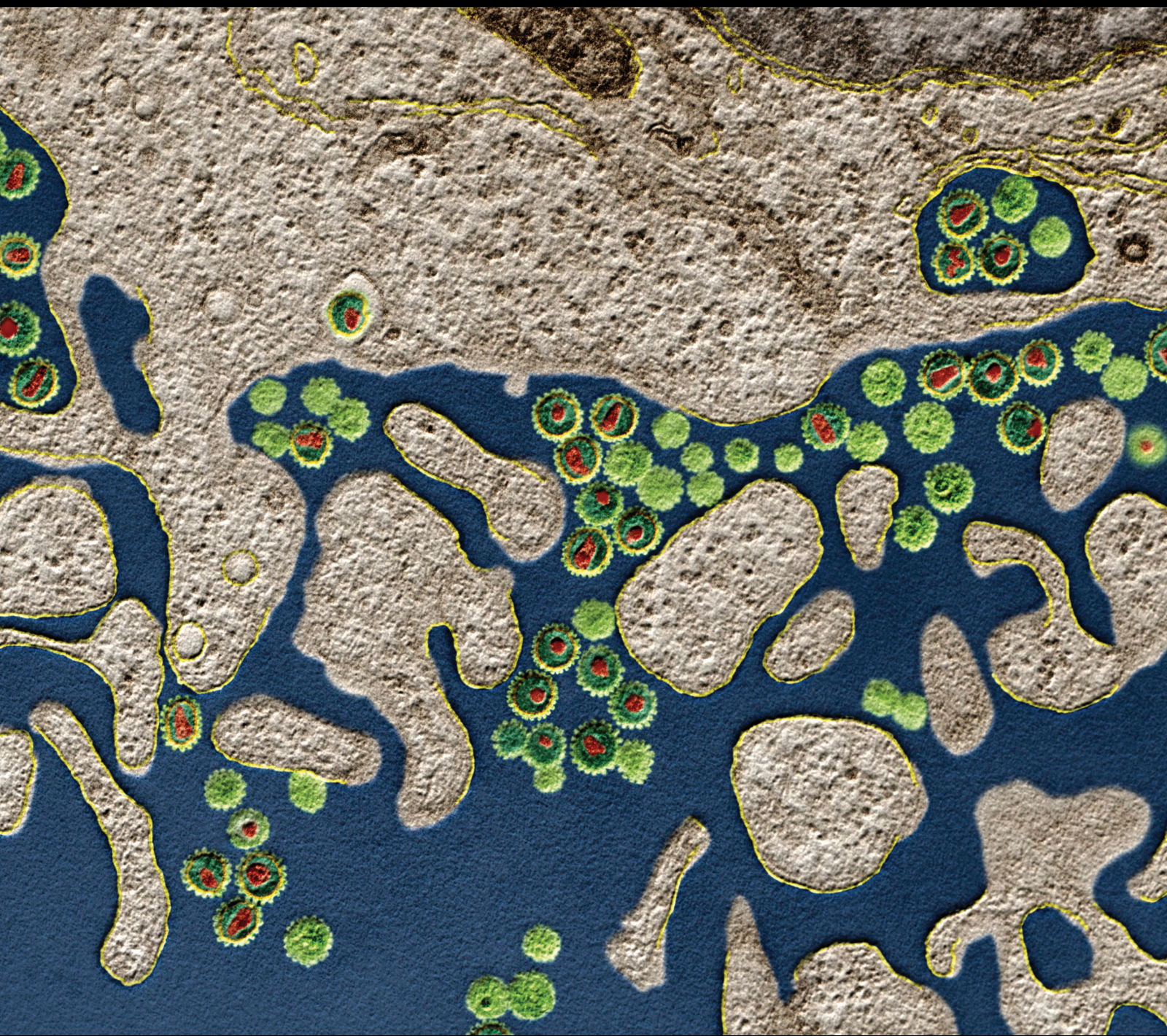
Journal of Immunology Research

Natural Immunomodulators

Lead Guest Editor: Pushpa Hegde
Guest Editors: Srinivas Kaveri, Kurt Zänker, Ajay K. S. Rawat, and Daniel Ortuño-Sahagún



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Editorial
Natural Immunomodulators

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In this special collection of research results, we present a group of original works performed by employing natural compounds as tools to approach immunomodulation at different levels and against different diseases.

Natural compounds have contributed enormously to immunomodulatory therapeutics. Since ancient times, natural medicines have constituted treatments with minimal side effects. There are thousands of natural compounds that are known to influence the immune system by either affecting the functions of immune cells or affecting antibody secretion to control the infection and to maintain immune homeostasis. The relevance of this research would be crucial to the search for better treatments, both to complement those that already exist and to develop new strategies for the prevention and treatment of immune-related diseases. Therefore, it is interesting to dissect the molecular mechanisms of the immunomodulatory effects of natural compounds and to discover novel promising candidates that can be used in the future immunotherapeutic strategies.

Immunomodulation is a key issue in tissue homeostasis for the physiological stability of organisms. Consequently, it is important to search for immunoregulators, such as those derived from natural immunomodulators, with less severe side effects. This is the case for the work of Y.-H. Cheng et al. in which the authors demonstrate Th-1 selective immunomodulatory activity for crude leaf extracts from *Neolitsea* spp., which contains phytochemicals meriting further research as potentials for development as selective immunomodulators. Additionally, M. O. Arruda et al. reported the modulatory activity of *Mentha piperita* (peppermint) leaf hydroalcoholic extract on macrophages, which are essential cells against bacterial infection, by attenuating their oxidative stress and improving their survival. Furthermore, S. Lewicki et al. explored the immunomodulatory properties of the *Rhodiola kirilowii* aqueous extract to stimulate innate immunity in an attempt to avoid, or to limit, the excessive use of antibiotics during pregnancy and lactation, which has been associated with a risk for immune system developmental disorders.

The immune system encounters one of its great challenges against cancer. In the beginning, cell proliferation dysregulations begin in a small microenvironment, and distinct immune cell populations are recruited to the vicinity of tumor [1], thus the high relevance of cancer immunotherapy and the need to conduct exploration for new natural compounds, or derived compounds, with antitumor properties, as that which the aqueous extract of *Viscum album* exhibits in the work presented by R. M. Stammer et al. against alveolar rhabdomyosarcoma. In addition, J.-T. Yang et al. demonstrated that propyl gallate, a phytochemical polyphenolic compound, can affect migration of malignant glioma cells through inhibition of ROS and the NF-κB pathway.

Cytokines and chemokines regulate immune responses by signaling, through membrane receptors, whose signaling pathways can be evaded or mimicked by viruses [2]. Consequently, research on natural compounds that can prevent,

reduce, or counter viral infective process acquires relevance, in diseases such as that presented by S. Feustel et al. in which an extract of *Moringa oleifera* exhibits protective effects against hepatitis B virus (HBV) infection, particularly for genotypes C and H.

The largest organ in the human body is the skin, and it represents the greatest upon which the immune system needs to act. Therefore, an imbalance or lack of adjustment of innate or adaptative immunity leads to immune diseases. Three of the works presented herein approach immunity modulation in this organ's epithelium, dealing with atopic dermatitis and psoriasis.

Atopic dermatitis is not a life-threatening disease; however, it severely affects patients' quality of life. Again, it is derived from immune-response deregulation and with an undefined etiology to date. Recently, air pollutants have been involved as inductive factors [3]; therefore, it became relevant to find some natural elements that can counteract this pathology, as F. C. Muñoz et al. present in their results on glycomacropeptide, a dairy bioactive peptide derived from milk, κ-casein, which possesses prebiotic, anti-inflammatory, and immunoregulatory properties that can lead to prophylactic and therapeutic actions.

Likewise, psoriasis is the most common type of chronic inflammatory dermatosis, derived from a disorder of the immune system. Several genetic-susceptibility factors have been discovered to be involved in this disease [4]. However, their treatment comprises a search for compounds able to prevent or thwart its effects. Here, C.-Y. Lai et al. present a comprehensive review focusing on the natural modulators able to inhibit endosomal TLR activation that can represent candidate drugs for developing novel treatment options for psoriasis. In addition, S. J. Kim et al. revealed promissory experimental results with the use of the *Euphorbia kansui* methanol extract, which ameliorates the symptoms of psoriasis through inhibition of Th17 differentiation and dendritic cell activation.

Undoubtedly, there remains much more work to be done, but this avenue of natural immunomodulators is one highly promissory pathway to continue to explore and investigate. Many more surprises are awaiting to be discovered in the prevention and treatment of inflammatory and immune-regulated diseases. In the future, an extensive investigation is required with respect to their mechanisms of action at the systemic, cellular, and molecular levels and extension to a broad spectrum clinical trial. Given the multiple biological effects of natural compounds, it is exceedingly interesting to design future therapeutic strategies for inflammatory pathologies and malignant diseases with a synergistic combination of natural products together and various conventional therapies. We hope that researchers enjoy the analysis of this special issue.

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D. Ortuño-Sahagún
K. Zänker
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Research Article

Administration of *Rhodiola kirilowii* Extracts during Mouse Pregnancy and Lactation Stimulates Innate but Not Adaptive Immunity of the Offspring

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The use of antibiotics during pregnancy and lactation is associated with an increased risk of developmental disorders. One of the natural medicinal plants—*Rhodiola kirilowii*, widely used as an immunostimulant in adults—might be a good alternative to antibiotic treatment. The aim of present study was to assess whether daily oral administration of 20 mg/kg of *Rhodiola kirilowii* aqueous (RKW) or 50% hydroalcoholic (RKW-A) extracts affected hematological and immunological parameters of 6-week-old mouse progeny. There was no significant change in hematological parameters of blood with the exception of hemoglobin, which was significantly higher (about 4%) in RKW group. Offspring of mothers fed *Rhodiola kirilowii* extracts had increased percentage of granulocytes and decreased percentage of lymphocytes. These changes correlated with decreased percentage of $CD3^+/CD4^+$ T-cells (RKW and RKW-A), decrease of $CD8^+$ cells, and increase percentage of NK cells in RKW group. In addition, both types of *Rhodiola kirilowii* extracts stimulated granulocyte phagocytosis and increased level of respiratory burst. In conclusion, the long-term supplementation of mouse mothers during pregnancy and lactation with RKW or RKW-A extracts affects the immune system of their progeny. These results should be taken into consideration before administration of *Rhodiola kirilowii* to pregnant and lactating women.

1. Introduction

Treatment of illnesses during pregnancy and in early infancy can be problematic because of its potential effects on development of infant immune system [1]. Vast majority of infections are treated with antibiotics that cause plethora of side effects. FDA (Food and Drug Administration) of the U.S. Department of Health and Human Services created a list of antibiotics, divided into 5 groups (A, B, C, D, and X) depending on the potential risk of side effects. Only antibiotics from group A are considered as being safe to use during pregnancy and lactation [2]. The validity of such a classification was confirmed in studies conducted on mouse model. Using experimental model of pregnant mice, Skopińska-Różewska and collaborators showed that prenatal exposure to antibiotics modifies the immune system in progeny [3, 4]. Moreover, the offspring of mothers treated during pregnancy with antibiotics from penicillin and cephalosporin group (group B) showed an altered reactivity of the immune system manifested by decreased intensity of cellular response and increased intensity of humoral immune response to pathogens. In addition, a prenatal and postnatal exposure to antibiotics is associated with an increased risk of asthma and obesity in childhood [5].

The overuse of antibiotics not only in medicine but also in food industry causes increasing antibiotic resistance and other side effects [6–8]. Thus, there is an increasing demand for a natural antimicrobial and immunostimulatory

medications, which could be safely administered during pregnancy; the herbs from the *Rhodiola* genus seem to be an excellent candidate.

Rhodiola genus from *Crassulaceae* family consists of more than 200 species out of which over 20 species have medicinal properties. They were used in traditional medicine of Asia and Europe as tonic, adaptogen, antidepressant, and anti-inflammatory drugs, and for these properties, they are being tested recently in some clinical trials [9–11]. Clinical trials showed lack of adverse effects and interactions between *Rhodiola* extracts and other drugs. Plants from *Rhodiola* species are known not only as a diet supplement that supports mental and physical performance of the body but also for the beneficial antitumor, antimicrobial, and immunomodulatory activity [12, 13]. Importantly, *Rhodiola* has antiviral and antimicrobial properties against hepatitis C virus (HCV) and *Mycobacterium tuberculosis* [14, 15]. Currently, several novel clinical therapies based on the abilities of *Rhodiola* extracts to stimulate the immune system are under development [16].

All these properties make *Rhodiola* an attractive source for producing various medications. Biological activity of *Rhodiola* spp. depends on the phytochemicals such as polyphenols, phytosterols, caretonoids, saponines, and alkaloids. Using mouse model, we showed that *Rhodiola kirilowii*, *R. rosea*, and *R. quadrifida* extracts stimulated granulocyte and lymphocyte activity. Feeding mice with *Rhodiola* extracts for 7 days lowered intensity of *Pseudomonas aeruginosa* infection, increased blood leukocyte number, and had modulatory effect on their metabolic activity [17–22].

The aim of the present study was to investigate if and how 50% hydroalcoholic (RKW-A) and aqueous (RKW) extracts of underground (roots and rhizomes) parts of *Rhodiola kirilowii* administrated to pregnant and lactating mice stimulate innate immunity of the offspring.

2. Material and Methods

2.1. Plant Cultivation. *Rhodiola kirilowii* (Crassulaceae) plants were collected from the Institute of Natural Fibers and Medical Plants (Poznań, Poland) field cultivations of herbal plants. The taxonomic status of plant was confirmed with *Flora of the Soviet Union* (Vol. 9, 1939) and *Flora of China* (Vol. 8, 2001). A voucher specimen is kept in the herbarium of the Department of Botany, Breeding and Agriculture in Plewiska near Poznań.

2.2. Preparation of Extracts. Extracts have been prepared as previously described [23]. Briefly, for RKW extract production, the finely powdered *R. kirilowii* roots were extracted twice with water (first for 2 h and then for 1 h) in a raw material to solvent ratio of 1 : 5, at temperature between 40 and 45°C. Supernatants from each extraction were mixed together, centrifuged (15 min, room temperature, 2000 ×g), and lyophilized. RKW extract contained about 14 μg of analyzed polyphenols (kaempferol, epicatechin, quercetin, (+)-catechin, salidroside, fisetin, naringenin, luteoli, p-coumaric acid, ellagic acid, epigallocatechin, ferulic acid, chlorogenic acid, epicatechin gallate, and epigallocatechin gallate) per mg of dry extract. For RKW-A extract production, the finely powdered *R. kirilowii* roots were extracted with a 50% ethanol, in a raw material to solvent ratio of 1 : 10 using the percolation method. The percolates were lyophilized following distillation at 40–45°C. RKW-A extract contained about 21 μg of analyzed polyphenols.

Dry extract ratio (g of medical herb to g final extract) was 5.09 : 1 for RKW and 3.27 : 1 for RKW-A. Extracts were stored at −70°C until further use.

2.3. Animals. All animal experiments were conducted according to the Polish regulation and standards of the wellness of laboratory animals. All experiments were accepted by and conducted according to the ethical guidance of Local Bioethical Committee (permission 73/2011).

Experiments were performed on 6-week-old progeny of 70 adult inbred female BALB/c mice (8-9 weeks old; ~20 g weight; purchased from Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland), which were mated with adult males of the same strain. After confirmation of pregnancy (the presence of copulatory plug), Balb/c females were fed daily (up to the 28th day after delivery) with lyophilized RKW or RKW-A extracts (20 mg of extract/kg of body weight) dissolved in sterile water. The control group received sterile water only. To avoid stress related to gavage feeding and handling that can lead to miscarriage, 20 microliters of tested substance dissolved in sterile water was placed on one corn chip and served to the mouse in a petri dish. Female and male progenies were housed separately.

Mice were maintained under conventional conditions (22.5–23°C, relative humidity 50–70%, 12 h day/night cycle) with free access to breeding rodent feed (Labofeed H, Wytwórnia Pasz "Morawski") and water. Pups were withdrawn from mothers 24 days after delivery.

2.4. Blood Isolation. Mice were anesthetized (intraperitoneal injection of ketamine (120 mg/kg) and xylazine (12 mg/kg); Polypharm S.A., Warsaw, Poland), and retro-orbital blood was collected in the EDTA-containing tubes (for further hematological and immunological analysis) and heparin-containing tubes (for further phagocytosis and oxidative burst analysis).

2.5. Hematological Analysis. Blood (50 μl from EDTA tubes) was analyzed in a hematological analyzer (Exigo veterinary hematological system; Boule Medical AB, Stockholm, Sweden). The following parameters were evaluated: WBC (white blood cells), RBC (red blood cells), HGB (hemoglobin), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW% (red cell distribution width (%)), RDW-a (red cell distribution width absolute), PLT (platelets), and MPV (mean platelet volume). Additionally, WBC number and cell percentage from flow cytometry analysis were used to calculate a total number of lymphocytes, lymphocyte subpopulations, monocytes, and granulocytes per μl of blood. The results are presented as the mean ± standard error.

2.6. Determination of White Blood Cell Phenotype. Determination of WBC phenotype was performed as described previously for splenocyte phenotyping [24]. Briefly, the cells in whole blood (100 μl from EDTA tubes) were labeled with the following fluorochrome-conjugated anti-mouse monoclonal antibodies: Mouse T lymphocyte Subset Antibody Cocktail with Isotype Control (hamster anti-mouse cluster of differentiation (CD) 3e, rat anti-mouse PE CD4, and rat anti-mouse CD8a; cat. number 558431), Mouse B Lymphocyte Activation Antibody Cocktail with Isotype Control (rat anti-mouse CD25, hamster anti-mouse CD69, and rat anti-mouse CD19), and rat anti-mouse CD335 (natural killer cell p46-related protein all purchased from BD Biosciences, Warsaw, Poland). Staining (20 min incubation at room temperature) was performed according to the manufacturer's instructions. Subsequently, the red blood cells were lysed (10 min, Lysing Solution 10x Concentrate; BD Biosciences) and remaining cells were washed twice with PBS. Phenotypic analysis was performed by flow cytometry (FACSCalibur; BD Biosciences). Additional phenotypic determination of WBC population (lymphocytes, monocytes, and granulocytes) was made using FSC/SSC parameters. The results are presented as the mean % ± standard error of the mean of WBC (for lymphocytes, monocytes, and granulocytes) or lymphocytes (for CD3, CD4, CD8, CD19, and CD335 analysis) and also as mean ± standard error of cell number in liter of blood.

2.7. Treg Analysis. Mouse Treg cell evaluation was performed analogously as previously described for Treg cells [25]. Briefly, cells in whole blood samples (100 μl) were immunostained with 20 μl of primary antibodies CD4-PerCP and CD25-APC (extracellular staining, BD Biosciences, Poland) or an appropriate isotype control for 20 minutes at room temperature. Subsequently, erythrocytes were lysed in BD FACS Lysing Solution for 10 minutes at room temperature. Remaining immune cells were fixed, permeabilized (fixation/permeabilization buffer) in accordance with the manufacturer's protocol (BD Pharmingen, Poland), and stained with 20 μl of FoxP3 PE or isotype IgG1 kappa PE antibody (45 min/RT in the dark). Afterwards, the cells were washed twice with PBS and fixed in 300 μl of 1% PFA in PBS solution. The cells were counted by flow cytometry—10000 counts of CD4-PerCP-positive cells stopped the acquisition. Evaluation of $CD4^+/CD25^+$ cells or Treg cells ($CD4^+/CD25^+$ and $FoxP3^+$) was performed using CellQuest Pro software (BD). The results are presented as the mean percentage of $CD4^+$ cells ± standard error of the mean.

2.8. Phagocytosis. Blood cell phagocytosis was assessed by PHAGOTEST™ (BD Biosciences, Warsaw, Poland) according to the manufacturer's protocol. 100 μl of whole blood (collected in heparin tube) was used per test. Blood was incubated for10 min. With opsonized GFP-stained *E. coli* in control (0°C) and experimental (37°C) conditions, then quenching solution was added, and red blood cells were lysed in lysing solution. Level of phagocytosis (in granulocytes or monocytes) was measured by flow cytometry (FACSCalibur). The results are presented as the mean % of cells (granulocytes or monocytes) containing phagocytized *E. coli* ± standard error of the mean.

2.9. Oxidative Burst. Quantitative determination of leukocyte oxidative burst was performed in heparinized whole blood using PHAGOBURST™ assay (BD Biosciences, Warsaw, Poland) according to the manufacturer's protocol, with our own modification. Standard kit contains unlabeled opsonized *E. coli* bacteria as particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) as high stimulus, and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) as low physiological stimulus. We modified protocol by replacing PMA with the zymosan (20 μg/ml). Oxidative burst analysis was performed by flow cytometry (FACSCalibur). The results are presented as the mean % of cells (granulocytes or monocytes) with oxidative burst ± standard error of the mean.

2.10. Statistical Analysis. Statistical evaluation of the results obtained, from the control and experimental groups, was performed using unpaired *t*-tests and one- or two-way analysis of the variance, followed by the Tukey test or Bonferroni correction (in the case of a normal distribution) or nonparametric Kruskal-Wallis and Mann–Whitney *U* tests (in the case of abnormal distribution). Assessment of the distribution of the data was evaluated using the Shapiro-Wilk test. GraphPad Prism software was used to carry out these tests (version 5; GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Hematological Analysis. There was no significant difference in RBC, HCT, MCH, MCHC, RDW%, RDWa, PLT, and MPV parameters between control and experimental groups. The offspring of mice fed during pregnancy and lactation with water extract of *Rhodiola kirilowii* exhibited slightly higher (about 4%, $P = 0.0325$, statistically significant) concentration of hemoglobin in blood in comparison to control group. There was also a slight but statistically significant ($P = 0.0354$) difference between RKW and RKW-A group in mean corpuscular volume. The results of hematological analysis are presented in Table 1.

3.2. WBC Analysis. Water and hydroalcoholic extract of *Rhodiola kirilowii* did not affect the total number of WBC populations (lymphocytes, monocytes, and granulocytes) or percentage of monocytes in blood when compared to control group. Both extracts of *Rhodiola kirilowii* decreased proportion of lymphocytes and increased proportion of granulocytes. However, statistically significant differences (approximately 7% for lymphocytes ($P = 0.0288$) and 12% for granulocytes ($P = 0.0243$)) were observed for water extract of *Rhodiola kirilowii*. Described results are presented in Table 2.

3.3. Lymphocyte Phenotyping. The offspring of mice that received *Rhodiola kirilowii* extracts during pregnancy and nursing period exhibited significantly lower percentage of $CD3^+$ (approximately RKW-14%, $P = 0.0434$; RKW-A-10%,

Table 1: Blood hematological analysis. Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*. Bold font indicates statistically significant differences in comparison to control group ($P < 0.05$).

Parameters	Unit	Control		RKW		RKW-A	
		Mean	SEM	Mean	SEM	Mean	SEM
RBC	$\times 10^{12}$/l	10.3	0.1	10.5	0.1	10.3	0.1
HGB	g/dl	15.4	0.2	**16.0**	0.2	15.7	0.2
HCT	%	55.8	0.8	57.1	0.8	56.0	0.7
MCV	fl	54.2	0.3	54.1	0.3	54.4	0.3
MCH	pg	15.3	0.1	15.4	0.1	15.3	0.1
MCHC	g/dl	28.3	0.2	28.5	0.1	28.1	0.1
RDW	%	20.6	0.4	20.5	0.6	20.4	0.3
RDWa	fl	39.1	0.5	38.4	0.6	39.1	0.3
PLT	$\times 10^9$/l	510.1	47.8	521.6	62.2	487.2	32.9
MPV	fl	5.8	0.2	5.9	0.1	6.1	0.2

Table 2: Blood WBC analysis. Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*. Bold font indicates statistically significant differences in comparison to control group ($P < 0.05$).

Parameters	Unit	Control		RKW		RKW-A	
		Mean	SEM	Mean	SEM	Mean	SEM
WBC	$\times 10^9$/l	5.49	0.57	4.66	0.30	5.36	0.44
Lymphocytes	$\times 10^9$/l	3.70	0.37	3.00	0.21	3.51	0.33
	%	68.62	1.63	**64.02**	1.06	64.17	1.64
Monocytes	$\times 10^9$/l	0.37	0.05	0.27	0.03	0.32	0.03
	%	6.60	0.62	5.71	0.37	5.97	0.43
Granulocytes	$\times 10^9$/l	1.00	0.14	0.98	0.07	0.99	0.07
	%	17.82	1.10	**21.49**	1.11	19.91	1.66

$P = 0.0337$) and $CD4^+$ cells (15%, $P = 0.0184$ and 13%, $P = 0.0116$, resp.) in comparison to control group. Feeding with RKW extract caused statistically significant decrease in percentage of CD8-positive cells ($P = 0.0342$) and increase in percentage of CD335-positive NK cells ($P = 0.0134$) in comparison to control. There was no significant change in B-cell percentage between all studied groups (Figure 1).

There were some slight changes in the total number of lymphocytes in each studied subpopulation; however, the differences between control and extract-fed groups were not statistically significant (Figure 2).

3.4. Treg Analysis. There was no statistically significant difference in the percentage of $CD4^+/CD25^+$ and Treg cells between examined groups (Figure 3).

3.5. Phagocytosis. Both extracts of *Rhodiola kirilowii* significantly stimulated granulocyte phagocytosis as measured by the number of cells containing opsonized *E. coli*, when compared to control group. The stimulation was approximately 7% higher in RKW group ($P = 0.0311$) and 6% in RKW-A group ($P = 0.0370$). Interestingly, monocytes isolated from blood of the offspring, which mothers were fed during pregnancy and nursing with water extract of *Rhodiola kirilowii*, exhibited significantly lower level of monocyte phagocytosis (about 12%, $P = 0.0075$). Level of monocyte phagocytosis in RKW-A group remained unaffected (Figure 4).

3.6. Oxidative Burst. Similar to phagocytosis, extracts of *Rhodiola kirilowii* administrated to pregnant and lactating mice increased level of respiratory burst stimulated by exposure to *E. coli* or fMLP. The granulocytes from RKW group exposed to *E. coli* (strong stimulant of oxidative burst) showed approximately 26% enhancement ($P = 0.0021$) of oxidative burst when compared to control. Addition of low physiological stimulant, fMLP caused significant increase of granulocyte respiratory burst in both RKW and RKW-A groups (46% with $P = 0.0010$ and 40% with $P = 0.0066$, resp.). There was no significant change after zymosan (all groups) and *E. coli* (RKW-A group) exposure (Figure 5).

There was also no significant difference between control and *Rhodiola kirilowii* groups in monocyte oxidative burst after *E. coli* and fMLP stimulation. The statistically significant decrease of oxidative burst occurred only after zymosan stimulation and only in RKW (around 28%, $P = 0.0098$) group. There was no difference in RKW-A group (Figure 5).

4. Discussion

The immune system of fetus develops slowly during pregnancy and rapidly during the first period after birth [26]. Proper functioning of the immune system is essential for defense against pathogens and regulation of homeostasis. Our previous experiments in mouse model showed that *Rhodiola kirilowii* extract affects certain parameters of pregnancy such as litter size and number of females without progeny [23, 27].

In this study, we showed that the *Rhodiola kirilowii* extracts administrated to the mouse mothers during lactation and nursing did not affect the hematological parameters. The only exception was a slight but statistically significant increase in the concentration of hemoglobin in the group supplemented with an aqueous extract of *Rhodiola kirilowii*. Similar results were obtained by Gupta et al. [28] with another *Rhodiola* species. In these studies, rats treated with *Rhodiola imbricate* did not show significant changes in the blood. Also, Senthilkumar et al. [29] had not observed any significant differences in blood parameters of Wistar rats after administration of the acetone extract of *Rhodiola imbricata* (200 and 400 mg/kg). Moreover, *Rhodiola imbricata* supplementation showed hepatoprotective properties against paracetamol-induced liver toxicity.

There are many factors, which are able to modify (increase or decrease) the population of WBC cells. Such biologically active compounds are probably present in water extract and hydroalcoholic extract of *Rhodiola kirilowii*. In our previous study, we showed that both extracts of *Rhodiola kirilowii* contain kaempferol, epicatechin quercetin, (+)-catechin, salidroside, fisetin, naringenin, luteolin, p-

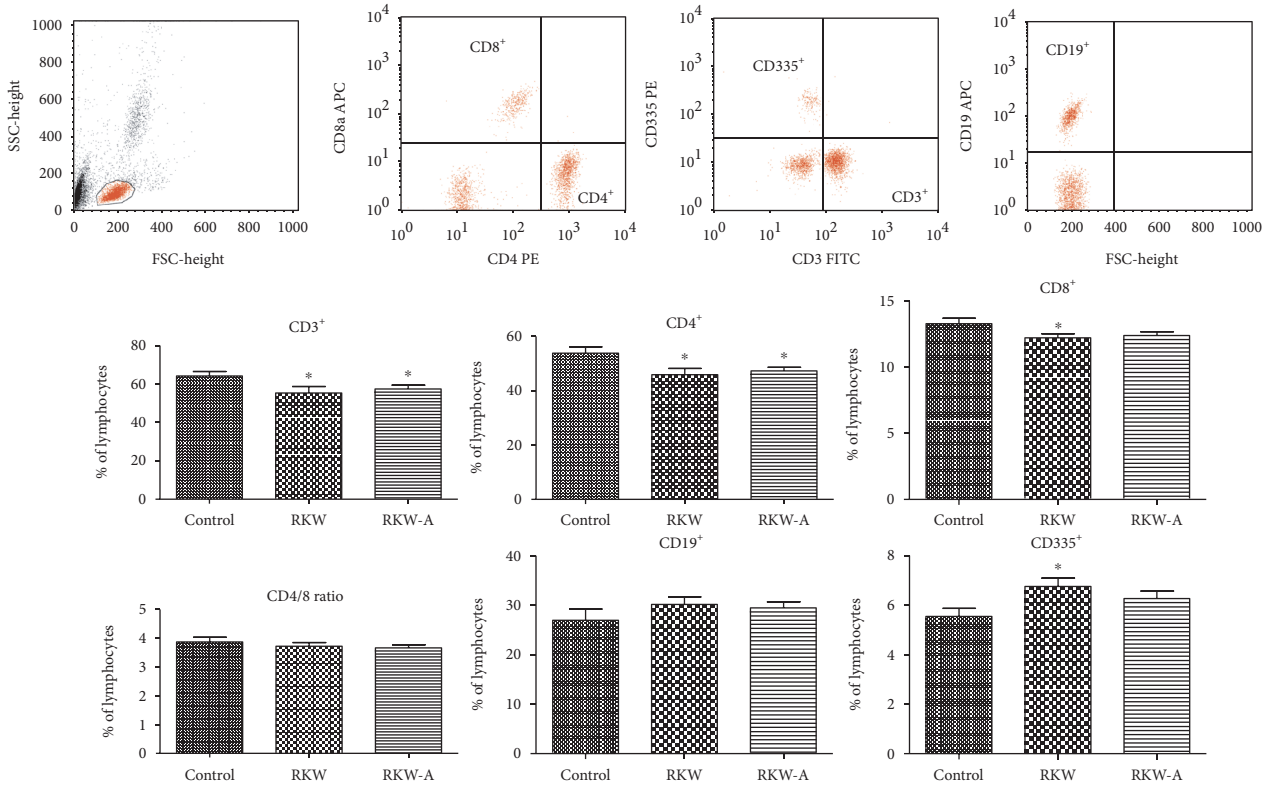

Figure 1: Blood lymphocyte phenotype analysis (mean % ± SEM). Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*. $^*P < 0.05$.

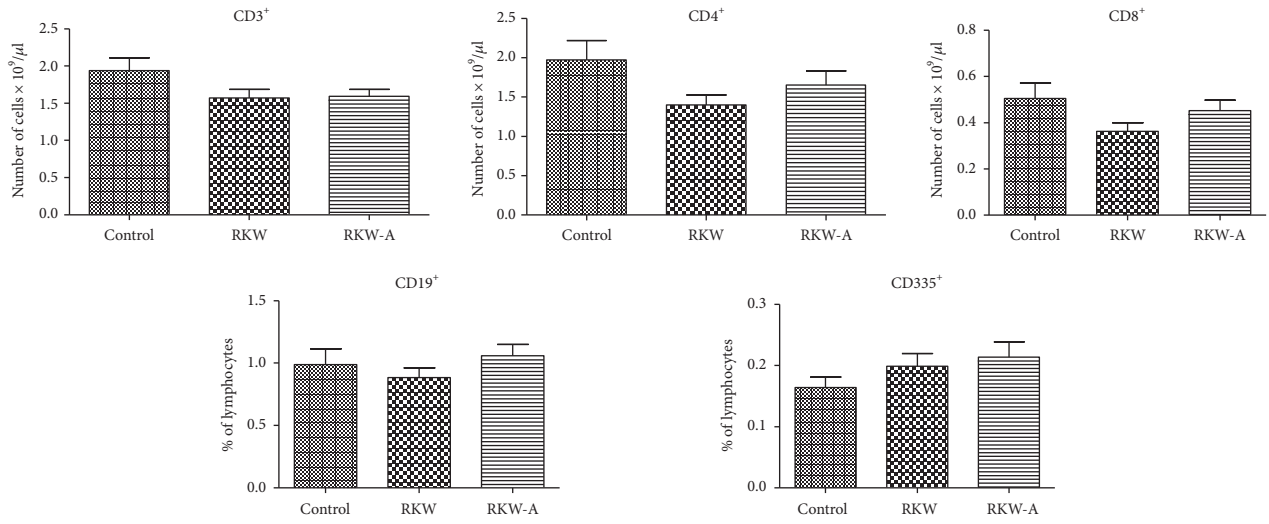

Figure 2: Blood lymphocyte phenotype analysis (cell number per μl). Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*.

coumaric acid, ellagic, acid epigallocatechin, ferulic and chlorogenic acid, epicatechin gallate, and epigallocatechin gallate [27]. Some of these compounds were also found in sera of mouse mothers [30]. It is known that some of biological compounds found in *Rhodiola kirilowii* may affect the number, percentage distribution, and activity of WBC populations. For example, the salidroside modulates mouse inflammatory responses, both in the number of immunological cells and in secretion of inflammatory cytokines [31]. It also positively affects bone marrow (BM) function by modulating the number of peripheral white blood cells in bone marrow-depressed mice [32].

We showed here that the offspring of mice that received *Rhodiola kirilowii* extracts during pregnancy and nursing

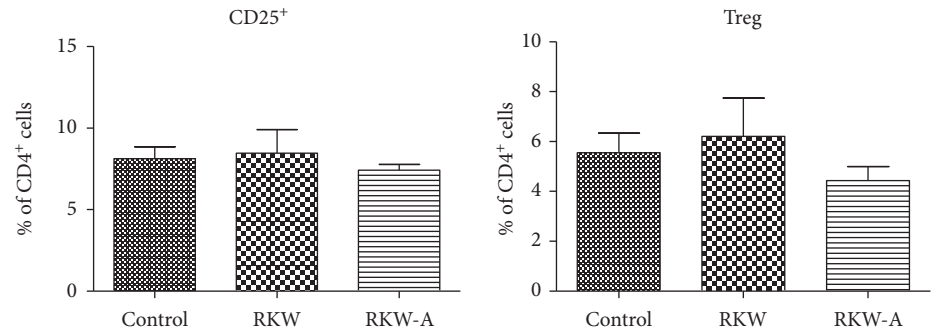

Figure 3: Treg cells in peripheral blood. Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*.

