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Protection of *Monascus*-Fermented Dioscorea against DMBA-Induced Oral Injury in Hamster by Anti-inflammatory and Antioxidative Potentials

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Monascus-fermented products offer valuable therapeutic benefits and have been extensively used in East Asia. This study investigated the prevention of oral tumor formation and antioxidative ability of the ethanol extracts from red mold dioscorea (RMDE) on 7,12-dimethyl-1,2-benz[*a*]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis. The HBP was painted with DMBA three times per week for 14 weeks, and animals were painted with celecoxib, RMDE (50, 100, and 200 mg/kg of bw), and ethanol extracts from dioscorea (200 mg/kg of bw) on days alternate to the DMBA application. The results demonstrated that RMDE attenuated tumor formation by elevating the antioxidase activity and suppressing the overproduction of reactive oxygen species, nitric oxide, prostaglandin E_2, and pro-inflammatory cytokines in the HBP caused by DMBA induction. These results indicated that RMDE exerted anti-inflammatory and antioxidative activity to prevent oral cancer. Therefore, the metabolite from *Monascus* fermentation may serve as a possible functional edible substance for the prevention of oral cancer.

KEYWORDS: Ethanol extract of red mold dioscorea (RMDE); oral cancer; hamster buccal pouch (HBP); antioxidase activity; inflammatory cytokines

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the fifth most common neoplasm worldwide, accounting for over 500,000 cases annually (*1*). In Taiwan, oral cancer has the fastest rising incidence and mortality rate of any cancer and is the sixth leading cause of cancer deaths. Tobacco and alcohol consumption have been reported to be the major factors for the development of oral cancer (*2*). Diets low in carotenoids and vitamin A, poor oral hygiene, and indoor air pollution are also recognized to be factors for oral cancer (*3*,*4*). However, betel quid chewing is one of the most important factors of oral cancer in Taiwan, with high mortality and poor prognosis. Therefore, in an effort to improve patient survival and quality of life, new therapeutic approaches focusing on the molecular target and mechanism that mediate tumor cell growth have gained much attention.

Chemoprevention has evolved as a novel approach to control the incidence of oral cancer. Therefore, it is important to establish chemoprevention in an experimental animal tumor model that mimics specific characteristics of human OSCC. The hamster buccal pouch (HBP) carcinogenesis model is the most well characterized animal system for the investigation of OSCC development and intervention by chemopreventive agents. Squamous cell carcinomas (SCCs) induced by application of 7,12-dimethylbenz-[*a*]anthracene (DMBA) to the HBP share many morphological and histological similarities to human OSCCs (*5*). The major risk factors associated with human oral cancer such as betel quid, tobacco, and alcohol also promote HBP carcinogenesis (*6*,*7*). We have used this model to test the antitumor effect of ethanol extract of red mold rice (RMRE) (*8*).

Chemoprevention by dietary agents has evolved as an effective strategy to control the incidence of oral cancer. *Monascus* species have been used as traditional food fungi in East Asia for several centuries. *Monascus*-fermented rice, known as red mold rice (RMR), has been proved to show antioxidative ability and anticancer progression in our previous studies (*9*, *10*). RMR has many functional secondary metabolites; for example, yellow pigments such as monascin and ankaflavin have been reported to have cancer cell cytotoxic activities (*11*).

Monascus-fermented dioscorea, known as red mold dioscorea (RMD), comprises a dioscorea root substance as well as several *Monascus* metabolites. Dioscorea is regarded as a beneficial herb because of the inclusion of many beneficial ingredients for the prevention of various diseases (*12*). Dioscorin, polysaccharides, flavones, vitamin C, and polyphenol of dioscorea are proven to exhibit great antioxidative ability (*12*). In addition, dioscorea is proven to have antitumor ability (*13*, *14*). Thus, the *Monascus* fermentation of dioscorea may lead to stronger anticancer effects. The present study was designed to evaluate the inhibitory effects of the ethanol extract of red mold dioscorea (RMDE) on DMBA-induced HBP carcinogenesis.

