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Anti-inflammatory Activities of Mogrosides from *Momordica grosvenori* in Murine Macrophages and a Murine Ear Edema Model

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ABSTRACT: *Momordica grosvenori* (*Luo Han Guo*), grown primarily in Guangxi province in China, has been traditionally used for thousands of years by the Chinese to make hot drinks for the treatment of sore throat and the removal of phlegm. The natural noncaloric sweetening triterpenoid glycosides (mogrosides) contained in the *M. grosvenori* fruits are also antioxidative, anticarcinogenic, and helpful in preventing diabetic complications. The aim of this study was to assess the anti-inflammatory properties of mogrosides in both murine macrophage RAW 264.7 cells and a murine ear edema model. The results indicate that mogrosides can inhibit inflammation induced by lipopolysaccharides (LPS) in RAW 264.7 cells by down-regulating the expression of key inflammatory genes *iNOS*, *COX-2*, and *IL-6* and up-regulating some inflammation protective genes such as *PARP1*, *BCL2l1*, *TRP53*, and *MAPK9*. Similarly, in the murine ear edema model, 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation was inhibited by mogrosides by down-regulating *COX-2* and *IL-6* and up-regulating *PARP1*, *BCL2l1*, *TRP53*, *MAPK9*, and *PPARδ* gene expression. This study shows that the anticancer and antidiabetic effects of *M. grosvenori* may result in part from its anti-inflammatory activity.

KEYWORDS: *Momordica grosvenori*, Luo Han Guo, mogrosides, anti-inflammation, TaqMan gene expression assay

INTRODUCTION

Momordica grosvenori (*Siraitia grosvenorii* Swingle), or *Luo Han Guo* in Chinese, is a member of the Curcubitaceae. It is primarily grown in Guangxi province in southern China.[1] The fruit of *M. grosvenori* has been traditionally used for thousands of years by the Chinese to make hot drinks for the treatment of sore throat and the removal of phlegm.[2] The sweetness of *M. grosvenori* fruit comes from a mixture of triterpenoid glycosides, mogrosides I−V, with mogroside V as the major component. It has been estimated that mogrosides are 100 times sweeter than sucrose. *M. grosvenori* extracts were recently granted GRAS (Generally Regarded As Safe) status to be used as food additives by the U.S. Food and Drug Administration (FDA) in 2010 (No. GRN 000301).

As a natural sweetener, mogrosides were shown to effectively regulate the immune imbalance in diabetic mice by inducing and up-regulating heme oxygenase-1 (HO-1) and, hence, up-regulating the $CD4^+$ T lymphocyte subsets.[3] Administration of mogrosides to diabetic mice was found to be helpful in preventing diabetic complications through their strong antioxidant activities.[4,5] Additionally, mogrosides have been investigated from the perspective of their anticarcinogenic characteristics. Ukiya et al.[6] reported that mogrosides exhibited potent inhibitory effects on the induction of Epstein−Barr virus early antigen, equivalent to that of β-carotene. Takasaki et al.[7] later demonstrated that mogrosides significantly inhibited the two-stage carcinogenesis of mouse skin tumors induced by peroxynitrite as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter.

Due to the traditional use of Luo Han Guo for sore throat treatment, the anti-inflammatory potential of mogrosides was evaluated in this study. Inflammation has been implicated in human illnesses including cardiovascular diseases, arthritis, and cancer.[8] One of the major factors involved in the inflammation response is nitric oxide synthase (iNOS), which is induced by lipopolysaccharide (LPS) and various inflammatory mediator cytokines such as interferons (IFs), interleukins (ILs), and tumor necrosis factor (TNF)-α.[9] Cyclooxygenase (COX) and lipoxygenase (LOX) are two other major inflammation factors, which metabolize arachidonic acid (AA) released from the lipid bilayer of cell membrane during inflammation into prostaglandins (PGs) and leukotriens (LTs).[10] Mitogen-activated protein kinases (MAPK)[11] are also implicated in inflammation. Recently, Pan et al.[12] have demonstrated by regular reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting that mogrosides can reduce LPS-induced inflammation in murine macrophage cells by blocking protein and mRNA expression of iNOS and COX-2, reducing the nuclear translocation of nuclear factor-κB and inhibiting LPS-induced activation of PI3K/Akt, extracellular signal-regulated kinase 1/2, and p38 MAPK.

However, there are other factors that have been identified as prosurvival and inflammation protective, such as poly ADP-ribose polymerase family, member 1 (PARP1),[13] BCL2-like 1 (BCL2l1),[14] transformation-related protein 53, p53 (TRP53),[15] mitogen-activated protein kinase 9 (MAPK9),[16] andperoxisome proliferator activator receptor δ (PPARδ).[17] It has not been

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studied if these inflammation protective factors play any role in the anti-inflammation of mogrosides. In this study, we used the TaqMan Gene Expression Assay (Applied Biosystems Inc.) with custom-designed TaqMan probes and sensitive quantitative (q)RT-PCR analysis to evaluate the effects of mogrosides on the inflammatory and anti-inflammatory factors in murine macrophage RAW 264.7 cells and in a murine ear edema model.