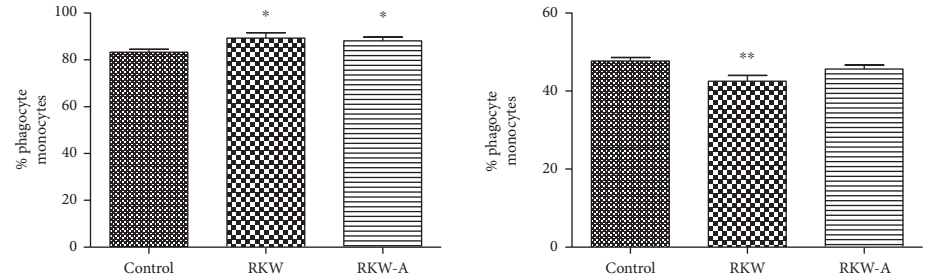

Figure 4: Blood cell phagocytosis. Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*. $^{*}P < 0.05$; $^{**}P < 0.01$.

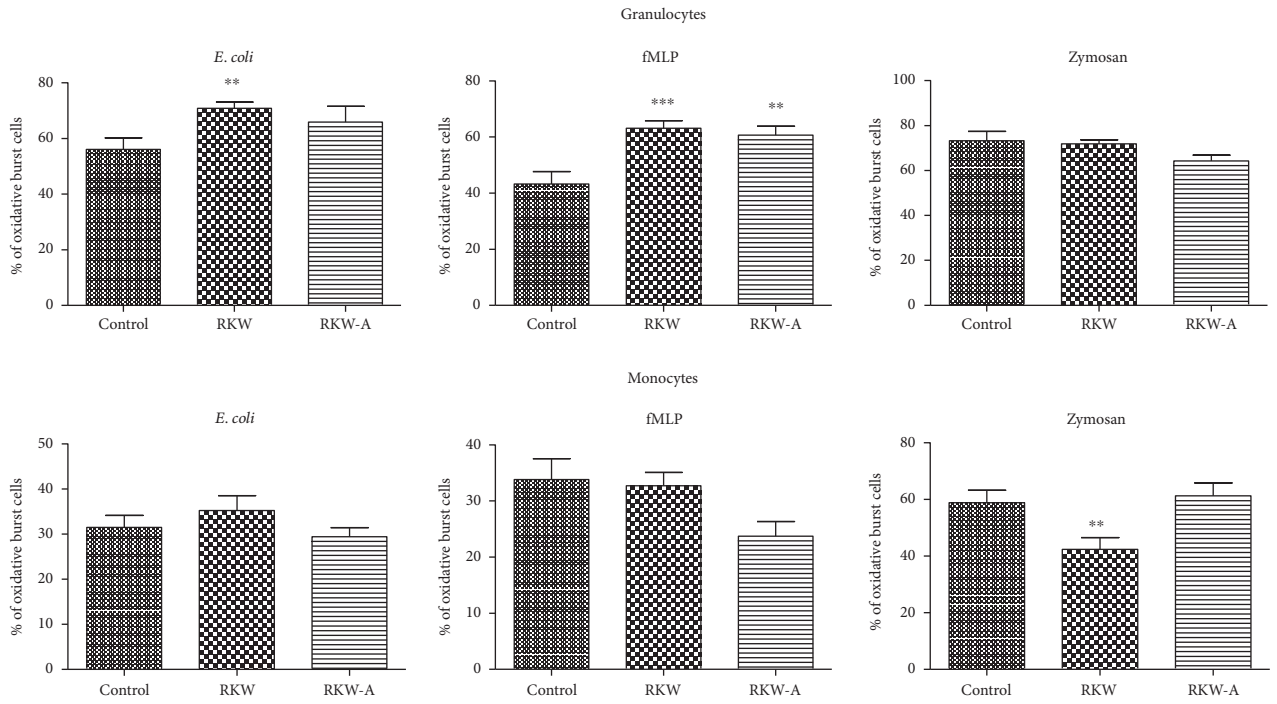

Figure 5: Oxidative burst. Analysis was performed in the offspring of mothers fed during pregnancy and lactation with water (RKW) or hydroalcoholic (RKW-A) extract of *Rhodiola kirilowii*. $^{**}P < 0.01$; $^{***}P < 0.001$.

period exhibited statistically significant lower percentage of $CD3^+$ T-cells and decrease of $CD4^+$ T-cells, in comparison to control group. $CD4^+$ T-cells are important for adaptive immunity response [33]. These cells recognize MHC class II protein complex and are involved in the induction and the restraint of most immunological functions. A deficiency

of $CD4^+$ T-cells causes significant attenuation of adaptive response. This is especially visible in HIV patients where reduction of $CD4^+$ T-cells causes immunological failure [34]. To confirm this conjectures, in our previous studies [24], we checked the functionality of the components of adaptive response in the progeny after *Rhodiola* extract administration. Spleen cell number and spleen cell phenotype ($CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$) were not affected after RKW treatment. Also, spleen cell proliferation (after lipopolysaccharide and phytohaemagglutinin) was not affected; however, we noted significant reduction of spleen cell proliferation after concanavalin A stimulation in RKW group, which is somewhat worrying. Reduction in the percentage of $CD4^+$ cells observed in previous work was also not associated with the significant reduction in the total number of $CD4^+$ cells. $CD4^+$ T-cells cooperate with B-cells and together promote strength and duration of adaptive immune response. Here, we showed that there was no significant difference in B-cell percentage and number between *Rhodiola*-treated and control groups. All these data indicated that RKW extract did not have negative effect on the adaptive functionality. Administration of SRBC to the progeny which mothers were fed with RKW extracts during pregnancy and nursing did not influence antibody production [24]. In contrast, the hydroalcoholic extract of *Rhodiola kirilowii* significantly decreased the level of antibodies in serum (SRBC test) and significantly reduced spleen cell proliferation. This suggests that about 33% increase of polyphenols in mothers' diet (differences between RKW and RKW-A) significantly affects adaptive immune response.

Similar situation (significantly lower percentage of $CD8^+$ T-cells in the blood) was observed in RKW group. Decreased level of both T-cell populations suggests their faulty production in bone marrow or faulty development in the thymus. However, the results of our previous studies indicated that feeding pregnant and lactating mothers with water or hydroalcoholic extract of *Rhodiola kirilowii* decreased thymus T lymphocyte apoptosis and did not significantly affect IL-7 expression in progeny's thymocytes [35]. Similar results were reported by Liu et al. [36] in the model of septic rats after administration of *Rhodiola rosea* (50 mg/kg body weight). These changes correlated with downregulation of tumor necrosis factor-α-induced protein 8-like 2. Taken together, these results indicate that the extracts from *Rhodiola* help to preserve thymus function in the progeny of treated animals. We believe that a decreased level of $CD8^+$ T-cells in the blood may be a consequence of the increased mobility of these cells. Our previous studies showed significantly higher number of $CD8^+$ T-cells in the spleen of the progeny whose mothers were fed with *Rhodiola kirilowii* extracts [37]. Moreover, $CD8^+$ T-positive cells were found in the nontypical location within the spleen, that is, not only in the PALS but also in the B-cell follicle and in the red pulp.

The lack of effect of RKW extracts on the adaptive immunity is also confirmed by the results of Treg cell analysis. Our present study showed that there was no significant difference in the total number and percentage of regulatory T-cells (Treg) between control and *Rhodiola*-treated groups. Also, Xu et al. [38] did not observe significant changes in the ratio of circulating Tregs or Treg cell differentiation in the mice treated with *Rhodiola rosea*. These results should be seen as positive outcome of the treatment, because changes in the number and percentage of Tregs may be one of the reasons for the development of autoimmune, allergic, or malignant diseases [39].

Another positive outcome of the *Rhodiola* treatment is visible in the behavior of NK cells. We observed significant increase of NK cell percentage in the blood of mice whose mothers were fed during pregnancy and lactation with water extract of *Rhodiola kirilowii*. The NK cells exhibit cytotoxic properties and are the part of the innate immune system. The NK cells survey the body for aberrant expression of MHC class I molecules and stress markers on the autologous cells [40]. Increased number and activity of NK cells are especially desirable for negative regulation of growth and metastasis of tumors. Lee et al. [41] showed that NK cells isolated from human blood reduce systemic metastasis and inhibit development of glioblastoma cells in nude mouse. Also, Diwaker et al. [42] noted increased number of NK cells after *Rhodiola* treatment in dengue-virus-infected human PBMCs.

Phagocytosis is a major process in which granulocytes and monocytes eliminate pathogens [43]. Several studies showed that the granulocytes exhibit higher level of phagocytosis than monocytes [44, 45]. Filias et al. [46] showed that phagocytotic ability of granulocytes and monocytes in the neonates is fully functional few days after birth. Although the standard isolation procedures do not affect phagocytosis of *Escherichia coli* bacteria [47], the isolation procedures may affect the granulocyte respiratory burst but only after anti-CD15 antibody-conjugated microbead isolation (positive selection).

We observed an increased level of phagocytic activity in granulocytes from *Rhodiola* extract group. Additionally, there were changes in oxidative burst after strong (*E. coli*) and weak (fMLP) stimulation. RKW groups showed higher stimulation of phagocytosis and oxidative burst than RKW-A groups. There is strong evidence that herbs from *Rhodiola* species stimulate innate immunity by enhancement of phagocytosis. These effects were found in several mammalian species (mice, rats, and pigs) after administration of *Rhodiola quadrifida* or *Rhodiola rosea* [20, 21]. Administration of *Rhodiola kirilowii* to the mouse mothers during pregnancy and nursing significantly improves activity of innate immunity cells of their offspring. These are very promising results; however, further studies are needed to elucidate the mechanisms of *Rhodiola* action in pregnancy and lactation.

Cytokines are hormone-like messengers regulating development and functionality of both adaptive and innate immune responses [48, 49]. There are several studies describing the effects of *Rhodiola* spp. on the cytokine secretion. Lin et al. [50] showed increased secretion of both Th1- and Th2-pattern cytokines in a dose- and time-dependent manner after *Rhodiola rosea* treatment. The *Rhodiola rosea* supplementation enhanced production of interferon (IFN) β and other cytokines, including IL-1β, TNF-α, IL-6, and IL-8, in the monocytes infected with dengue virus [42]. We also checked the expression of cytokines in the serum of the progeny which mothers were fed during pregnancy and

lactation with the RK extracts. The RKW and RKW-A extracts supplementation did not change the expression of IFN γ, IL-2, IL-4 or IL-6 in progeny sera compared with the control. However, TNF-α and IL-10 expression was higher in the progeny of mice fed with RKW-A extract [24]. The RKW administration lowered the concentration of interleukin-17a. It is known that IL-17a induces and mediates the proinflammatory responses and promotes production of IL-6, TNF-α, GCF, GM-CSF, IL-1β, TGF-β, and IL-8 [51, 52].

Our present and previously published studies tried to assess, which one of the two extracts, water (RKW) or hydroalcoholic (RKW-A) of *Rhodiola kirilowii* when given during pregnancy and lactation period that are less detrimental to the health of the offspring. We showed that the water extract (RKW) is less detrimental to the health of the offspring. This conclusion is based on the following data:

(i) The offspring of mothers fed during pregnancy and nursing period with water extract (RKW) of *Rhodiola kirilowii* exhibits stimulation of innate immune response (granulocytes and NK cells) and slight decrease of monocyte activity (present work), without changes in adaptive immune response (SRBC blood test). In contrast, feeding with RKW-A impaired adaptive response [24].

(ii) There were no significant differences in spleen cell response after lipopolysaccharide and phytohaemagglutinin, and there was a decreased response after concanavalin A stimulation in RKW group. In contrast, feeding with RKW-A diminished response to all these stimulants [24].

(iii) Both extracts protected thymus cells from apoptosis [35].

(iv) The offspring of RKW-fed group, in contrast to RKW-A-fed group, had no significant differences in kidney structure and function [53]. In addition, mice from RKW-A group had higher level of angiogenic factors (VEGF and bFGF) in serum [30].

(v) There were no neonatal deaths in RKW and control groups and several neonatal deaths in RKW-A group [23].

Taking together, these results suggest that water extract of *Rhodiola kirilowii* is safer to use during pregnancy and lactation.

5. Conclusions

Long-term supplementation of mouse mothers during pregnancy and lactation with water extract or hydroalcoholic extract of *Rhodiola kirilowii* affects some parameters of the immune system of their progeny. These treatments increase the number and activity of innate immune cells in blood and slightly decrease the percentage of T-cells (mainly $CD4^+$). The results obtained in the present work provide evidence that plant-derived biologically active compounds administrated to the mothers during pregnancy and nursing period may significantly and permanently affect the immune response of their offspring. These results can be useful for creation of guidelines for pregnant and lactating women's diet.

Conflicts of Interest

The authors certify that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

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Research Article

Protective Effects of *Moringa oleifera* on HBV Genotypes C and H Transiently Transfected Huh7 Cells

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Chronic hepatitis B infection treatment implicates a long-lasting treatment. *M. oleifera* extracts contain compounds with antiviral, antioxidant, and antifibrotic properties. In this study, the effect of *M. oleifera* was evaluated in Huh7 cells expressing either HBV genotypes C or H for the antiviral, antifibrotic, anti-inflammatory, and antioxidative responses. Huh7 cells were treated with an aqueous extract of *M. oleifera* (leaves) at doses of 0, 30, 45, or 60 μg/mL. The replicative virus and *TGF-β1*, *CTGF*, *CAT*, *IFN-β1*, and pgRNA expressions were measured by real time. HBsAg and IL-6 titers were determined by ELISA. *CTGF*, *TGF-β1*, *IFN-β1*, and pgRNA expressions decreased with *M. oleifera* treatment irrespective of the HBV genotype. HBsAg secretion in the supernatant of transfected Huh7 cells with both HBV genotypes was decreased regardless of the dose of *M. oleifera*. Similar effect was observed in proinflammatory cytokine IL-6, which had a tendency to decrease at 24 hours of treatment. Transfection with both HBV genotypes strongly decreased *CAT* expression, which is retrieved with *M. oleifera* treatment. *M. oleifera* treatment reduced fibrosis markers, IL-6, and HBsAg secretion in HBV genotypes C and H. However, at the level of replication, only HBV-DNA genotype C was slightly reduced with this treatment.

1. Introduction

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family. It is a noncytopathic and hepatotropic virus and causes inflammation, cirrhosis, and, eventually, hepatocellular carcinoma (HCC). There are around 2 billion people infected with the virus worldwide, and more than 240 million people are chronic carriers of HBV [1]. There are ten different recognized genotypes of HBV. In Mexico, the most prevalent genotype is H followed by G and A [2, 3]. When HBV infects a host cell, the partial double-stranded DNA is repaired generating the covalently closed circular DNA (cccDNA), and then, this DNA is transcribed into different mRNAs; one is the pregenomic RNA (pgRNA). This RNA represents a replication intermediate and is reverse transcribed into viral DNA by viral polymerase. Together with translated surface antigens (HBsAg), the virus is able to leave the host cell for further infection [4, 5].

Nowadays, there are five antiviral drugs (lamivudine, adefovir, entecavir, telbivudine, and tenofovir) and IFN (conventional or pegylated) approved by the United States Food and Drug Administration for HBV treatment. According to international guidelines, treatment with antiviral drugs implicates a long-lasting administration. On one side, in the United States, treatment is for the entire life, while in Europe, treatment is prescribed until complete virus elimination and antigen disappearance. During long-term treatment, there is a risk of developing resistant HBV mutants. Favorable IFN

treatment is limited to less than 30% of the patients. Treatment success is defined with normalization of functional hepatic tests, seroconversion, decrease of viral load, and histological improvement. The loss of HBsAg is the best predictor of sustained remission after treatment but is not achieved very frequently with the actual HBV treatment [6–11].

The *Moringa oleifera* (*M. oleifera*) tree belongs to the family of Moringaceae [12]; its origin is in the north of India, and the cultivation is possible in tropical and subtropical countries [13]. The different parts of the tree are used especially in traditional medicine. Extracts of leaves of *M. oleifera* have shown the highest antioxidant activity [14]. Additionally to antioxidative properties, *M. oleifera* leaves are rich in vitamins, carotenoids, polyphenols, phenolic acids, flavonoids, alkaloids, glucosinolates, isothiocyanates, tannins, saponins/oxalates, and phytates [15]. There is only one report about antiviral effect of *M. oleifera* in cccDNA of HBV with no conclusive evidence [16]. In that paper, buffered and alcoholic extracts of *M. oleifera* (leaves) were evaluated as a preventive therapy. Since, extracts were added previously to transfection at HepG2 cells and only cccDNA was measured. In our study, *M. oleifera* was added after transfection of Huh7 cells, and antiviral, antifibrotic, and antioxidative responses in Huh7 cells expressing genotypes C or H of HBV were evaluated.

2. Materials and Methods

2.1. M. oleifera. The *M. oleifera* leaves were obtained from the south coast of Jalisco, Mexico and were validated by the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). The leaves were pulverized and dissolved in sterile water at a final concentration of 10 mg/mL. This aqueous extract was left to sit for 65 h at 4°C in the dark and was centrifuged 2 times at 13,000 ×g for 10 min, and then the supernatant was filter-sterilized (0.2 μm). The extracts were stored at −80°C.

2.2. Cell Culture. Huh7 cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (Gibco™) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Medium was changed every third day, and when cells reached a confluence of 80 to 90%, they were passaged to a new culture flask.

2.3. Viability. 5×10^4 Huh7 cells were seeded in 6-well plates. After 24 h of culture, the first dose of aqueous *M. oleifera* extract (0, 30, 45, 60, 120, 250, and 500 μg/mL) was added and repeated two times every 24 h until cell viability was determined at 72 h with a Guava ViaCount (Guava Technologies) flow cytometer. The cell concentration of stained cells was between 1×10^4 and 5×10^5 cells/mL in accordance with the recommendations of the manufacturer.

2.4. Plasmid Construction. The complete genome of HBV genotype H was amplified from DNA obtained from serum of an infected patient. The full genome of HBV was cloned in pGEM-T EASY (Promega). The clones were analyzed by restriction enzymes. Clones with the HBV were selected to subclone into pHY-106 vector (kindly donated by William Delaney). This vector contains the minimum HBV sequence necessary for viral transcription and replication after insertion of a full length HBV genome [17]. Then, selected clones (pHY-H) were analyzed by restriction enzymes (*EcoRI*/*XhoI*) (ChemiDoc™ XRS+, Bio-Rad), and DNA sequencing was performed to verify the integrity of the insert. The plasmids were analyzed by CLC Sequence Viewer and genotypified by NCBI genotyping. Plasmids with the complete genome of genotype C cloned into pHY-106 vector were a kind gift from Dr. Jake Liang.

2.5. Transfection. 2×10^5 Huh7 cells were seeded in 6-well plates. When the cells reached a confluence of 80 to 90%, cells were transfected transiently with 0.7 μg of plasmid pHY-C, pHY-H, or pHY-106 (control) and lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Transfection efficiency and green fluorescent protein (GFP) were detected by fluorescent microscopy. All transfections were performed in triplicate.

2.6. M. oleifera Treatment. After 6 h of transfection, cells were washed with PBS, then DMEM plus either 0, 30, 45, or 60 μg/mL of *M. oleifera* was added. This treatment was repeated further at 24 and 48 h, and the supernatant was stored at −80°C. Cells were harvested after 72 h.

2.7. Quantitative Real-Time Reverse Transcriptase PCR. RNA extraction was performed with TRIzol reactive according to the manufacturer's instruction. Briefly, 500 ng of total RNA was treated with DNase I (Thermo Fisher Scientific) and reverse transcribed using M-MLV (Invitrogen) according to the manufacturer's instruction, respectively. The resulting cDNA was used for real-time PCR using LightCycler® 96 (Roche), LightCycler TaqMan® Master (Roche), and appropriate primers for pgRNA (S: 5′-GTT CAT GTC CYA CTG TTC AAG CC, AS: 5′-TAG AGG GCT GAA GCG GTG TC). *CAT* (S: 5′-CTG ACA CTC ACC GCC ATC GCC, AS: 5′-GCT GTG CTC CAG GGC AGA AGG), *CTGF* (S: 5′-AAT GCT GCG AGG AGT GGG, AS: 5′-TGG CTC TAA TCA TAG TTG GGT CT), and *TGF-beta1* (S: 5′-CAC TGC TCC TGT GAC AGC AG, AS: 5′-GGT GGC CAT GAG AAG CAG GA) and *IFNbeta1* (Applied Biosystems: Hs01075529_m1). The relative expression was normalized by *GAPDH* (S: 5′-CAT GAG AAG TAT GAC AAC AGC CT, AS: 5′-AGT CCT TCC ACG ATA CCA AAG T) expression.

2.8. HBV-DNA Quantification. A mixture from the triplicates from the cell culture supernatant of transfected Huh7 cells was prepared with proportional volumes. DNA was extracted from 200 μL of the mixture using QIA amp minElute Virus Spin Kit (Qiagen). 2.5 μL was used as a template for real-time PCR using 480 SBYR Green I master (Roche Molecular Systems, Inc.), according to the manufacturer's instruction. Two standard curves were generated: one for HBV-DNA quantitation and the other to quantify the plasmid to be subtracted to avoid interference with the quantity of the viral DNA. Appropriated primers were PHY-106, S: 5′ GTAAACTGC CCACTTGGCAG, R: 5′ AGCGATGACTAATACGTAGAT G. HBV, S: 5′ TTCGCAGTCCCCAAYCTC (310-327), and R: 5′ CAMACGGGCAACATACCTTG (474-455).

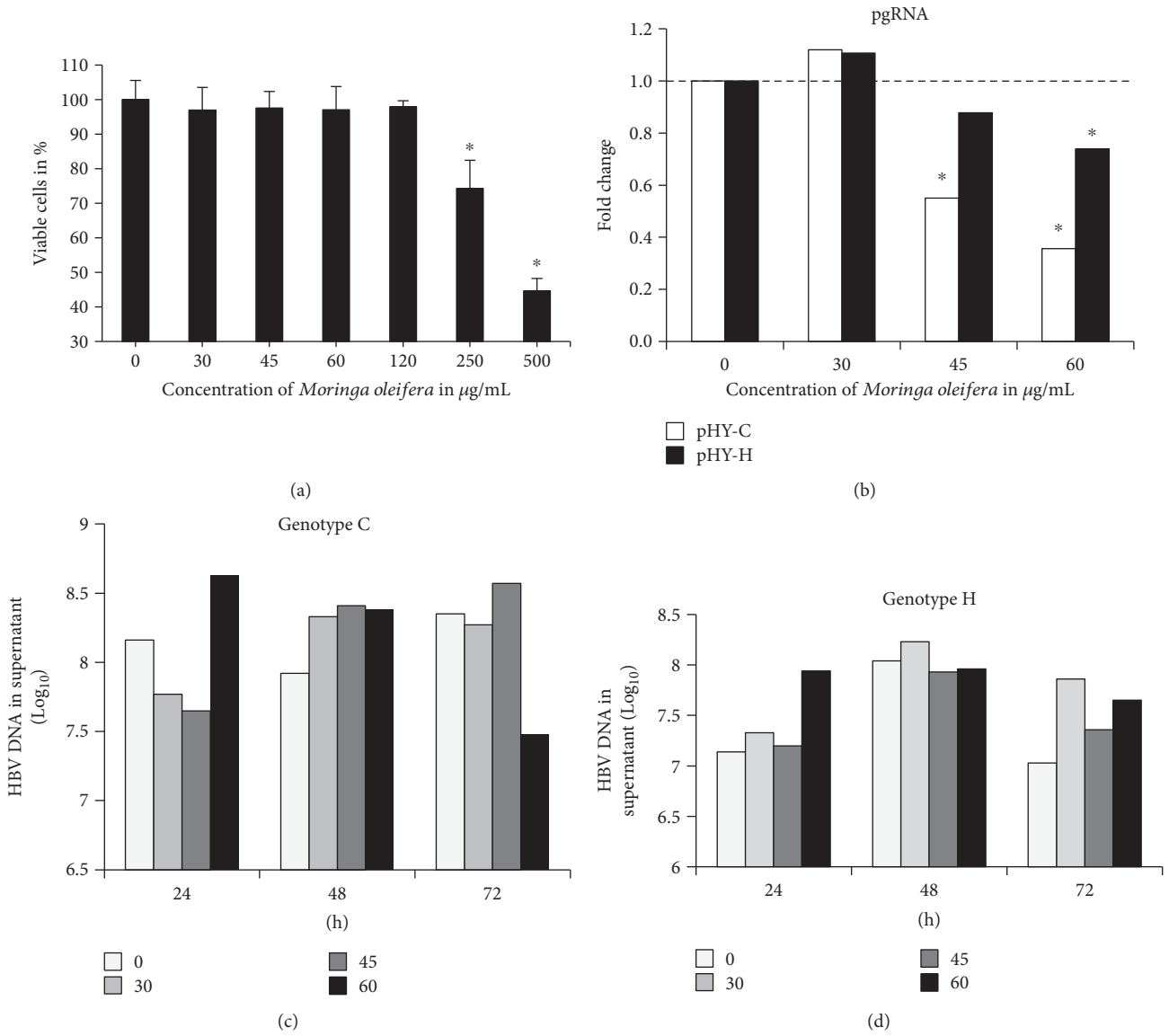

Figure 1: (a) Effect of aqueous leaf extract of *Moringa oleifera* on the viability of Huh7 cells. $^{*}p < 0.05$. (b) pgRNA expression of Huh7 cells transfected with genotype C (white bars) or H (black bars) of HBV and treated with *Moringa oleifera*. Fold change ($2^{-\Delta\Delta Ct}$) was calculated with reference data without treatment. $^{*}p < 0.05$ based on ΔCt values. (c and d) HBV-DNA was quantified from supernatants recovered at 24, 48, and 72 hours after treatment by real-time PCR (copy number expressed in Log_{10}); *x*-axis specifies the *Moringa oleifera* dose at μg/ml.

2.9. Secretion of HBsAg and IL-6. HBsAg secretion was detected with Monolisa™ HBsAg ULTRA (Bio-Rad) in 100 μL of the cell culture supernatant of transfected Huh7 cells according to the manufacturer's instruction. The OD was read at 450/700 nm. IL-6 was determined by competitive ELISA (R&D Systems) according to the manufacturer's instructions. Levels of IL-6 and HBsAg were determined in an ELISA reader (μQuant Bio-Tek, Instruments, Inc.).

2.10. Statistical Analysis. To compare the data between two independent groups, the *t*-test was used. For no parametric data, Kruskal-Wallis and Mann–Whitney *U* tests were applied. Values with $^{*}p < 0.05$ were considered statistically significant. For statistical analysis, SPSS version 20 and Microsoft Office Excel 2010 were used.

3. Results

3.1. Viability. The aqueous *M. oleifera* extract decreased the viable cell number ($p < 0.05$) at concentrations of 250 and 500 μg/mL (74.3 and 44.7%, resp.) (Figure 1(a)). In general, the lower the concentration of *M. oleifera*, the lower was the number of death cells. Concentrations of 30, 45, 60, and 120 μg/mL of *M. oleifera* decreased the number of viable cells in an average of 2.8%. Thus, for further experiments, the three lowest concentrations of *M. oleifera* (30, 45, and 60 μg/mL) were used to test our proof of concept.

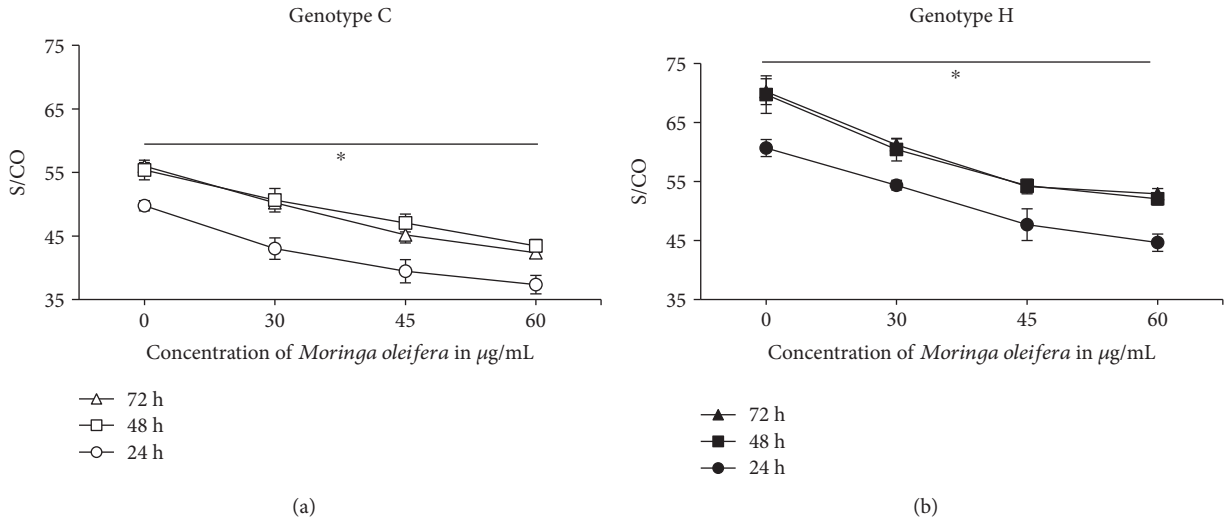

Figure 2: HBsAg secretion after *Moringa oleifera* treatment. Huh7 cells transfected with genotype C (a) and genotype H (b) of HBV and treated with 0, 30, 45, and 60 μg/mL of *Moringa oleifera*. $^*p < 0.05$.

3.2. Pregenomic RNA Expression. There was a slight tendency of pgRNA expression reduction in cell cultures of transfected cells with HBV genotypes C and H and the treatment of 30 μg/mL of *M oleifera* (Figure 1(b)). The doses of 45 and 60 μg/mL of *M. oleifera* reduced ($p < 0.05$) the pgRNA expression with HBV genotype C-transfected cells (0.55 and 0.36, resp.). Cells transfected with HBV genotype H showed a decrease (0.74, $p < 0.05$) at the highest concentration of *M. oleifera* studied (60 μg/mL).

3.3. HBV-DNA Quantitation. Treatment with *M. oleifera* reduced HBV-DNA from genotype C, 1.09 Log_{10} at 60 μg/mL at 72 h of treatment (Figure 1(c)). Viral DNA from genotype H was not reduced by the treatment at any dose and time (Figure 1(d)).

3.4. HBsAg Secretion. The HBsAg secretion in the supernatant of transfected Huh7 cells with genotypes C and H of HBV was decreased ($p < 0.05$) independently of the dose of *M. oleifera* treatment (Figures 2(a) and 2(b)). At 24 h, the HBsAg secretion was lower than at 48 and 72 h, though *M. oleifera* treatment displayed the same effect. The ratio of immunoassay signal strength of the sample to cut-off (S/CO) at 24 h without treatment was 49.8 with genotype C of HBV. *M. oleifera* treatment of 30, 45, and 60 μg/mL reduced the S/CO to 43.0, 39.5, and 37.3, respectively. This data represents a decrease up to 25.1% of HBsAg secretion with the highest dose of *M. oleifera* at 24 h. At 48 and 72 h, the reduction of secretion was similar (21.5 and 24.3%, resp.). Compared with genotype H of HBV, HBsAg secretion was higher but treatment with *M. oleifera* also reduced the S/CO value. At 24 h, the S/CO without treatment was 60.6 and decreased with *M. oleifera* treatment of 30, 45, and 60 μg/mL (54.4, 47.7, and 44.6, resp.). Independently of the time point of measurement, *M. oleifera* treatment reduced the HBsAg secretion between 24.6 and 26.4% (72 and 24 h, resp.).

3.5. CTGF and TGF-β1 Gene Expression. The gene expression of *CTGF* and *TGF-β1* decreased with *M. oleifera* treatment independently of the genotype of HBV (Figures 3(a) and 3(b)). There was no difference in *CTGF* expression in Huh7 cells compared with Huh7 cells transfected with the plasmid without HBV insert (pHY-106; Figure 3(a)). Transfection of the cells with genotypes C and H of HBV increased the expression (1.64 and 1.2 times, resp.). *M. oleifera* treatment with 60 μg/mL significantly reduced *CTGF* expression (genotype C up to 0.83 times and genotype H up to 0.71 times). *TGF-β1* expression was induced by the transfection of the plasmid without HBV insert (pHY-106) compared with Huh7 cells ($0.71 < 1$; Figure 3(b)). The transfection with HBV genotypes C and H enlarged this induction of expression (1.63 and 1.32 times, resp.). *M. oleifera* treatment of 30, 45, and 60 μg/mL significantly reduced *TGF-β1* expression with genotype C of HBV (1.01, 0.95, and 0.41, resp.). With genoytpe H of HBV, the decrease ($p < 0.05$) of *TGF-β1* expression started with 45 μg/mL of *M. oleifera* treatment (0.81 and 0.58 times with 60 μg/mL dose).

3.6. CAT Gene Expression. The gene expression of *CAT* was decreased in Huh7 cells transfected with the plasmid without HBV insert (pHY-106) compared with Huh7 cells ($1 > 1.59$; Figure 3(c)). Transfection with genotypes C and H of HBV strongly decreased *CAT* expression (0.19 and 0.41 times). Interestingly, treatment with *M. oleifera* restored this reduction ($p < 0.05$) starting with 45 μg/mL dose with both genotypes of HBV. The increase of *CAT* expression was more marked with genotype H than with genotype C of HBV (45 μg/mL dose $0.94 > 0.53$ and 60 μg/mL dose $1.63 > 0.84$).