MATERIALS AND METHODS

Chemicals and Reagents. DMBA, nitro blue tetrazolium (NBT), mineral oil, glutathione (GSH), glutathione reductase (GR), glutathione

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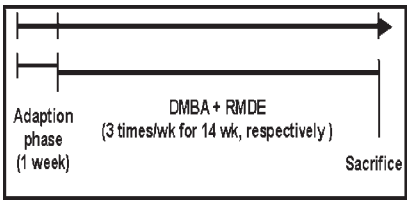

Figure 1. Animal experimental design for the evaluation of chemopreventive effect of RMDE against oral carcinogenesis. To induce oral tumor formation (DMBA-induced phase), 0.5% DMBA solution was prepared in mineral oil and applied to the entire mucosal surface of the left buccal pouches three times per week for 14 weeks. In addition, the animals were painted with 50, 100, 200 mg/kg RMDE or 200 mg/kg DE on days alternate to the DMBA application.

disulfide (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), 5,5-dithiobis[2-nitrobenzoic acid] (DTNB), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), 1-chloro-2,4-dinitrobenzene (CDNB), NaN_3, and $MgCl_2 \cdot 6H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO). Celecoxib was purchased from Pfizer (New York, NY). Prostaglandin E_2 immunoassay kit and nitric oxide (NO) assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI). SOD assay kit were purchased from Randox Laboratories Ltd. (Antrim, U.K.).

Preparation of RMDE. The *Monascus purpureus* NTU 568 culture strain was maintained on potato dextrose agar (PDA, Difco Co., Detroit, MI) slanted at 10 °C and transferred monthly. The dioscorea (*Dioscorea batatas* Dence) root was used to produce RMD using the method of solid-state culture (*14*). Briefly, a 500 g substrate was soaked in deionized water for 8 h, and excess water was removed with a sieve. The substrate was autoclaved (HL-341 model, Gemmy Corp, Taipei, Taiwan) for 20 min at 121 °C in a "koji dish", which was made of wood with the dimensions of 30 × 20 × 5 cm. After cooling, the substrate was inoculated with a 5% (v/w) spore suspension and cultivated at 30 °C for 10 days. During the culturing stage, 100 mL of water was added daily to the substrate from the second day to the fifth day. At the end of cultivation, the crushed and dried product with the mold was extracted by 95% ethanol at 50 °C for 3 days. The extracts were further freeze-dried to powder and stored at −20 °C until used. Samples were dissolved in mineral oil.

Determination of Monascin and Ankaflavin Concentration. RMD (1 g) was extracted with 10 mL of ethanol at 60 °C for 30 min. The extracts (10%, w/v) were further filtered with a 0.45 μm pore size filter and analyzed by high-performance liquid chromatography (HPLC, model L-6200; Hitachi Co., Tokyo, Japan), as described previously (*15*). A chromatographic eluent pump (PU2089 plus, Jasco Co., Tokyo, Japan), injector (7725i, Rheodyne Co., Robert Park, CA), and column (C_{18}, 25 cm × 4.6 mm inner diameter, 5 μm, Discovery, Supelco, Inc., Bellefonte, PA) were used in this study. Monascin and ankaflavin were detected using a UV detector UV2075 plus (Jasco Co.) set at 231 nm.

Animals and Experimental Design. The experiment was carried out with male Syrian hamsters aged 5 weeks obtained from National Laboratory Animal Center (Taipei, Taiwan). Hamsters were subjected to a 12 h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C (protocol complied with guidelines described in the "Animal Protection Law", amended January 17, 2001; Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, ROC). The model of DMBA-induced HBP carcinogenesis was modified from Salley (*16*). As shown in **Figure 1**, the animals were treated by painting the entire mucosal surface of the left buccal pouches three times a week for continuous 14 weeks with a 0.5% solution of DMBA dissolved in mineral oil. Also, the animals were painted with RMDE (50, 100, 200 mg/kg of bw) or ethanol extracts from dioscorea (DE) (200 mg/kg of bw) on days alternate to the DMBA application. Each animal of the positive control group was treated with 100 μL of 6% celecoxib, which is a cyclooxygenase-2 inhibitor and has a chemopreventive action against DMBA-induced buccal pouch carcinogenesis (*17*). RMDE, DE, and celecoxib were dissolved in mineral oil. In addition, animals in another three groups were painted with 100 or 200 mg of RMDE and 200 mg of DE/kg of bw alone, respectively. Animals were sacrificed after 14 weeks, and the tumor volume and burden were determined as follows (*18*). Tumor volume was measured using the formula $V = 4/3(D_1/2)(D_2/2)(D_3/2)$, where D_1, D_2, and D_3 are the three diameters (mm) of the tumor. Tumor burden = total numbers of tumors × mean volume.