MATERIALS AND METHODS

Anti-inflammatory Effects of Mogrosides in Murine Macrophage RAW 264.7 Cells. Mogrosides were isolated from Luo Han Guo fruits as described.[12] The final percentage of mogroside V was determined as 25.9% by HPLC. Mogrosides are water-soluble and were dissolved in cell culture medium. Biochanin A was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) as a 100 mM stock solution. Murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 20 units/mL penicillin, and 20 μg/mL streptomycin. Cells were maintained at 37 °C with 5% CO_2. Prior to induction of inflammation, 6×10^5 cells were plated in 60 mm cell culture dishes and cultured in serum-free DMEM overnight. Inflammation was induced by incubating the cells with 2 μg/mL LPS (Sigma-Aldrich) for 2 h at 37 °C. Cells were then washed with phosphate-buffered saline (PBS; Fisher Scientific, Pittsburgh, PA) and treated with mogrosides or biochanin A in the culturing medium for 24 h or without these agents in DMEM/FBS medium (the LPS-only treated cells).

Cell Viability Assay by MTS. The viability of RAW 264.7 cells after LPS, mogroside, and biochanin A treatment was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) reagent using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). In brief, 20 μL of MTS reagent was added into 100 μL of RAW 264.7 cells in DMEM culturing medium. The cells were incubated at 37 °C for 30−60 min. The absorbance was measured at 490 nm with a plate reader. The level of A_{490} was directly proportional to the cell viability.

Treatment of RAW 264.7 Cells with Mogrosides and Its Inhibitory Effects on the Formation of Nitric Oxide (NO). RAW 264.7 cells were plated into 24-well plates at the density of 3×10^5 cells/well in serum-free DMEM for overnight growth. Cells were stimulated with 2 μg/mL LPS for 2 h. Cells were washed with PBS and treated with mogrosides or biochanin A for 24 h in the culturing medium. The extracellular media (50 μL) were collected to measure NO production as the stabilized oxidation product, nitrite, by the Griess Reagent System (Promega) using nitrite standard dilutions as specified by the manufacturer.

TPA Induction of Inflammation and the Inhibitory Effects of Mogrosides in Murine Ear Edema Model. Female CD-1 mice were treated with 10 μL of acetone or mogrosides in acetone 20 min prior to the application of acetone or 1.5 nM TPA (Sigma-Aldrich) in acetone.[18] The mice were treated for 4 days to induce inflammation. The mice were sacrificed 6 h after the last TPA treatment. Ear punches, 6 mm in diameter, were taken, weighed, and homogenized in PBS.

Isolation of RNA from RAW 264.7 Cells and Murine Ear Tissue. The RNeasy RNA isolation kit (Qiagen, Valencia, CA) was used to isolate total RNA from treated RAW 264.7 cells and murine ear tissues. The cells were washed with PBS and harvested in lysis buffer. The murine ear tissue was ground into powder in liquid nitrogen and then resuspended in lysis buffer. The cells or the ground murine ear tissues in lysis buffer were applied to the RNA-binding column. They were centrifuged at 14000 rpm for 1 min. The column was washed as suggested by the supplier. RNA was eluted with diethyl pyrocarbonate (DEPC)-treated water and quantitated by a Nanodrop (ThermoFisher, Waltham, MA) spectrophotometer.

Reverse Transcription Real-Time Polymerase Chain Reaction (RT-qPCR). RNA samples (2 μg/each) were used to produce cDNA by reverse transcriptase with the cDNA synthesis kit and random primers (Applied Biosystems, Carlsbad, CA) in a volume of 20 μL. The concentration of cDNAs produced was measured spectrophotometrically by a Nanodrop (ThermoFisher). One hundred nanograms of cDNA from each sample was applied to the 96-well custom-designed TaqMan Gene Assay Plate that contained TaqMan probes and gene-specific primers (assays with specific identification numbers, ID #) for the following 16 genes: *iNOS* (inducible nitric oxide synthase 2, ID # Mm01309897_m1)), *COX-1* (prostaglandin-endoperoxide synthase 1, ID # Mm0477214_m1), *COX-2* (prostaglandin-endoperoxide synthase 2, ID # Mm01307334_g1), *ALOX12* (arachidonate 12-lipoxygenase, ID # Mm00545033_m1), *TNF* (tumor necrosis factor, ID # Mm00443259_g1), *MAPK1* (mitogen-activated kinase 1, ID # Mm00466437_s1), *MAPK3* (mitogen-activated kinase 3, ID # Mm00662375_g1), *MAPK8* (mitogen-activated kinase 8, ID # Mm00489514_m1), *IL1r1* (interleukin 1 receptor, type 1, ID # Mm00434237_m1), and *IL-6* (interleukin 6, ID # Mm99999064_m1) were chosen for their known pro-inflammation activities. *PARP1* (poly ADP-ribose polymerase family, member 1, ID # Mm00500154_m1), *BCL2l1* (BCL2-like 1, ID # Mm00437783_m1), *TRP53* (transformation related protein 53, ID # Mm00441964_g1), *MAPK9* (mitogen-activated protein kinase 9, ID # Mm0444231_m1)m and *PPARδ* (peroxisome proliferator activator receptor δ, ID # Mm1305434_m1) were chosen for being prosurvival and inflammation protective. The 18S rRNA gene was selected as the endogenous control (ID # Hs99999901_s1). The validated qPCR primers and TaqMan probes (assays) were synthesized by Applied Biosystems and applied dry into the 96-well plate with each gene in six replicates. The qPCR reactions were carried out with the StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA). The gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ relative quantification method [19] using the 18S rRNA gene as the endogenous control.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA kits were used to measure levels of inflammation mediators IL-6 (Biosource, Camarillo, CA), PGs (Cayman, Ann Arbor, MI), and LTs (Cayman). One hundred microliters of medium from the LPS inflammation-induced and mogrosides-treated RAW 264.7 cells cultured in 24-well plate were added into the antibody-coated ELISA plates. Manufacturer's instructions were followed to analyze the levels of interleukins and prostaglandins.

