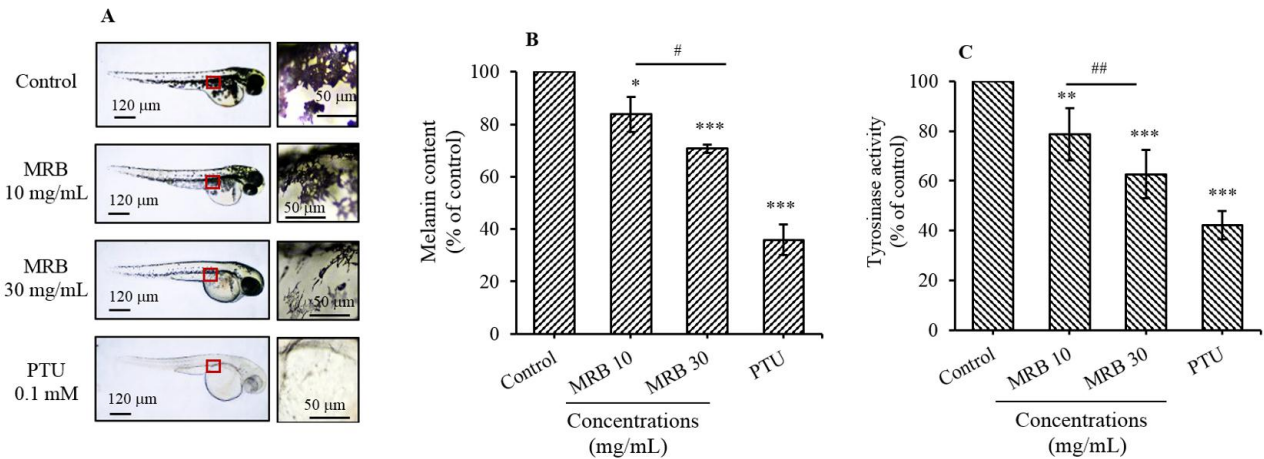


Fig. 4. Effect of MRB on melanogenesis in zebrafish embryos. Six-h post ferritization of embryonic zebrafish was treated with MRB at 10 and 30 mg/mL until 48-h post ferritization. (A) The phenotype-based melanin pigmentation was observed under a stereomicroscope (40×). Dorsal melanophores were visualized (inserted panel, 400×). (B) Melanin content and (C) tyrosinase activity were determined in the lysate samples. PTU at 0.1 mM was used as the positive control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared with the control. MRB, Modified Riceberry rice; PTU, phenylthiourea.