Table 1. Monascin and Ankaflavin Concentration in RMDE[a]

	monascin (g/kg)	ankaflavin (g/kg)
RMDE	137.2 ± 7.68	34.14 ± 0.84

[a] Results are expressed as the mean ± SD (n = 3).

Table 2. Body Weight and Daily Intake of Control and Experiment Animals[a]

	body weight (g)		
group	initial	final	daily intake (g/day)
control	77.31 ± 10.15	132.46 ± 12.51 a	7.48 ± 0.57 a
DMBA	76.03 ± 5.18	107.11 ± 11.37 b	6.46 ± 0.16 b
DMBA + celecoxib	77.55 ± 8.23	116.60 ± 14.71 ab	7.02 ± 0.99 ab
DMBA + 50 RMDE	76.69 ± 8.24	108.81 ± 10.22 b	6.88 ± 0.61 ab
DMBA + 100 RMDE	76.80 ± 4.44	125.51 ± 05.20 ab	7.88 ± 0.57 a
DMBA + 200 RMDE	76.46 ± 6.07	128.31 ± 10.85 ab	7.57 ± 0.26 a
DMBA + 200 DE	75.49 ± 7.71	119.74 ± 15.91 ab	7.10 ± 0.27 a
100 mg/kg RMDE	75.35 ± 7.53	122.23 ± 12.27 ab	7.77 ± 0.31 a
200 mg/kg RMDE	76.78 ± 11.49	133.45 ± 08.50 a	7.60 ± 0.68 a
200 mg/kg DE	75.73 ± 5.14	131.03 ± 08.72 a	7.99 ± 0.82 a

[a] DMBA, 7,12-dimethyl-1,2-benz[*a*]anthracene treatment; RMDE, ethanol extract of red mold dioscorea treatment; DE, ethanol extract of dioscorea treatment. Results are expressed as the mean ± SD (n = 8). Different letters indicate statistically significant differences between the means ($p < 0.05$) for each group.

Determination of Reactive Oxygen Species (ROS), Nitric Oxide (NO), and Prostaglandin E_2 (PGE_2) Levels in Tissue of Hamster Buccal Pouch Homogenates. In the measurement of ROS, 100 μL homogenates of tissue were added to 96-well plates and reacted with 25 μL of NBT (10 mg/mL) at 37 °C for 2 h and measured by absorbance at 570 nm. In the measurement of NO or PGE_2, 100 μL homogenates were measured by the NO kit or a PGE_2 immunoassay kit.

Assay for Cytokines. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and interferon-γ (IFN-γ) levels in the hamster buccal pouches were determined with ELISA kits from Peprotech (Rocky Hill, NJ).

Assay for Glutathione (GSH). Tissue homogenate was mixed with TCA (50 mg/mL) mixture, incubated for 5 min, and centrifuged at 8000*g* for 10 min under 4 °C. The homogenate was reacted with DTNB for 5 min under 4 °C. The absorbance was measured at 412 nm, and the concentration of GSH was calculated using the absorbance expressed by micromoles per milligram of protein.

Assay for Antioxidant Enzymes. Glutathione peroxidase (GPx) activity was determined as previously described (*19*). Briefly, 0.1 mL of homogenate was mixed with 0.8 mL of 100 mM potassium phosphate buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GR, and 1 mM GSH, pH 7.0) and incubated for 5 min at room temperature. Thereafter, the reaction was initiated after the addition of 0.1 mL of 2.5 mM hydrogen peroxide (H_2O_2). GPx activity was calculated by the change of absorbance at 340 nm for 5 min. To another reaction containing 0.1 M phosphate buffer (1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH, pH 7.0) was added 0.1 mL of homogenate for GR activity determination, and the decreased absorbance at 340 nm was measured for 3 min (*20*). The catalase (CAT) activity was determined according to the method of Aebi (*21*). Fifty microliters of homogenate was mixed with 950 μL of 0.02 M H_2O_2 and incubated at room temperature for 2 min. CAT activity was calculated by the change of absorbance at 240 nm for 3 min. The glutathione-*S*-transferase (GST) activity was determined as previously described (*22*). One hundred microliters of homogenate was mixed with 20 μL of 50 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 880 μL of 100 mM phosphate buffer (pH 6.5) containing 1 mM GSH. The GST activity was calculated by the change of absorbance at 340 nm for 5 min. The assay of superoxide dismutase (SOD) activity was accomplished with a commercial kit (Randox Laboratories Ltd.).