RESULTS

Mogrosides Inhibited Several Key Inflammation Genes in Murine Macrophage RAW 264.7 Cells. To evaluate the anti-inflammatory characteristics of mogrosides, RAW 264.7 cells were stimulated with LPS and then treated with mogrosides. Because biochanin A has been shown by Jun et al.[20] to strongly inhibit the release of arachidonic acid and the formation of NO as a result of LPS induction in RAW 264.7 cells, it was selected as a positive control to assess the effects of mogrosides in this study. Cell morphology after LPS induction and mogrosides or biochanin A treatment was monitored with an inverted microscope. It was noted that LPS stimulation resulted in the elongation of RAW 264.7 cells as shown by Jun et al.[20] Cells treated with mogrosides and biochanin A alone without the LPS stimulation, however, looked similar to the unstimulated and untreated cells (data not shown). MTS reagent from the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit (Promega) was

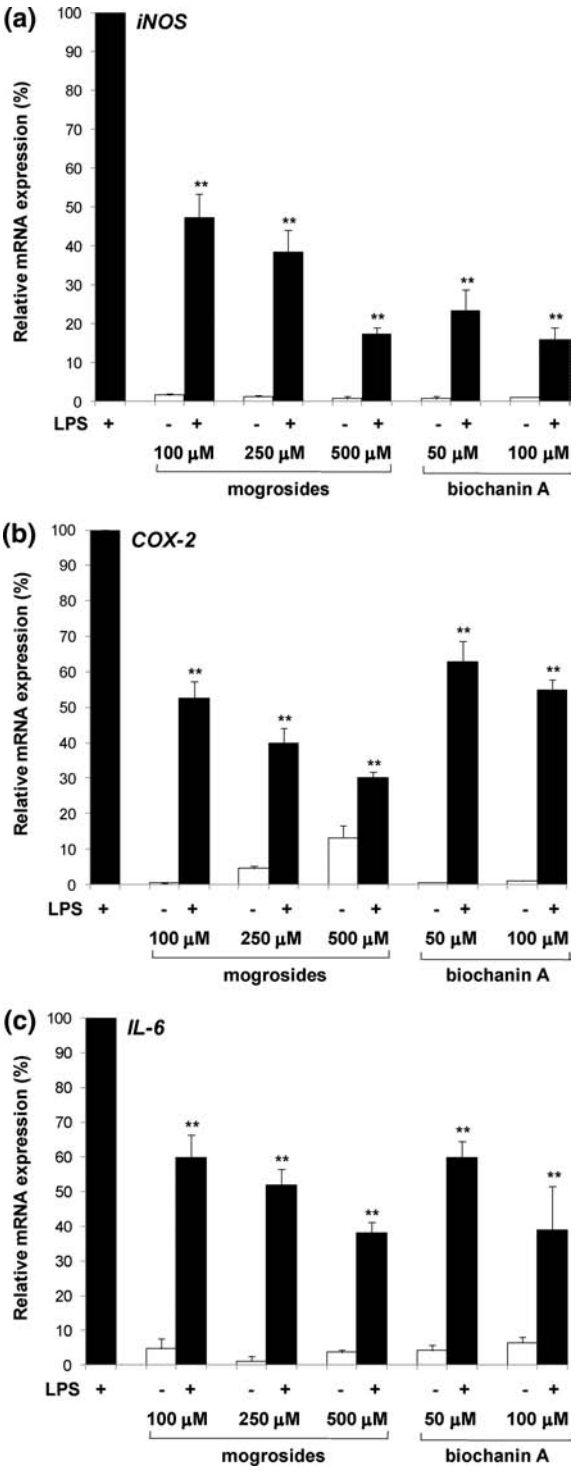

Figure 1. Inhibitory effects of mogrosides and biochanin A on inflammation genes in murine macrophage RAW 264.7 cells. Inflammation was induced with lipopolysaccharide (LPS) for 2 h. Cells were washed and incubated with mogrosides or biochanin A for 24 h. Total RNA was isolated and subjected to reverse transcription with random primers. One hundred nanograms of cDNA from each sample was assayed with TaqMan probes and primers designed specifically for *iNOS* (a), *COX-2* (b) and *IL-6* (c) by Applied Biosystems (Carlsbad, CA) in qPCR analysis. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method using 18S rRNA as the endogenous control and compared to cells that were not treated with LPS, mogrosides or biochanin A. Analysis was carried out with at least three independent experiments with standard deviations and statistical Student *t* test. **, $P < 0.01$.

used to assay cell viability after treatments. It was demonstrated that 100−500 μM mogrosides or 100 μM biochanin A did not cause any inhibitory effects on cell growth (data not shown).