3.7. IFNbeta1 Gene Expression. The gene expression for *IFNβ1* was increased by transfection of Huh7 cells with HBV genotypes C and H compared with the plasmid without insert (pHY-106; $1 < 1.34 < 1.60$; Figure 3(d)). *M. oleifera* treatment reduced the *IFNβ1* expression ($p < 0.05$) with 45 and 60 μg/mL doses with HBV genotype H (0.95 and 0.74

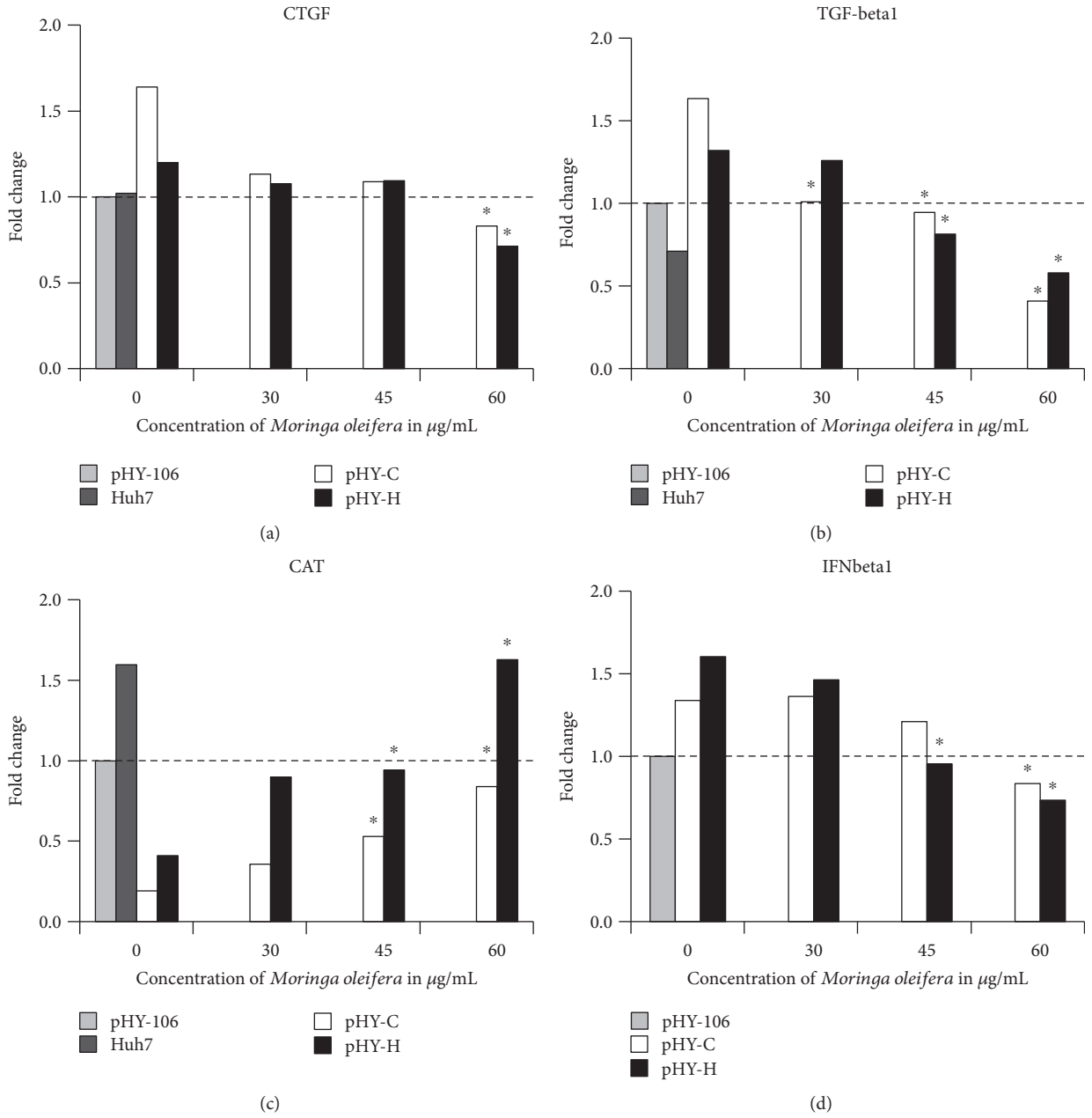

Figure 3: (a) *CTGF*, (b) *TGF-beta1*, (c) *CAT*, and (d) *IFNbeta1* gene expressions of Huh7 cells transfected with genotype C (white bars) or H (black bars) of HBV and treated with *Moringa oleifera*. Fold change ($2^{-\Delta\Delta Ct}$) was calculated with reference data without treatment and plasmid pHY-106 (without HBV genome). $^{*}p < 0.05$ based on ΔCt values.

times, resp.). With genoytpe C of HBV, the highest dose of *M. oleifera* treatment decreased ($p < 0.05$) the *IFNβ1* expression (0.83 times).

3.8. IL-6 Quantitation in the Supernatant of Huh7-Transfected Cells. IL-6 levels determined by ELISA in the supernatant of transfected Huh7 cells were detected only at 24 hours (genotype C from 3.7 to 6.14 ng and genotype H from 0 to 4.89 ng). The quantitation of IL-6 at 48 and 72 hours after *M. oleifera* did not show positive values. Interestingly, after *M. oleifera* treatment, IL-6 showed a tendency to decrease in a dose-dependent manner in the supernatant of Huh7-transfected cells with genotypes C and H (Figure 4).

4. Discussion

The viability of Huh7 cells was not affected with the aqueous extract of the *M. oleifera* leaves at the concentrations of 30, 45, 60, and 120 μg/mL after 72 h (Figure 1). There is no report about the cytotoxic effect of *M. oleifera* in Huh7 cells. Waiyaput et al. [16] studied HepG2 and COS-7 cell viability after 5 days of treatment with an ethanolic leaves extract of *M. oleifera* (50, 150, and 300 μg/mL). The viable cell number

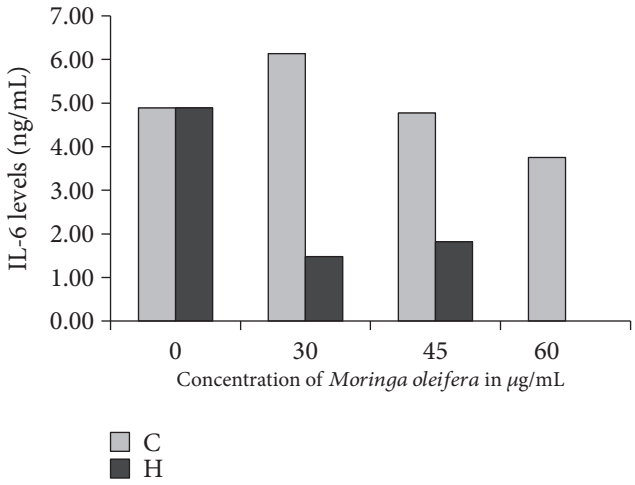

Figure 4: IL-6 levels after *Moringa oleifera* treatment determined in the supernatant of Huh7 cells transfected with genotypes C and H. A mixture from the triplicates from the cell culture supernatant of transfected Huh7 cells was prepared with proportional volumes. ELISA was performed with a capture-specific mouse anti-human IL-6 antibody (100 μL at 2 μg/mL). After incubation and washing steps, detection was performed with Biotinylated Goat anti-human IL-6 detection antibody (100 μL at 50 ng/mL) using streptavidin-HRP.

of HepG2 cells decreased about 15.5, 27.9, and 65%, respectively. Compared with COS-7 cells, the cell viability was reduced significantly in comparison with HepG2 cells with the treatment of 150 μg/mL (63.5 > 27.9%). In a different study, Sangkitikomol et al. [18] reported the cell viability of HepG2 cells treated with an ethanolic extract of *M. oleifera* leaves at 29 h. *M. oleifera* concentrations of 100, 200, and 400 μg/mL had no cytotoxic effect on the cells, and higher concentrations, such as 600, 800, and 1000 μg/mL, induced only a slight tendency in cell death (5–10% of viable cell reduction). Regarding this issue, scientific literature shows us a report about cell viability of HeLa, HepG2, MCF-7, CACO-2, and L929 cells at 24 h with treatment with an essential oil of *M. oleifera* seeds [19]. In all cell lines, viability decreased with the increase of *M. oleifera* concentration. Our results shown here are in accordance with the published data. Cell viability is affected by the *M. oleifera* in a dose-dependent manner. The different extracts showed at various times of measurement an increase of cytotoxicity in distinct cell lines. The extract preparation is not standardized between the studies. In dependence of the preparation of the extract and the buffer used, there is a divergence of components in the extracts. This variation of the components can cause distinct cytotoxic effects in the same cell line.

The pgRNA expression decreased with an increase of *M. oleifera* treatment in accordance with the HBsAg secretion, which was reduced independently of *M. oleifera* concentration and HBV genotype (Figure 2). There are no reports about the effect of *M. oleifera* on pgRNA expression and/or HBsAg secretion. There is only one study published about HBV and *M. oleifera* [16]. They measured the cccDNA expression in HepG2 cells treated with a buffer or alcoholic extract of *M. oleifera* leaves and further transfected with HBV. The alcoholic extract of *M. oleifera* (30 μg/mL) had no effect on the cccDNA expression, meanwhile 0.3 μg of total protein of a buffer extract of *M. oleifera* decreased significantly the cccDNA expression (0.2 times). The methodology of the experiment varies with our study. In the present study, we transfected first Huh7 cells with two different genotypes of HBV and then treated with *M. oleifera*. Thus, our proof of concept was rationalized in a different manner, since we studied *M. oleifera* effect as a therapeutic agent instead of a preventive action as Waiyaput et al. [16] reported. Additionally, our data shows that pgRNA did not differentiate the replicative virus from the transfected plasmid. However, it is very important to state that the drop in pgRNA levels were statistically evidenced by repeating the experiment three times in triplicate cultures as well as by real-time PCR. Nevertheless, this pgRNA level reduction was only supported by a slightly reduction in HBV-DNA levels in the supernatant of Huh7 cells transfected with genotype C at 60 μg/mL after 72 hours of treatment (Figure 1(c)). Probably, the reduction observed in the pgRNA of genotype H was not enough to reduce the rate of replication of the virus, but the antifibrotic and antioxidant effects deduced by the changes in *CTGF*, *TGFβ*, and *CAT* gene expressions with *M. oleifera* treatment support the fact that Moringa might be used as a valuable nutritional supplement for patients with chronic hepatitis B. There are additional studies, both *in vivo* and *in vitro*, reporting effects of *M. oleifera* in different viruses, like herpes simplex virus type 1 [20], Epstein-Barr virus [21], Newcastle disease virus [22], and infectious bursal disease virus [23]. Nevertheless, the complete elucidation of the molecular mechanisms driving *M. oleifera* action remains to be defined.

CTGF expression was not modified by transfection with the plasmid without HBV insert (pHY-106) in contrast to an increase of expression when transfected with HBV (Figure 3(a)), though expression of *TGF-β1* was enhanced in cells transfected with the HBV-less plasmid (pHY-106; Figure 3(b)). This effect was amplified when cells were transfected with HBV genotypes C and H. *M. oleifera* decreased the expression of *CTGF* and *TGF-β1* in Huh7 cells transfected with genotypes C and H of HBV (Figures 3(a) and 3(b)).

HBV infection causes a profibrogenic environment with increase of CTGF and TGF-β1 protein expressions in human hepatic stellate cells (HSCs) cocultivated with HBx [24]. Pan et al. reported an increase of TGF-β1 protein expression in HepG2 cells expressing HBxAg [25]. *CTGF* and *TGF-β1* expressions activate HSCs and triggers a profibrogenic response, increase of extracellular matrix, and expression of collagen 1 alpha [26, 27]. In this study, we detected antifibrogenic potential of *M. oleifera*. There is no data about the possible mechanism of regulation of *CTGF* and *TGF-β1* expressions by *M. oleifera*. However, it is known that quercetin is one of the components of *M. oleifera* [28, 29], and our group [30] reported the potential quercetin effect to regulate the balance of pro- and antifibrogenic stimuli, rendering reduction of activated HSCs and ameliorating fibrosis in rats.

Quercetin in *M. oleifera* leaves moderates inflammation of high-fat-fed mice [28]. Park and Chang [31] showed a

decrease of mRNA and protein expression of fibronectin, type I collagen, and plasminogen activator inhibitor I in a rat kidney fibrosis model. On the other hand, hepatoprotective effects in rats by *M. oleifera* were reported by Fakurazi et al. [32]. Our results are in accordance with that published data. Furthermore, it is necessary to measure more inflammation and fibrosis markers and oxidative stress molecules to obtain a wider spectrum about the potential of *M. oleifera* in cells transfected with HBV.

The expression of *CAT* was decreased in transfected Huh7 cells with HBV (Figure 3(c)). After *M. oleifera* treatment, the *CAT* expression was reestablished. There are studies *in vivo* and *in vitro* about antioxidant components of *M. oleifera* leaves [33]. Sreelatha and Padma [34] reported an antioxidative activity in vitro arisen from phenol and flavonoid components of different parts of the *M. oleifera* plant. *In vitro*, the higher the total content of polyphenols the higher the antioxidant activity [35, 36]. Verma et al. showed potential of *M. oleifera* to increase enzymatic levels of catalase and superoxide dismutase in rats [37]. It is known that infection with HBV decreases antioxidative components [38] and increases reactive oxygen species (ROS) and prooxidants [39]. The endoplasmic reticulum of the host cell is saturated because of the high amount of proteins being synthesized for the viral particle assembly. This provokes an imbalance in the transport of calcium to the mitochondria, which results in inflammation, oxidative stress, and increase of ROS [39–42]. *M. oleifera* acts on the side of antioxidants restoring the imbalance by increasing *CAT* expression. Furthermore, it would be interesting to study the protein expression of catalase and the expression of other anti- and prooxidative components, which could be modified in response to *M oleifera*.

The gene expression of *IFNβ1* was increased in Huh7 cells transfected with both genotypes of HBV and further decreased with increasing *M. oleifera* treatment (Figure 3(d)). It is reported that cells in response to a virus attack enhance IFNβ production as a defense [43, 44]. Patients infected with HBV genotype C are lower responders for IFNα treatment, probably the same resistance is for IFNβ treatment. In this study, in spite that *M. oleifera* treatment decreased mRNA-IFNβ, the decrease in cellular pgRNA and in the HBV-DNA observed in the supernatant from Huh7-transfected cells with genotype C could be influenced by a different mechanism not by IFNβ production. In the case of genotype H, there are no reports about the reaction to interferon type 1 treatment. In this regard, we have previous unpublished results showing that genotype H responds to IFNβ in a similar way as genotype A which is more sensitive to IFNα treatment. Probably, the decrease observed in the mRNA-IFNβ nullifies the antiviral activity of *M. oleifera* in Huh7 cells transfected with genotype H.

Also, in this study, *M. oleifera* showed a tendency to decrease IL-6 in Huh7 cells transfected with genotypes C and H (Figure 4). This interleukin is produced mainly by Kupffer cells and regulates HBV gene expression and replication shortly after infection [45]. Complete neutralization of IL-6 for treatment of certain diseases may represent a risk if the patient is HBV infected [45]. Nevertheless, a balance of IL-6 is very important since transcriptionally enhanced IL-6 strongly facilitates the activation of human hepatic stellate cells favoring the advance of liver fibrosis. In animal models using rats with hepatotoxicity and diabetes induced by streptozotocin, *M. oleifera* showed a hepatoprotective, anti-inflammatory, and lipid-lowering effect in the treated diabetic rats compared to controls. IL-6 was returned to normal levels as well as TNF-α in the rats treated with Moringa [46]. It is possible that in patients with chronic hepatitis B, supplementation with Moringa could delay fibrosis progression.

5. Conclusion

Aqueous leaf extract of *M. oleifera* had no cytotoxic effect on Huh7 cells at selected concentrations (30, 45, and 60 μg/mL) but modulated the pgRNA levels and HBsAg secretion positively in Huh7 cells transfected with genotypes C and H of HBV. The antiviral effect determined by reduction of DNA-HBV in the supernatant was only evidenced by genotype C and probably was independent of *IFNβ1* activity. In spite of the differences in the antiviral activity between genotypes C and H, gene expression modification of *CTGF*, *TGF-β1*, and *CAT* was modulated by *M. oleifera* treatment, favoring an antifibrotic, antioxidative, and anti-IL-6 environment. Further experiments are necessary to extend the obtained benefits of *M. oleifera* on HBV.

Ethical Approval

The study was approved by the ethics committee of *"Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara."*

Disclosure

An earlier version of this work was presented as an abstract at the XII National Meeting of the Mexican Association of Hepatology.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

The Hydroalcoholic Extract Obtained from *Mentha piperita* L. Leaves Attenuates Oxidative Stress and Improves Survival in Lipopolysaccharide-Treated Macrophages

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Mentha piperita L. (peppermint) possesses antimicrobial properties, but little is known of its ability to modulate macrophages. Macrophages are essential in bacterial infection control due to their antimicrobial functions and ability to link the innate and adaptive immune responses. We evaluated the effects of the peppermint leaf hydroalcoholic extract (LHAE) on cultured murine peritoneal macrophages stimulated or not with lipopolysaccharide (LPS) *in vitro*. Vehicle-treated cells were used as controls. The constituents of the extract were also identified. Epicatechin was the major compound detected in the LHAE. LPS-induced macrophage death was reversed by incubation with LHAE (1–30 μg/ml). Higher concentrations of the extract (≥100 μg/ml) decreased macrophage viability (49–57%) in the absence of LPS. LHAE (1–300 μg/ml) attenuated H_2O_2 (34.6–53.4%) but not nitric oxide production by these cells. At similar concentrations, the extract increased the activity of superoxide dismutase (15.3–63.5-fold) and glutathione peroxidase (34.4–73.6-fold) in LPS-treated macrophages. Only LPS-unstimulated macrophages presented enhanced phagocytosis (3.6–6.6-fold increase) when incubated with LHAE (3–30 μg/ml). Overall, the LHAE obtained from peppermint modulates macrophage-mediated inflammatory responses, by stimulating the antioxidant pathway in these cells. These effects may be beneficial when the excessive activation of macrophages contributes to tissue damage during infectious disease.

1. Introduction

Macrophages are on the first line of the host's immune response to bacterial infection. Indeed, these cells play detrimental roles in pathogen recognition, bacterial killing, and antigen presentation, leading to further activation of adaptive immune responses (see for review [1–3]). Gram-negative bacterial strains are major pathogens causatives of severe infectious diseases in humans, associated with high mortality rates [4, 5]. This is due not only to their ability to become resistant to the available antimicrobials [4] but also depends on an effective macrophage response to these pathogens [6].

The production of oxidant species by macrophages is a hallmark of the inflammatory response to infection (see for

review [7, 8]). Oxidant species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are produced following phagocytosis of the pathogen by these cells as part of their machinery to respond to harmful insults [9]. Alongside an excessive nitric oxide (NO) production, increased levels of prooxidant species may lead to damage and poor perfusion of vital organs of the host, contributing to multiple organ failure; thus, to counteract this response, antioxidant pathways are activated [10].

Natural antioxidants including phenolic compounds have been identified in a variety of plants. Additionally, antimicrobial properties have been attributed to these compounds, suggesting them to be potential therapies for bacterial infections. *Mentha piperita* L., a member of the family Lamiaceae and popularly known as peppermint, is native to the Mediterranean region and has been spread worldwide due to its medicinal properties, taste, and aroma [11]. Its medicinal properties include antitumor, antimicrobial, and antioxidant actions and have been reported especially for its essential oil [12–17]. Of importance, *M. piperita* essential oil was previously shown to be effective against Gram-negative and Gram-positive bacteria and to act as a potential antioxidant *in vitro* [12]. This essential oil was also shown to reduce the numbers of leukocytes in a murine model of skin inflammation [18] and modulate cytokine production *in vivo* [19]. However, the underlying mechanisms of the effects of *M. piperita* on macrophages remain unclear. Considering peppermint antioxidant and anti-inflammatory potentials, we hypothesized whether its leaf hydroalcoholic extract (LHAE) is able to modulate macrophage-mediated inflammatory responses. Therefore, the aim of this study was to investigate the effects of the peppermint leaf hydroalcoholic extract (LHAE) on cultured murine peritoneal macrophages *in vitro*.

2. Material and Methods

2.1. Plant. The leaves of *M. piperita* were collected in September at Santa Luzia, Maranhão, Brazil (4°4′8″S, 45°41′24″W). A voucher specimen (number 01275) was deposited in the herbarium Ático Seabra of the Federal University of Maranhão, São Luís, Brazil.

2.2. Preparation of the Crude Hydroalcoholic Extract. The collected leaves were washed in running water before being dried under forced air circulation at 45°C. The dried leaves were triturated, and the resulting powder was macerated for 10 days in 70% ethyl alcohol (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. The mixture was filtered through cellulose filter paper (Whatman No. 4, GE Healthcare UK, Amersham, UK) and evaporated to dryness under reduced pressure using a rotary evaporator (Eyela N-1200BV-W, Tokyo, Japan) at 40°C. The residual solvent was removed in a vacuum centrifuge at 40°C to yield crude ethanol extracts of leaves.

2.3. Chemical Characterization by High-Performance Liquid Chromatography (HPLC). For HPLC analysis, the peppermint LHAE was dissolved in methanol and water to a final concentration of approximately 5 mg/ml and filtered through a 0.22 μm nylon filter. An HPLC (Surveyor Plus/Finnigan) coupled to an ultraviolet-visible detector (HPLC-UV-Vis), with an ACE 5 C18 reverse phase analytical column (250 × 4.60 mm, 5 μm, ACE) protected by a C18 precolumn (4 × 3 mm, 5 μm, Gemini, Phenomenex), was used for the analysis. Compounds were separated at room temperature using an elution gradient at a flow rate of 0.6 ml/min. Mobile phases consisted of purified water containing 0.1% acetic acid (A) and methanol (B). The following gradient was used: 0–2 min, 5% B; 2–10 min, 25–40% B; 10–20 min, 40–50% B, 20–30 min, 50–60% B; 30–40 min, 60–70% B; and 40–50 min, 70–80% B. Injection volume was 10 μl and UV-Vis detection was performed at 254 nm. Ursolic acid, epicatechin, caffeic acid, rutin, quercetin, naringenin, and kaempferol standards were diluted and analyzed under the same conditions.

2.4. Macrophage Assays

2.4.1. Animals. Nonfasted outbred male Swiss mice (2-3 months old) were used. Mice were obtained from the animal's facility of the Universidade CEUMA (UNICEUMA). Mice were kept in a climatically controlled environment (room temperature of 22 ± 2°C and humidity of around 60%) under 12 : 12 h light-dark cycle (lights on 07:00 h). All procedures were approved by the Ethics Committee of UNICEUMA and carried out in accordance with the Brazilian Society for Animal Welfare (SBCAL).

2.4.2. Macrophage Culture and Viability. Peritoneal cells were collected from animals injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS, Sigma-Aldrich, Brazil) containing 1% oyster glycogen (Sigma-Aldrich, Brazil). Briefly, 18 h following injection, the peritoneal cavity was washed with 10 ml of cold PBS and the peritoneal cells were harvested, centrifuged (10 min, 4°C), and resuspended (final concentration of 2×10^6 cells/ml) in DMEM-Glutamax® (Life Technologies, Brazil) containing 10% FCS (v/v, Life Technologies, Brazil) and 1x penicillin-streptomycin (Sigma-Aldrich, Brazil). Cells (6×10^5/well) were incubated in 96-well plates, at 37°C under 5% CO_2, and after 2 h, nonadhered cells were removed and the adherent cells (macrophages) were incubated with either peppermint LHAE (1–300 μg/ml) or vehicle (1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Brazil) in PBS), and after 15 min, stimulated with *K. pneumoniae* lipopolysaccharide (LPS, 100 ng/ml in PBS, Sigma-Aldrich, Brazil) or PBS for 24 h. After this period, the supernatant was collected and stored at −80°C for further analysis of NO_2^-/NO_3^- (NO end products) and H_2O_2 concentrations. For analysis of macrophage viability, the remaining cells were incubated with PrestoBlue® reagent (1 : 10, Life Technologies, Brazil) for 90 min; and then, the absorbances were read at 550 and 650 nm. Results were calculated according with the manufacturer's instructions and are expressed as absorbance in percentage (%) of cell viability in relation to vehicle/PBS-treated cells.

2.4.3. Phagocytosis. In a separate series of experiments, peritoneal macrophages were obtained and cultured (6×10^5/

well) as described above in eight chamber culture slides (BD Falcon). Just after removal of nonadherent cells, macrophages were incubated with 2 μm fluorescent latex beads (1 : 100; 5 μl/well; Sigma-Aldrich, Brazil), for 24 h as described by Fernandes et al. [20]. After the incubation period, the cell culture medium was removed and each well was washed three times with PBS. Wells were fixed in 2% paraformaldehyde for 10 min and washed three times with PBS for the removal of excessive paraformaldehyde. Then, 10 μl PBS were added per well and slides were covered with a glass slip. Slides were analyzed in a fluorescence microscope (Zeiss Axio Image Z2, German, ×40 objective, bright field). Two lots of 100 cells were counted for each well, and the average for each well was considered as an *n* number. Results are expressed as percentage of cells containing beads and number of phagocytosed beads per 100 cells.

2.4.4. NO End Product (Nitrate NO_3^- plus Nitrite NO_2^-) Measurement. The NO_2^-/NO_3^- content was measured by the Griess reaction assay as an indicator of NO production in supernatant samples as previously described [21]. NO_3^- was reduced to nitrite (NO_2) by incubating 80 μl of the sample with 20 μl of 1 U/ml nitrate reductase and 10 μl of 1 mM NADPH for 30 min at 37°C in a 96-well plate. Next, 100 μl Griess reagent (5% v/v H_3PO_4 containing 1% sulfanilic acid and 0.1% N-1-napthylethylenediamine) was added and incubated for 15 min at 37°C. Absorbance at 550 nm was immediately measured using a spectrophotometer (Plate reader MB-580; Heales, Shenzhen, China). After subtraction of background readings, the absorbance in each sample was compared with that obtained from a sodium nitrite (0–100 μM) standard curve and expressed as NO^x concentrations (μM).

2.4.5. Measurement of H_2O_2 Concentrations. H_2O_2 production by macrophages was measured by using a H_2O_2/peroxidase assay kit (Amplex Red H_2O_2/Peroxidase assay kit, Invitrogen, Brazil), as described by Mendes et al. [21]. Briefly, 50 μl of the supernatants were incubated with 50 μl of a 0.05 M $NaPO_4$ (pH 7.4) solution containing 0.2 U/ml horseradish peroxidase (HRP) and 25.7 mg/ml Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) for 2 h, at 37°C. Samples incubated with 0.05 M $NaPO_4$ only were used as controls. After incubation, the absorbance was read at 560 nm. After subtraction of background readings, the absorbance in each sample was compared with that obtained from a H_2O_2 standard curve (0–40 μM). H_2O_2 concentrations are expressed in μM.

2.4.6. Antioxidant Enzyme Activities

(1) Sample Preparation. In another series of experiments, macrophages were obtained, isolated, cultured (6×10^5/well) in 24-well plates, and stimulated as described above. Following incubation with LPS (24 h), the supernatant was removed and 500 μl of 0.05 M $NaPO_4$ (pH 7.4) (containing ethylenediaminetetraacetic acid (EDTA), 1 mM) was added to each well. Plates were placed on ice for 15 min. Then, cells were scraped from each well, transferred to tubes, and lysed by three snap freezing/defrosting times. Tubes were centrifuged at 10,000 ×g for 10 min at 4°C, and the supernatants were used for the enzyme activity assays.

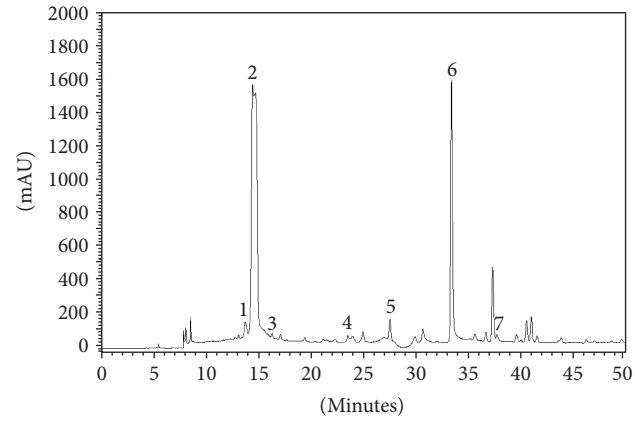

Figure 1: HPLC analysis of the peppermint LHAE. Peaks are numbered 1–7 and were shown to coelute with (1) ursolic acid, (2) epicatechin, (3) caffeic acid, (4) rutin, (5) quercetin, (6) naringenin, and (7) kaempferol.

Table 1: Registered retention times obtained by HPLC analysis of the peppermint LHAE. Retention times in minutes were registered for each peak.

Peak number	Retention time in min	Compound
1	13.8	Ursolic acid
2	14.5	Epicatechin
3	16.3	Caffeic acid
4	23.6	Rutin
5	27.6	Quercetin
6	33.5	Naringenin
7	37.8	Kaempferol

(2) Superoxide Dismutase (SOD) Activity Assay. SOD activity was measured as described by Ukeda et al. [22], with modifications. Briefly, 20 μl of sample were incubated with 200 μl of a solution containing 2.5 ml sodium carbonate buffer (50 mM; pH 9.4) and 0.1 ml of a mixture containing xanthine (3 mM), EDTA (3 mM) and 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, 153 mU/ml), in the presence and absence of SOD. Samples (200 μl/well) were added in 96-well plates and the absorbance was read at 470 nm for 20 min. Results are expressed as milliunits (mU) of SOD/mg of protein. One unit of SOD was defined as the amount of enzyme capable of dismutating 1 μmol of O_2^-/min.

(3) Glutathione Peroxidase (GPx) Activity Assay. GPx activity was determined as described by Paglia and Valentine [23]. For this, 30 μl of sample per well (diluted 1 : 3) was incubated for 5 min at 37°C, with 145 μl per well of 0.05 M phosphate buffer (pH 7.4) containing 0.1 M EDTA, 5 μl of glutathione (GSH, 80 mM), and 5 μl glutathione reductase (0.0096 U/μl). After incubation, 5 μl of 0.46% *tert*-butyl

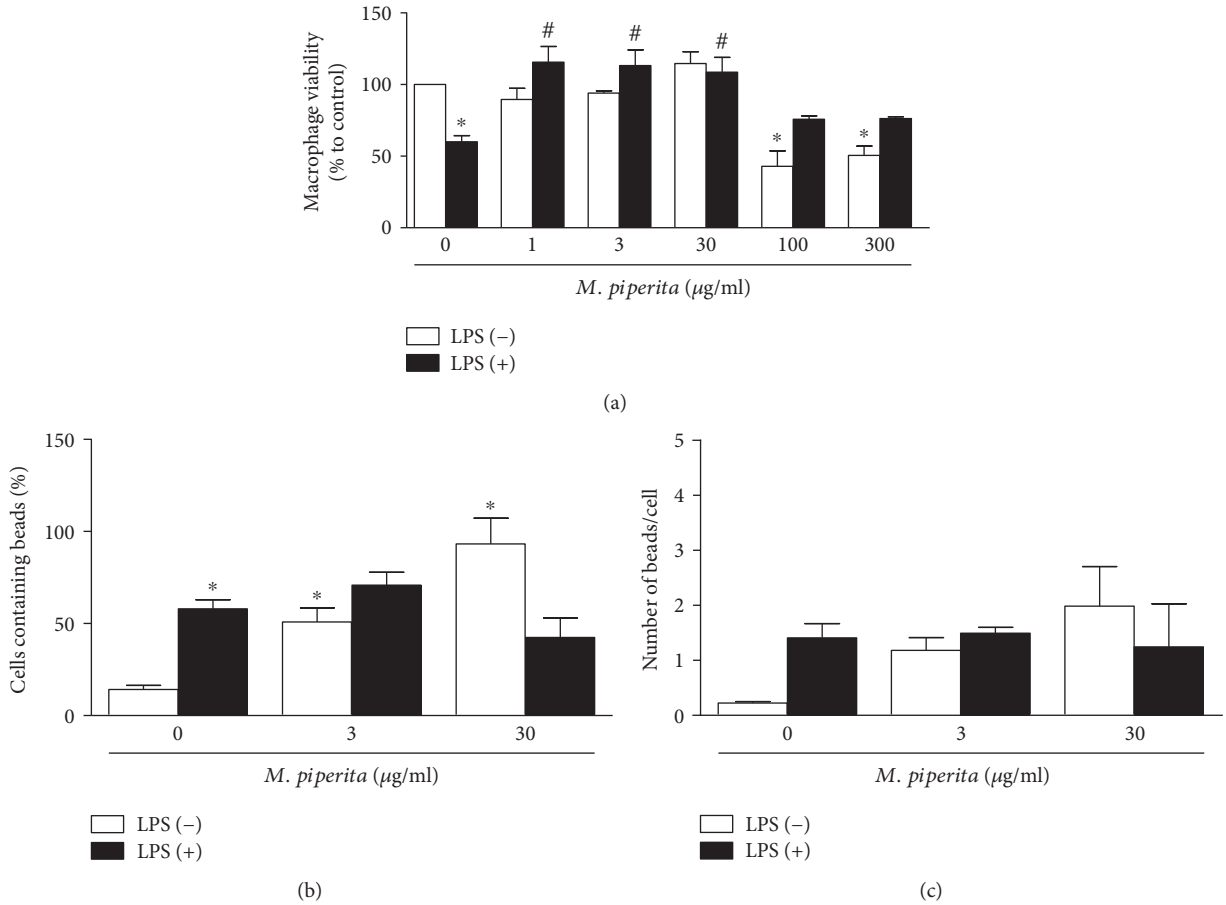

Figure 2: Effect of peppermint LHAE on peritoneal macrophage viability and phagocytosis. Cell viability (a), number of cells containing beads (b), and number of beads per cell (c) were quantified on peritoneal macrophages pretreated with peppermint LHAE (1–300 μg/ml, in 1% DMSO in PBS) and stimulated with LPS for 24 h. Vehicle-treated cells were used as controls. Data are expressed as mean ± SEM. $^{*}p < 0.05$ compared with vehicle-treated cells; $^{\#}p < 0.05$ compared with LPS-treated cells.

hydroperoxide solution and 10 μl of 1.2 mM NADPH were added to each well. Absorbances were monitored at 340 nm for 10 min. The results are expressed as μmol of GSH/min/mg of protein.

2.5. Statistical Analysis. Data are expressed as mean ± standard error (SEM). Differences between groups were analysed by two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison tests, or paired *t*-test as appropriate. Percentages of inhibition were calculated as the mean of the inhibitions obtained for each individual experiment. p values < 0.05 were considered statistically significant.

3. Results

3.1. Chemical Analysis. HPLC analysis of peppermint LHAE detected the presence of seven peaks that coeluted with ursolic acid, epicatechin, caffeic acid, rutin, quercetin, naringenin, and kaempferol (Figure 1). Epicatechin and naringenin were the major compounds, with retention times of 14.5 min and 33.5 min, respectively (Table 1).

3.2. Peppermint LHAE Modulates Macrophage Viability. Peppermint LHAE effects were evaluated on macrophage viability stimulated or not with LPS. As expected, LPS reduced macrophage viability by 40% (Figure 2(a)). LPS-induced macrophage death was reversed by incubation with LHAE (1–30 μg/ml; Figure 2(a)). At higher concentrations (≥100 μg/ml), the extract decreased (49–57%) the viability of macrophages cultured in the absence of LPS (Figure 2(a)).