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). The statistical significance was determined by one-way

Table 3. Incidence of Oral Neoplasm in Control and Experiment Animals[a]

group	no. of tumors	mean tumor volume (mm^3)	tumor burden (mm^3)
control	0		
DMBA	32	130.11 ± 40.94 a	4163.64 ± 169.33 a
DMBA + celecoxib	15	61.48 ± 46.21 a	922.25 ± 128.39 c
DMBA + 50 RMDE	17	81.78 ± 37.54 a	1390.18 ± 156.51 b
DMBA + 100 RMDE	14	49.15 ± 21.19 b	688.10 ± 82.24 d
DMBA + 200 RMDE	10	33.65 ± 36.19 b	336.51 ± 64.23 e
DMBA + 200 DE	16	98.60 ± 85.99 a	1577.63 ± 101.12 b

[a] DMBA, 7,12-dimethyl-1,2-benz[*a*]anthracene treatment; RMDE, ethanol extract of red mold dioscorea treatment; DE, ethanol extract of dioscorea treatment. Tumor volume was measured using the formula $V = 4/3(D_1/2)(D_2/2)(D_3/2)$, where D_1, D_2, and D_3 are the three diameters (mm) of the tumor. Tumor burden = total numbers of tumors × mean volume. Results are expressed as the mean ± SD (n = 8). Different letters indicate statistically significant differences between the means ($p < 0.05$) for each group.

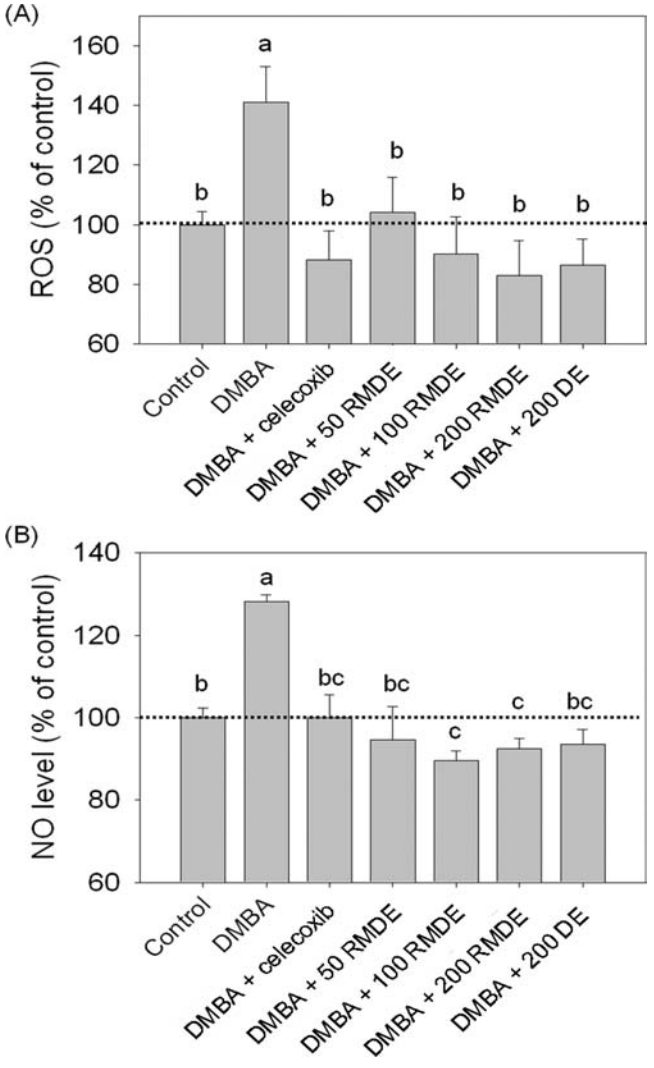

Figure 2. Inhibitory effects on ROS and NO expression of hamster buccal pouches by different treatments. RMDE decreased (**A**) ROS and (**B**) NO levels in experimental hamsters. Results are expressed as the mean ± SD (n = 8); different letters indicate statistically significant differences between the means ($p < 0.05$) for each group. DMBA, 7,12-dimethyl-1,2-benz-[*a*]anthracene; RMDE, ethanol extract of red mold dioscorea; DE, ethanol extract of dioscorea.

analysis of variance (ANOVA) using the general linear model procedure of SPSS software (SPSS Institute, Inc., Chicago, IL), followed by ANOVA with Duncan's test. The results were considered to be statistically significant if the p value was < 0.05.