By taking advantage of the TaqMan Gene Expression Assays from Applied Biosystems Inc. for all known genes in mouse, a customized TaqMan gene array with available TaqMan probes was designed to analyze the inhibitory effects of mogrosides to several genes known to be involved in inflammation. Gene expression levels in the LPS-induced, mogroside- or biochanin A-treated samples were compared to those in the sample that was not LPS-induced or treated (the control sample). Under the general concept that a 2-fold (2×) increase in gene expression is considered to be a significant enhancement, our results indicated that *COX-1*, *ALOX12*, *TNF*, *MAPK1*, *MAPK3*, *MAPK8*, and *IL1r1* gene expressions were not induced by LPS when LPS-induced RAW 264.7 cells were compared to the control RAW 264.7 cells.

The gene expression levels of *iNOS*, *COX-2*, and *IL-6*, however, were greatly induced by LPS in RAW 264.7 cells. In three separate experiments, the up-regulation of *iNOS* gene expression by LPS was 122−212-fold compared to the noninduced control RAW 264.7 cells. The elevations of *COX-2* and *IL-6* genes by LPS were 12−23- and 13−41-fold, respectively. The gene expression levels of these three genes in the LPS-induced RAW 264.7 cells were adjusted to 100% in each individual experiment. The inhibitory effects of mogrosides and biochanin A on these three genes in three separate experiments are individually expressed as the percentage reductions compared to the cells induced by LPS only in the same experiment. The average percentage reductions from three separate experiments were statistically analyzed by Student *t* test and are presented in Figure 1. It is shown in Figure 1a that mogrosides alone at 100, 250, and 500 μM did not affect the expression level of *iNOS*, nor did biochanin A alone at 50 and 100 μM. However, the *iNOS* level in the RAW 264.7 cells induced by 2 μg/mL LPS and then treated with 100 μM mogrosides for 24 h was reduced to 47.3% of the *iNOS* level in the cells induced by LPS only. That is, 100 μM mogrosides could reduce the LPS-induced up-regulation of *iNOS* in RAW 264.7 cells by 52.7%. Furthermore, when higher concentrations of mogrosides were tested, 500 μM mogrosides could reduce the LPS up-regulation of *iNOS* as well as 100 μM biochanin A. Statistical analysis indicates that the inhibitory effects of mogrosides and biochanin A are highly significant when compared to the LPS-only induced RAW 264.7 cells.

Figure 1b demonstrates that the up-regulation of *COX-2* gene expression by LPS induction was also greatly reduced by 100 μM mogrosides. The inhibitory effect of 100 μM mogrosides on *COX-2*'s expression was even higher than that of 100 μM biochanin A. Although the *COX-2* level in the 500 μM mogrosides only treated sample was relatively higher than the negligible levels in those samples treated by 100 and 250 μM mogrosides and by 50 and 100 μM biochanin A, the *COX-2* expression in the LPS-induced and then 500 μM mogroside-treated sample was still the lowest at 30%, indicating the strong inhibitory effect of mogrosides on this highly important pro-inflammatory gene.

Mogrosides were shown to be highly inhibitory to another important pro-inflammatory gene, *IL-6* (Figure 1c). Mogrosides at 100 μM could inhibit the up-regulation of *IL-6* by LPS by approximately 40%, similar to the inhibitory effect by 50 μM biochanin A. Mogrosides at 500 μM could further reduce the up-regulation of the *IL-6* gene as well as 100 μM biochanin A.

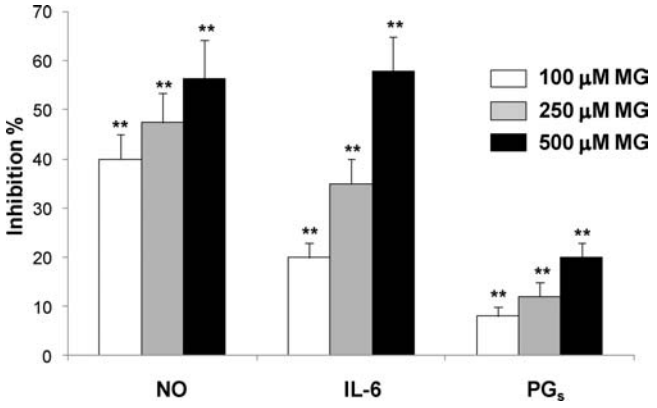

Figure 2. Inhibitory effects of mogrosides on the formation of nitric oxide (NO), interleukin 6 (IL-6), and prostaglandins (PGs) in murine macrophage RAW 264.7 cells. After LPS induction and mogroside treatment, supernatants of RAW 264.7 cell cultures were assayed for the formation of NO by the Griess Reagent System (Promega, Madison, WI), IL-6 by an ELISA kit from Biosource (Camarillo, CA), and PGs by an ELISA kit from Cayman (Ann Arbor, MI). Results were compared to cells that were induced with LPS only and plotted as inhibition percentages.