3.3. Macrophage-Mediated Phagocytosis. LPS stimulated phagocytosis in comparison with vehicle-treated cells, as denoted by an increase in the percentage of cells containing beads (4.1-fold increase) and in the number of beads per cell (6.2-fold increase; Figures 2(b) and 2(c)). Peppermint LHAE potentiated the ability of macrophages to phagocytose in the absence but not in the presence of LPS (Figures 2(b) and 2(c)). This potentiation was as high as 6.6- and 8.8-fold for the percentage of macrophages containing beads and number of beads per cell, respectively (Figures 2(b) and 2(c)).

3.4. Peppermint LHAE Reduces H_2O_2 but Not NO Production. Figures 3(a) and 3(b) show the measured concentrations of H_2O_2 and NO, respectively, in supernatant samples from macrophages incubated or not with LPS and LHAE.

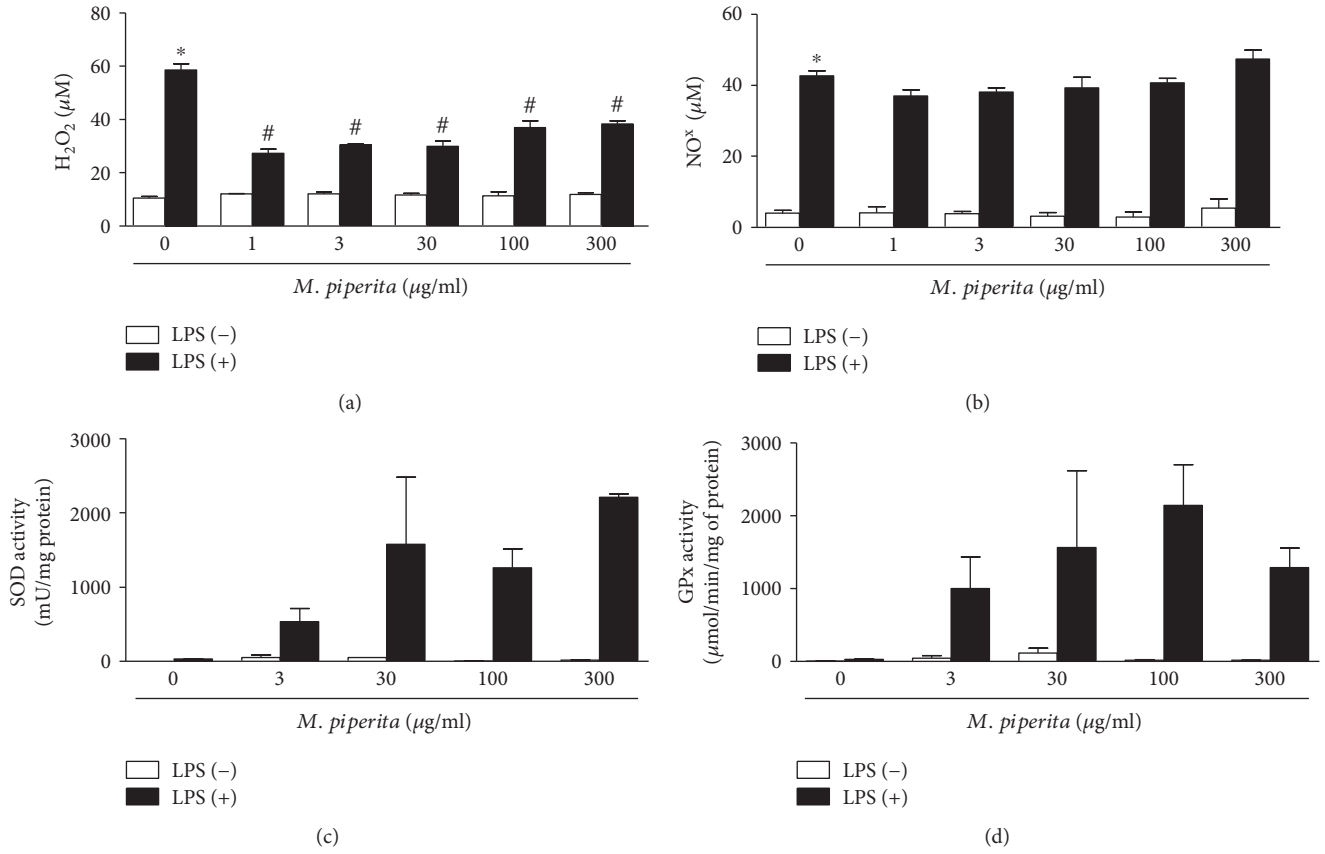

Figure 3: Effect of peppermint LHAE on H_2O_2 and NO release. H_2O_2 (a) and NO^x (b) concentrations in supernatant samples of cultured peritoneal macrophages. SOD (c) and GPx (d) activities in cultured peritoneal macrophages. Cells were pretreated with peppermint LHAE (1–300 μg/ml, in 1% DMSO in PBS) and stimulated with LPS for 24 h. Vehicle-treated cells were used as controls. Data are expressed as mean ± SEM. $^*p < 0.05$ compared with vehicle-treated cells; $^\#p < 0.05$ compared with LPS-treated cells.

Incubation of macrophages with LPS triggered the release of both H_2O_2 and NO by these cells, with fold increases of 5.6 and 10.0, respectively, for LPS-treated cells in comparison with vehicle controls. H_2O_2 but not NO release was reduced (34.6–53.4%) in LHAE-treated macrophages.

3.5. SOD and GPx Activities Are Increased in LPS-Stimulated Macrophages Treated with Peppermint LHAE. Figures 3(c) and 3(d) show the measured activities of SOD and GPx in cultured macrophages. Peppermint LHAE increased the activation of both enzymes in LPS-treated macrophages in comparison with vehicle controls. SOD activity was increased by 15.3–63.5-fold (Figure 3(c)), whilst GPx activity was raised by 34.4–73.6-fold (Figure 3(d)).

4. Discussion

M. piperita was previously suggested to have antimicrobial activity against both Gram-negative and Gram-positive bacteria [12], in addition to presenting with antioxidant potential *in vitro* [12, 17, 24]. *In vivo* anti-inflammatory actions were also reported for this plant in murine models of infection and inflammation. However, little is known on the modulatory effects of this plant in inflammatory cells. Here, we investigated the effects of a peppermint LHAE on cultured macrophages stimulated or not with LPS from *K. pneumoniae*. We found that this extract is able to modulate macrophage responses to LPS.

Mentha spp. effects on macrophage viability *in vitro* have been suggested to be concentration dependent. Indeed, RAW264.7 macrophage viability was previously shown not to be affected by treatment with *M. piperita* essential oil at concentrations as high as 100 μg/ml [16]. On the other hand, extracts from different *Mentha* species were found to be cytotoxic in both macrophage and monocyte cell lines when assessed at concentrations >200 μg/ml [25]. Thus, we initially evaluated the effects of LHAE on peritoneal macrophage viability. LPS-stimulated cells had their viability increased when incubated with LHAE in comparison with LPS controls. This was observed for the smallest concentrations tested and did not affect macrophage's ability to phagocytose when stimulated with LPS. On the other hand, at higher concentrations (≥100 μg/ml), LHAE caused cytotoxicity in cells not stimulated with this endotoxin. Additional effects were also observed for LPS-untreated cells, as they presented increased phagocytosis. To the best of our knowledge, we present here the first evidence on that *M. piperita* affects the viability and phagocytosis of LPS-stimulated murine peritoneal cells. This set of results allows us to suggest that *M. piperita* effects on macrophage may be not only

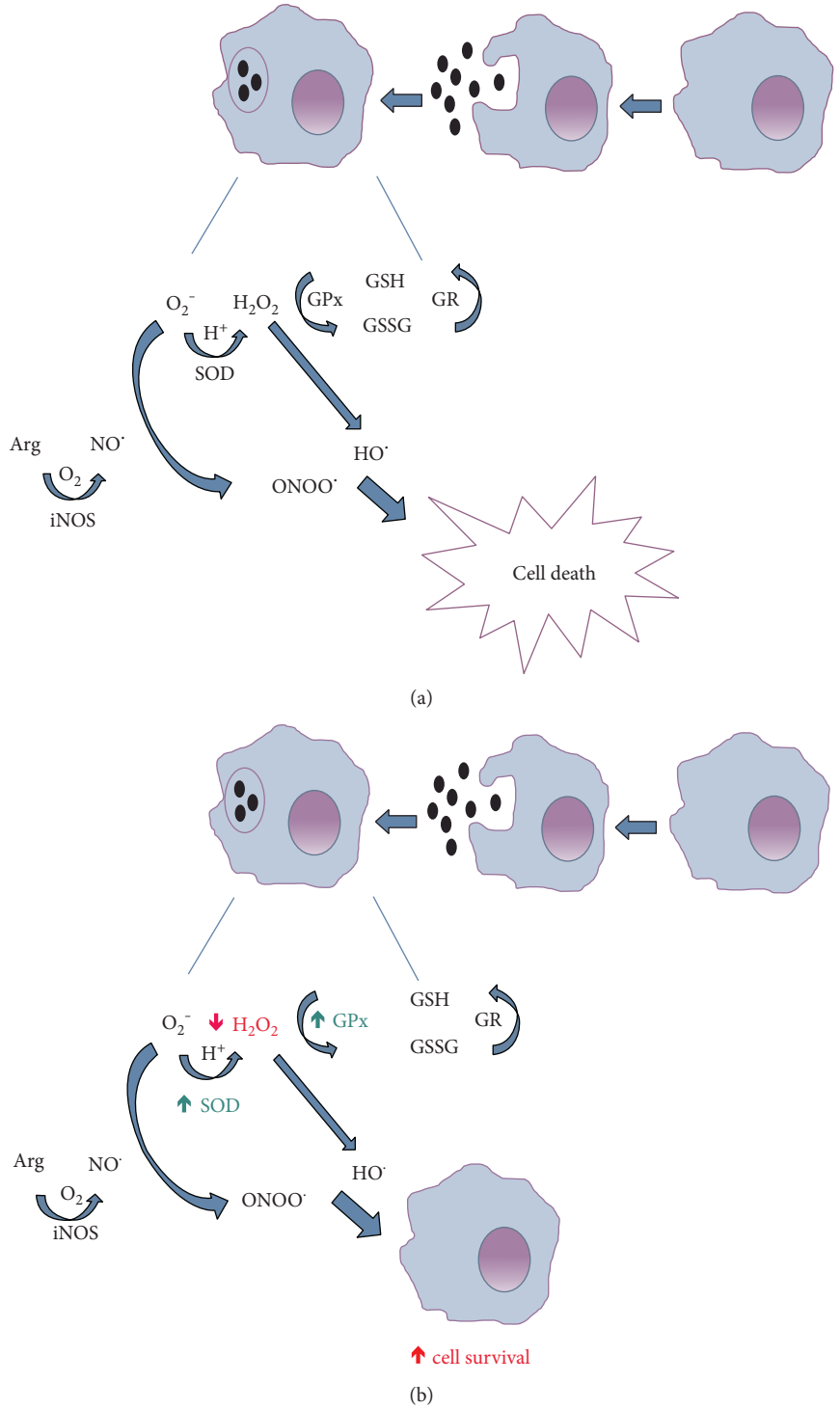

Figure 4: Mechanisms of action of LHAE on macrophage-mediated responses. (a) As part of the host's immune response during bacterial infections, macrophages are activated by bacteria-derived products, such as lipopolysaccharide (LPS); and as a result of this activation, reactive species are formed. Superoxide (O_2^-) anion produced by NADPH oxidase is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 can, in turn, be further reduced to H_2O by glutathione peroxidase (GPx) or even render hydroxyl radical ($HO^·$), a much more potent oxidant that can lead to diminished cell survival via peroxidation (and further breakdown) of lipids, as well as oxidation of protein and DNA bases. In parallel, nitric oxide ($NO^·$) continuously produced by inducible NO synthase (iNOS) can react with O_2^- and form peroxynitrous acid (ONOOH) which, after homolytic breakdown, can also render $HO^·$ in addition to the highly reactive nitro ($NO_2^·$) radical (a potent modifier of proteins and lipids), thus potentiating cell death. (b) The incubation of LPS-stimulated macrophages with LHAE does not affect NO formation but rather increases SOD and GPx activities (thus lowering O_2^- and H_2O_2 availability). As a consequence, OH and/or $NO_2^·$ formation is avoided, thus improving macrophage survival.

dependent on concentration but also on the culture conditions (presence versus absence of LPS).

In a recent study by Sun et al. [16], a peppermint essential oil reduced LPS-induced NO production by naïve RAW264.7 macrophages at similar concentrations to those tested for LHAE herein. A similar result was observed for an aqueous extract from *Mentha haplocalyx* when incubated with LPS-stimulated macrophages [26]. These studies and others [12, 17, 24] also suggested an antioxidant potential for peppermint and other plants from the same genus. We found that H_2O_2, but not NO production, was decreased in LHAE-treated cells stimulated with LPS at concentrations as low as 1 μg/ml. The same cells presented increased SOD and GPx activities, as key antioxidant enzymes. Increased SOD activity was previously reported in mice treated with peppermint aqueous extract [27]. More recently, peppermint essential oil was shown to act as a scavenger of hydroxyl radicals and to be an antioxidant at concentrations $\geq$200 μg/ml [16]. These results allow us to suggest that peppermint antioxidant actions on macrophages may be due to increased activation of SOD and GPx, which in turn, leads to decreased H_2O_2 production by these cells. These evidences, in addition to recent reports on that peppermint LHAE increases serum concentrations of anti-inflammatory cytokines in *Schistosoma mansoni*-induced infection [19], indicate an important anti-inflammatory action for *M. piperita.*

In regards to NO production, our results contradicted those described for *Mentha* spp. in the literature [28] as LHAE did not affect its levels upon macrophage stimuli with LPS. However, the inhibitory effects of *Mentha* spp. on NO release by LPS-stimulated macrophages were shown for hexane and ethyl acetate fractions [28], in addition to aqueous extract [26], suggesting that compounds found in different fractions and extractions of *Mentha* spp. may present different actions on NO production.

Different compounds were detected in essential oils obtained from peppermint leaves in previous studies [12, 17, 24, 29]. *M. piperita* antioxidant actions were previously suggested to be due to the presence of phenolic constituents in its leaves including rosmarinic acid and different flavonoids such as rutin, naringin, eriocitrin, luteolin, and hesperidin [30–33]. Here, HPLC analysis of the peppermint LHAE detected some peaks that coeluted with pure ursolic acid, epicatechin, caffeic acid, rutin, quercetin, naringenin, and kaempferol. These compounds were previously shown to act as anti-inflammatory and/or antioxidants [30, 33, 34]. It is possible that all these compounds contribute to the modulatory actions of LHAE observed in our study. However, we observed an unexpected lack of effect for LHAE on NO release by LPS-stimulated cells. This was rather surprising as it's detected compounds are known as potent inhibitors of NO production [35–40]. On the other hand, ursolic acid effects on NO release by macrophages are controversial and may be concentration dependent. Indeed, some evidences suggest this compound increases NO production by both infected [41] and resting [42] macrophages, whilst others show ursolic acid inhibits NO release by LPS-stimulated cells [43]. We suggest that, although the different compounds detected in the LHAE may contribute synergistically to its antioxidant effects, it is possible they counteract each other's abilities to stimulate or inhibit NO production by macrophages depending on their bioavailability in the extract.

Overall, our data show that the peppermint LHAE modulates macrophage-mediated inflammatory responses, by stimulating the antioxidant pathway in these cells (Figure 4). These effects may be beneficial when the excessive activation of macrophages contributes to tissue damage in diseases in which there is an unbalanced oxidative stress, such as those of infectious nature.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Propyl Gallate Exerts an Antimigration Effect on Temozolomide-Treated Malignant Glioma Cells through Inhibition of ROS and the NF-κB Pathway

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In this study, we demonstrated that temozolomide (TMZ) and propyl gallate (PG) combination enhanced the inhibition of migration in human U87MG glioma cells. PG inhibited the TMZ-induced reactive oxygen species (ROS) generation. The mitochondrial complex III and NADPH oxidase are two critical sites that can be considered to regulate antimigration in TMZ-treated U87MG cells. PG can enhance the antimigration effect of TMZ through suppression of metalloproteinase-2 and metalloproteinase-9 activities, ROS generation, and the NF-κB pathway and possibly provide a novel prospective strategy for treating malignant glioma.

1. Introduction

Malignant gliomas, the most common primary brain tumor with an annual incidence of about 5.26 cases per 100,000 people, have high growth rates and necrotic characteristics [1]. Glioblastoma multiforme (GBM), a type of malignant glioma, is one of the most difficult cancers to treat, and median survival is about 14.6 months in spite of aggressive treatment with surgery, radiotherapy, chemotherapy, and immunotherapy [2]. Temozolomide (TMZ) is an oral alkylating and well-tolerated chemotherapeutic drug and has been approved for the treatment of malignant glioma clinically for a long time. However, O^6-methylguanine-DNA methyltransferase gene (MGMT) promoter methylation status influences the efficacy of the drug, and therapeutic outcomes are still unsatisfactory [3]. In addition, GBM is highly neovascularizated with the appearance of angiogenesis and vasculogenesis. The modification of preexisting blood vessels combined with antiangiogenesis drugs for blocking new vessel formation may possibly improve the therapeutic outcome [4]. Bevacizumab, a vascular endothelial cell proliferation inhibitor, is currently an alternative treatment choice for GBM but fails to extend overall survival time [5, 6]. In experimental studies, drugs such as betulinic acid, lonidamine, and CD437, which target the mitochondrial pore [7–9], and crizotinib, which inhibit mesenchymal-epithelial transition (MET) expression [10], possibly provide a strategy for GBM therapy. Our previous study also demonstrated that valproic acid could enhance the apoptotic effect of TMZ through a redox regulation mechanism [11].

Propyl gallate (PG), a polyphenolic compound family that is synthesized by the condensation of propanol and gallic acid, has an antiproliferation effect on tumor cells [12]. In one study, PG could modulate heme oxygenase-1 (HO-1)

activation and decrease lung cancer cell survival [13]. PG also induces apoptosis in human leukemia cells [14] and HeLa cells [15] by increasing reactive oxygen species (ROS) levels and glutathione (GSH) depletion. In brain ischemia, PG inhibits the activity of NF-κB, reduces COX-2 and TNF-alpha G expression, and decreases ischemic-reperfusion injury [16]. However, PG effects on brain glioma cells are still questionable and not well investigated. In this study, we examined whether PG can potentiate the antimigration effects of TMZ on malignant glioma cells and elucidate the possible molecular mechanisms.

2. Materials and Methods

2.1. Cell Lines, Reagents, and Chemicals. U87MG (a human primary glioblastoma cell line) was obtained from the Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). HBVP (a human brain vascular pericyte cell line), HA (a human astrocyte cell line), and HBMEC (a human brain microvascular endothelial cell line) were purchased from ScienCell Research Laboratories Inc. (Carlsbad, CA, USA). Gibco™ Eagle's minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Pericyte medium (PM), astrocyte medium (AM), and endothelial cell medium (ECM) were obtained from ScienCell Research Laboratories Inc. (Carlsbad, CA, USA). Primary antibodies against $I_\kappa B$ kinase (IKK), $I_\kappa B$, p65, p-IKK, p-$I_\kappa B$, p-p65, lamin B, and β-actin were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The Bradford protein assay reagent for protein concentration determination was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). PG, propidium iodide (PI), TMZ, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), dimethyl sulfoxide (DMSO), Trypan blue solution, crystal violet, and other chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

2.2. Cell Culture and Drug Treatment. U87MG cells were cultured in MEM containing 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin G, and 100 μg/mL streptomycin and placed in a 5% CO_2/95% incubator at 37°C. HBVP cells, HA cells, and HBMEC cells were cultured in PM, AM, and ECM, respectively, and placed in a 5% CO_2/95% incubator at 37°C. PM consists of 500 mL of basal medium, 10 mL of FBS, 5 mL of pericyte growth supplement, and 5 mL of penicillin/streptomycin solution. AM consists of 500 mL of basal medium, 10 mL of FBS, 5 mL of astrocyte growth supplement, and 5 mL of penicillin/streptomycin solution. ECM consists of 500 mL of basal medium, 25 mL of FBS, 5 mL of endothelial cell growth supplement, and 5 mL of penicillin/streptomycin solution. The stock solutions of PG or TMZ were prepared in DMSO, and all treated concentrations were diluted in culture medium. The concentration of DMSO should not be exceeding from 0.05%.

2.3. Cytotoxicity Assay. Cytotoxicity was evaluated using a cell proliferation kit (Biological Industries; Kibbutz Beit Haemek, Israel). A total of 1×10^4 cells per well were seeded in a flat bottom 96-well plate before treatment. After treatment, cells in a 100 μL medium were incubated with 50 μL XTT reagent for 2 h, and the absorbance of the sample (450 nm) was measured against a background control medium as a blank. The nonspecific absorbance (690 nm) was measured and subtracted from the 450 nm measurement in an EnSpire® multimode plate reader (PerkinElmer; Billerica, MA, USA).

2.4. Transwell Migration Assay. Transwell migration assay was carried out using 24-well Millicell® hanging cell culture inserts with a polyethylene terephthalate (PET) membrane pore size of 8 μm (EMD Millipore; Billerica, MA, USA). Cells (1×10^4 cells/well) were seeded in a serum-free medium to triplicate wells of cell culture inserts, and a complete medium containing 10% FBS was added to the lower chamber. After 48 h, the interior of the inserts was swabbed to remove nonmigratory cells, and those migratory cells that passed through the PET membrane were fixed and stained using crystal violet solution. At least three individual fields per insert were counted for cell number using a phase contrast microscope. Then, each insert was transferred to an empty well containing 200 μL extraction solution (33% acetic acid) to lyse the cells, and 100 μL from each sample was transferred and measured at 560 nm using an EnSpire multimode plate reader (PerkinElmer).

2.5. Analysis of the Activities of Matrix Metalloproteinase-(MMP-) 2 and MMP-9 in Human U87MG Cells. U87MG cells (2×10^6) were cultured in 60 mm culture dishes for 48 h. The cells were then exposed to various concentrations of TMZ alone, PG alone, or combined PG and TMZ for appropriate times. After treatment, the cultured media were collected and then centrifuged at 12,000 ×g for 10 min at 4°C to obtain the supernatant. The protein concentrations of supernatant were measured with a Bio-Rad protein assay reagent (Hercules, CA, USA). The enzyme activities of MMP-2 and MMP-9 were evaluated by zymography. Fifty micrograms of the supernatant protein were subjected to electrophoresis in a 10% SDS-PAGE gel copolymerized with gelatin (1 mg/mL). After electrophoresis, the gels were then washed in distilled water for 5 min and then incubated for 1 h in 2.5% Triton-X solution in order to remove the SDS [17]. It would restore the protein 3D structure and functionality. The gelatinolytic activities of MMP-2 and MMP-9 were evaluated as transparent bands against a background of coomassie brilliant blue-stained gelatin and quantified using Bio-Rad Quantity One® 1-D analysis software (Hercules, CA, USA).

2.6. Intracellular ROS Measurement. The production of intracellular ROS was detected by flow cytometry using 2′,7′-dichlorofluorescin diacetate (DCFDA). After drug treatment, the cells were stained with 20 μM DCFDA for 30 min at 37°C and then washed with 1X PBS twice to remove the DCFDA. All cells were trypsinized to obtain a single-cell suspension. Intracellular ROS levels, which were indicated by the fluorescence of dichlorofluorescein (DCF), were

measured through an excitation/emission = 485 nm/535 nm using a BD FACSCanto™ II flow cytometer (San Jose, CA, USA). Ten thousand cells were collected and analyzed per experimental conditions using mean fluorescent intensity.

2.7. Preparation of Nuclear Protein Extracts from U87MG Cells. After treatment, cells were washed with cold 1X PBS and suspended in 500 μL of hypotonic lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl) containing protease inhibitors for 30 min. Then, 30 μL of 10% NP-40 was added to the swollen cells in the lysis buffer and vortexed vigorously for 10 seconds. The homogenate was centrifuged immediately for 30 seconds at 11,000 ×g, and the supernatant containing the cytoplasmic extracts was stored at −80°C for further analysis. The nuclear pellet was resuspended in ~70 μL of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, and 25% (v/v) glycerol). After 30 min of intermittent mixing, the extract was centrifuged at 20,000 ×g for 5 min, and the supernatants containing the nuclear extracts were secured. Nuclear p65 expression was evaluated by Western blotting.

2.8. Western Blot Analysis. For crude total protein extraction, cells were lysed in 1X buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) for 10 min and centrifuged at 12,000 ×g for 10 min at 4°C to obtain the soluble proteins. Twenty-five to fifty micrograms of protein were separated on a 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% nonfat milk, incubated with various primary antibodies overnight, and then washed with 1X PBST solution (0.05% Tween 20 in 1X PBS). After washing, the appropriate secondary antibodies, which were each labeled with horseradish peroxidase, were added to the membrane for 1 h and then washed with 1X PBST solution. The antigen-antibody complexes were detected using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences; Uppsala, Sweden). Autoradiographic signals were detected by an X-ray film (Roche Applied Science, Mannheim, Germany). The signal intensity was quantitated by GeneTools analysis software (SYNGEN, Cambridge, UK).

2.9. Statistical Analysis. Data are presented as the mean ± standard deviation from at least three independent experiments and were analyzed using Student's *t*-tests. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. The Effect of Combined TMZ and PG on Cell Viability. To exclude any confounding factors in the determination of migration inhibition by cell death, we first evaluated the effect of drug concentration on cell viability in U87MG cells. The U87MG cells maintained a cell viability of at least 85% when treated with TMZ (200 μM) alone, PG alone (50 and 100 μM), and combined TMZ and PG after 48 h (Figure 1).

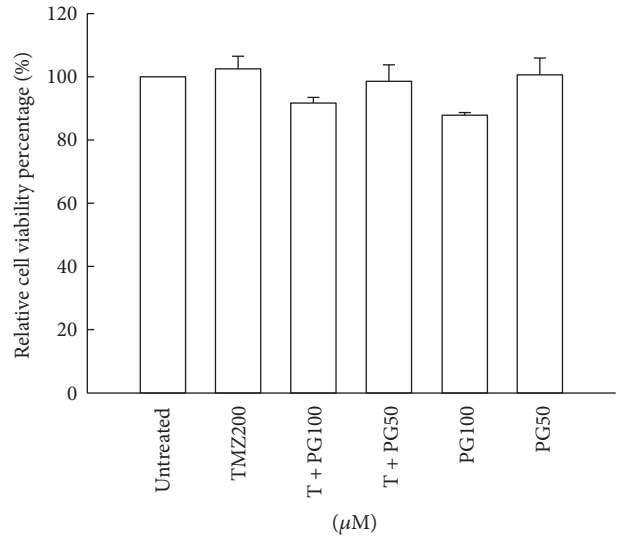

Figure 1: Evaluation of cell viability with TMZ, PG, or TMZ/PG treatment. U87MG cells (1×10^4) were seeded in each well of a 96-well cultured plate for 24 h and then treated with TMZ (200 μM), PG (50 or 100 μM), or their combination for 48 h. After treatment, cell viability was determined by an XTT-based assay. The values are represented as mean ± standard deviation ($n = 5$–8) of individual experiments.

Concentrations of 50 and 100 μM for PG and 200 μM for TMZ were used in further studies.

3.2. PG Enhanced the Antimigration Effect of TMZ. To determine whether PG has a potential role, either alone or in combination with TMZ, in the inhibition of migration in the treatment of glioma cells, the transwell migration assay and crystal violet staining method were used. There were no significant difference on antimigration in TMZ (200 μM) alone or PG (50 μM) alone compared with untreated cells (Figures 2(a) and 2(b)). PG (100 μM) alone produced a significant effect on antimigration. The antimigration effect was further increased when PG (50 μM) and TMZ (200 μM) or PG (100 μM) and TMZ (200 μM) in U87MG cells are combined. To evaluate the effect of PG pretreated TMZ-treated glioma cells, the PG was pretreated for 6 h and then treated with TMZ for 48 h and then antimigration was analyzed. As shown in Figures 2(c) and 2(d), there were no significant statistic differences between PG-alone treatment and pretreated PG plus TMZ treatment suggesting the antimigration should be cotreated with PG and TMZ.

3.3. PG Enhanced the TMZ Inhibition of MMP Activities in U87MG Cells. MMP-9 and MMP-2 are important matrix metalloproteinases in the migration of glioma cells. MMP-9 and MMP-2 degrade the extracellular matrix and result in the migration of glioma cells to other normal tissue area. To determine whether PG has a potential role in the inhibition of MMP-9 or MMP-2 activity in U87MG cells either PG alone or PG/TMZ combination, we used zymographic analysis. As shown in Figure 3, the MMP-9 and MMP-2 activities were significant inhibition in PG/TMZ combination as compared with untreated cells. It is interesting to note

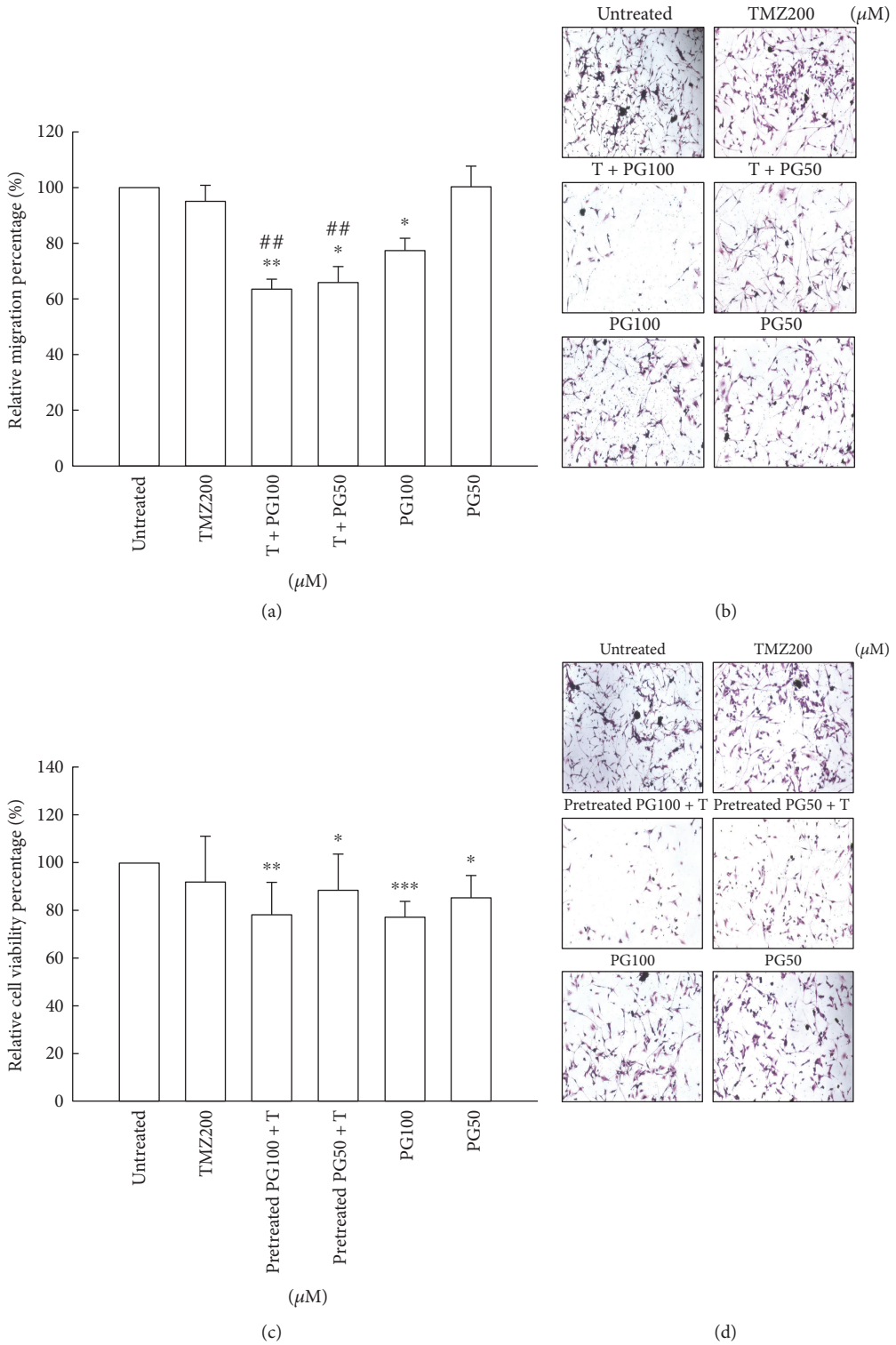

Figure 2: Evaluation of migration with TMZ, PG, or TMZ/PG treatment. (a, b) U87MG cells (1×10^4) were plated in 24-well Millicell hanging cell culture inserts with an 8 μm pore size membrane for 24 h and then treated with TMZ (200 μM), PG (50 or 100 μM), and their combination for 48 h. (c, d) U87MG cells (1×10^4) were plated in 24-well Millicell hanging cell culture inserts with an 8 μm pore size membrane for 24 h and then treated with TMZ (200 μM) or PG (50 or 100 μM) for 48 h or pretreated with PG (50 or 100 μM) for 6 h and then treated with (200 μM) for 48 h. (a, c) Transwell migration assay was carried out. The migrated cells were stained with crystal violet. The dye was eluted with 33% acetic acid, and crystal violet absorbance was measured at 570 nm using a microplate reader. The values are presented as mean ± standard deviation ($n = 5$–8) of individual experiments. Significant differences for the untreated group and TMZ group were $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{\#\#}P < 0.01$. (b, d) Random fields from each of the triplicate migration assays were counted using phase contrast microscopy (magnification 200x).