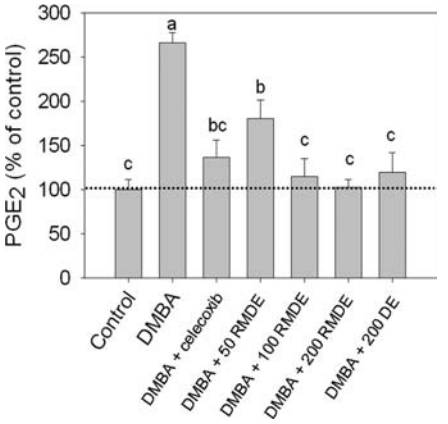

Figure 3. Inhibitory effect on the PGE_2 expression of hamster buccal pouches by different treatments. RMDE decreased PGE_2 levels in experimental hamsters. Results are expressed as the mean ± SD (n = 8); different letters indicate statistically significant differences between the means ($p < 0.05$) for each group. DMBA, 7,12-dimethyl-1,2-benz[*a*]-anthracene; RMDE, ethanol extract of red mold dioscorea; DE, ethanol extract of dioscorea.

RESULTS

Monascin and Ankaflavin Concentration of RMDE. Several yellow pigments such as monascin and ankaflavin from *Monascus* have been reported to have anti-inflammatory potential and cancer cell cytotoxic activities (*11*, *23*). **Table 1** shows that RMDE contained monascin (137.2 g/kg) and ankaflavin (34.14 g/kg), respectively.

RMDE Reduced Carcinogenesis in DMBA-Induced HBP Model. In our previous study, animals were treated by painting DMBA for 6 weeks before treatment with RMRE for 8 weeks (*8*). Different from the experimental schedule used in RMRE, this study focused on the chemopreventive efficacy of RMDE rather than the therapeutic effect. Therefore, HBPs were painted with DMBA for 14 consecutive weeks and painted with RMDE on days alternate to the DMBA application. **Table 2** shows the body weight and daily intake of hamsters in the control and experimental groups. At the end of 14 weeks, the mean final body weights and the amounts of diet consumed were significantly decreased in the DMBA-treated group compared to the control group ($p < 0.05$). However, RMDE-treated groups (100 and 200 mg/kg of bw) maintained the body weights and consumptive amounts compared to the DMBA group. In the experimental groups, tumor formation was induced in the hamster buccal pouch by topical application of DMBA for a continuous 14 weeks, whereas in the control group, no tumor formation was induced by mineral oil treatment (**Table 3**). Treatment with celecoxib, RMDE, and DE grossly reduced the number of tumors and the mean tumor volume, particularly in the 100 and 200 mg/kg of bw RMDE groups, and significantly decreased tumor burden ($p < 0.05$) in a dose-dependent manner. The DE treatment group also displayed inhibition of tumor formation. However, RMDE exerted stronger antitumor activity than DE.

RMDE Decreased the ROS, NO, and PGE_2 Levels in DMBA-Induced HBP Model. As shown in **Figure 2**, when compared to the control group, the DMBA group showed a significant increase in the ROS and NO levels in the hamster buccal pouches. Celecoxib, RMDE, and DE treatments significantly reduced the DMBA-induced increase in ROS and NO levels. Painting