Mogrosides Inhibited the Formation of NO and the Production of Prostaglandins and Interleukins in Murine Macrophage RAW 264.7 Cells. Because mogrosides have been shown to inhibit the mRNA expression of three key pro-inflammatory genes, *iNOS*, *COX-2*, and *IL-6*, studies were carried out to further investigate if mogrosides would affect the downstream events of these genes. Nitric oxide (NO), a reactive free radical and cytotoxic to cells, is produced from L-arginine by NO synthases including iNOS.[9] Therefore, the formation of NO in RAW 264.7 cells as a result of LPS induction was evaluated by the Griess Reagent System (Promega). The NO levels in the RAW 264.7 cells induced by LPS and then incubated with mogrosides at 100, 250, and 500 μM concentrations were compared with cells induced with LPS only and expressed as the inhibition percentage in Figure 2. Our results demonstrated that LPS induced a 10-fold increase in NO and that 100, 250, and 500 μM mogrosides inhibited NO formation by 40, 47.5, and 56.3%, respectively, consistent with a dosage-dependent inhibition pattern. Our data seemed to agree with what Pan et al.[12] have shown, that 500 μg/mL (equivalent to 344.8 μM) mogrosides inhibited NO production by >50%.

Our ELISA data showed that the production of IL-6, one of the most common inflammatory cytokines, was elevated by approximately 20-fold after incubation with 2 μg/mL LPS for 2 h in RAW 264.7 cells compared to the uninduced control cells. However, upon treatment with 500 μM mogrosides, the IL-6 level was brought down by 58% in RAW 264.7 cells, followed by 250 and 100 μM mogrosides at 35 and 20%, respectively. This level of inhibitory effects of mogrosides is highly significant ($P < 0.01$).

During inflammation, arachidonic acid (AA) is released from the lipid bilayer of the cell membrane by phosphlipase A_2 (PLA_2) from membrane-bound arachidonate.[20,21] The enzymes cyclooxygenase (COX) and arachidonate lipoxygenase (ALOX) further metabolize the released AA into eicosanoids including PGs and LTs.[10] Accordingly, the inhibitory effects of mogrosides on the production of PGs and LTs were assessed in our study by ELISAs. Because LPS did not seem to induce the gene expression of *ALOX12* in RAW 264.7 cells, the production of LTs was not increased. However, LPS treatment increased the production of PGs by 6-fold when compared to the noninduced control cells. When LPS-induced RAW 264.7 cells were incubated with mogrosides, the 100 μM mogroside treatment inhibited PGs production by 8% (Figure 2). Additionally, mogrosides at 250 and 500 μM inhibited PGs production by 12 and 20%, respectively. Statistical analysis indicates that these inhibitory effects of mogrosides on PGs production are highly significant, with $P < 0.01$, and that mogrosides exert their anti-inflammation action through the *COX* pathway.

Mogrosides Enhance the Expression of Inflammation-Protective Genes in RAW 264.7 Cells. In a dextran sulfate sodium (DSS)-induced colitis rat model, resveratrol up-regulated genes such as *MAPK9*.[22] These genes are down-regulated in a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced inflammation model, ulcerative colitis, or Croh's disease. Other genes such as *PARP1*, *BCL2l1*, *TRP53*, and *PPARδ* have been considered prosurvival and inflammation-protective.[23] The up-regulation of these genes could be viewed as means to combat inflammation and to help the cells survive. To determine if mogrosides elicit the overexpression of inflammation-protective genes in RAW 264.7 cells, TaqMan probes were designed for *PARP1*, *BCL2l1*, *TRP53*, *MAPK9*, and *PPARδ*. The regulation of these genes by mogrosides was analyzed by RT-qPCR.

Compared to the nontreated control RAW 264.7 cells, LPS treatment alone neither induced nor suppressed the expression of *PARP1*, *BCL2l1*, *TRP53*, *MAPK9*, and *PPARδ* genes, so the fold levels of these genes in LPS-treated cells were adjusted to 1. However, the expression levels of these genes in cells that were treated with mogrosides alone or induced by LPS, and then treated by mogrosides, were more or less elevated. Our results indicate that *PPARδ* was not induced by mogrosides treatment only, or the combination of LPS induction and mogrosides treatment in RAW 264.7 cells. On the other hand, mogrosides at 250 and 500 μM elevated *PARP1* expression by 3.9- and 5.3-fold, respectively, compared to nontreated control cells (Figure 3). Even though in the cells induced by LPS and then treated with mogrosides the *PARP1* expressions were reduced slightly, compared to cells treated with mogrosides alone, the elevation of *PARP1* was still significant ($P < 0.01$).