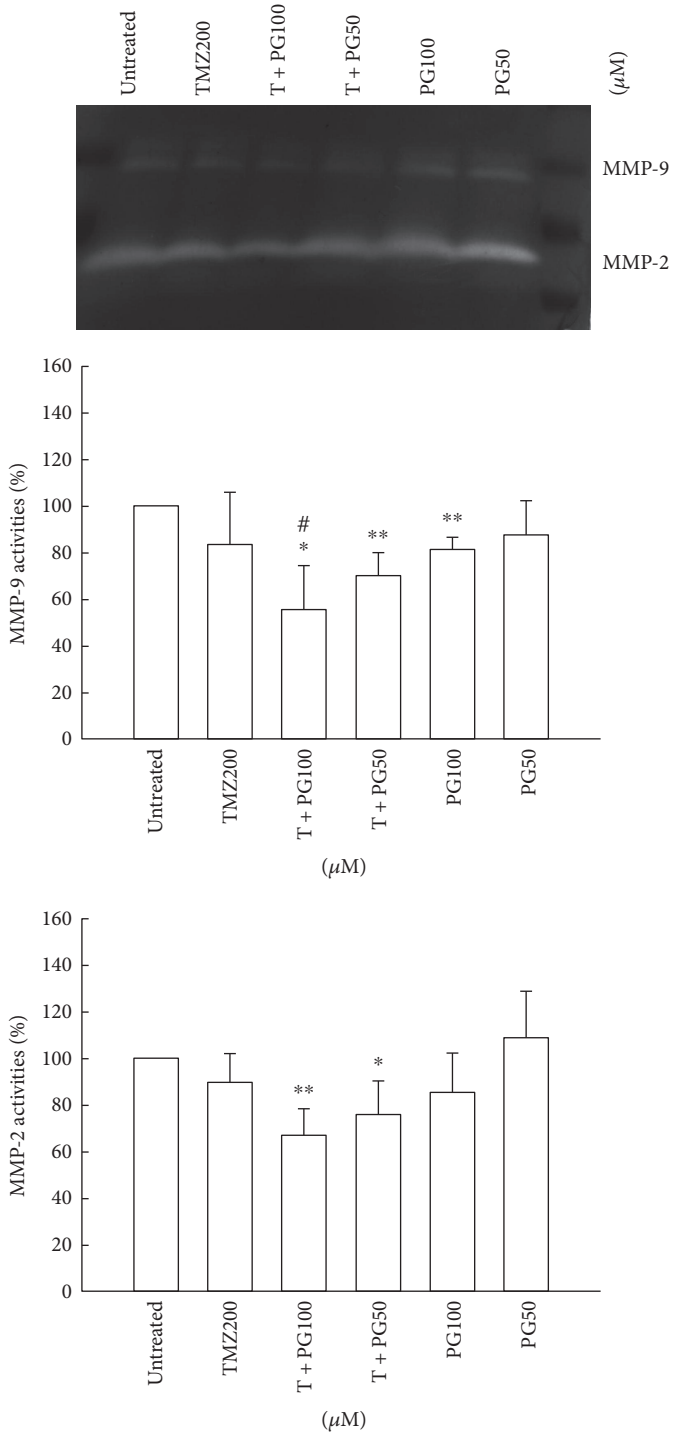

Figure 3: Analysis of MMP-2 and MMP-9 activities with TMZ, PG, or TMZ/PG treatment. U87MG cells (2×10^6) were plated in 100 mm cultured dishes for 24 h and then treated with TMZ (200 μM), PG (50 or 100 μM), or their combination for 48 h. After treatment, the cultured media were collected and then centrifuged at 12,000 ×g for 10 min at 4°C to obtain supernatants. Aliquot protein (50 μg) was used to evaluate the activities of MMP-2 and MMP-9 by zymography. These experiments were performed at least three times; a representative experiment is presented. Data indicate the densitometric values of various treated groups normalized to their corresponding untreated group. Significant differences for the untreated group and TMZ group were $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\#}P < 0.05$.

that PG (100 μM)/TMZ (200 μM) combination resulted in a significant inhibition on MMP-9 activity as compared with TMZ (200 μM) alone treatment. PG (50 μM or 100 μM) alone did not inhibit the activity of MMP-2.

3.4. PG Decreased ROS Production in TMZ Treatment. To fully evaluate the possible involvement of ROS in the antimigration effects of PG/TMZ-combined treatment, we detected ROS accumulation in the treated cells. Increased ROS

accumulation occurred in the TMZ-alone group (Figure 4). Treatment with PG (50 μM or 100 μM) alone had an inhibitory effect on ROS accumulation compared to that of TMZ alone. However, ROS inhibition was little enhanced by combined treatment with PG (50 μM or 100 μM) and TMZ. These results indicate that ROS generation may partially obstruct the antimigration effect of TMZ, and the inhibition of ROS by PG may be a critical event contributing to the antimigration effect of TMZ treatment.

3.5. PG/TMZ Combination Suppressed NF-κB Activity. NF-κB is known to regulate the expression of a number of MMPs. We therefore investigated the effect of PG and TMZ on the NF-κB pathway in U87MG cells. The expression of NF-κB pathway-related proteins such as p-IKK, p-IκB, and p-p65 was examined by Western blot analysis. PG alone or in combination was quite effective in suppressing the constitutive activation of the NF-κB pathway, including p-IKK, p-IκB, and p-p65 in U87MG cells, but TMZ treatment alone had no marked effect on the constitutive NF-κB activation pathway in U87MG cells (Figure 5).

3.6. Inhibition of Critical ROS-Generated Sites Enhanced the Antimigration Effect of TMZ on U87MG Glioma Cells. The antioxidant effect of PG enhanced the antimigration effect of TMZ treatment. We further evaluated the critical ROS-generated sites in TMZ-treated U87MG cells. Various specific ROS-generated inhibitors were pretreated for 1 h and then treated with TMZ for 48 h. Finally, the cells were evaluated for intracellular ROS levels. All ROS-generated inhibitors reduced TMZ-induced ROS production (Figure 6(a)). The inhibition of migration with TMZ treatment was enhanced in the presence of carboxin (a complex II inhibitor), antimycin A (a complex III inhibitor), or apocynin (a NADPH oxidase inhibitor), but not in the presence of rotenone (a complex I inhibitor) (Figures 6(b) and 6(c)). These results indicated that ROS production induced by TMZ treatment occurs through mitochondrial respiratory chain complex I, complex II, complex III, and NADPH oxidase. Inhibition of ROS generated from complex II and III, and NADPH oxidase can enhance the antimigration effect of TMZ on U87MG cells.

3.7. PG Did Not Induce Cytotoxicity in the Normal Cell Lines. To evaluate whether 50 or 100 μM PG induced toxic in normal neuron and glia cells, three human normal cell lines, HBVP (human brain vascular pericytes), HA (human astrocytes), HBMEC (human brain microvascular endothelial cells), and XTT analysis were used. As shown in Figure 7, TMZ treatment resulted in 80–90% cell viability in three normal cell lines. However, treatment of 50 or 100 μM PG did not induce cytotoxicity in the three cell lines suggesting PG may protect normal cells during TMZ treatment.

4. Discussion

A critical step in cancer invasion is breaking through the ECM and invading the neighboring stroma. Control of the invasive nature of GBM cells may offer hope for more efficacious local therapy and improve the patient's quality of life

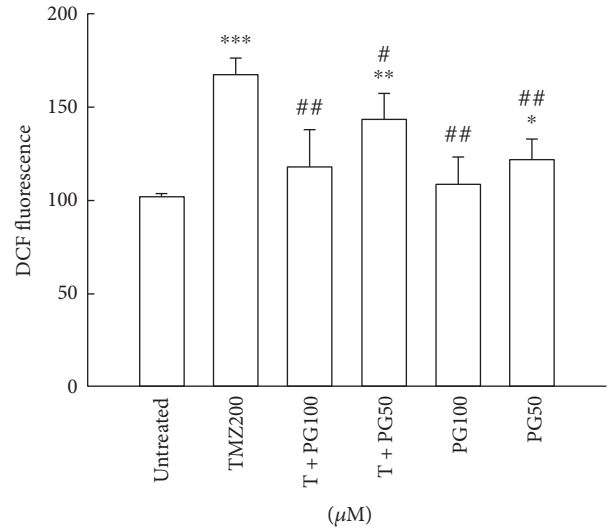

Figure 4: Evaluation of intracellular ROS with TMZ, PG, or TMZ/PG treatment. U87MG cells (1×10^6) were plated in 60 mm cultured dishes for 24 h and then treated with TMZ (200 μM), PG (50 or 100 μM), or their combination for 48 h. After treatment, the cells were stained with 2′,7′-dichlorfluorescein-diacetate (DCFDA) for ROS analysis and were then evaluated by flow cytometry. Data represent the mean fluorescence intensity within the cells. The values are presented as mean ± standard deviation ($n = 5$–8) of individual experiments. Significant differences for the untreated group and TMZ group were $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$.

[18]. Neutral proteases can alter capillary permeability by attacking ECM around the blood vessels. MMP-2 and MMP-9 destroy ECM and help breast and prostate cancer cells migrate to new places [19, 20]. In glioma cells, MMP-2 and MMP-9 are highly expressed and are involved in GBM migration and invasion [21]. Rojiani et al. reported that enhanced vascular proliferation, particularly at the brain-tumor interface, increased the capability of tumors to grow and invade in brain malignancy, possibly modulated by MMP-2 [22].

ROS regulation of cell migration and adhesion in endothelial-derived cells is an important mechanism [23], and it also provides a common trigger for many downstream pathways that directly mediate BBB oxidative damage [24]. The role of ROS in tumor metastasis involves complicated processes, including MET, migration, invasion by tumor cells, and angiogenesis around the tumor lesion [25, 26]. ROS generation may be induced intracellularly in either a NADPH oxidase-dependent manner or a mitochondria-dependent manner, by growth factors and cytokines (such as TGF beta and HGF) or by tumor promoters (such as TPA) capable of triggering cell adhesion, MET, and migration [27–29]. ROS has been found to directly regulate the expression and activity of MMPs in human endothelial cells [30], and several reports indicated that ROS was involved in the abnormal activation of these MMPs in several types of cancer cells, including glioblastoma [31]. Our results revealed that PG inhibited the expression of MMP-2 and

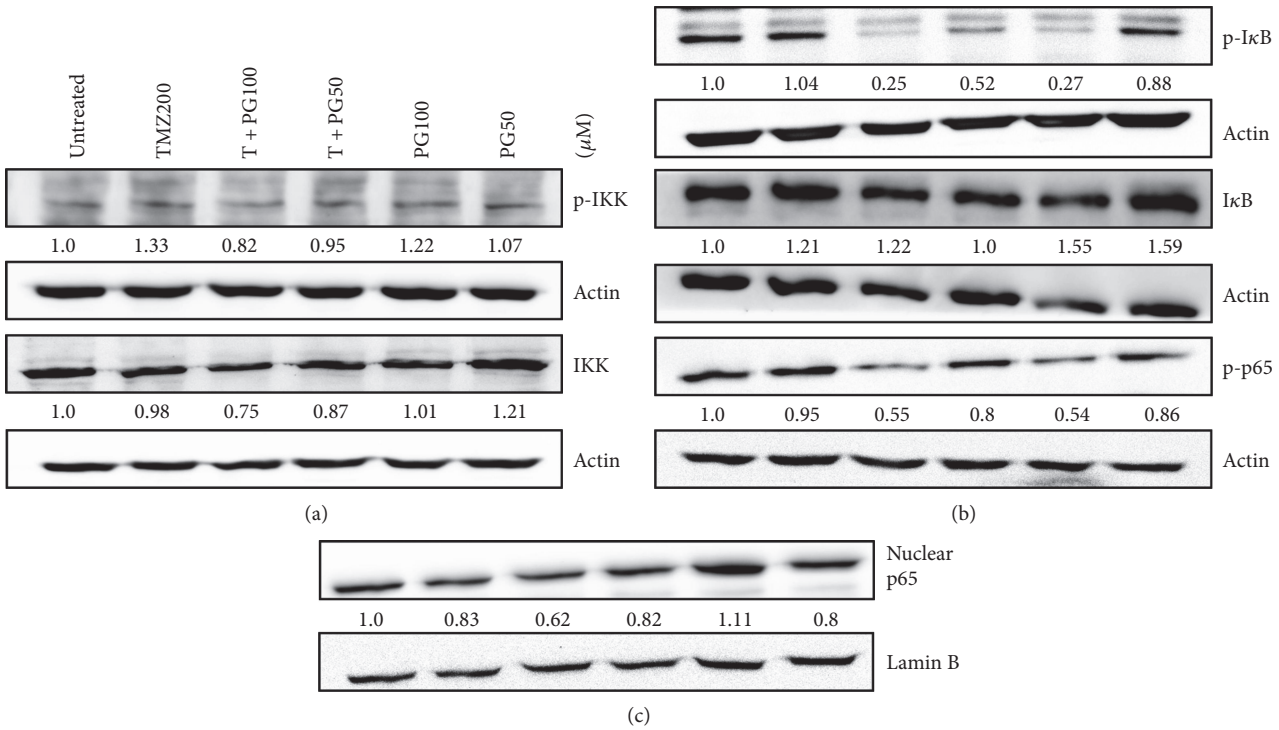

Figure 5: Expressions of (a) p-IKK and IKK; (b) p-IκB, IκB, and p-p65; (c) nuclear p65 with TMZ, PG, or TMZ/PG treatment. U-87 MG cells (2×10^6) were plated in 100 mm cultured dishes for 24 h and then treated with TMZ (200 μM), PG (50 or 100 μM), or their combination for 48 h. After treatment, total proteins or nuclear proteins were extracted to assess various protein expressions. Fifty micrograms of protein were loaded onto a 12% SDS-polyacrylamide gel and evaluated by Western blotting. These experiments were performed at least three times; a representative experiment is presented. Data indicate the densitometric values of various treated groups normalized to their corresponding untreated group.

MMP-9 and exerted an antimigration effect on TMZ-treated U87MG cells. TMZ induced an increased in ROS production by about 1.6-fold, and PG (100 μM) significantly inhibited the ROS level in TMZ treatment. This seems to suggest that downregulation of the ROS level with TMZ treatment can enhance the inhibition of migration.

The ROS-dependent intracellular pathway activation relationship with tumor invasion includes the regulation of NF-κB transcription factor located upstream of MMPs [30]. The elevation of the NF-κB level in cancer may be the result of either exposure to proinflammatory stimuli in the tumor microenvironment or upregulation of the signaling pathway by upstream regulators [32]. In addition, the NF-κB pathway is activated in glioblastoma-initiating cells undergoing differentiation, and blockade of this activation promoted the senescence of differentiating cells [33]. Appropriate control of NF-κB activity, which can be achieved by gene modification or pharmacological strategies, would provide a potential approach to the management of NF-κB-related tumors, including glioblastoma.

The NF-κB-induced expression of MMPs has been found to be regulated by ROS generated from mitochondria and NADPH oxidase [34]. PG also possessed anti-inflammatory activity via downregulation of the NF-κB pathway [35], but its potential effect on the NF-κB signaling cascade in glioma cells has never been evaluated. Our previous study found that PG could reduce the proliferation and augment the chemosensitivity of a THP-1 leukemia cell line via extrinsic and intrinsic apoptotic pathways [14]. In this study, we noticed that the effect of PG/TMZ-combined treatment may be mediated by inhibition of the NF-κB pathway, such as p-IKK, p-IκB, and p-p65, in U87MG glioma cells. We also found that both p-IκB and nuclear p65 in U87MG glioma cells were more suppressed by combined PG and TMZ. This indicates that the inhibition of the NF-κB pathway enhanced by PG is a critical mechanism of antimigration in TMZ treatment. Furthermore, blockade of NADPH oxidase by diphenyleneiodonium abolishes NF-κB activation and inhibits MMP-9 expression, indicating the role of ROS in NF-κB-induced MMP expression [35]. In agreement with our results, the inhibitors of mitochondrial complex III (carboxin and antimycin A) and NADPH oxidase (apocynin) significantly inhibited the ROS level and migration with TMZ treatment, further indicating that inhibition of the ROS-mediated NF-κB pathway in TMZ treatment is an important antimigration mechanism in glioma cells.

Many polyphenol compounds have been found to pass the blood-brain barrier, including epigallocatechin-3-gallate [36, 37]. PG is a polyphenol compound which contains hydrophobic propyl group and phenol group, and the compound structure of PG is smaller than epigallocatechin-3-gallate. However, Wu et al. demonstrated that the mean

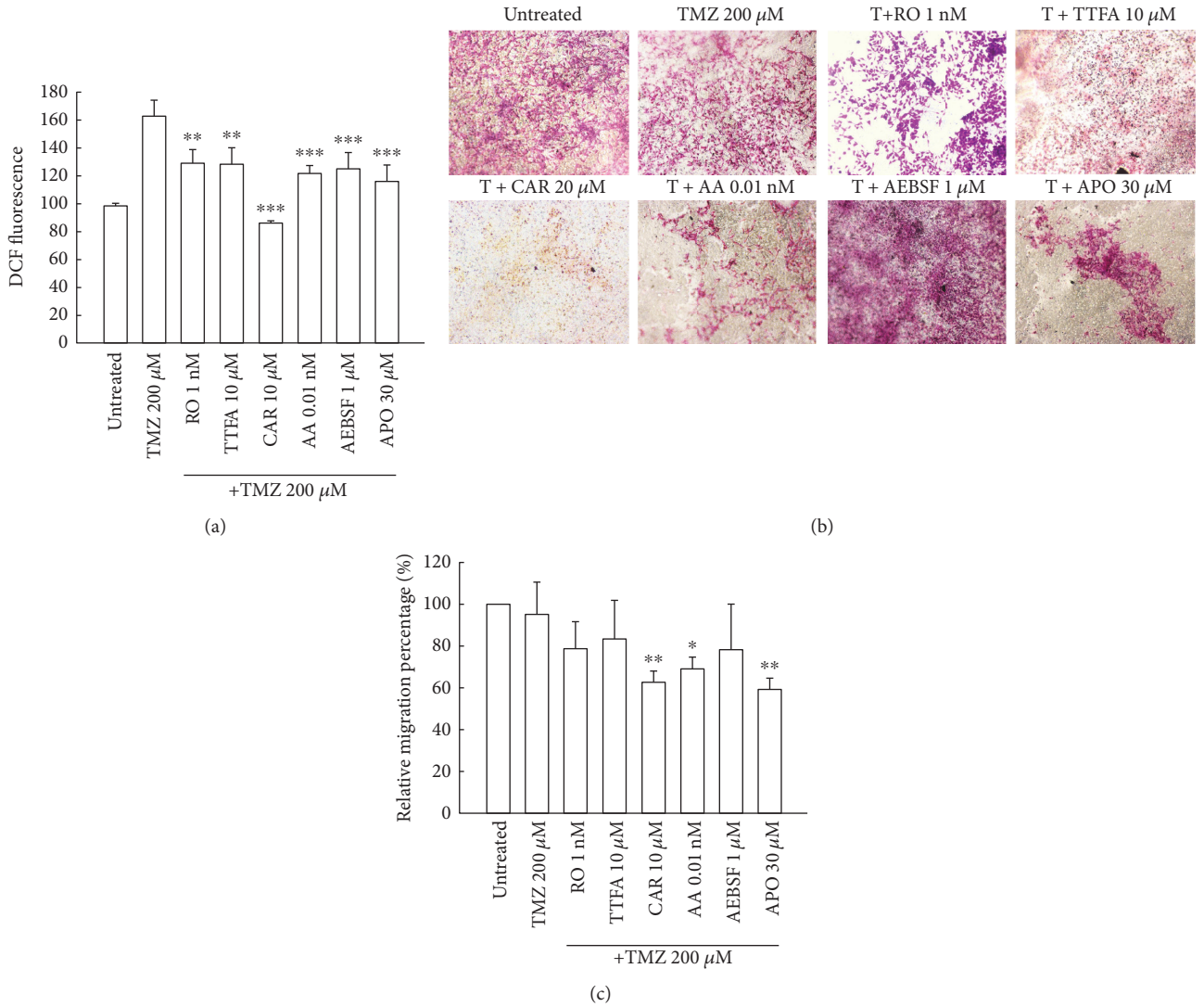

Figure 6: Inhibition of critical ROS events enhances antimigration with TMZ treatment. U-87 MG cells (1×10^6) were plated in 60 mm cultured dishes for 24 h and then treated with TMZ (200 μM) alone for 48 h or pretreated with 1 nM rotenone (RO, a complex I inhibitor), 10 μM 2-thenoyltrifluoroacetone (TTFA, a complex II inhibitor), 10 μM carboxin (CAR, a complex II inhibitor), 0.01 nM antimycin A (AA, a complex III inhibitor), 1 μM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, a NADPH oxidase inhibitor), and 30 μM apocynin (APO, a NADPH oxidase inhibitor) for 1 h, followed by TMZ (200 μM) for 48 h. After treatment, the cells were stained with 2′,7′-dichlorfluorescein-diacetate (DCFDA) for ROS analysis and were then evaluated by flow cytometry. Data represent the fluorescence intensity within the cells. The values are presented as mean ± standard deviation ($n = 5$–8) of individual experiments. Significant differences for the TMZ group were $^{**}P < 0.01$ and $^{***}P < 0.001$. (b) U87MG cells (1×10^4) were plated in 24-well Millicell hanging cell culture inserts in an 8 μm polyethylene terephthalate membrane for 24 h and then treated with TMZ (200 μM) alone for 48 h or pretreated with 1 nM rotenone (RO, a complex I inhibitor), 10 μM 2-thenoyltrifluoroacetone (TTFA, a complex II inhibitor), 10 μM carboxin (CAR, a complex II inhibitor), 0.01 nM antimycin A (AA, a complex III inhibitor), 1 μM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, a NADPH oxidase inhibitor), and 30 μM apocynin (APO, a NADPH oxidase inhibitor) for 1 h, followed by TMZ (200 μM) for 48 h. Transwell migration assay was carried out. The migrated cells were stained using crystal violet. Random fields from each of the triplicate migration assays were counted using phase contrast microscopy (magnification 200x). (c) The dye was eluted with 33% acetic acid, and crystal violet absorbance was measured at 570 nm using a microplate reader. The values are presented as mean ± standard deviation ($n = 5$–8) of individual experiments. Significant differences for the TMZ group were $^{*}P < 0.05$ and $^{**}P < 0.01$.

in vivo recoveries of both polyphenol compounds (+)-catechin and (−)-epicatechin in microdialysis probes were 38.3% and 29.1% in the brain [37]. We speculate that 50 or 100 μM of PG may partially pass through the blood-brain barrier. The correct concentration of PG in the brain must be further detected. In our previous study demonstrated that U87MG cells express high oxidative stress status as compared with that of other glioma cells [38]. Our results show that TMZ induced ROS generation in glioma by 1.6-fold of untreated cells. The increased ROS generation in TMZ-treated U87MG cell may not further induce the migration activity.

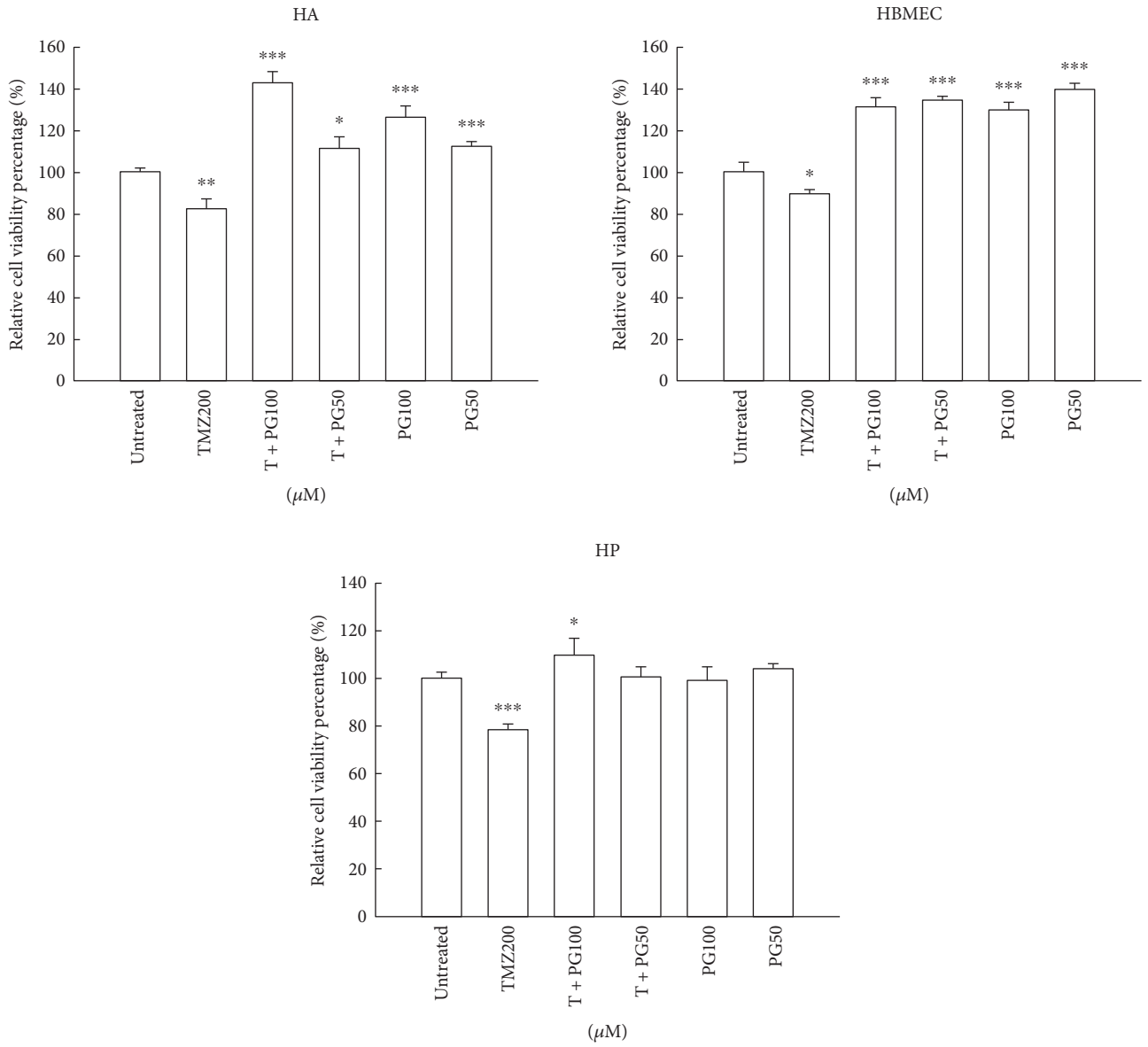

Figure 7: Evaluation of cell viability with TMZ, PG, or TMZ/PG treatment in normal cell lines. HA, HBMEC, and HP cells (1×10^4) were seeded in each well of a 96-well cultured plate for 24 h and then treated with TMZ (200 μM), PG (50 or 100 μM), or their combination for 48 h. After treatment, cell viability was determined by an XTT-based assay. The values are represented as mean ± standard deviation ($n = 5$–8) of individual experiments. Significant differences for the untreated group were $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Wu et al. demonstrated that a strong linear correlation between the cell migration rate and the amount of intracellular ROS suggests that ROS is an intermediate product to enhance cell migration [39]. Furthermore, an antioxidant α-tocopherol can decrease the migration rate by quenching the ROS production [39]. Our results showing that PG could block ROS generation induced by TMZ treatment suggested that ROS blocking by PG may provide partially antimigration effect. Accumulating reports indicate the transcription factor NF-κB as a positive mediator of tumor metastasis, and the NF-κB signaling pathway provides critical roles in neuroblastoma migration and invasion [40]. Our results demonstrating that PG inhibited the NF-κB pathway resulted in MMP activity inhibition suggest that NF-κB pathway inhibition may be the main mechanism on antimigration in PG/TMZ combination. Morgan and Liu indicate that certain NF-κB-regulated genes play a major role in regulating the amount of ROS in the cell and ROS would modulate an NF-κB response and that NF-κB target genes would attenuate ROS to promote survival [41]. Our results found that PG/TMZ combination resulted in NF-κB pathway inhibition but did not increase ROS generation suggesting those two phenomena (block ROS generation and NF-κB pathway inhibition) may be independent events.

In conclusion, our findings provide further scientific evidence that (1) PG enhances the antimigration effect of

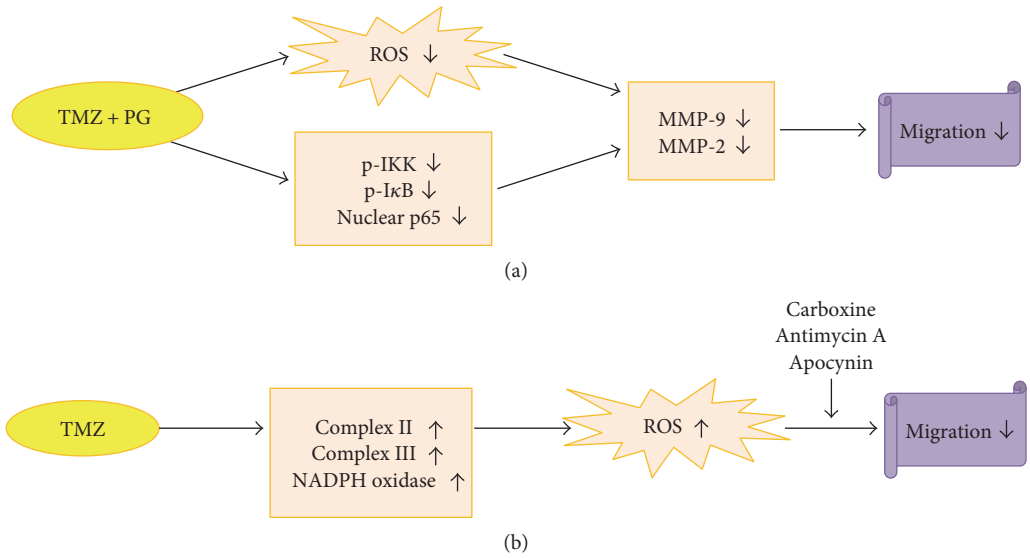

Figure 8: Diagrammatic scheme denoting the cascade of events in antimigration. (a) PG enhances the effect of TMZ on antimigration through inhibition of ROS generation, the NF-κB pathway, and MMP activities; (b) inhibition of mitochondrial complex II, III, and NADPH oxidase contributes to the antimigration effect of TMZ.

TMZ through inhibition of ROS generation, the NF-κB pathway, and MMP activities (Figure 8(a)); and (2) TMZ induces ROS generation from mitochondrial complex I, II, and III and NADPH oxidase, and inhibition of complex III and NADPH oxidase enhances the antimigration effect of TMZ (Figure 8(b)).

Conflicts of Interest

The authors declared no conflicts of interest.

Authors' Contributions

Jen-Tsung Yang and I-Neng Lee contributed equally to this work.

Acknowledgments

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Review Article

Natural Modulators of Endosomal Toll-Like Receptor-Mediated Psoriatic Skin Inflammation

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Psoriasis is a chronic inflammatory autoimmune disease that can be initiated by excessive activation of endosomal toll-like receptors (TLRs), particularly TLR7, TLR8, and TLR9. Therefore, inhibitors of endosomal TLR activation are being investigated for their ability to treat this disease. The currently approved biological drugs adalimumab, etanercept, infliximab, ustekinumab, ixekizumab, and secukizumab are antibodies against effector cytokines that participate in the initiation and development of psoriasis. Several immune modulatory oligonucleotides and small molecular weight compounds, including IMO-3100, IMO-8400, and CPG-52364, that block the interaction between endosomal TLRs and their ligands are under clinical investigation for their effectiveness in the treatment of psoriasis. In addition, several chemical compounds, including AS-2444697, PF-05387252, PF-05388169, PF-06650833, ML120B, and PHA-408, can inhibit TLR signaling. Although these compounds have demonstrated anti-inflammatory activity in animal models, their therapeutic potential for the treatment of psoriasis has not yet been tested. Recent studies demonstrated that natural compounds derived from plants, fungi, and bacteria, including mustard seed, *Antrodia cinnamomea* extract, curcumin, resveratrol, thiostrepton, azithromycin, and andrographolide, inhibited psoriasis-like inflammation induced by the TLR7 agonist imiquimod in animal models. These natural modulators employ different mechanisms to inhibit endosomal TLR activation and are administered via different routes. Therefore, they represent candidate psoriasis drugs and might lead to the development of new treatment options.

1. Introduction

Psoriasis is a common immune-mediated chronic inflammatory skin disease that affects the quality of life of 2%-3% of the global population. Psoriasis is typically associated with red, scaly, raised plaques resulting from a marked thickening of the epidermis induced by enhanced keratinocyte proliferation, leukocyte infiltrates in the epidermis and dermis, and inflammation [1–5]. Leukocyte infiltrates in psoriatic lesions primarily comprise dendritic cells (DCs), macrophages, neutrophils, and T cells. DCs generate multiple proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-23, that promote the development of psoriasis. TNF-α is a potent proinflammatory stimulus that promotes IL-23 production in DCs. IL-1β can activate IL-17 secretion from Th17 cells. IL-6 protects cutaneous T cells from Treg suppression and promotes Th17 participation in inflammation. Together, these immune cells and cytokines promote the inflammatory responses that underlie the development of psoriatic lesions.

Psoriasis can result from an interplay between genetic factors and external factors, including microbial infections, skin injuries, immune disorders, environmental influences, weather, and stress [6–15]. Nevertheless, the molecular mechanisms underlying the pathogenesis of this disease are not yet fully understood. TLRs are a family of pattern recognition receptors (PPRs) that localize to the cell surface or intracellular vesicles and are responsible for recognizing pathogen-associated molecular patterns (PAMPs) associated with microbes and danger-associated molecular patterns (DAMPs) released from dead cells in damaged tissues. A

group of intracellular TLRs referred to as endosomal TLRs contributes to the pathogenesis and development of psoriasis by sensing endogenous DNA and RNA released from dead cells. In this review, we discuss current knowledge on the mechanism underlying endosomal TLR activation and the link between endosomal TLR activation and the pathogenesis of psoriasis. This mechanism can inform the development of therapeutics for psoriasis that target endosomal TLRs. Synthetic antagonists of endosomal TLRs are currently being developed. Natural products from plants, fungi, and bacteria are promising candidate drugs in this context because of their diverse structures and bioactivities. Many natural compounds have demonstrated acceptable safety profiles and immunomodulatory activity [16, 17]. We also discuss recently identified natural compounds that inhibit endosomal TLRs and reduce psoriatic inflammation via different mechanisms.

2. Toll-Like Receptors

The innate immune system is the first line of host defense to microbial infections. Innate immune cells use a diverse variety of PPRs including TLRs, nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), C-type lectin-like receptors (CLRs), retinoic acid-inducible gene- (RIG-) I-like receptors (RLRs), and intracellular DNA sensor proteins to detect a wide variety of microbial PAMPs that initiate intermediate innate immune responses and lead to the development of adaptive immune responses [18–29]. Of them, TLRs are the most well-characterized PRRs. Thirteen TLRs have been identified in mammals, and ten of these (TLR1–10) are expressed in humans [30–35]. Human TLRs are strongly expressed in multiple types of immune cells, including DCs, macrophages, monocytes, natural killer cells, B cells, and T cells. They are also expressed in other cell types, including keratinocytes, chondrocytes, endothelial cells, and fibroblasts. Human TLRs are type I transmembrane receptors that feature an extracellular domain, a transmembrane region, and a highly conserved cytoplasmic region. The extracellular domain consists of multiple leucine-rich repeats (LRRs). The cytosolic region contains a Toll/interleukin-1 receptor (TIR) domain that mediates protein-protein interactions with the TIR domains of MyD88 adaptor protein family members, and these interactions initiate downstream intracellular signaling pathways [35–41].