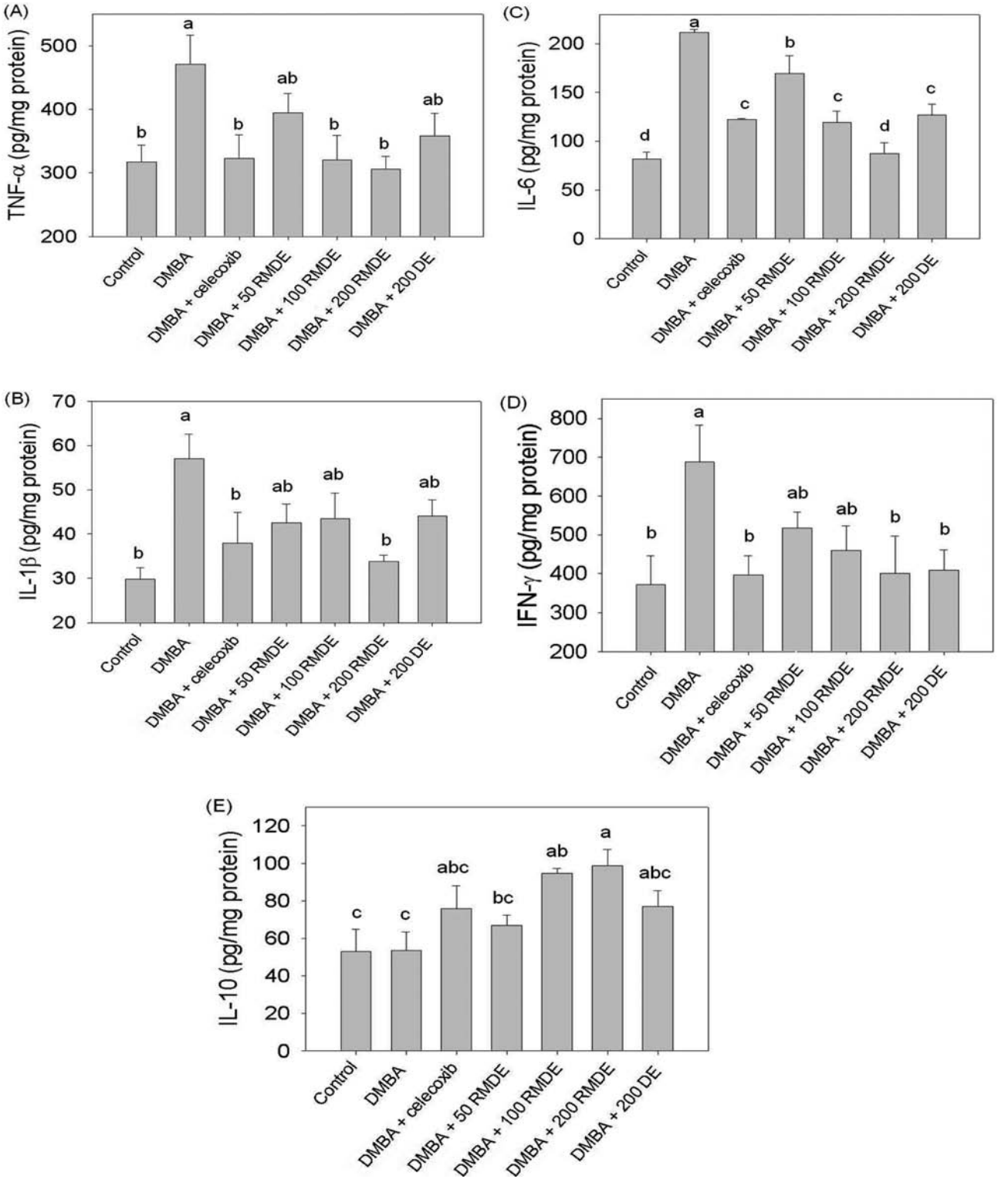

Figure 4. Effects of RMDE on inflammatory cytokines expression in the serum from experimental animals. RMDE decreased (**A**) TNF-α, (**B**) IL-1β, (**C**) IL-6, and (**D**) IFN-γ and increased (**E**) IL-10 levels in experimental animals. Results are expressed as the mean ± SD ($n = 8$); different letters indicate statistically significant differences between the means ($p < 0.05$) for each group. DMBA, 7,12-dimethyl-1,2-benz[*a*]anthracene; RMDE, ethanol extract of red mold dioscorea; DE, ethanol extract of dioscorea.

with RMDE lowered the levels in ROS and NO production, thereby reducing the DMBA-induced oxidative damage in hamster buccal pouches.

PGE_2 facilitates the invasiveness, progression, and growth of cancer (*24*, *25*). The PGE_2 level in the buccal pouches showed a significant increase in the DMBA group when compared with that in the control group (**Figure 3**). RMDE treatment decreased the PGE_2 levels in the buccal pouches. The inhibitory effect of RMDE (100 and 200 mg/kg of bw) on PGE_2 levels was greater than that of celecoxib, indicating that RMDE attenuated the associated factor during the development of oral tumor.