For *BCL2l1* gene, mogrosides at 250 and 500 μM alone enhanced its expression by 2.8- and 5.5-fold (Figure 3). In cells that were induced by LPS and then treated with 250 and 500 μM mogrosides, *BCL2l1* expression levels were elevated by 1.5- and 2.7-fold, respectively, compared to nontreated control cells ($P < 0.01$). Similarly, the expression levels of *TRP53* were enhanced significantly by the treatment of mogrosides alone and by the combination of LPS induction and mogroside treatment, especially at higher concentrations (Figure 3). Finally, the expression levels of *MAPK9* gene were also enhanced by the treatment of mogrosides at 250 and 500 μM, and the combination of LPS induction followed by mogroside treatment (Figure 3). These results imply that mogrosides can not only inhibit gene expression of a few key pro- inflammatory genes after LPS induction in murine macrophage RAW 264.7 cells but also can elicit the gene expression of some inflammation-protective genes such as *PARP1*, *BCL2l1*, *TRP53*, and *MAPK9*.

Mogrosides Inhibited Inflammation in Murine Ear Tissues by Down-regulating Key Inflammatory Genes and Up-regulating Several Inflammation-Protective Genes. To investigate the anti-inflammation activity of mogrosides in vivo, the ears of female CD-1 mice were stimulated with TPA followed by the treatment of mogrosides.[18] As murine ear tissues are inflamed, the weight increases. The results in Table 1 show that mogrosides

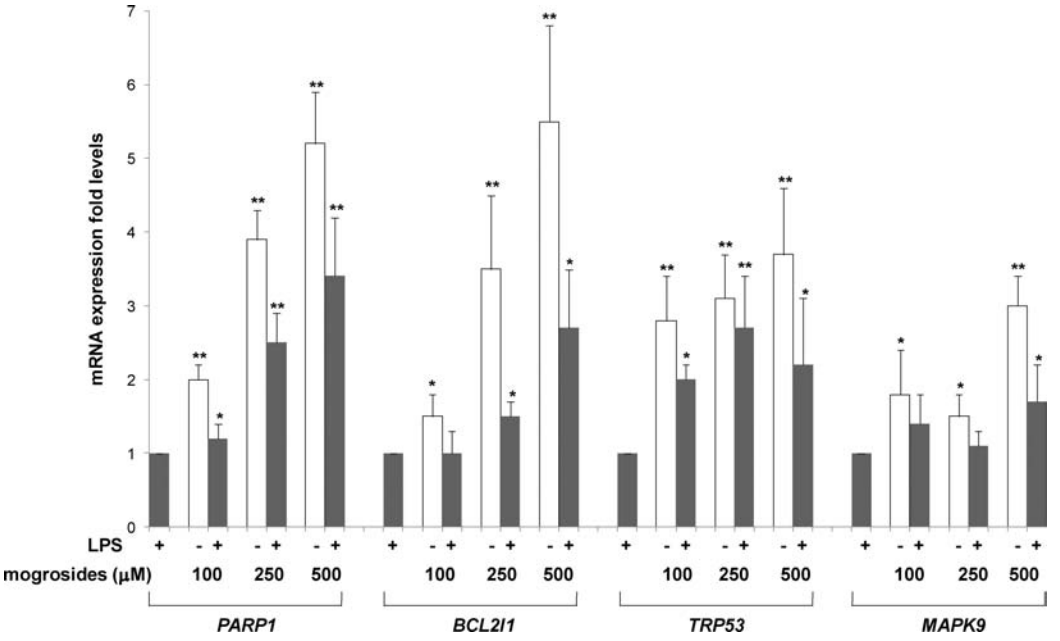

Figure 3. Up-regulation of inflammation protective gene expression by mogrosides in RAW 264.7 cells. Inflammation was induced with lipopolysaccharide (LPS) for 2 h. Cells were washed and incubated with mogrosides for 24 h. Total RNA was isolated and subjected to reverse transcription with random primers. One hundred nanograms of cDNA from each sample was assayed with TaqMan probes and primers designed specifically for *PARP1*, *BCL2l1*, *TRP53*, and *MAPK9* by Applied Biosystems (Carlsbad, CA) in qPCR analysis. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method using 18S rRNA as the endogenous control and compared to cells that were not treated with LPS and mogrosides. Analysis was carried out with at least three independent experiments with standard deviations and statistical Student *t* test. *, $P < 0.05$. **, $P < 0.01$.

at 0.4 μM reduced the inflammation of murine ear tissues by 28.3% as measured by the reduction in ear punch weight. Higher concentration of mogrosides at 0.8 μM significantly reduced the inflammation by 52.2% ($P < 0.01$).

When cDNAs produced from the total RNAs isolated from treated murine ear tissues were applied to RT-qPCR using TaqMan probes, *iNOS*, *COX-1*, and *MAPK8* gene expressions were not elevated by TPA treatment in mouse ear tissues. However, the expression of *ALOX12*, *TNF*, *MAPK1*, and *IL1r1* were elevated by TPA in murine ear tissues compared to murine ears treated by acetone alone, and mogrosides did not seem to have much effect. Remarkably, *COX-2* expression was induced by TPA by approximately 18-fold, and 0.4 μmol of mogrosides applied to the murine ears reduced the *COX-2* level by 17.7%. Furthermore, in murine ear tissues treated with 0.8 μmol of mogrosides, the *COX-2* gene expression level was significantly reduced by 31.7% ($P < 0.01$). The gene expression of the key inflammation player *IL-6* was dramatically increased by 65.6-fold through the application of TPA to murine ears. However, it was reduced by a following application of 0.4 μmol of mogrosides to 57.5-fold, a reduction of 13%, and further reduced by 24.1% by 0.8 μmol of mogrosides treatment ($P < 0.05$). These results demonstrate that mogrosides can reduce the TPA-induced inflammation in murine ears through the inhibition of *COX-2* and *IL-6*.