TLRs interact with a diverse variety of microbial PAMPs via their extracellular domain (Figure 1). TLR2 recognizes a broad range of microbial components, including peptidoglycan, lipoteichoic acids, lipoproteins, lipoarabinomannan, glycophosphatidylinositol anchors, prions, and zymosan [42–48]. TLR2 and TLR6 form a complex that selectively recognizes mycoplasma macrophage-activating lipopeptide 2, whereas a heterodimer composed of TLR2 and TLR1 selectively recognizes bacterial lipoproteins and triacyl lipopeptides. Natural ligands of TLR10 have not yet been identified [49–51]. TLR4 is the primary receptor responsible for recognizing lipopolysaccharides on the outer membrane of gram-negative bacteria, and TLR5 recognizes flagellin, a component of bacterial flagella [52, 53]. The binding of members of the TLR3, TLR7, TLR8, and TLR9 subfamilies to their ligands is mediated by the recognition of nucleic acid-derived structures. TLR3 recognizes double-stranded RNA (dsRNA) generated during viral replication in infected cells [54]. TLR7 and TLR8 recognize single-stranded RNA viruses such as vesicular stomatitis virus and the influenza virus [55, 56]. TLR9 is required for the response to microbial unmethylated CpG DNA [57, 58]. Most CpG sites in mammalian cells are methylated, whereas microbial CpG sites are typically unmethylated; therefore, unmethylated CpG DNA is a microbial PAMP [59, 60]. In addition, TLRs recognize a wide variety of DAMPs released from dead cells at inflammatory sites (Figure 1). DAMPs recognized by TLRs include cellular components and stress-induced gene products such as extracellular matrix components, extracellular proteins, intracellular proteins, and nucleic acids [61, 62]. TLR2 recognizes heat shock proteins (HSPs), Gp96, biglycan, hyaluronic acid, hyaluronan, high-mobility group box 1 (HMGB1), versican, and monosodium urate crystal [63–71]. TLR4 recognizes HSPs, Gp96, HMGB1, oxidized phospholipids, heparan sulfate, fibrinogen, fibronectin, tenascin-C, hyaluronic acid, and hyaluronan [64, 69–79]. TLR3, TLR7, TLR8, and TLR9 are activated by host RNA and host DNA from necrotic cells [80–83].

Upon activation by PAMPs or DAMPs, TLR monomers dimerize, and their cytosolic domains subsequently recruit adaptor proteins from the MyD88 family (MyD88, TRIF/TICAM-1, TIRAP/Mal, TIRP/TRAM, and SRAM), thereby initiating downstream signaling pathways [84] (Figure 1). With the exception of TLR3, which signals via a TRIF-dependent signaling pathway, all TLRs signal via a MyD88-dependent pathway. In the MyD88-dependent pathway, a MyD88/IRAK1/IRAK4/TRAF6 complex activates TAK1, thereby promoting the activation of several transcription factors, including NF-κB and AP-1. TLR3 and TLR4 recruit TRIF to activate NF-κB, AP-1, and IRF3/7. NF-κB and AP-1 activation involves TRAF6 and RIP, and IRF3/7 activation is mediated by a TBK1-IKKε/IKKi complex (Figure 1). These transcription factors are key regulators of the expression of adhesion and costimulatory molecules and the production of various inflammatory cytokines required for the maturation, differentiation, and proliferation of DCs, natural killer cells, and cytotoxic T cells [41, 85–88].

3. Endosomal Toll-Like Receptors

The ten human TLRs are divided into three phylogenetic subfamilies. The first subfamily comprises TLR1, TLR2, TLR6, and TLR10. The second subfamily comprises TLR4 and TLR5, and the third subfamily comprises TLR3, TLR7, TLR8, and TLR9 (Figure 2(a)). TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface. In contrast, TLR3, TLR7, TLR8, and TLR9 localize to the endoplasmic reticulum and are trafficked to the endosomal/lysosomal compartment where they initiate cellular responses upon their activation by PAMPs and DAMPs. Therefore, these four TLRs are referred to as endosomal TLRs [89, 90]. In addition to their unique mechanism of ligand recognition,

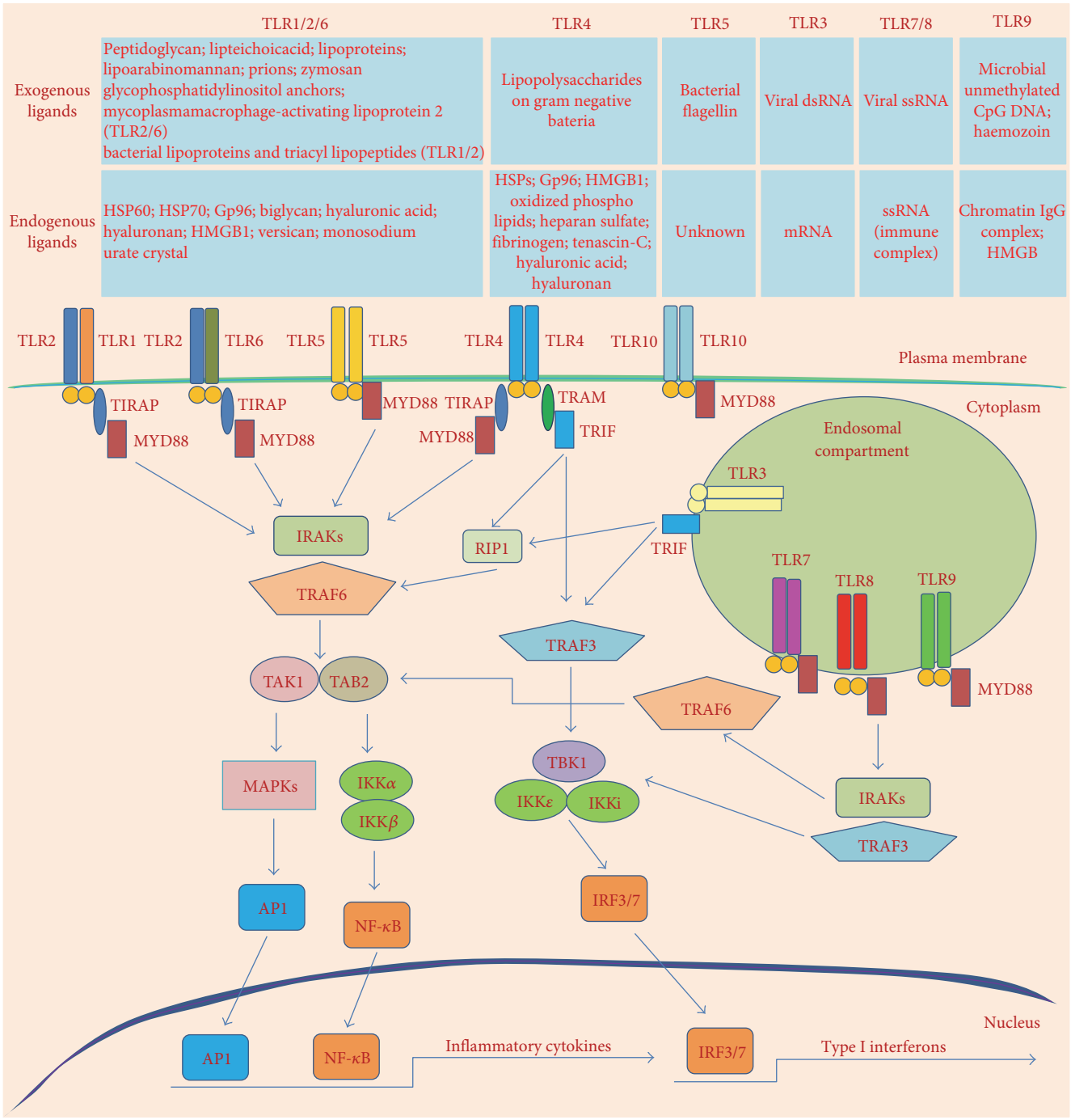

Figure 1: TLR ligands and signaling pathways. TLRs localize to the cell surface and to intracellular vesicles such as endosomes where they respond to their exogenous and endogenous ligands as shown. The TLRs utilize adaptor proteins of the MyD88 family, including MyD88, TRIF, TIRAP, and TRAM, to initiate downstream signaling pathways that induce the activation of various transcription factors, including NF-κB, AP-1, and IRF3/7, and the production of inflammatory cytokines and type I interferons.

the four endosomal TLRs are also distinct from other TLRs with respect to protein length. Specifically, TLR7, TLR8, and TLR9 are composed of more than 1000 amino residues (Figure 2(a)), and their extracellular domain is longer than that of other TLRs. Most TLRs contains 19–25 LRRs arranged in a horseshoe-shaped solenoid structure that mediates ligand binding. TLR7, TLR8, and TLR9 have 25 LRRs and a unique undefined region/Z-loop between LRR14 and LRR15 (Figure 2(b)). Previous studies have shown that mouse and rat TLR8 has low activity levels and can only be activated by agonists in the presence of PolyT-ODN. The lack of a five-amino-acid motif in the undefined region/Z-loop is proposed to account for the weak activity of these two TLR8 homologues, suggesting that the undefined region/Z-loop plays a role in the activation of TLR7, TLR8, and TLR9 [91, 92].

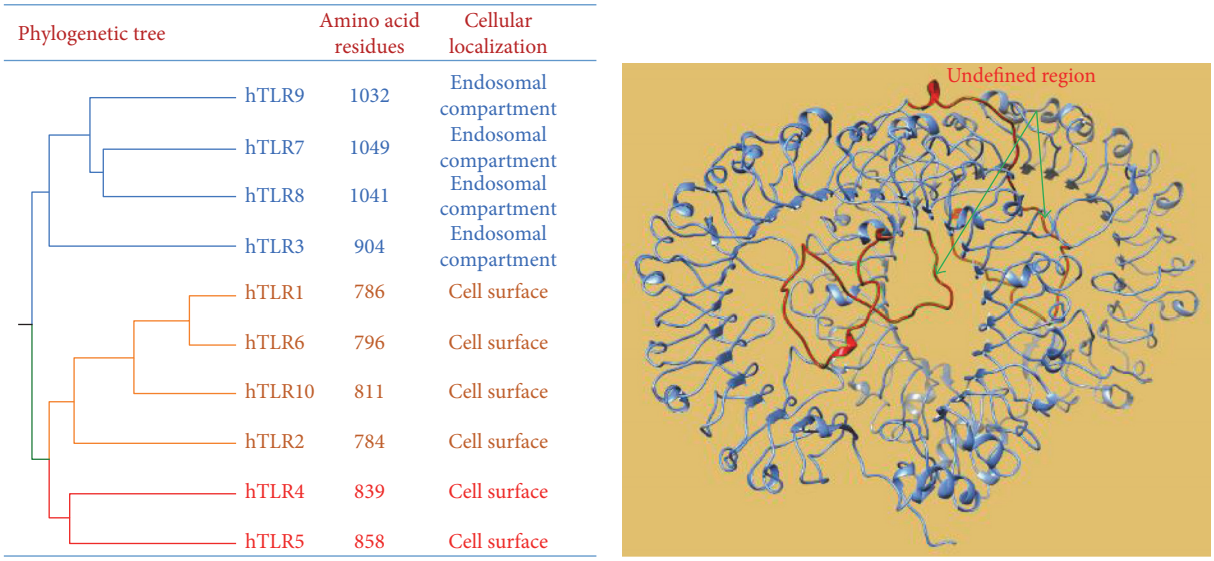

Figure 2: Subfamilies and the extracellular structure of human TLRs. (a) Human TLRs are divided into three phylogenetic subfamilies shown with different colors. The number and cellular location of amino acid residues are shown in the middle and right columns, respectively. (b) Computational modeling of the ectodomain structure of dimerized TLR7. Blue color shows the horseshoe-shaped solenoid structure of TLR ectodomain. Arrowheads indicate undefined regions (red color).

Endosomal TLRs must be trafficked from the endoplasmic reticulum to specific cellular locations in order to be activated. The intracellular trafficking of endosomal TLRs is regulated by accessory proteins such as UNC-93 homolog B1 (UNC93B1) and specific adaptor proteins (APs). UNC93B1 directly interacts with endosomal TLRs in the endoplasmic reticulum, facilitates their transport to the Golgi apparatus via coat protein complex II (COPII) vesicles, and remains associated with them in endosomes. AP1 and AP2 are required for UNC93B1-mediated endosomal TLR trafficking. AP3 facilitates TLR9 trafficking from endosomes to lysosome-related organelles (LROs). In contrast to the effects of TLR9 activation at endosomes, TLR9 activation at LROs induces the production of type I interferons rather than proinflammatory cytokines [93–95].

After reaching the endosomal compartment, TLRs are cleaved by specific proteases, including asparagine endopeptidase (AEP) and cathepsins such as cathepsin B, cathepsin H, cathepsin K, cathepsin L, and cathepsin S [96–98]. For example, TLR9 is cleaved at its undefined region/Z-loop in endosomes, and this cleavage is a prerequisite for its activation. Interestingly, following proteolytic cleavage, the N-terminal and C-terminal portions remain associated with one another, and this association is required for protein function [98–102]. This raises the question of what the role of proteolytic cleavage in TLR activation is. Recent studies have shown that the cleaved and uncleaved receptors bind with equal affinity to their ligand [101, 103]. Nevertheless, ligand-induced TLR dimerization requires proteolytic cleavage to relieve steric hindrance at the undefined region/Z-loop. This finding is consistent with the observation that endosomal acidification is required for endosomal TLR activation, as an acidic pH is required for the activation of cathepsins and most endosomal and lysosomal proteases [99–101].

4. The Role of Endosomal Toll-Like Receptors in the Pathogenesis of Psoriasis

In recent years, significant progress has been made in our understanding of the molecular mechanisms underlying the pathogenesis of psoriasis and the role of endosomal TLRs in this process. As shown in Figure 3, in the initiation phase, external triggers such as microbial infections and skin injuries induce the release of the antimicrobial peptide LL37 from keratinocytes and the release of self-DNA and self-RNA from dying cells to activate endosomal TLRs [104–106]. These TLRs can typically distinguish pathogen-derived nucleic acids from self-derived nucleic acids. Nucleic acids derived from viruses during cytosolic replication can be transported into endosomes during the process of autophagy where they activate endosomal TLRs. However, the localization of endosomal TLRs to intracellular compartments prevents their activation by self-nucleic acids under physiological conditions, because self-nucleic acids from dead cells in damaged tissues are unable to passively enter other cells and endosomes [107, 108]. Nevertheless, tolerance to self-nucleic acids can be evaded under some pathological conditions. For example, the antimicrobial peptide LL37 is upregulated and delivered to inflammatory sites in psoriatic skin where it forms complexes with self-nucleic acids to facilitate their entry into DCs and the subsequent activation of endosomal TLRs. These events render nonstimulatory self-nucleic acids into potent immune stimuli [104–106, 109, 110].

Endosomal TLRs are differentially expressed in different subsets of DCs. Plasmacytoid DCs (pDCs) express TLR7

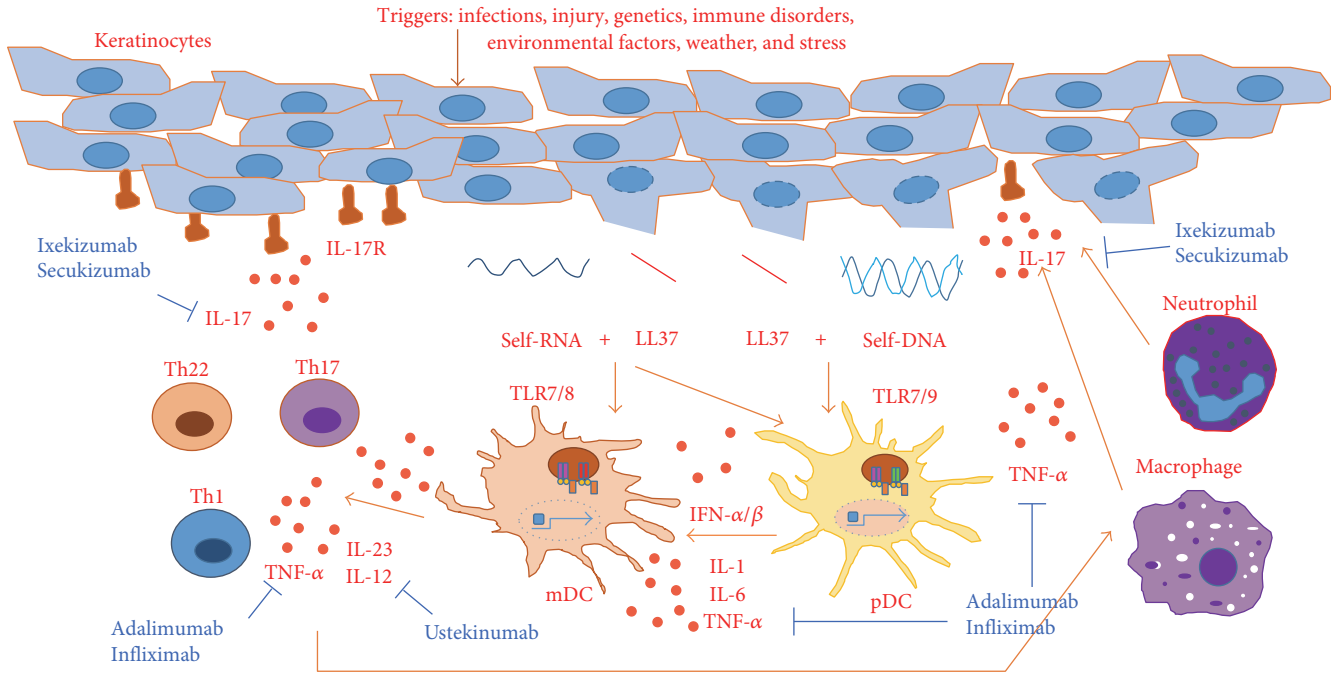

Figure 3: The role of endosomal TLRs in the development of psoriasis and the mechanism of action of biological drugs. Endosomal TLRs in plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) can be triggered by self-DNA and self-RNA that forms complexes with LL37 upon its release from necrotic cells. Cytokines released by DCs further drive T cell-mediated inflammation by activating cytokines that promote leukocyte recruitment and keratinocyte activation and proliferation. Blue font: biological drugs inhibit effector cytokines in psoriatic inflammation.

and TLR9, and myeloid DCs (mDCs) express TLR7 and TLR8 [111]. Thus, LL37/RNA and LL37/DNA complexes can trigger the production of various proinflammatory cytokines, including TNF-α, IL-1, and IL-6, as well as type I interferons in pDCs, by activating TLR7 and TLR9. Cytokines produced by pDCs in turn promote the activation of mDCs. In addition, LL37/RNA complexes can directly activate mDCs via TLR7 and TLR8, thereby inducing the production of IL-12 and IL-23 in mDCs at psoriatic inflammatory sites (Figure 3). These cytokines activate T cells into Th1, Th22, and Th17 cells, thereby further activating cytokines that promote keratinocyte activation and proliferation and the recruitment of inflammatory cells such as neutrophils and macrophages to psoriatic lesions [112, 113]. Together, these events result in chronic cutaneous inflammation.

5. Evidence for the Involvement of Endosomal TLRs in Psoriatic Inflammation

The involvement of endosomal TLRs in the pathogenesis of psoriasis is supported by studies of imiquimod in mouse models of psoriasis. Imiquimod is a small molecular weight agonist of TLR7. Aldara™ is a 5% imiquimod cream approved for the treatment of genital warts and superficial basal cell carcinoma. In mouse models, consecutive topical application of Aldara cream to the ear or shaved back skin causes inflammation, and the responses to imiquimod in mice closely resemble symptoms of human psoriasis, including skin thickening and erythema. Aldara not only causes phenotypic changes consistent with psoriasis but it also induces leukocyte infiltration and activation of the IL-23/Th17 axis, suggesting that the mechanism of imiquimod-induced pathogenesis is similar to the pathogenesis of human psoriasis [114–116]. Consistent with these findings, there have been reports of psoriasis associated with the clinical application of imiquimod in patients with basal cell carcinoma or actinic keratosis with or without a history of psoriasis [116–118].

Direct evidence that endosomal TLRs are potential therapeutic targets of psoriasis treatment stems from clinical investigation of TLR antagonists. In a phase 2 clinical trial in patients with moderate to severe psoriasis, immune modulatory oligonucleotide- (IMO-) 3100, an antagonist of TLR7 and TLR9, was associated with a reduction in Psoriasis Area Severity Index (PASI) score. In an animal model of psoriasis established by intradermal injection of IL-23 in the dorsum, IMO-3100 inhibited epidermal hyperplasia. IL-23 injection altered the expression of more than 5000 genes and upregulated the expression of genes associated with IL-17 signaling [119]. Treatment with IMO-3100 modulated the expression of 1900 of genes and downregulated the expression of IL-17-regulated genes. IMO-8400 is a second generation IMO that antagonizes TLR7, TLR8, and TLR9. Similar to IMO-3100, IMO-8400 inhibited symptoms of psoriasis; however, IMO-8400 had a broader effect on the expression of IL-23-induced genes. In a phase 2a clinical trial evaluating the safety and efficacy of IMO-8400 compared with placebo in patients with moderate to severe plaque psoriasis, IMO-8400 did not cause any serious or severe adverse effects and it demonstrated clinical improvements. PASI-50 with IMO-8400 was

Table 1: Inhibitors for endosomal TLR-mediated inflammation.

Inhibitor	Natural/synthetic	Target and mechanism of action
IMO-3100	Synthetic	Antagonist of TLRs
IMO-8400	Synthetic	Antagonist of TLRs
IRS-954	Synthetic	Antagonist of TLRs
DV117	Synthetic	Antagonist of TLRs
INH-ODN-24888	Synthetic	Antagonist of TLRs
CPG-52364	Synthetic	Antagonist of TLRs
Chloroquine	Synthetic (quinine derivative)	Inhibits endosomal acidification or sequester TLR ligands
Hydrochloroquin	Synthetic (quinine derivative)	Inhibits endosomal acidification or sequester TLR ligands
Quinacrine	Synthetic (quinine derivative)	Inhibits endosomal acidification or sequester TLR ligands
Bortezomib	Synthetic	Inhibits TLR trafficking
SM934	Synthetic	Promotes downregulation of TLRs
ST-2825	Synthetic	MyD88 inhibitor
AS-2444697	Synthetic	IRAK4 inhibitor
PF-05387252	Synthetic	IRAK4 inhibitor
PF-05388169	Synthetic	IRAK4 inhibitor
PF-06650833	Synthetic	IRAK4 inhibitor
ML120B	Synthetic	IKK2 inhibitor
PHA-408	Synthetic	IKK2 inhibitor
Mustard seed	Natural (mustard plant product)	Inhibit NF-κB activation and cytokine expression
Antrodia cinnamomea extract	Natural (*A. cinnamomea* product)	Inhibit cytokine expression
Curcumin	Natural (grapes product)	Inhibit NF-κB activation and cytokine expression
Resveratrol	Natural (grapes product)	Inhibit NF-κB activation and cytokine expression
Thiostrepton	Natural (*Streptomyces* product)	Inhibits endosomal acidification and proteasomal activity
Azithromycin	Natural (*Streptomyces* product)	Inhibits endosomal acidification and proteasomal activity
Andrographolide	Natural (*A. paniculata* product)	Promotes downregulation of myD88

38% compared with 1% with placebo, and PASI-75 and PASI-90 with IMO-8400 were 17% and 2%, respectively, compared with 0% with placebo [119, 120]. These findings support the hypothesis that blocking endosomal TLR activation is a promising therapeutic approach for the treatment of psoriasis.

6. Strategies for Blocking Endosomal TLR-Mediated Inflammation

The design of strategies to block inflammatory responses elicited by the activation of distinct endosomal TLRs can be based on their unique functions and signaling mechanisms. These strategies are (1) neutralizing cytokines that mediate the effects of endosomal TLRs, (2) blocking TLR ligand interactions using TLR antagonists, (3) blocking TLR ligand interactions by sequestering TLR ligands, (4) blocking TLR activation by inhibiting proteasomal activity and endosomal acidification, (5) downregulating TLRs and their downstream signaling molecules, and (6) inhibiting signal transduction downstream of endosomal TLR activation. The feasibility of these strategies to treat psoriasis and other autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), has been demonstrated by the action mechanisms of the biological drugs and synthetic compounds (shown in Figure 3 and Table 1) that are currently being used or investigated.

6.1. Biological Drugs That Neutralize Effector Cytokines. Several biological drugs targeting cytokines that can be directly or indirectly generated by the activation of endosomal TLRs have been approved by FDA for the treatment of psoriasis. These biological drugs can be divided into three classes: TNF-α antagonists, IL-12/IL-23 inhibitors, and IL-17A inhibitors. The TNF-α antagonists adalimumab, etanercept, and infliximab have demonstrated strong efficacy in the treatment of moderate to severe psoriasis. Adalimumab is a fully humanized monoclonal antibody [121–123]. Etanercept is a recombinant fusion protein containing a TNF-α receptor ligand-binding domain and a human IgG Fc domain [124–126]. Infliximab is a chimeric monoclonal antibody [127–129]. TNF-α antagonists reduce inflammatory responses at psoriatic sites and downregulate the differentiation and function of Th17 cells. These findings suggest that TNF-α functions upstream of the IL-23/Th17 axis. Ustekinumab is a member of the class of psoriasis drugs that target IL-12/IL-23. It is a humanized monoclonal antibody that neutralizes the p40 subunit common to both IL-12 and IL-23, thereby preventing the binding of these cytokines to their receptors and the subsequent initiation of Th1- and Th17-mediated signaling pathways [130, 131]. Ustekinumab demonstrated a superior

clinical effect to etanercept, suggesting that IL-23 plays a key role in the pathogenesis of psoriasis [132, 133]. The human monoclonal antibodies ixekizumab and secukizumab are members of the class of psoriasis drugs that target IL-17A. Multiple studies have shown that ixekizumab and secukizumab inhibit the expression of a wide variety of genes associated with Th17- and Th1-mediated inflammatory responses. Although these biological drugs have demonstrated efficacy in the treatment of psoriasis, their use is limited by their high cost and the fact that they must be administered by injection. Therefore, synthetic chemical drugs and natural inhibitors continue to be investigated and developed.

6.2. TLR Antagonists. IMO-3100 and IMO-8400 (discussed in Section 5) bind to endosomal TLRs, thereby preventing the interaction of endosomal TLRs with their agonists. Other immune inhibitory oligonucleotides that directly interact with TLR7 and TLR9, including IRS-954, DV117, and INH-ODN-24888, have also been developed [134–137]. The binding of these compounds to endosomal TLRs blocks their access to the agonists that trigger their activation. Although their therapeutic potential for the treatment of psoriasis has not yet been evaluated, these compounds have demonstrated immune inhibitory effects in preclinical and clinical SLE trials. CPG-52364 is another endosomal TLR inhibitor that blocks ligand-induced activation of TLR7, TLR8, and TLR9. Structurally distinct from other inhibitory oligonucleotides, CPG-52364 is a derivative of the small molecular weight chemical compound quinazoline. CPG-52364 has been reported to be well tolerated in clinical trials evaluating its effect in the treatment of several inflammatory autoimmune diseases, including psoriasis, RA, and SLE [137–139].

6.3. Compounds That Inhibit Endosomal Acidification, Proteasomal Activity, and Sequestering TLR Ligands. The antimalarial drugs chloroquine, hydroxychloroquine, and quinacrine are derivatives of quinine, a natural alkaloid isolated from the South American cinchona bark tree. Although they are primarily used to treat malaria, these drugs have long been used for treating skin diseases and reducing inflammation in RA and SLE. More recent studies demonstrated that these compounds function as inhibitors of TLR7, TLR8, and TLR9 [140, 141]. They are weak bases; therefore, their ability to inhibit endosomal TLR activation has been attributed to their ability to inhibit endosomal acidification. These antimalarial compounds have also been shown to directly interact with nucleic acid-based TLR ligands, thereby sequestering these ligands and preventing them from binding to endosomal TLRs. Bortezomib (Velcade) is one example of a drug that targets proteasomal activity. Bortezomib is a proteasome inhibitor approved for the treatment of multiple myeloma, and it has demonstrated inhibitory effects in several autoimmune disorders, including psoriasis, RA, and SLE, in animal models [142–144]. In addition, bortezomib has been shown to suppress the trafficking of TLR9 to endolysosomes, inhibit TLR9 activation, and reduce lupus- and psoriasis-associated inflammation [145, 146].

6.4. Compounds That Downregulate Endosomal TLRs and Inhibit TLR Signaling. Other small molecular weight chemical compounds that inhibit TLR signaling and TLR-mediated inflammatory responses in autoimmune diseases include SM934, ST-2825, IRAK4 inhibitors, and IKK2 inhibitors. SM934 (β-aminoarteether maleate), a derivative of artemisinin, possesses potent antiproliferative and anti-inflammatory properties. In a preclinical study, SM934 provided a significant protective effect in a mouse model of SLE. SM934 inhibits TLR activation by promoting the downregulation of TLR7, TLR9, and MyD88 mRNA expression and the inhibition of NF-κB phosphorylation [147–149]. Other compounds that block NF-κB activation and cytokine production by targeting molecules associated with TLR signaling have also been developed. For example, the peptide mimetic ST-2825 targets MyD88. ST-2825 interferes with MyD88-mediated recruitment of IRAK1 and IRAK4 to the TLR signalsome, thereby inhibiting TLR-mediated inflammatory responses. This compound has been shown to inhibit TLR9 activation and block the production of autoantibodies in B cells in SLE patients [150, 151]. AS-2444697, PF-05387252, PF-05388169, and PF-06650833 target the kinase activity of IRAK4 and have been investigated in preclinical or clinical studies for the treatment of multiple inflammatory and autoimmune diseases, including gout, sepsis, AR, and SLE [152, 153]. IKK2 is a subunit of IκB kinase, a protein that controls NF-κB activation and the production of TLR-induced inflammatory cytokines. The chemical compounds ML120B and PHA-408 inhibit IKK2 kinase activity and have exhibited anti-inflammatory effects in an animal model of arthritis [154–156].

7. Natural Inhibitors of Endosomal TLR-Mediated Psoriatic Inflammation

Many natural compounds derived from bacteria, fungi, and plants have long been known to exhibit immunomodulatory activity and have been used for the treatment of inflammation-related disorders [16, 17]. For example, retinoids, vitamin D, and their corresponding analogs are used in topical treatments for psoriasis [157, 158]. These compounds bind to their endogenous cellular receptors, thereby initiating the transcription of genes that suppress inflammation and inhibit cell proliferation. In addition, the following plant extracts and natural compounds have been reported to inhibit endosomal TLR-mediated psoriatic inflammation in animal models via different mechanism of actions shown in Table 1. These natural products have therapeutic potential in the treatment of psoriasis.

7.1. Mustard Seed. Mustard seed from mustard plant is a popular food seasoning worldwide, especially in Japan, India, and China. Mustard seed possesses several biological effects, including anti-inflammatory, antioxidant, and antitumor effects. These effects are mediated by multiple active components, including erucic acid, isothiocyanate, phenols, and phytin [159]. In mice, a diet supplemented with 5% mustard seed for three weeks attenuated imiquimod-induced psoriasis-like inflammation. Mustard seed inhibited the

infiltration of various types of leukocytes, including DCs, macrophages, and T cells, into psoriatic lesions. In addition, PASI score significantly decreased in mustard seed-fed mice compared with control mice. Furthermore, NF-κB, IFN-α, IL-17, and IL-22 levels decreased in the psoriatic lesions of mustard seed-fed mice compared with control mice [160]. However, the specific component mediating this effect and its molecular target in this context remains unclear.

7.1.1. Antrodia cinnamomea (A. cinnamomea) Extract. *A. cinnamomea* is a species of fungus commonly used in Asia as a medicinal herb. It possesses a broad range of biological effects, including anti-inflammatory, antioxidant, antifatigue, and antitumor effects [161–163]. A previous study evaluated the effects of orally administered lyophilized extract from the fruit body of *A. cinnamomea* on disease severity in an animal model of imiquimod-induced psoriasis. *A. cinnamomea* extract reduced psoriasis-like inflammation, infiltration of CD4+ T cells, CD8+ T cells, and neutrophils, and the expression of TNF-α, IL-17A, and IL-22 in imiquimod-induced psoriatic skin lesions [164]. Although these observations support a role for *A. cinnamomea* in the treatment of endosomal TLR-mediated psoriasis inflammation, the effective component mediating these effects and the molecular target of *A. cinnamomea* in this context remain unclear.

7.2. Curcumin. Curcumin is a bright yellow powder obtained from the rhizome of several types of ginger plants. Chemically, curcumin is a diarylheptanoid belonging to the curcuminoid group. It has diverse bioactive effects, including anti-inflammatory, antioxidant, antitumor, and antiatherosclerotic effects [165–167]. Topical use of a curcumin-formulated gel has been shown to inhibit imiquimod-induced psoriasis-like inflammation. Curcumin treatment significantly inhibited imiquimod-induced epidermal hyperplasia and TNF-α, IL-1β, IL-6, IL17A, IL-17F, and IL-22 production in psoriatic lesions [168]. Curcumin is known to inhibit NF-κB activation by inhibiting IκB phosphorylation and degradation. NF-κB signaling mediates the production of the inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-23, which cooperatively induce the production of IL-17 cytokines and IL-22 from dermal $\gamma\delta$ T cells. Therefore, curcumin might inhibit endosomal TLR-induced psoriatic inflammation by targeting NF-κB signaling. Consistent with this hypothesis, curcumin-mediated inhibition of NF-κB activation resulted in the downregulation of IL-17 and IL-22.

7.3. Resveratrol. Resveratrol is a stilbenoid, which is a type of natural phenol found in grapes, berries, and nuts. It possesses anti-inflammatory and antioxidant effects and is used as a dietary supplement [169, 170]. Oral administration of resveratrol diminished the severity of imiquimod-induced psoriasis-like inflammation in an animal model, and microarray analysis revealed that resveratrol treatment inhibited imiquimod-induced expression of IL-17A, IL-19, and IL-23p19. Resveratrol has been shown to inhibit LPS- and TNF-α-induced NF-κB activation [171]. Thus, resveratrol might inhibit imiquimod-induced psoriatic inflammation by directly or indirectly targeting signaling of NF-κB.

7.4. Thiostrepton. Thiostrepton, an antibiotic derived from several strains of *Streptomycetes*, functions as an antagonist of TLR7, TLR8, and TLR9. It was identified using a connectivity map screen for functional analogs of bortezomib. Subsequent studies demonstrated that thiostrepton inhibited TLR7-, TLR8-, and TLR9-mediated NF-κB activation in a cell-based assay. It also reduced the production of TNF-α and IL-12/23p40 induced by R848 and LL37/RNA complexes (TLR7 and TLR8 ligands) and by CpG-ODN and LL37/DNA complexes (TLR9 ligands) in DCs. This natural antibiotic inhibited imiquimod-induced psoriasis-like inflammation in mice. Specifically, thiostrepton inhibited the accumulation of monocytes and DCs and the expression of TNF-α, IL-1β, and IL-8 in inflammatory lesions. Thiostrepton uses two mechanisms to block endosomal TLR activation. One mechanism depends on its proteasomal inhibitory activity, similar to the mechanism underlying bortezomib-mediated inhibition of endosomal TLR activation. The other mechanism depends on its ability to inhibit endosomal acidification [172].