Effects of RMDE on Inflammatory Cytokines Expression. TNF-α, IL-1β, IL-6, and IFN-γ are pro-inflammatory cytokines; their secretion is known to be up-regulated by carcinogens. These play

Table 4. Antioxidase and GSH Activities of Buccal Pouches in Control and Experimental Animals[a]

group	CAT (nmol of H_2O_2/min/mg of protein)	GST (nmol of NADPH/min/mg of protein)	GPx (nmol of NADPH/min/mg of protein)	GR (nmol of NADPH/min/mg of protein)	SOD (U/mg of protein)	GSH (μg/mg of protein)
control	153.1 ± 27.0 b	101.6 ± 24.3 b	15.7 ± 2.9 b	33.8 ± 5.7 b	8.5 ± 3.0 b	28.5 ± 9.7 b
DMBA	95.1 ± 36.8 c	58.3 ± 10.4 c	9.2 ± 0.8 c	13.0 ± 4.1 d	3.1 ± 1.9 c	9.2 ± 5.6 c
celecoxib	215.6 ± 51.2 ab	111.9 ± 26.5 b	14.5 ± 3.7 b	22.5 ± 6.5 bc	5.7 ± 2.4 bc	34.6 ± 11.4 b
DMBA + 50 RMDE	140.7 ± 45.4 b	114.0 ± 29.9 b	11.5 ± 2.5 bc	20.8 ± 3.9 c	5.1 ± 1.9 bc	9.83 ± 3.4 c
DMBA + 100 RMDE	186.5 ± 48.1 ab	122.0 ± 69.4 b	12.7 ± 5.1 bc	23.1 ± 7.4 bc	5.5 ± 2.8 bc	13.3 ± 2.8 c
DMBA + 200 RMDE	277.4 ± 56.4 a	128.5 ± 29.4 b	15.2 ± 1.7 b	23.9 ± 7.5 bc	8.2 ± 2.2 b	30.9 ± 4.7 b
DMBA + 200 DE	185.9 ± 81.6 ab	86.8 ± 26.0 c	13.7 ± 2.3 bc	19.2 ± 5.0 c	5.6 ± 1.6 bc	25.6 ± 8.7 b
100 mg/kg RMDE	194.3 ± 75.7 ab	100.2 ± 29.3 b	16.6 ± 5.8 b	43.8 ± 9.6 a	9.2 ± 5.0 bc	40.6 ± 10.9 b
200 mg/kg RMDE	216.2 ± 77.2 ab	166.2 ± 28.1 a	20.3 ± 5.4 a	45.4 ± 9.8 a	14.5 ± 5.0 a	64.4 ± 21.9 a
200 mg/kg DE	198.7 ± 63.4 ab	107.0 ± 30.3 a	20.0 ± 6.2 a	37.5 ± 9.9 b	10.5 ± 6.7 bc	36.9 ± 14.3 b

[a] CAT, catalase; GST, glutathione *S*-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; GSH, glutathione; DMBA, 7,12-dimethyl-1,2-benz[*a*]anthracene treatment; RMDE, ethanol extract of red mold dioscorea treatment; DE, ethanol extract of dioscorea treatment. Results are expressed as the mean ± SD (n = 8). Different letters indicate statistically significant differences between the means ($p < 0.05$) for each group.

an important role in tumorigenesis (*26*). When compared to the control group, the DMBA group showed a significant increase in the levels of TNF-α, IL-1β, IL-6, and IFN-γ (**Figure 4A−D**). However, compared to DMBA treatment, celecoxib, RMDE, and DE treatments obviously lowered the TNF-α, IL-1β, IL-6, and IFN-γ levels, especially the RMDE treatment group (200 mg/kg of bw), which exerted better effects than celecoxib and DE treatments. In addition, RMDE elevated anti-inflammatory cytokine (IL-10) expression, indicating that RMDE had anti-inflammatory effects on DMBA-induced hamster buccal pouch carcinogenesis.

Effects of RMDE on GSH Level and Antioxidase Activities. **Table 4** shows the effect of RMDE on GSH contents. The DMBA-treated group reduced the GSH level significantly. Treatment with celecoxib and RMDE (200 mg/kg of bw) significantly protected the GSH depletion and recovered the GSH levels. In addition, the 200 mg/kg of bw RMDE treatment (without painting with DMBA) promoted the GSH level compared to the control group significantly (**Table 4**).

In the DMBA treatment group, the activities of CAT, GST, GPx, GR, and SOD in the hamster buccal pouches showed notable decreases (**Table 4**). However, the activities of CAT, GST, GPx, GR, and SOD in celecoxib, RMDE, and DE treatment groups were higher than those of the DMBA treatment group, especially in the 200 mg/kg of bw RMDE treatment group ($p < 0.05$). These findings indicate that RMDE played an antioxidative role in DMBA-induced oxidative injury. In addition, the 100 and 200 mg/kg of bw RMDE and the 200 mg/kg of bw DE treatment (without painting with DMBA) exerted higher antioxidase activities compared to the control group (**Table 4**). Although no significant difference in the CAT activity in each group was observed, administration of 200 mg/kg of bw of RMDE significantly promoted GST, GPx, GR, and SOD activities compared to the control group ($p < 0.05$).