As with murine macrophage RAW 264.7 cells, the expression levels of five inflammation-protective genes, *PARP1*, *BCL2l1*, *TRP53*, *MAPK9*, and *PPARδ*, were evaluated in murine ear tissues after TPA induction and mogroside treatments by RT-qPCR. TPA treatment alone did not change the gene expression levels of these five genes compared to acetone-treated murine ear tissues (Table 1). However, in murine ears induced by TPA followed by mogroside treatments, the expression levels of *PARP1*, *BCL2l1*, *MAPK9*, and *PPARδ* were significantly up-regulated by 0.4 and 0.8 μmol of mogrosides (Table 1). Again, our data indicate that mogrosides can suppress inflammation not only through the inhibition of pro-inflammation gene expression but also through the up-regulation of several inflammation-protective genes such as *PARP1*, *BCL2l1*, *MAPK9*, and *PPARδ* in murine ears.

DISCUSSION

This study demonstrates for the first time that mogrosides from *M. grosvenori* not only inhibit the expression of several key inflammation genes but also enhance the expression levels of some inflammation-protective genes. Our results showed that mogrosides at a concentration as high as 500 μM did not have any adverse effect on RAW 264.7 cell growth after 24 h of treatment. At 500 μM, mogrosides were shown to inhibit the expression of *iNOS* and *IL-6* genes similarly to 100 μM biochanin A after LPS induction in RAW 264.7 cells by RT-qPCR assay (Figure 1a,c). However, at 100 μM, mogrosides could inhibit *COX-2* gene expression at a similar level as 100 μM biochanin A (Figure 1b). Consequently, it was shown that 100 μM mogrosides could inhibit the release of NO (40%), the production of IL-6 protein (20%), and the formation of PGs metabolized from AA by COX (8%) (Figure 2). It has been shown that 50 μM genistein from kudzu[24] could inhibit the production of NO in RAW 264.7 cells by 56%.[20] Genestein, a health beneficial polyphenol component of soybean, is normally present as 250 μg per gram of dry powder (0.025%).[25] Mogrosides are usually present at 1% of the dry weight of the fruit.[1] As a natural noncaloric sweetener, mogrosides and *M. grosvenori* fruit can certainly be more appealing to nonsoy product consumers.

Table 1. Effects of Mogrosides (MG) on Inflammation in Murine Ears

	treatment			
	acetone + acetone	acetone + TPA	MG (0.4 μmol) + TPA[a]	MG (0.8 μmol) + TPA[a]
(A) Inflammation				
av weight[b] (mg)	8.9 ± 0.5	15.8 ± 0.5	13.8 ± 0.7	12.2 ± 0.6
% inhibition[c]			28.3*	52.2**
(B) Pro-inflammation Gene Expression Levels (Fold) Compared to Acetone-Only Treated Murine Ears				
COX-1	1 ± 0	2.9 ± 0.1	3.1 ± 0.2	3.0 ± 0.3
COX-2	1 ± 0	18.0 ± 0.6	15.0 ± 0.8 (↓17.7%)*	12.3 ± 1.0 (↓31.7%)**
ALOX1	1 ± 0	3.0 ± 0.3	3.1 ± 0.4	2.9 ± 0.5
TNF	1 ± 0	5.4 ± 1.0	5.8 ± 0.7	5.2 ± 0.9
MAPK1	1 ± 0	2.6 ± 0.3	2.8 ± 0.5	2.1 ± 0.2
MAPK8	1 ± 0	1.8 ± 0.2	2.0 ± 0.5	1.9 ± 0.2
IL1r1	1 ± 0	2.3 ± 0.4	3.3 ± 0.7	3.1 ± 0.3
IL-6	1 ± 0	65.6 ± 4.9	57.5 ± 3.7 (↓13%)	49.8 ± 3.3 (↓24.1%)[a]
(C) Pro-survival Gene Expression Levels (Fold) Compared to Acetone-Only Treated Murine Ears				
PARP1	1 ± 0	2.6 ± 0.3	3.7 ± 0.6	4.3 ± 0.6*
BCL211	1 ± 0	2.9 ± 0.6	3.8 ± 0.6	4.6 ± 0.4*
TRP53	1 ± 0	1.5 ± 0.3	2.8 ± 0.4	2.8 ± 0.6
MAPK9	1 ± 0	0.8 ± 0.1	3.5 ± 0.6*	3.8 ± 0.6**
PPARδ	1 ± 0	3.1 ± 0.6	4.2 ± 0.8*	5.7 ± 1.0*

[a] *, significant difference compared to the TPA-induced mice ($P < 0.05$); **, highly significant difference compared to the TPA-induced mice ($P < 0.01$). [b] Average weight of ear punches from six individually treated mice. [c] Percentage of inhibition was calculated by comparing each punch weight to that of the control (acetone-only treated) mice.