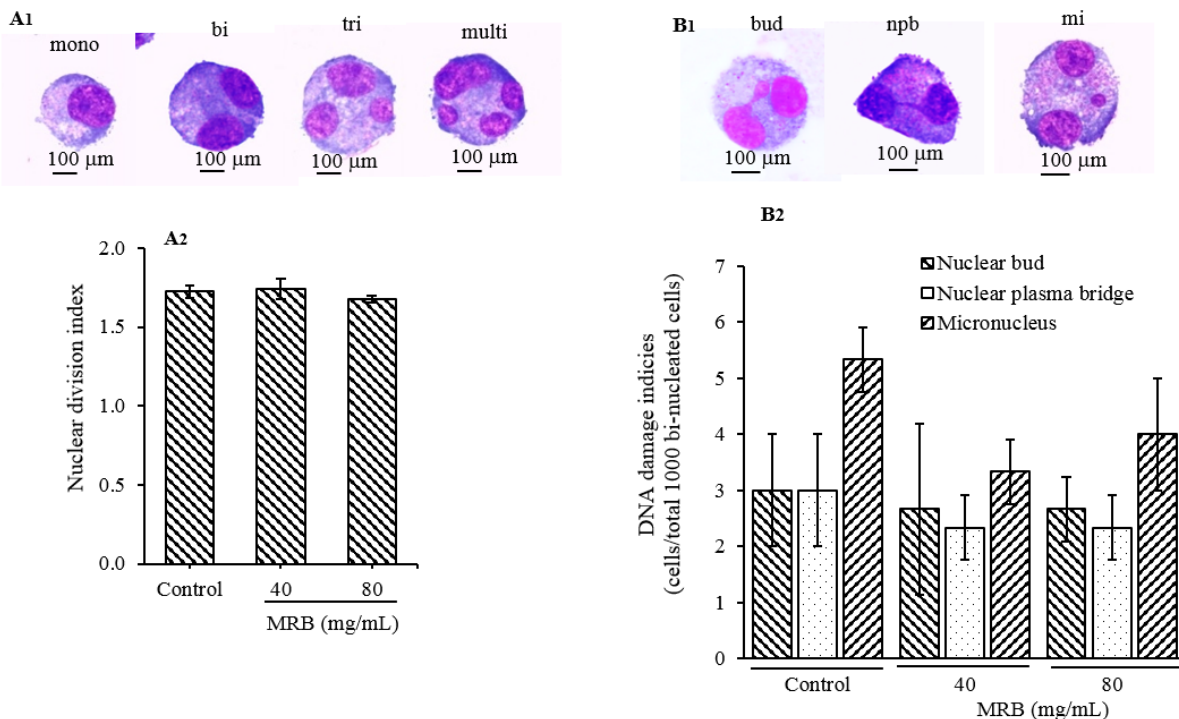


Fig. 5. Genotoxic effect of MRB on V79 cells. The cells were treated with MRB at 40 and 80 mg/mL before blocking the cytokinesis by mitomycin C. (A₁) The cells were differentiated into mono-nucleated cell (mono), bi-nucleated cell (bi), tri-nucleated cell (tri), and multi-nucleated cell (multi) under a light microscope at magnification 400×. (A₂) The cytostatic effect was calculated as a nuclear division index. (B₁) Nuclear bud (bud), nuclear plasma bridge (npb), and micronucleus (mi) were observed on bi-nucleated cells. (B₂) The DNA damage cells were expressed in the bar graph. Results are expressed in a bar graph as mean ± SD compared with the control. MRB, Modified Riceberry rice.

Non-genotoxic and mutagenic effects of modified Riceberry rice extract

As mentioned, this study aimed to develop a novel, effective, and safe agent for hyperpigmentation control. Thus, the genotoxicity of MRB was investigated by cytokinesis-block micronucleus cytochrome assay.

In this experiment, V79 cells were treated with the MRB extract at 40 and 80 mg/mL, and then cytokinesis was blocked by mitomycin C. To investigate the cytostatic effect, the number of mono-nucleated cells, bi-nucleated cells, tri-nucleated cells, and multi-nucleated cells (Fig. 5A) were observed under a light

microscope. Treatment of V79 cells with MRB at 40 and 80 mg/mL showed no cytostatic effect by providing a nuclear division index of 1.75 ± 0.05 and 1.73 ± 0.05 , respectively, which were not significantly different compared with the control (1.72 ± 0.04). Moreover, DNA damage indices including nuclear bud, nuclear plasma bridge, and micronucleus formation (Fig. 5B) were also observed in a total of 1,000 binucleated cells. The treatment of V79 cells with MRB at 40 and 80 mg/mL showed no significant increase in the number of DNA-damaged V79 cells when compared with the control.

The mutagenicity of MRB was then

investigated by Ames's test. In the study, TA98 and TA100 strains of *Salmonella typhimurium* were used for testing with the MRB extract. As shown in Fig. 6A, the revertant colonies growing on the agar plates of MRB treatment groups were counted and compared with the spontaneous control. The results show that TA98 and TA100 had 30.83 ± 5.57 and 147.17 ± 30.70 spontaneous colonies, respectively. Treatment of MRB extract at 200 and 400 mg/mL did not increase the number of revertant colonies for both the TA98 (Fig. 6B) and TA100 strains (Fig. 6C) when compared with the spontaneous control.