DISCUSSION

The Syrian golden hamster buccal pouch carcinogenesis model closely mimics events in the development of precancerous lesions and epidermoid carcinomas of the oral cavity in humans using topically and chronically applied DMBA (*27*, *28*). DMBA is a member of the polycyclic aromatic hydrocarbons that are present in the environment as products of incomplete combustion of complex hydrocarbons. Being an indirect carcinogen, DMBA, after metabolic activation, has been suggested to induce cancer through an oxidatively mediated genotoxicity by incorporating diolepoxide and other ROS into DNA (*29*, *30*).

Oxidative damage to cellular macromolecules may occur through the overproduction of ROS and faulty antioxidant and/or DNA repair mechanisms that result in cancer (*31*). Several transcriptional factors such as nuclear factor kappa B (NFκB) and AP-1 are induced by ROS and are known to have a direct impact on inflammation, cellular proliferation, and apoptosis, thus making ROS instrumental in tumor promotion (*32*). Therefore, administration of antioxidants may retard this process. The administration of RMDE decreased the levels of ROS, thereby reducing the oxidative damage caused by DMBA. NO levels showed a significant increase in the OSCC cells when compared with the levels in normal epithelial cells (*33*). Chronic inflammation can lead to the production of chemical intermediates such as NO, which in turn can mediate DNA damage and block the DNA repair system (*34*). The potential role of PGE_2 in tumor progression has been well studied. Its role has been implicated in the regulation of tumor-associated angiogenesis, cell migration or invasion, and the inhibition of apoptosis. PGE_2 is also involved in chronic inflammation and is a facilitator of cancer progression, growth, and proliferation (*24*, *25*). In this study, we found that RMDE significantly inhibited the DMBA-induced increase in the ROS, NO, and PGE_2 levels in the homogenates of oral tissue. These findings imply that RMDE might inhibit DMBA-induced oral tumor carcinogenesis through its antiinflammatory and antioxidative effects.

The chemopreventive potential of RMDE may be attributed to its anti-inflammatory effects and antioxidative properties; therefore, we further investigated the inflammatory cytokine expression and antioxidase activities in hamster buccal pouches. Chronic inflammation leads to the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IFN-γ, which in turn leads to oxidative stress (*26*). RMDE was found to inhibit the pro-inflammatory cytokines and promoted anti-inflammatory cytokine production, which means that RMDE had anti-inflammatory effects on DMBA-induced hamster buccal pouch carcinogenesis and, therefore, mitigated OSCC.

GSH participates in the elimination of reactive intermediates by conjugation and hydroperoxide reduction as well as by scavenging free radicals against toxicity (*35*). The oxidative stress of the tissue generally involves the GSH system. DMBA treatment decreased the GSH level and antioxidase activities (CAT, GST, GPx, GR, and SOD); however, RMDE treatment elevated the GSH level and the antioxidase activities, thereby reducing DMBA-induced oxidative damage and then promoting the antioxidative ability in hamster buccal pouches. In addition, topical application of RMDE without painting with DMBA also led to an increase in antioxidase activities (**Table 4**), indicating that

RMDE might have antioxidative potential and therefore prevent oxidative injury due to DMBA-induced oxidative stress.

Monascus sp. produce several antioxidative and anti-inflammatory pigments such as monascin and ankaflavin, which, in this study, were present in concentrations of 137.2 and 34.14 g/kg in *Monascus*-fermented dioscorea, respectively (**Table 1**). Dioscorea has proven antitumor abilities (*13*, *14*), and ethanol extracts from *Monascus*-fermented dioscorea have stronger anticancer activity than the dioscorea used in this study. We suggest that the greater anticancer activity of RMDE compared to DE might be attributable to RMDE containing more anti-OSCC substances such as the high content of monascin and ankaflavin.

Taken together, RMDE exerts a chemoprevention activity in the inhibition of inflammation and oxidation, which is possible for oral cancer prevention. Therefore, RMDE may serve as a possible functional food in the development of human oral cancer adjuvant chemotherapy.

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