7.5. Azithromycin. Similar to thiostrepton, azithromycin is an antibiotic isolated from *Streptomycetes*. Azithromycin possesses anti-inflammatory and immunomodulatory properties and is used to treat bacterial infections [173, 174]. Azithromycin inhibited imiquimod-induced expression of costimulatory molecules (CD40 and CD80) and cytokines (TNF-α, IL-10, IL-12p40, IL-12p70, and IL-23p19) in bone marrow-derived DCs (BMDCs), and topical treatment with azithromycin attenuated the severity of imiquimod-induced skin inflammation in an animal model of psoriasis. Azithromycin treatment inhibited keratinocyte hyperproliferation and the accumulation of DCs, CD4+ T cells, and CD8+ T cells in psoriatic lesions. The functional mechanism of azithromycin in this context is similar to that of thiostrepton. Azithromycin inhibited lysosomal acidification and the proteolytic processing of TLR7, thereby blocking imiquimod-induced NF-κB and IRF7 activation in DCs [175]. In addition, a clinical study demonstrated that long-term oral azithromycin treatment improved PASI score in patients with chronic plaque psoriasis, further confirming the antipsoriatic function of this antibiotic [176].

7.6. Andrographolide. Andrographolide is a labdane diterpenoid isolated from the stem and leaves of *Andrographis paniculata*. This natural compound possesses anti-inflammatory activity and is currently used as a prescription medicine in China for the treatment of laryngitis, diarrhea, and RA. Intragastric administration of andrographolide alleviated imiquimod-induced psoriasis, but not IL-23-induced psoriasis, in mice. The therapeutic effect was dose-dependent, and treatment with 10 mg/kg andrographolide was as effective as treatment with 10 mg/kg etanercept in improving clinical scores in mice with imiquimod-induced psoriasis. Andrographolide inhibited imiquimod-induced expression of the genes encoding CD80, CD86, IL-1β, IL-6, and IL-23 in

Table 2: Key points of natural modulator in inhibition of psoriatic skin inflammation.

(1) Less cost
(2) More choice of administration routes
(3) More diverse sources
(4) More diverse chemical structures
(5) More diverse targets in endosomal TLR-mediated inflammatory signaling pathways
(6) More diverse mechanisms to block endosomal TLR-mediated inflammation

BMDCs. Treating BMDCs with andrographolide promoted MyD88 degradation and blocked the recruitment of TRAF6 to form signalsomes. Inhibiting autophagic proteolysis in BMDCs using NH_4Cl or deleting the gene encoding microtubule-associated protein 1 light chain 3 (MAP1LC3B) abolished andrographolide-induced MyD88 degradation [177]. These findings suggested that andrographolide induces autophagic proteolysis of MyD88, thereby reducing psoriatic inflammation by inhibiting TLR-mediated cytokine production.

8. Conclusion

As summarized in Table 2, the studies described in this review indicate that natural products from plants, fungi, and bacteria inhibit the activation of endosomal TLRs via mechanisms that block their functions in the initiation and development of psoriasis. Compared with biological drugs, these natural modulators can be more cost-effective and are administered via different routes. Therefore, they are promising candidate drugs for the treatment of psoriasis, and they might inform the development of multiple treatment options.

Conflicts of Interest

The authors declare that they have no conflict of interests regarding the publication of this manuscript.

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Research Article

Synergistic Antitumour Properties of *viscumTT* in Alveolar Rhabdomyosarcoma

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Aqueous mistletoe extracts from the European mistletoe (*Viscum album*) contain mainly mistletoe lectins and viscotoxins as cytotoxic compounds. Lipophilic triterpene acids, which do not occur in conventional mistletoe preparations, were solubilised with β-cyclodextrins. The combination of an aqueous extract (*viscum*) and a triterpene-containing extract (*TT*) recreated a whole mistletoe extract (*viscumTT*). These extracts were tested on rhabdomyosarcoma in vitro, ex vivo, and in vivo with regard to anticancer effects. *Viscum* and *viscumTT* inhibited cell proliferation and induced apoptosis effectively in a dose-dependent manner in vitro and ex vivo, whereas *TT* showed only moderate inhibitory effects. *viscumTT* proved to be more effective than the single extracts and displayed a synergistic effect in vitro and a stronger effect in vivo. *viscumTT* induced apoptosis via the extrinsic and intrinsic pathways, evidenced by the loss of mitochondrial membrane potential and activation of CASP8 and CASP9. CASP10 inhibitor inhibited apoptosis effectively, emphasising the importance of CASP10 in *viscumTT*-induced apoptosis. Additionally, *viscumTT* changed the ratio of apoptosis-associated proteins by downregulation of antiapoptotic proteins such as XIAP and BIRC5, thus shifting the balance towards apoptosis. *viscumTT* effectively reduced tumour volume in patient-derived xenografts in vivo and may be considered a promising substance for rhabdomyosarcoma therapy.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents in Germany, accounting for 3.1% of all paediatric tumours [1]. Alveolar RMS (ARMS) is one of the two main histopathological subtypes and occurs in approximately 25% of the cases [2–4]. ARMS is more common in adolescents and young adults and associated with aggressive clinical behaviour and poor treatment response and outcome [5]. Most of ARMS are characterised by the balanced translocations t(2;13)(q35;q14) or t(1;13)(q36;14) [6], resulting in the PAX3/FOXO1 (~55%) or PAX7/FOXO1 (~25%) fusion genes, respectively [7]. Despite aggressive multimodal treatment regimes, the prognosis of fusion-positive ARMS (65%) [8] and patients with metastatic disease (<20%) [9] remains poor and has not changed in years [10], highlighting the need for new therapy approaches.

Aqueous extracts of the European mistletoe (*Viscum album*) are widely used in complementary cancer therapy in German-speaking countries, despite their controversial efficacy since 1920. *Viscum album* contains a large variety of biological active compounds including mistletoe lectins (ML) [11], viscotoxins (VT) [12–14], triterpene acids [15, 16], flavonoids [17, 18], and polysaccharides [19, 20]. Commercially available mistletoe preparations are based on aqueous extracts and contain mainly ML and VT as active compounds. Mistletoe extracts have been shown to exert cytotoxic effects on acute leukaemia [21], multiple myeloma [22], head and neck squamous cell carcinomas [23], and bladder carcinoma [24] in vitro as well as acute leukaemia

[25], breast cancer [26], malignant melanoma [27], and pancreatic carcinoma [28] in vivo. Furthermore, there is clinical evidence that *Viscum album* extracts prolong the overall survival of patients with pancreatic cancer [29].

Lipophilic triterpene acids, such as oleanolic acid (OA) and betulinic acid (BA), represent another group of mistletoe-derived substances with known cytotoxic effects. Previous studies on neuroblastoma [30, 31], osteosarcoma [32], acute leukaemia [33], breast cancer [34], and gastric cancer [35] showed that OA and its derivatives inhibit cell proliferation and induce apoptosis effectively in vitro. Moreover, in vivo experiments have shown cytotoxic effects of OA on osteosarcoma [36], gallbladder carcinoma [37], hepatocellular carcinoma [38], and pancreatic carcinoma [39]. Due to their low solubility, triterpene acids do not occur in significant amounts in commercial mistletoe extracts [40]. Using cyclodextrins, it was possible to solubilise triterpene acids and therefore to overcome their loss in standardised aqueous extracts.

Previous studies showed the efficacy of a combined mistletoe extract produced by addition of solubilised triterpene acids (*TT*) to aqueous mistletoe extracts (*viscum*), creating a whole plant extract (*viscumTT*). This whole plant extract effectively induced apoptosis in acute myeloid and lymphoblastic leukaemia [41, 42], Ewing-sarcoma [43], and osteosarcoma [44] and inhibited tumour growth in murine melanoma in vivo [45]. The aim of this study was to analyse the effect of the whole mistletoe extract, *viscumTT*, on rhabdomyosarcoma in vitro, ex vivo, and in vivo.

2. Materials and Methods

2.1. Material and Reagents. RPMI 1640, penicillin, streptomycin, trypsin (0.05%), and phosphate-buffered saline (PBS) were purchased from Gibco®, Life Technologies (Darmstadt, Germany). Foetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). RIPA buffer, protease inhibitors, molecular mass standards for SDS-PAGE, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), 5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and propidium iodide (PI) were purchased from Sigma-Aldrich (München, Germany). Tween, acrylamide, and dithiotreitol (DTT) were purchased from Carl Roth GmbH (Karlsruhe, Germany).

2.2. Viscum album Extracts. Viscum album extracts were kindly provided by Birken AG (Niefern-Öschelbronn, Germany) and prepared from *Viscum album* harvested from apple trees (*Malus domestica* Borkh.) as previously described [41, 42, 46]. Intact mistletoe lectin-I (A + B chain) was quantified by ELISA in the *viscum* extract as described before [47]. OA and BA were quantified in the *TT* extract by gas chromatography-flame ionisation detector (GC-FID) and external calibration with OA as reference substance [40]. Both extracts, *viscum* and *TT*, were reconstituted in PBS to a final concentration of 1 μg/mL intact ML-I and <1 μg/mL VT in *viscum* and 4000 μg/mL OA and 172 μg/mL BA in *TT* (Table 1).

Table 1: Composition of mistletoe extracts.

Extract	CD [μg/mL]	ML [μg/mL]	VT [ng/mL]	BA [μg/mL]	OA [μg/mL]
viscum	—	1	221.3	—	—
TT	230	—	—	172	4000
viscumTT	230	1	221.3	172	4000

2.3. Cell Culture. Two human ARMS cell lines were used in this study. RH-30 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and RMS-13 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were maintained in RPMI 1640 medium with L-glutamine, supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. For experimental settings, 2×10^5/mL cells were seeded onto 6-well plates with a final volume of 2 mL/well. Cell numbers were adjusted to sizes of well plates according to experimental settings. Cells were cultured 24 h to allow cell attachment, then *viscum*, *TT*, and *viscumTT* were added in defined concentrations for another 24 h.

2.4. Determination of Cell Proliferation and Measurement of Early Cytotoxicity. Cell counts were determined with the CASY® Cell Counter and Analyser System of Schärfe System GmbH (Reutlingen, Germany). Alterations of cell proliferation were indicated as the percentage of untreated control cells (100% viability). To rule out early cytotoxicity, lactate dehydrogenase (LDH) release was measured after 2 h of incubation with *viscum*, *TT*, and *viscumTT* using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

2.5. Determination of Apoptosis. After 24 h incubation with *viscum*, *TT*, and *viscumTT* in increasing concentrations, cells were stained with APC-conjugated annexin V (BD Biosciences, Heidelberg, Germany) and 1 mg/mL PI as described before [42]. Cells were analysed by flow cytometry (FACSCalibur™, Becton Dickinson, Heidelberg, Germany), and results were evaluated with FlowJo® Software (Tree Star, Inc., Ashland, USA).

2.6. Assessment of Mitochondrial Membrane Potential ($\Delta\Psi_m$). Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were detected by JC-1 staining and flow cytometry, according to the manufacturer's instructions. Subsequently, results were evaluated with FlowJo Software. 50 μM depolarising CCCP was used as a reference control.

2.7. Determination of Caspase Activity. Activity of caspase-3 (CASP3), caspase-8 (CASP8), and caspase-9 (CASP9) was measured using the Green Caspase Staining Kit (Promokine, Heidelberg, Germany) according to the manufacturer's protocol. Subsequently, cells were analysed by flow cytometry and results were evaluated with FlowJo Software.

2.8. Caspase Inhibitor Assays. Cells were pretreated for one hour with 50–100 μM caspase inhibitors, before 24 h

incubation with *viscum* (2.5–5 ng/mL) and *viscumTT* (2.5–5 ng/mL *viscum* + 35–40 μg/mL *TT*). DMSO was added as a solvent control. Induction of apoptosis was determined by annexin V/PI staining and flow cytometry analysis as described above. In this experimental setting, we used, in addition to a pan-caspase inhibitor (Z-VAD-FMK), inhibitors of CASP8 (Z-IETD-FMK), CASP9 (Z-LEHD-FMK), and caspase-10 (CASP10; Z-AEVD-FMK) (R&D Systems, Minneapolis, MN, USA).

2.9. Protein Extraction and Western Blot Analyses. Cells were incubated with *TT* (OA 40 μg/mL), *viscum* (ML 5 ng/mL), and *viscumTT* (OA 40 μg/mL + ML 5 ng/mL) for 24 h. After washing twice with PBS, cells were lysed for 30 min in RIPA buffer containing protease inhibitors. Protein lysates were centrifuged at 14000 *rpm* for 5 min at 4°C, then protein concentrations were determined by Bradford Reagent (Bio-Rad, München, Germany). Lysates (20–25 μg protein/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Blots were incubated with primary antibodies overnight at 4°C. After incubation with HRP-conjugated secondary antibodies (Bio-Rad), protein bands were visualized by ECL (Thermo Fisher Scientific, Bonn, Germany) and Molecular Imager® ChemiDoc™ (Bio-Rad). The following primary antibodies were used: β-actin (#A3854, Sigma-Aldrich), B-cell lymphoma 2 (BCL2; #2870, Cell Signaling Technology, CST, Danvers, MA, USA), BCL2 like 1 (BCL2L1; #2764, CST), baculoviral IAP repeat containing 5 (BIRC5; #2803, CST), myeloid cell leukemia 1 (MCL1; sc-819, Santa Cruz Biotechnology, Santa Cruz, CA, USA), poly(ADP-ribose) polymerase 1 (PARP1; #9542, CST), and X-linked inhibitor of apoptosis protein (XIAP; #610716, BD Biosciences).

2.10. Ex Vivo Cultured RMS Primary Cells. Tumour samples were obtained as a treatment residue from three different patients with ARMS during routine surgical resection. Tumour tissue was not explicitly collected for this study, and diagnoses were confirmed by histopathology by the institute of pathology, Charité - Universitätsmedizin Berlin. Immediately after surgical excision, tumour tissue from patient no. 1 (11 years, female, recurrent disease), patient no. 2 (12 years, male, primary disease), and patient no. 3 (13 years, female, primary disease) was cut into smaller pieces and cultured as primary explants in RPMI 1640 base medium with L-glutamine supplemented with 20% heat-inactivated FCS and 1% penicillin/streptomycin solution. When cells dissociated from the explant and formed a confluent monolayer culture, apoptosis induction was investigated. Ex vivo cell cultures were used for experimental settings within seven trypsinised passages. Because of slow or bad cell growth, experiments could only be performed twice. For analyses, cells were seeded into 12-well microtiter plates at 1.5×10^5–3×10^5 cells/well and incubated with increasing concentrations of *viscum*, *TT*, and *viscumTT* as described above. Subsequently, apoptosis was measured by annexin V/PI assay. Further, JC-1 measurement was carried out with cells from patient no. 1. In accordance with the Declaration of Helsinki, written informed consent was obtained from the patients and experiments were approved by the local ethics committee of the Charité - Universitätsmedizin Berlin.

2.11. RMS Xenografts and Experimental Procedures. Patient-derived RMS tissue was subcutaneously transplanted into the left flank of eight-week-old NMRI-nu/nu mice. The mice were obtained from Charles River Laboratories (Sulzfeld, Germany), housed in a pathogen-free facility under pathogen-free conditions, and fed autoclaved standard diet (Sniff, Soest, Germany) with acidified drinking water ad libitum. The tumour was propagated in vivo, and tumour tissue from one in vivo passage was used for s.c. implantation in the inguinal region of seven (no. 1) or five (no. 2) mice per treatment or control group. The same patient material was used for ex vivo as well as in vivo experiments (no. 1 ex vivo = no. 1 in vivo, no. 2 ex vivo = no. 2 in vivo). Treatment with the test substances or controls started on day 12 when tumours were palpable. Mice were treated with *viscum*, *TT*, *viscumTT*, and cyclodextrins (control group) intratumourally (i.t) and doxorubicin (Doxo) intravenously (i.v.) in no. 1. In experiment no. 2, mice received *viscumTT* i.t. and *viscumTT* and cyclodextrins i.v. The mice were treated every two to three days in rising concentrations, and each dose was given twice. The administered concentrations were 40/60/80 mg/kg OA (*TT*), 0.5/1.0/1.5 μg/kg ML (*viscum*), or a combination thereof (*viscumTT*). Body weight was measured before each treatment, and mice were carefully monitored for health and symptoms of toxicity. The animals were sacrificed by cervical dislocation at the end of the experiment or if the mice were moribund (tumour volume > 1.2 cm^3 or >10% body weight loss). The animal experiments were carried out in accordance with the German legislation on the care and use of laboratory animals and in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of animals in Experimental Neoplasia to minimise suffering. Approval for the study was obtained from the Regional Office for Health and Social Affairs (LaGeSo, approval G-0030/15).

2.12. Statistics. All in vitro experiments were performed in independent experiments at least three times, for which means ± standard error are displayed in bar graphs. Ex vivo experiments were carried out only in sets of two independent experiments due to low cell growth. To assess whether the effect of *viscumTT* was synergistic, Webb's fractional product (*Fp) [48] was calculated as described before [42]. Values greater than one (*Fp > 1) display a synergistic effect. Two-way ANOVA and Bonferroni post hoc tests were applied to determine differences between mouse xenograft treatment groups. All results with $p \leq 0.05$ were considered significant.

3. Results

3.1. viscumTT Inhibits Cell Proliferation and Induces Apoptosis Synergistically In Vitro. To assess the antiproliferative effects of mistletoe extracts, RMS-13 and RH-30 cells were incubated with increasing concentrations of *viscum*, *TT*, and *viscumTT*. Both, *viscum* and *viscumTT*, effectively inhibited proliferation in a dose-dependent manner in

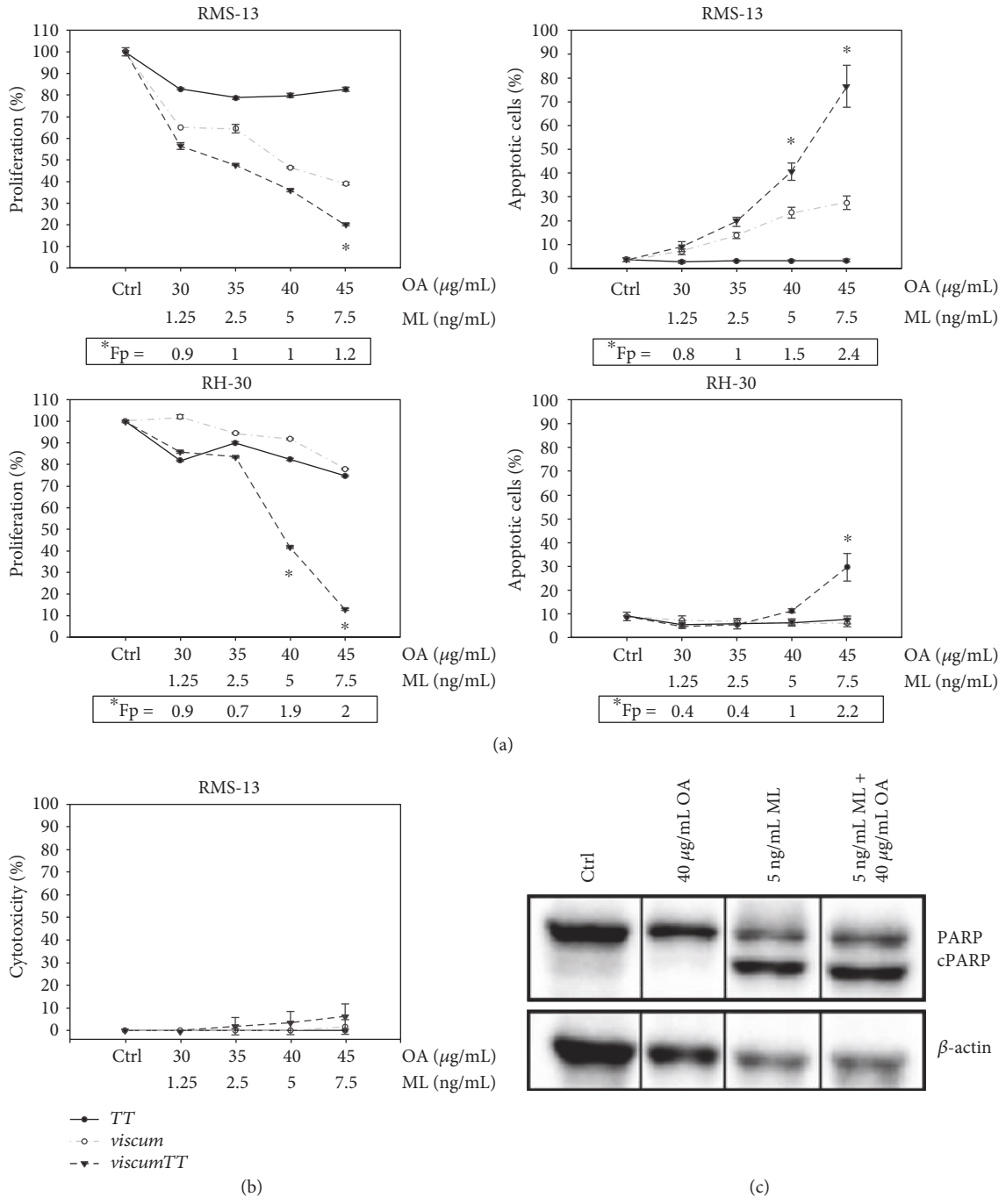


Figure 1: *viscumTT* inhibits cell proliferation and induces apoptosis synergistically in ARMS. (a) RMS-13 and RH-30 cells were incubated with *viscum*, *TT*, and *viscumTT* in increasing concentrations for 24 h. Cell proliferation was measured by CASY cell counter analysis, and the proliferation rate was calculated from the total cell numbers compared to untreated control cells. Apoptosis was assessed by annexin V/PI assay, and results were analysed by FlowJo Software. (b) RMS-13 cells were treated with *viscum*, *TT*, and *viscumTT* in increasing concentrations, and early cytotoxicity was measured by LDH assay. Results are presented as percentage of untreated control cells. (c) Apoptosis was confirmed by PARP cleavage. For this purpose, Western blot analyses were performed after treatment of RMS-13 cells with *viscum*, *TT*, and *viscumTT*. β-actin served as a loading control. Results are presented as means ± SD of three independent experiments. Webb's fractional product (Fp) was calculated to assess synergism, values $^*Fp > 1$ display synergism. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as marker substances for *viscum* and *TT*, respectively.

RMS-13 cells, whereas *TT* showed only moderate inhibitory effects (Figure 1(a)). RH-30 cells were more resistant towards treatment with *viscum* or *TT*, while *viscumTT* showed a strong inhibitory effect. Notably, treatment with *viscumTT* led to a synergistic inhibitory effect on cell proliferation in both cell lines in higher concentrations ($^*Fp > 1$).

To determine the induction of apoptosis, RMS-13 and RH-30 cells were analysed by annexin V/PI staining

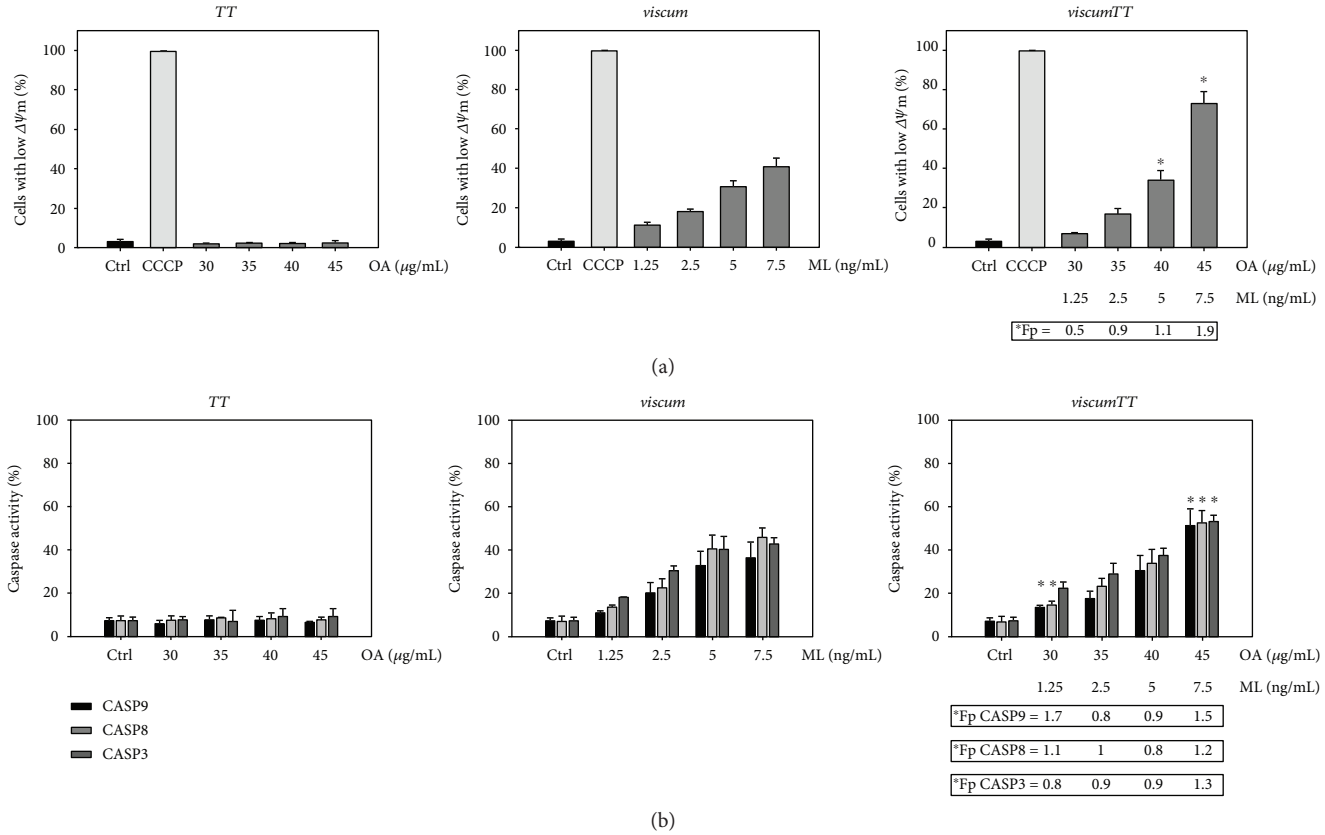


Figure 2: *viscumTT* displays synergistic effects on mitochondrial membrane ($\Delta\Psi_m$) depolarisation and activation of CASP3, CASP8, and CASP9. RMS-13 cells were incubated with *viscum*, *TT*, and *viscumTT* in increasing concentrations for 24 h. (a) $\Delta\Psi_m$ depolarisation was analysed by JC-1 staining and flow cytometry. The protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a mitochondrial membrane disruptor, was used as a positive control. (b) Active caspase assays were performed according to the manufacturer's instructions to monitor activation of caspase-3 (CASP3), caspase-8 (CASP8), and caspase-9 (CASP9). All results are presented as means ± SD of three independent experiments. Webb's fractional product (Fp) was calculated to assess synergism, values *Fp > 1 display synergism. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as marker substances for *viscum* and *TT*, respectively.

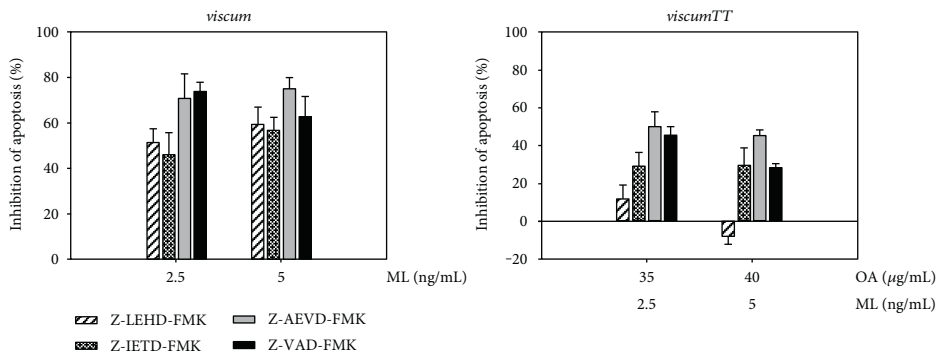


Figure 3: Caspase inhibitors effectively reduce *viscum*- and *viscumTT*-mediated apoptosis. RMS-13 cells were pretreated with 50–100 μmol of caspase inhibitors Z-VAD-FMK (pan inhibitor), Z-IETD-FMK (caspase-8), Z-LEHD-FMK (caspase-9), or Z-AEVD-FMK (caspase-10) for one hour. Subsequently, cells were treated with *viscum*, *TT*, and *viscumTT* for 24 h and analysed by annexin/PI assay and flow cytometry. Results are presented as percentage of untreated control cells and means ± SD of three independent experiments. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as marker substances for *viscum* and *TT*, respectively.

and FACS analysis after 24 h of incubation with *viscum*, *TT*, and *viscumTT*. Neither *viscum* nor *TT* affected apoptosis in RH-30 cells; however, *viscumTT* induced apoptosis at high concentration ranges (Figure 1(a)). In comparison, RMS-13 cells reacted more sensitively to incubation with *viscum* and *viscumTT*, but treatment with *TT* also proved to be ineffective. *viscumTT* again displayed a synergistic effect on apoptosis induction in both

cell lines at high concentration ranges. Detection of PARP cleavage by Western blot analysis confirmed apoptosis induction (Figure 1(c)).

To exclude an unwanted early cytotoxic effect via necrosis, LDH-release was measured after 2 h of incubation with *viscum*, *TT*, and *viscumTT* in RMS-13 cells. As shown in Figure 1(b), no significant LDH-release was detected; hence, a cytotoxic effect by necrosis could be excluded.

3.2. viscumTT Displays a Synergistic Effect on Mitochondrial Membrane Depolarisation and Caspase Activation In Vitro. To evaluate the underlying mechanisms of *viscum*-, *TT*-, and *viscumTT*-induced cytotoxicity more closely, we analysed the involvement of mitochondria and caspases in apoptosis. For this purpose, RMS-13 cells were treated as described above and $\Delta\Psi_m$ loss was detected by JC-1 staining and FACS analysis. Treatment with *viscum*, and to an even more pronounced extent with *viscumTT*, led to a dose-dependent loss of $\Delta\Psi_m$, whereas *TT* showed no significant effect (Figure 2(a)).

Caspases play an essential role in apoptosis, since they are initiators (CASP9, CASP8) as well as effectors (CASP3). After 24 h treatment with *viscum*, *TT*, and *viscumTT*, caspase activation was assessed by active caspase assays and FACS analysis in RMS-13 cells. *Viscum* as well as *viscumTT* activated CASP3, CASP8, and CASP9 in a dose-dependent manner (Figure 2(b)). *viscumTT* displayed a synergistic effect on mitochondrial membrane depolarisation and caspase activation, confirming observations made in the previous experiments.

To further determine caspase dependency in apoptosis induction, caspase inhibitor assays were performed and inhibition of apoptosis was estimated from untreated control cells. Interestingly, caspase inhibitors were more effective in preventing apoptosis in cells treated with *viscum* rather than *viscumTT*. The pan-caspase inhibitor achieved reduction of apoptosis by up to 73% in *viscum*- and 46% in *viscumTT*-treated cells (Figure 3). Inhibition of the extrinsic pathway by CASP10 and CASP8 inhibitors prevented apoptosis by up to 75% and 60%, respectively, after treatment with *viscum* and by 50% and 30%, respectively, in *viscumTT*-treated cells. Inhibition of the intrinsic apoptosis signalling pathway by CASP9 inhibitor was also effective in *viscum*-treated cells, but showed no effect after incubation with *viscumTT*. On the contrary, in increasing concentrations, CASP9 inhibitor enhanced *viscumTT*-induced apoptosis.

These results suggest that in addition to the intrinsic signalling pathway the extrinsic pathway plays a role in *viscum* and *viscumTT*-induced caspase-dependent apoptosis.

3.3. Viscum album Extracts Alter the Expression of Antiapoptotic Proteins. To further elucidate the underlying molecular mechanisms of *viscum*-, *TT*-, and *viscumTT*-induced apoptosis, we analysed protein expression of apoptosis-associated proteins by Western blot analysis after 24 h treatment with mistletoe extracts. *Viscum*, *TT*, and *viscumTT* suppressed the expression of the inhibitor of apoptosis protein (IAP) family members XIAP and BIRC5. Further, incubation with *viscum* and *viscumTT* led to downregulation of BCL2 family members BCL2, BCL2L1, and MCL1 (Figure 4).

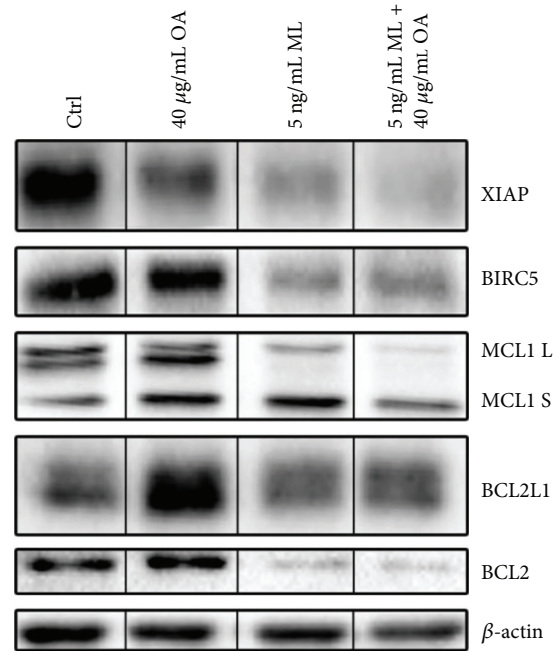


Figure 4: *viscumTT* alters the expression of antiapoptotic proteins. RMS-13 cells were treated with *viscum*, *TT*, and *viscumTT* in increasing concentrations for 24 h. Afterwards, cells were lysed and whole proteins lysates were analysed by Western blot for altered expression of X-linked inhibitor of apoptosis protein (XIAP), baculoviral IAP repeat containing 5 (BIRC5), myeloid cell leukemia 1 (MCL1), B-cell lymphoma 2 (BCL2), and BCL2 like 1 (BCL2L1). β-actin served as a loading control. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as marker substances for *viscum* and *TT*, respectively.

3.4. viscumTT Inhibits Proliferation and Induces Apoptosis Ex Vivo. To verify in vitro results, we next examined the effects of *viscum*, *TT*, and *viscumTT* in primary RMS tumour cells (ex vivo). For this purpose, ex vivo cultures of primary cells of three different patients were treated with *TT*, *viscum*, and *viscumTT* in increasing concentrations for 48 h (patient no. 1) and 24 h (patient no. 2; patient no. 3), respectively.

Patient no. 1 was incubated with lower concentrations of *viscum*, *TT*, and *viscumTT* than patient no. 2 and patient no. 3, but we changed the time course and extended the incubation time to 48 h. *Viscum* and to a more pronounced extent *viscumTT* effectively induced apoptosis and decreased $\Delta\Psi_m$, while *TT* showed no cytotoxic effect ex vivo (Figure 5(a)). Patient no. 2 showed only a moderate increase of apoptotic cells after treatment with *viscumTT*, while neither *TT* nor *viscum* inhibited cell proliferation or induced apoptosis effectively after 24 h incubation (Figure 5(b)). After 24 h incubation with *viscum* and *viscumTT*, we were able to detect a concentration-dependent inhibition of proliferation as well as induction of apoptosis in patient no. 3 (Figure 5(c)).