Our results indicate that 0.4 and 0.8 μmol of mogrosides applied to the TPA-induced inflamed murine ears significantly reduced the inflammation and the gene expression of *COX-2* and *IL-6* (Table 1), which is comparable to the effect of black tea theaflavin derivatives in a similar model.[18] Studies have shown that NO can stimulate tumor progression by promoting tumor cell migration and invasive and angiogenic abilities in concert with activation of COX-2.[26] IL-6 is one of the most common inflammatory cytokines.[27] Chronic inflammation resulting from unchecked production of IL-6 has been highly associated with many types of cancer. Therefore, it may be highly beneficial for any nutraceutical to inhibit the iNOS, COX-2, and IL-6 pathways. Ginseng, the "all healing" herb used in the Orient for thousands of years, has been demonstrated to be inhibitory to the p38 MAP kinase pathway, pro-inflammatory cytokines, and the induction of COX-2 and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB).[23] Resveratrol in grapes is antioxidative and anti-inflammatory by down-regulating an array of genes including *IL1β* and *COX-2* in TNBS-induced inflammation model.[22] Curcumin, the active component in the traditional Indian food ingredient turmeric, exerts its anti-inflammation and anticarcinogenic effects by inhibiting multiple molecular targets including iNOS, COX-2, NF-κB, and cytokines IL-10, IL-6, and IL-18.[28] The beneficial effects of curcumin seem to be concentration dependent, antioxidative at 10 μM and apoptosis-inducing at 50 μM. Human trials indicate that up to 8000 mg per day can be tolerated without adverse effects.[28]

Although concentrations of 100, 250, and even 500 μM mogrosides are required for inhibitory effects on the gene expression of *iNOS*, *COX-2*, and *IL-6* similar to that of 100 μM biochanin A (Figure 1), high concentrations of mogrosides produced no ill effects in RAW 264.7 cells (data not shown). The fact that Luo Han Guo consumption in the *M. grosvenori*-growing region in China is associated with an unusually high number of 100+-year-old residents,[1] suggests that mogrosides are well tolerated by humans and their health benefits should be explored in more detail.

Our study also demonstrated that mogrosides can not only inhibit key inflammatory genes but also enhance the expression of some genes that are known to be inflammation protective in both RAW 264.7 cells and mouse ears. PARP1 functions in detecting DNA damage, repairing DNA, and maintaining genomic stability.[13] The anti-apoptotic Bcl-2 family proteins are key regulators of mitochondrial-related apoptosis pathways.[14] TRP53 (p53) is a nuclear transcription factor that transactivates genes involved in the induction of cell cycle arrest, DNA repair, and apoptotic cell death.[15] It has been shown that *p53* mutation blocks the p53-dependent pro-apoptotic pathway and results in resistance of tumor cells to anticancer drugs.[29] MAPK9 or JNK2 (c-Jun NH_2-terminal kinase isoform) is involved in alleviating replicative stress by coordinating cell cycle progression and DNA damage repair.[16] PPARs (PPARα, β/δ, and γ) are members of the nuclear receptor superfamily of ligand-inducible transcription factors.[30] PPARs, activated by dietary fats, are important metabolic regulators[30] and may suppress inflammation through various mechanisms.[17] PPARδ is the predominant PPAR isotype in human skin keratinocytes.[31] Ginseng can activate PPARγ[23] and resveratrol can up-regulate MAPK9 in TNBS-induced rat inflammation model.[22] Curcumin is known to induce apoptosis by activating a PARP cleavage through caspase in human head and neck squamous cell carcinoma cells,[32] decreasing Bcl-X (L) mRNA and increasing Bcl-X (S) and c-IAP-2 mRNA.[33] Our data suggest that at 250 and 500 μM, mogrosides could significantly enhance the expression of *PARP1*,

BCL2l1, *TRP53*, and *MAPK9* when applied alone to RAW 264.7 cells or after the induction of LPS (Figure 3). In TPA-induced inflamed murine ears, mogrosides could increase the expression of these four genes plus *PPARδ*. This finding is particularly interesting because PPARs are key metabolic regulators[30] and mogrosides have been shown to be beneficial to prevent diabetic complications in a diabetic mouse model due to their antioxidant activities.[3]

Mogrosides are a mixture of triterpenoid glycosides with mogroside V as the major component, which may be metabolized in humans after oral ingestion. There is limited information on the bioavailability and biotransformation of mogrosides in the human body. Yang et al. reported in 2007 that mogroside III was converted to secondary glycoside mogroside II and aglycone mogrol when incubated with crude enzymes of human intestinal bacteria under anaerobic conditions at 37 °C.[34] In the work by Ukiya et al.,[6] it was shown that all 18 *M. grosvenori* triterpenoids and 11-oxomogrol, a hydrolysis product of 11-oxomogroside 1, exhibited inhibitory effects on the induction of Epstein−Barr virus early antigen by TPA. It will be important in the future to study the biotransformation and bioactivity of mogrosides in an animal model and human.

As more and more attention is shifted to searching for functional foods that benefit human health, our study has shown that *M. grosvenori* can be an excellent candidate because of its anti-inflammatory properties.

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