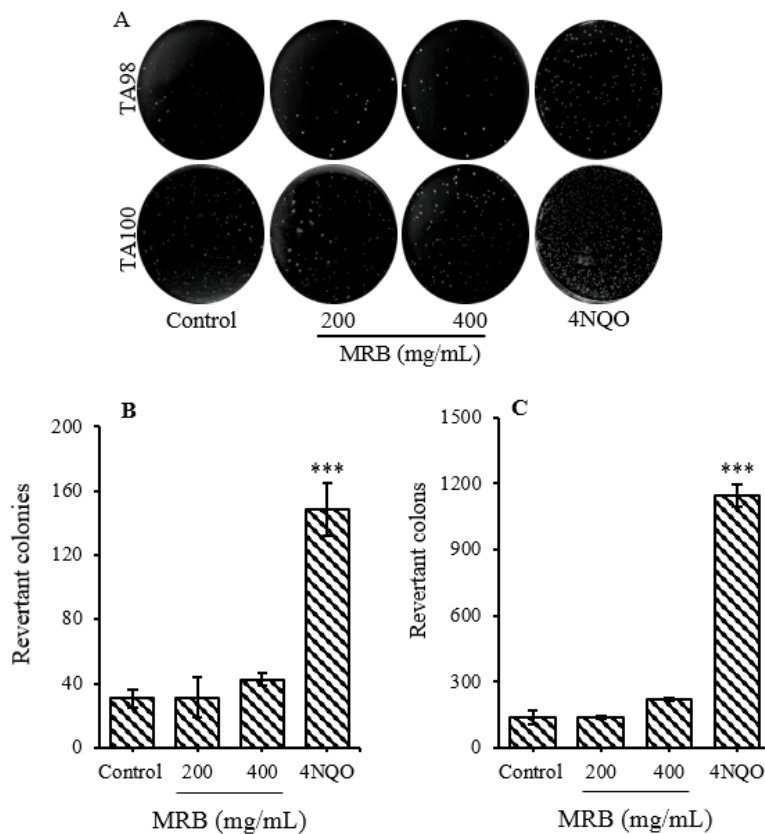


Fig. 6. Mutagenic effect of MRB. (A) The numbers of revertant colonies of the *Salmonella typhimurium* strains TA98 and TA100 were observed from agar plates after treatment with MRB at 200 and 400 mg/mL. In this study, 4NQO at 0.4 and 0.2 mg were used as a positive control for *Salmonella typhimurium* strains TA98 and TA100, respectively. The results are plotted in the bar graphs by mean \pm SD for both of (B) TA98 and (C) TA100 strains. *** $P < 0.001$ Indicates significant differences compared with the control. MRB, Modified Riceberry rice; 4NQO, 4-nitroquinoline N-oxide.

DISCUSSION

Hyperpigmentation can be caused by the overactivity of tyrosinase enzymes, which is a key targeting enzyme for melanin inhibition (22). Many studies have investigated the effectiveness of tyrosinase inhibitors with the aim of controlling hyperpigmentation disorder (6-8).

Rice seeds contain antioxidants and phenolic compounds-like tyrosinase inhibitors which can be promoted under the proper conditions of germination and fermentation processes (23,24). In a previous study, the researchers found that short-term germination of Riceberry rice promoted its melanin inhibitory effect through an antioxidant property (14). In this study, the fermentation process was used additionally with the aim to enhance the anti-melanogenesis-related tyrosinase inhibition of germinated Riceberry rice. Under the restricted conditions, MRB extract potentially suppressed melanogenesis and also affected melanin excretion-associated morphological dendritic, the hallmarks of B16 cell differentiation (14).

As mentioned, TYR and its-related proteins TRP-1 and TRP-2 are known to regulate melanogenesis and act as the biological markers of melanocyte differentiation through transcription protein MITF (3,22). In the present study, TYR, TRP-1, and its upstream MITF were suppressed at the protein level but did not affect TRP-2. The result can be supported by previously, expression, and function of TRP-2 protein can be compensated by another regulatory protein SOX-10, a melanocyte development regulator (25,26). In this study, MRB also downregulated the mRNA levels of TYR and MITF but did not affect TRP-1. The result suggests that MRB might be affected by TRP-1 post-translational inhibition (27).

Embryonic zebrafish has been recently recommended as an animal model suitable for studying of pigmentation control based on its correlation with human pigmentation (28).

The present study found that MRB effectively reduced melanogenesis through TYR inhibition in embryonic zebrafish *in vivo* without negative physiological or

histopathological effects. Melanocytes are differentiated from the neural crest during embryonic development, under the control of MITF (29). In this study, MRB affected cellular differentiation-associated melanophore shrinkage in zebrafish embryos.

As previously mentioned, this study aimed to develop a novel, effective, and safe anti-melanogenic product. The genotoxicity and mutagenicity of the MRB extract were further investigated to review safety concerns regarding natural products from The World Health Organization (30). The present study demonstrated that effective doses of MRB on TYR-related melanogenic inhibition provided favorable toxicity profiles. Therefore, MRB extract is suitable to be developed as a novel, effective, and safe anti-melanogenic product for hyperpigmentation control.

CONCLUSION

The MRB extract inhibited melanogenesis, melanin excretion, and TYR activity through the modulation of TYR and MITF. MRB also provided these efficiencies in *in vivo* zebrafish embryos without toxicity. Thus, MRB extract is suitable for application as an anti-melanogenic product for hyperpigmentation control.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this study.

AUTHORS' CONTRIBUTION

All authors conceived and planned the experiments. T. Rodboon performed the experimental studies, data acquisition, statistical analysis, and manuscript preparation. S. Sirilun participated in conducting rice fermentation. S. Okada and K. Ryusho participated in preparing facilities and conducting some molecular experiments. The animal studies were supervised by T. Chontanarith. P. Suwannalert supervised the project, participated in preparing facilities, and finalized the manuscript.

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