3.5. viscumTT Effectively Inhibits Tumour Growth In Vivo. To validate our in vitro and ex vivo findings, we established

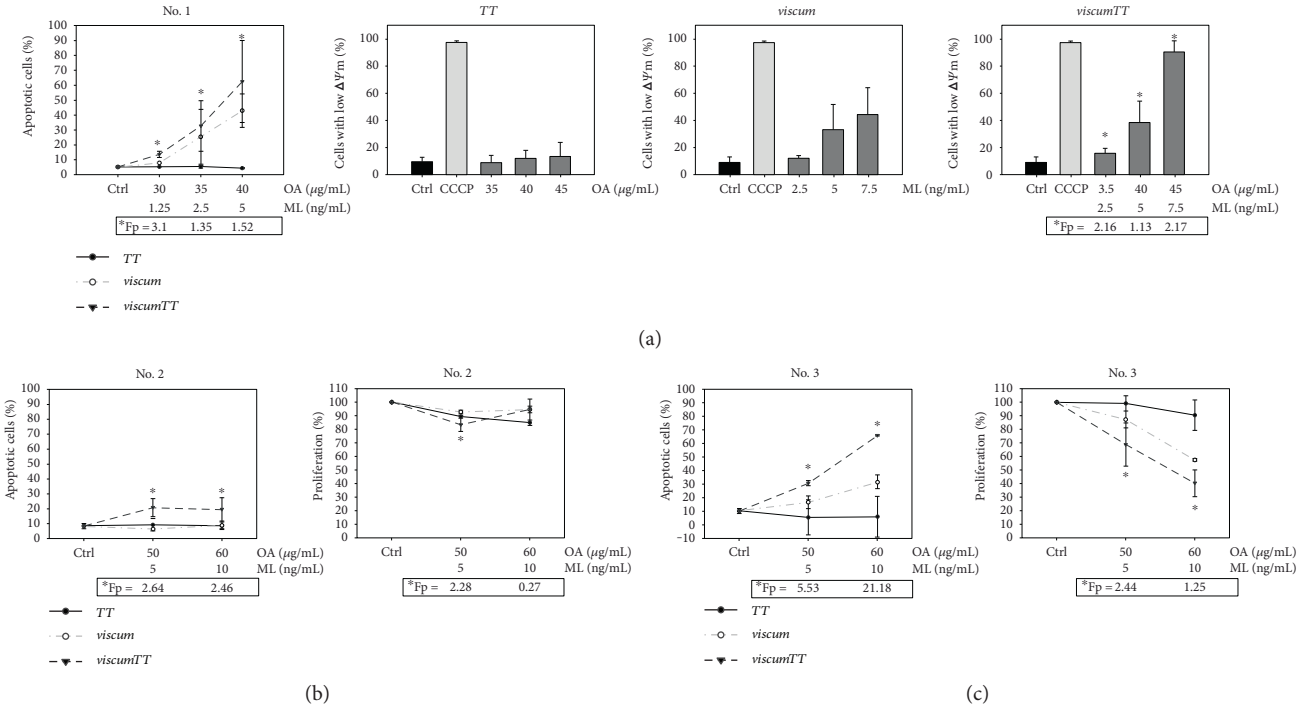


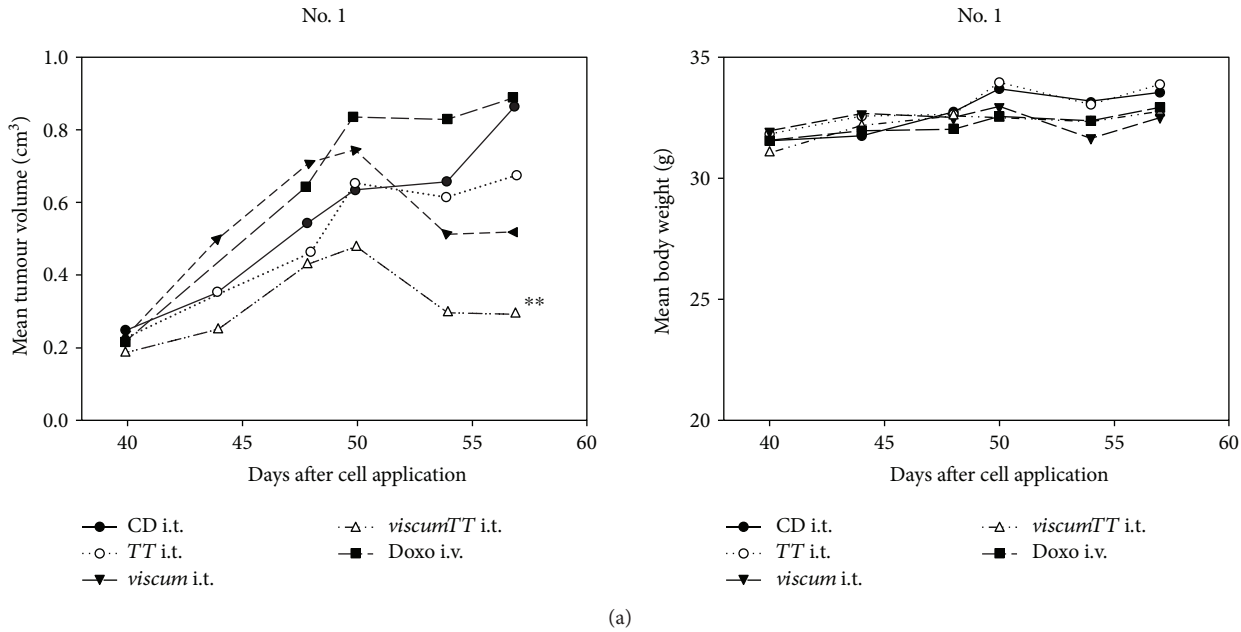
Figure 5: *viscumTT* induces apoptosis synergistically ex vivo. Ex vivo cultures from three different patients were incubated with *viscum*, *TT*, and *viscumTT* in increasing concentrations for 48 h (a) patient no. 1 or for 24 h, (b) patient no. 2, and (c) patient no. 3. Cell proliferation was measured by CASY cell counter analysis, and apoptosis was assessed by annexin V/PI assay and flow cytometry. All results are presented as means ± SD of two independent experiments. Webb's fractional product (*Fp) was calculated to assess synergism, values *Fp > 1 display synergism. Mistletoe lectin (ML) and oleanolic acid (OA) concentrations were used as marker substances for *viscum* and *TT*, respectively.

two patient-derived xenografts (PDX) to examine the effects of *viscum*, *TT*, and *viscumTT* in vivo. For this purpose, we focused on two different study designs. In PDX no. 1, we examined the therapeutic effect of i.t.-administered *viscumTT* compared to *viscum*, *TT*, and doxorubicin. In PDX no. 2, however, we investigated the therapeutic effectiveness of i.t. and i.v. application. Therefore, NMRI-nu/nu mice were treated with *viscum*, *TT*, and *viscumTT* (i.v. and/or i.t.). Control groups received the vehicle cyclodextrin i.v. or i.t. Extracts were administered every two to three days in increasing concentrations, and each concentration was given twice. During treatment, the mice showed no significant loss of body weight, and the administered extracts were well tolerated. The mean tumour volumes were calculated (Figure 6). In PDX no. 1, i.t. administration of *viscum* and *TT* reduced tumour volume effectively and *viscumTT* led to a significant inhibition of tumour volume in comparison to the control group (Figure 6(a)), whereas doxorubicin as a standard therapeutic drug had no effect. In PDX no. 2, *viscumTT* i.t. was more effective than *viscumTT* i.v. and both application forms inhibited tumour volume significantly compared to control mice (Figure 6(b)).

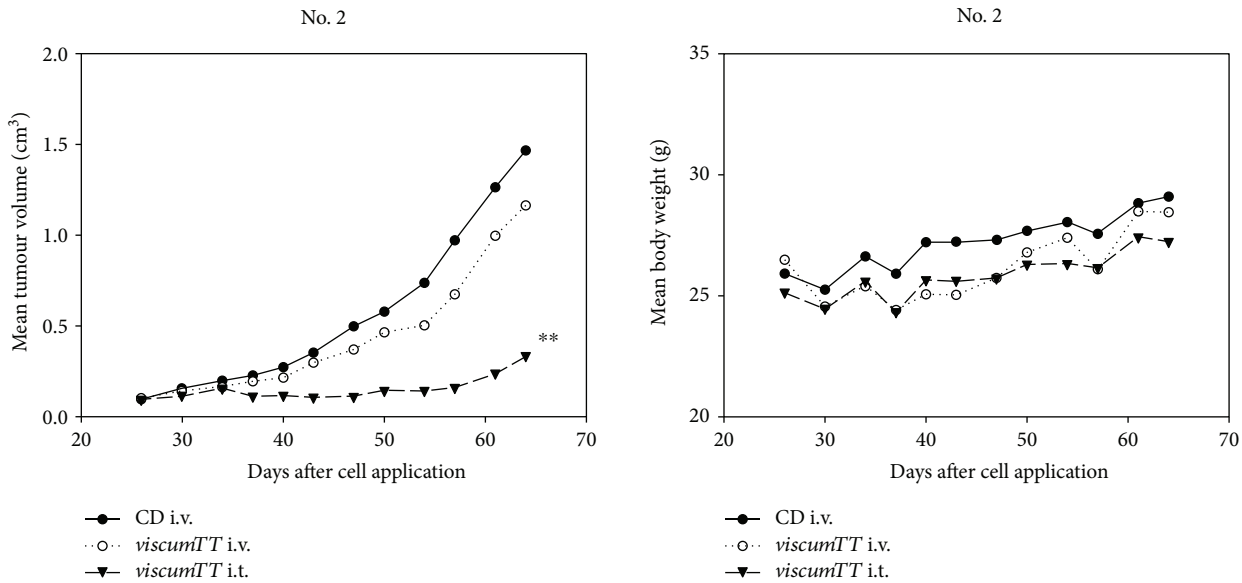
4. Discussion

This study shows that *viscum* and *viscumTT* exerted strong antiproliferative and apoptosis-inducing effects on RMS in vitro and ex vivo, whereas *TT* showed only moderate inhibitory effects. RH-30 cells were more resistant to *viscum* treatment than RMS-13 cells. It is known that cytotoxic effects of ML, the main component of the *viscum* extract, differ in cell lines, probably due to differences in expression of ML binding sites on the cell surface [49, 50]. The marker substance of the *TT* extract OA, which exert cytotoxic effects in various cancer cell lines [37, 46, 51], did not induce apoptosis in either RMS cell line effectively, which might be due to low OA concentrations. The whole mistletoe extract, *viscumTT*, proved to be more potent than the single extracts and displayed a synergistic effect on apoptosis induction in RMS cells in vitro and ex vivo. This result is in line with the assumption that whole plant extracts can be more effective than their single constituents [52–54]. Synergistic effects of *viscumTT* were already recently shown in other paediatric tumour cell lines [41–44].

Viscum and *viscumTT* triggered apoptosis in RMS via the extrinsic as well as the intrinsic apoptosis signalling pathway, evidenced by mitochondrial membrane depolarisation and activation of CASP8 and CASP9 (Figure 7). This is in line with results from several studies, which reported that ML as well as OA induced apoptosis by affecting different apoptosis pathways. Aqueous mistletoe extracts as well as purified ML proved to exert cytotoxic effects in various cancer cell lines either by the intrinsic [21, 25, 55] or additionally by the extrinsic [41, 56] apoptosis signalling pathway. OA and its derivatives are also known to invoke both the extrinsic [57–62] as well as the intrinsic [37–39] signalling pathway. Furthermore, BA could induce apoptosis via the mitochondrial signalling pathway in RMS [63] and neuroblastoma



(a)



(b)

Figure 6: *viscumTT* effectively reduces tumour volume in patient-derived RMS xenografts. Patient-derived RMS cells from (a) patient no. 1 and (b) patient no. 2 were used for s.c. implantation in the inguinal region of mice. Treatment started on day 12 when tumours were palpable, with (a) i.t. administration of *viscum*, *TT*, *viscumTT*, cyclodextrins (CD; control group), and i.v. doxorubicin (Doxo) and (b) i.t. administration of *viscumTT* and i.v. treatment with *viscumTT* and cyclodextrins. The mice were treated every two-three days in rising concentrations, and each dose was given twice. The administered concentrations were 40/60/80 mg/kg oleanolic acid (*TT*), 0.5/1.0/1.5 μg/kg mistletoe lectin (*viscum*), or a combination thereof (*viscumTT*). Two-way ANOVA and Bonferroni post hoc tests were applied to determine differences between mouse xenograft treatment groups ($^{**}p \leq 0.01$).

[64]. Additionally, BA and OA have been shown to interact directly with mitochondrial membranes [64–66].

Caspase inhibitory assays showed that *viscum*- and *viscumTT*-induced apoptosis was caspase-dependent. Interestingly, caspase inhibitors were more effective in preventing apoptosis in cells treated with *viscum* rather than *viscumTT*. Addition of triterpene acids to aqueous mistletoe extracts seems to activate as yet unknown mechanisms, which enhance apoptosis induction caspase independently. Caspase-independent apoptosis induction by OA was also observed by Konopleva et al. [66].

Inhibition of the extrinsic pathway by CASP8 and CASP10 inhibitors reduced apoptosis significantly. Notably, the CASP10 inhibitor was more effective in reducing apoptosis than the pan-caspase inhibitor, underlining the dependency of *viscum*- and *viscumTT*-mediated apoptosis induction on CASP10. CASP10 is a homologue of CASP8 and activated through the receptor-mediated pathway.

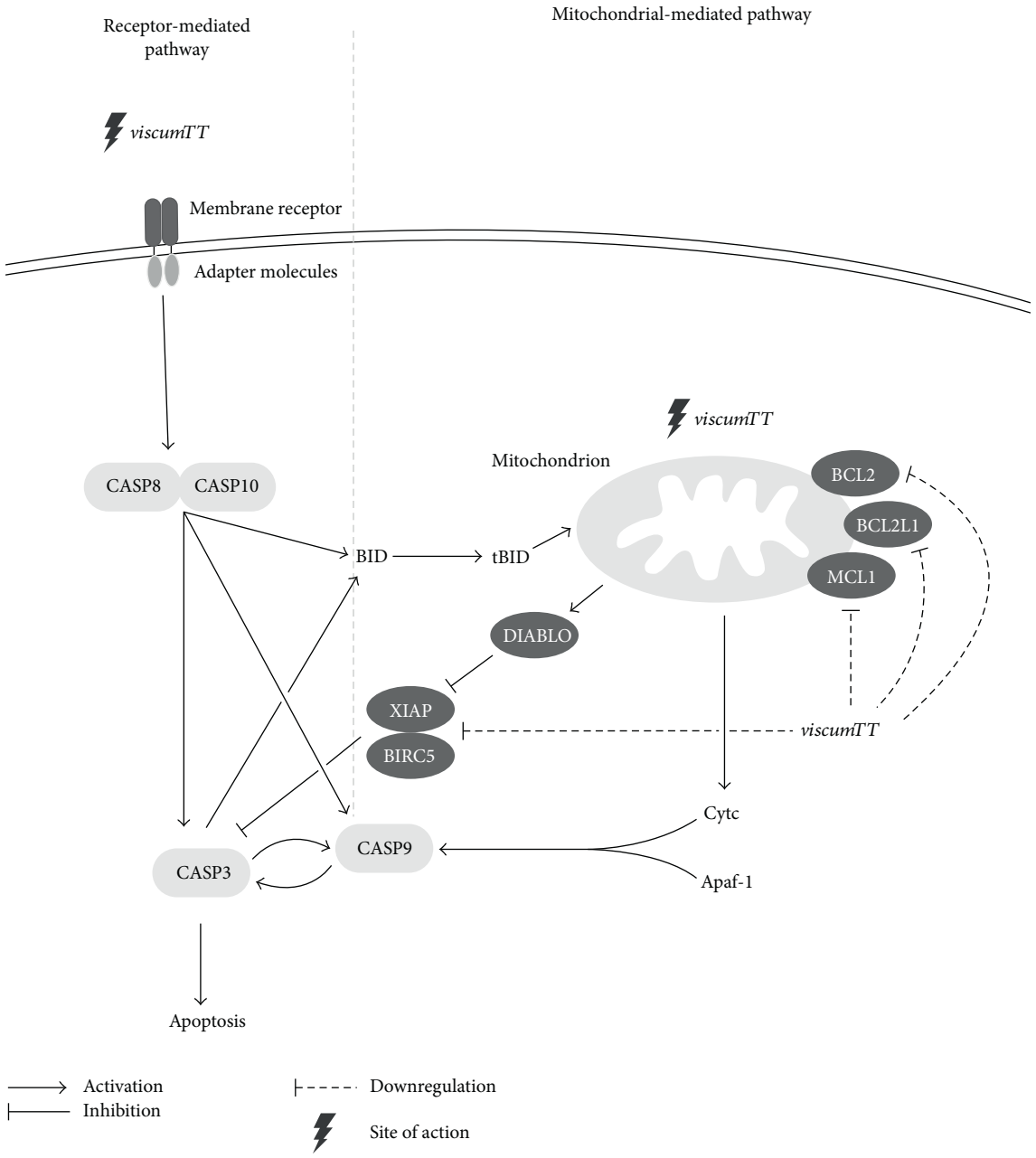


Figure 7: Scheme for *viscumTT*-induced apoptosis via the extrinsic and the intrinsic signalling pathways. Through yet unknown mechanisms, *viscumTT* activates caspase-8 (CASP8) and caspase-10 (CASP10), resulting in an activation of the caspase cascade. Caspase-9 (CASP9) acts as an effector downstream of CASP8 and CASP10 rather than an initiator of apoptosis. Further, *viscumTT* downregulates antiapoptotic proteins B-cell lymphoma 2 (BCL2), BCL2 like 1 (BCL2L1), and myeloid cell leukemia 1 (MCL1) as well as inhibitors of apoptosis proteins baculoviral IAP repeat containing 5 (BIRC5) and X-linked inhibitor of apoptosis protein (XIAP), thus shifting the balance towards apoptosis.

However, some studies indicate involvement of CASP10 in apoptosis induction by cytotoxic agents in a FADD-dependent but receptor-independent manner [67, 68].

Interestingly, the CASP9 inhibitor was not only ineffective in *viscumTT*-treated cells, but enhanced *viscumTT*-induced cell death. This result is in line with the previous results, which reported augmented apoptosis after treatment with inhibitors of CASP9 in combination with oridonin [69]. Also, CASP9 inhibition is suspected to block autophagic flux and thus to promote caspase-independent cell death [69, 70]. However, caspase inhibitor assays suggest that CASP9 does not act as an initiator but merely as an effector caspase in an amplification loop and is activated by CASP8 or CASP10 similar to TNF-α, which induced apoptosis via activation of CASP9 by CASP8 [71, 72]. Additionally, the mitochondrial and receptor-mediated pathways are crosslinked by the cleavage of BH3 interacting domain death agonist (BID) into truncated BID (tBID) by CASP8 or CASP10. Also, cotreatment with the OA derivative C-28 methyl ester of 2-cyano-3,12-

dioxoolean-1,9-dien-28-oic acid (CDDO-Me) and CASP8 inhibitor CASP9 activity was decreased, suggesting that CASP9 activation is downstream of CASP8 [73].

To illuminate the underlying mechanisms of *viscum*-, *TT*-, and *viscumTT*-induced apoptosis further, apoptosis-associated proteins were analysed by Western blot analyses to detect altered expression. The results showed that *viscum* and even more prominently *viscumTT* shifted the balance towards apoptosis by changing the ratio of pro- and antiapoptotic proteins.

After treatment with *viscum* and *viscumTT*, downregulation of the antiapoptotic BCL2 family members BCL2 and BCL2L1 was detected. Overexpression of BCL2 family members is often found in cancers and contributes to apoptosis resistance. Genetic mutations, for example, gli-amplification and a positive PAX3/FOXO1 fusion status, and increased activity of transcription factor signal transducer and activator of transcription 3 (STAT3) lead to overexpression of BCL2 and BCL2L1 in RMS [74]. Downregulation of BCL2 and BCL2L1 was found after treatment with *viscum* [42] and OA [75, 76]. MCL1, another antiapoptotic BCL2 family member, which is upregulated in RMS [77], is also downregulated by *viscum* and *viscumTT*. This is in line with the previous results of decreased expression of MCL1 after treatment with ML [21, 78] and *viscumTT* [43]. Furthermore, Ryu et al. have shown inhibition of the STAT3 pathway with downregulation of BCL2L1, BIRC5, and MCL1 after incubation with CDDO-Me [79]. Moreover, *viscum* and *viscumTT* effectively reduced expression of IAP family members BIRC5 and XIAP. IAP family members prevent apoptosis by directly inhibiting caspases or the assembly of proapoptotic complexes. BIRC5 is often highly expressed in malignant cells but not in differentiated tissues [80], and high expression levels are associated with poor prognosis [81] thus appointing it an attractive target in tumour therapy. RMS expresses high levels of BIRC5, and Caldas et al. were able to effectively reduce tumour growth by blocking BIRC5 in vivo [82]. OA treatment was able to effectively reduce BIRC5 expression in lung cancer [83], ovarian cell carcinoma [75, 84], and leukaemia [76] as well as XIAP expression in hepatocellular carcinoma [85]. Furthermore, we were able to show that *viscum*, *TT*, and *viscumTT* led to downregulation of BIRC5 and XIAP in AML [41], Ewing-sarcoma [43], and osteosarcoma [44].

Treatment with *viscumTT* seems to be more effective compared to the single compounds ex vivo and in vivo. Furthermore, *viscumTT* was more effective in reducing tumour volume than i.v. doxorubicin treatment in PDX no. 1. In addition, comparison of i.v. and i.t. treatment (PDX no. 2) showed that i.t. treatment is more effective. On the other hand, *viscumTT* can be administered in higher concentrations i.v. than i.t. Notably, *viscumTT* was more effective in vivo than in vitro. While *viscumTT* showed only moderate apoptosis-inducing properties ex vivo, tumour volume was effectively reduced in the xenograft derived from the same patient. Since mistletoe extracts are metabolised by mice, these in vitro results cannot be directly extrapolated to in vivo settings. However, *viscumTT* shows promising antitumour activity in ARMS.

5. Conclusions

The results show the high potential of *viscumTT* to induce apoptosis in ARMS without limiting toxicity. *viscumTT* is more effective than the single compounds and leads to a synergistic effect on apoptosis induction in vitro as well as ex vivo. In vivo *viscumTT* treatment resulted in an effective reduction of tumour volume compared to controls. In conclusion, addition of triterpene acids to aqueous mistletoe extracts enhances their cytotoxic effects in ARMS through an as yet unknown mechanism. However, *viscumTT* is a promising novel treatment approach for ARMS. Furthermore, ML exerts immunomodulating effects [86, 87]. The antiproliferative and apoptosis-inducing properties of *viscumTT* combined with the immunomodulating actions of ML provide an attractive research target not only for cancer therapy, but also for other research purposes such as rheumatic and cardiovascular diseases.

Ethical Approval

Written informed consent was obtained from the patient's parents and/or legal guardians in accordance with the Declaration of Helsinki, approved by the local ethics committee of Charité-Universitätsmedizin Berlin.

Conflicts of Interest

The authors have the following interests: J. Rolff is employed by EPO GmbH. Sebastian Jäger is employed by Birken AG. A patent, EP 1852105 A3, has been filed by this company. The patent affects commercial reproduction and use of triterpene containing plant extracts like *viscumTT*, while noncommercial use for research is not affected. There are no further patents, products in development, or marketed products to declare.

Authors' Contributions

Georg Seifert and Catharina I. Delebinski contributed equally to this work.

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Research Article

Immunomodulatory Effects of Taiwanese *Neolitsea* Species on Th1 and Th2 Functionality

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Neolitsea species, medicinal plants belonging to Lauraceae, contain rich alkaloids, steroids, sesquiterpenoids, and triterpenoids which possess antimicrobial, antioxidant, and anti-inflammatory bioactivities. However, species differences in the immunomodulatory effects and evidence pertaining to the effects of *Neolitsea* species on adaptive immunity are scarce. This study aimed to evaluate the immunomodulatory properties of ten Taiwanese *Neolitsea* plants on T helper (Th) cell functionality, especially Th1 and Th2. Most of the 29 crude extracts of *Neolitsea* were not toxic to splenocytes, except *N. buisanensis* roots. *N. aciculata* and *N. villosa* leaf extracts possessed differential immunomodulatory effects on Th1/Th2 balance. *N. aciculata* var. *variabillima* and *N. hiiranensis* leaf extracts attenuated both Th1 and Th2 cytokines while *N. konishii* dramatically suppressed IFN-γ production. As *N. aciculata* var. *variabillima* and *N. konishii* leaf extracts significantly attenuated Th1 functionality, we further evaluated their effects on CD4 cells under CD3/CD28 stimulation. *N. aciculata* var. *variabillima* significantly suppressed IFN-γ, IL-10, and IL-17, demonstrating the broad suppressive effects on T helper cells; *N. konishii* significantly suppressed IFN-γ and IL-10 production, while the production of IL-17 was not altered. Collectively, these data demonstrated that leaf extracts of Taiwanese *Neolitsea* species contain phytochemicals with potentials to be developed as selective immunomodulators.

1. Introduction

T cells play a pivotal role in the immune responses. They participate in a wide range of immune responses through a complicated cytokine network and via cell-cell interaction with other cells. Interleukin-2 (IL-2), a major autocrine and/or paracrine T-cell growth factor, is primarily produced by T helper cells and participates in the development and activation of T cells [1, 2]. T helper type 1 (Th1) cells produce interferon gamma (IFN-γ) to regulate immune responses and inflammation against viral and intracellular bacterial infections and inhibit tumor formation via the stimulation of antibody production and the activation of macrophage, cytotoxic T lymphocytes (CTL), and natural killer cells [3, 4]. On the other hand, Th2 cells induce interleukin-4 (IL-4) production to mediate the activation and maintenance of the humoral and/or allergy immune response against extracellular parasites, bacteria, allergens, and toxins [5]. The dysfunction of T cells, such as the imbalance of Th1/Th2 responses and abnormal immunostimulation, may lead to a variety of immune diseases. The excessive amounts of IFN-γ have been associated with several Th1-mediated immune disorders, such as delayed type hypersensitivity [6], Crohn's disease [7], and multiple sclerosis [8]. Immunosuppressants, developed for the treatment of these overreactive immune responses, also decrease normal immune responses and thereby increase the susceptibility of the patients to infections [9, 10]. Therefore, it is important to develop immunoregulators with less severe side effects. Natural compounds are under intensive investigation and showed promising progressions [11–13]. Several medicinal plants have been reported to regulate inflammatory responses in a variety of different animal models by

Table 1: The possible bioactivities of Taiwanese *Neolitsea* plants used in this study.

Scientific name	Parts of plant used	Known possible bioactivities
N. aciculata	Leaf, stem, and root	Antibacterial, antioxidant, and anti-inflammatory activities [38]
N. aciculata var. *variabillima*	Leaf, stem, and root	Need to be studied
*N. acuminatissima**	Leaf, stem, and root	Cytotoxicity [43]
N. buisanensis	Leaf, stem, and root	Need to be studied
*N. daibuensis**	Leaf, stem, and root	Anti-inflammation [40]
*N. hiiranensis**	Leaf, stem, and root	Anti-inflammatory, antimicrobial [34], and immunomodulatory activities [27]
N. konishii	Leaf, stem, and root	Vasoconstriction [25], cardiotonic, and anti-inflammatory effects [41, 60]
*N. parvigemma**	Leaf, stem, and root	Anti-inflammatory [39], antifungal [61], and antiplatelet activity [26]
N. sericea var. *aurata*	Leaf and stem	Antiradical activity [62]
N. villosa	Leaf, stem, and root	Cytotoxicity [44]

*Taiwanese endemic *Neolitsea* species.

attenuation of interleukin-2 (IL-2) and interferon-γ (IFN-γ) production [14–18].

Neolitsea, small evergreen trees or evergreen shrubs in the family Lauraceae, consists of about 100 species distributed in the tropics, especially in Brazil and Southern Eastern Asia. In Taiwan, there are 12 *Neolitsea* species and four of them, including *N. acuminatissima*, *N. daibuensis*, *N. hiiranensis*, and *N. parvigemma*, are endemic [19]. Recent studies on the diversity of phytochemical structures and bioactivities revealed the application potential of *Neolitsea* plants in industrial and medical fields [20, 21]. Parts of *Neolitsea* plants have been used in folk medicine for long periods of time in Asia. For example, the roots of *N. aurata* and the seeds of *N. chuii* are used to alleviate edema. The leaves of *N. cambodiana* are applied to treat furuncle and carbuncle. The roots of *N. zeylanica* have been shown to relieve rheumatic arthralgia [22]. Studies on the chemistry and pharmacology of *Neolitsea* species have led to the isolation and identification of more than 150 compounds including alkaloids, terpenoids, sterols, steroids and their derivatives, flavonoids, essential oils, and fatty acids with diverse activities [21, 23]. The essential oils of *N. pallens* exhibited antioxidant and antibacterial activities [24]. Alkaloids of *N. konishii* possessed vasoconstricting effects on rat aorta [25]. Sesquiterpenes of the *N. parvigemma* showed inhibitory effects on platelet aggregation [26]. Our previous study has shown that leaf extracts of *N. hiiranensis* and its derived terpenoids possessed immunomodulatory effects via regulation of IFN-γ production [27]. However, due to the complexity of compositions within *Neolitsea* species, it is still unclear how other Taiwanese *Neolitsea* species modulate the functionality of immune cells. The objective of this study aimed to examine the immunomodulatory effects of Taiwanese *Neolitsea* species on T-cell immunity.

To evaluate the immunomodulatory effects of Taiwanese *Neolitsea* species on T-cell immunity, we cultured and stimulated the mouse primary splenocytes with concanavalin A (ConA), a well-known T-cell mitogen, to stimulate cytokine production [28, 29]. Splenocytes are consist of antigen-presenting cells, B cells, and various type of T cells and have been widely used as primary immune cells for studying the functionality of T cells [30–33]. IL-2, IFN-γ, and IL-4 were evaluated to determine the effects of these plant extracts on Th1/Th2 functionality. In the present study, leaf extracts, including *N. aciculata* var. *variabillima*, *N. acuminatissima*, *N. hiiranensis*, *N. konishii*, and *N. villosa*, significantly inhibited IFN-γ production. These *Neolitsea* extracts showed potentials to be developed as new therapeutic immunomodulators. The chemical components and mechanisms of these medicinal plants to modulate Th1 functionality warrants further investigation.

2. Materials and Methods

2.1. Reagents and Chemicals. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Enzyme-linked immunosorbent assay (ELISA) sets for cytokine measurement were purchased from BD Biosciences (San Diego, CA). Fetal bovine serum (FBS) and cell culture supplies were from Hyclone (Logan, UT).

2.2. Plants and Extraction. Twenty-nine crude extracts were prepared and extracted with cold MeOH at room temperature [34]. These plants used here were identified by one of the authors, Prof. Ih-Sheng Chen, and the voucher specimens were deposited in the Herbarium of the College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan. Traditional usage and known possible bioactivities of these 29 *Neolitsea* extracts were shown in Table 1.

2.3. Animals. Male BALB/c mice (five weeks old) were obtained from BioLasco (Ilan, Taiwan). On arrival, mice were randomly transferred to plastic cages containing aspen bedding and quarantined for at least 1 week. Mice were housed in a temperature (22°C ± 2°C)-, humidity (50% ± 20%)-, and light (12 h light/dark cycle)-controlled environment. Food and water were supplied ad libitum. All animal experimental procedures and housing have been approved by the Institutional Animal Care and Use Committee (IACUC) of Kaohsiung Medical University, Taiwan (Protocol ID: 106014). The experimental mice were euthanized by carbon dioxide mixed with oxygen for anesthesia with approved IACUC protocol and regulations.

2.4. Splenocyte Isolation. The mice were sacrificed, and their spleens were harvested and made into single-cell suspensions. The isolation of splenocytes was according to the previous procedures which have been described in detail before [35, 36]. Briefly, spleens were gently dissociated by teasing on a sterile 60-mesh steel screen (Sigma-Aldrich). The cell suspensions were washed in incomplete RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 5% fetal bovine serum (Gibco, USA) and 1% penicillin and streptomycin (Amresco). Lymphocytes were enriched by removing red blood cells from splenocytes after treating with ACK lysis buffer. To activate splenocytes, cells were seeded in 48-well plates and stimulated with 5 μg/mL of ConA (Sigma-Aldrich, St. Louis, MO). The splenocytes (5×10^6 cells/mL) were either left untreated (control group) or exposed to crude extracts of *Neolitsea* extracts followed by stimulation with ConA (5 μg/mL) for 48 h. The supernatants of culture wells were collected to detect the cytokine levels by enzyme-linked immunosorbent assay (ELISA).

2.5. Cell Viability. Splenocytes (5×10^6 cells/mL) were seeded into 96-well plates. The cells were either left untreated or treated with crude extracts followed by stimulation with ConA for 48 h. The viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. A methyl-thiazol-tetrazolium stock solution (5 mg/mL in phosphate-buffered saline) was then added to each well (10 μL/well) and incubated for 4 h. The formed formazan was dissolved in a lysis buffer (10% SDS in *N,N*-dimethylformamide) overnight in the dark. The optical density was measured at 570 nm (and at 630 nm as a background reference) using a microplate reader (Dynatech Laboratories Inc., Chantilly, VA).

2.6. Isolation of CD4 T Helper Cells. Mouse CD4 T helper cells were purified from splenocytes by using magnetic cell separation with the CD4 T Lymphocyte Enrichment Kit (BD Biosciences). To induce the polarization of Th cells, CD4 T cells (5×10^5 cells/mL) were seeded in the 48-well flat bottom tissue culture plates which were precoated with anti-mouse CD3 antibody (1 μg/mL) overnight, and the cells were either left untreated or treated with *Neolitsea* extracts followed by stimulation with soluble anti-mouse CD28 antibody (1 μg/mL) for 48 h. The culture supernatants and the cells were collected for ELISA assay and intracellular cytokine staining, respectively.

2.7. Measurement of Th1/Th2 Cytokines of Total T Cells and CD3/CD28-Stimulated CD4 T Cells by Enzyme-Linked Immunosorbent Assay (ELISA). To examine the immunomodulatory activities of *N.* species on the total T cells, the splenocytes (5×10^6 cells/mL) and CD4 T cells were cultured in 48-well plates (300 μL/well) and either left untreated or treated with crude extracts followed by stimulation with ConA (5 μg/mL) or CD3/CD28 stimulation for 48 h. The supernatants were harvested and quantified for IL-2, IFN-γ, IL-4, IL-10, and IL-17 by sandwich ELISA kits according to the manufacturer's instructions (BD Biosciences).

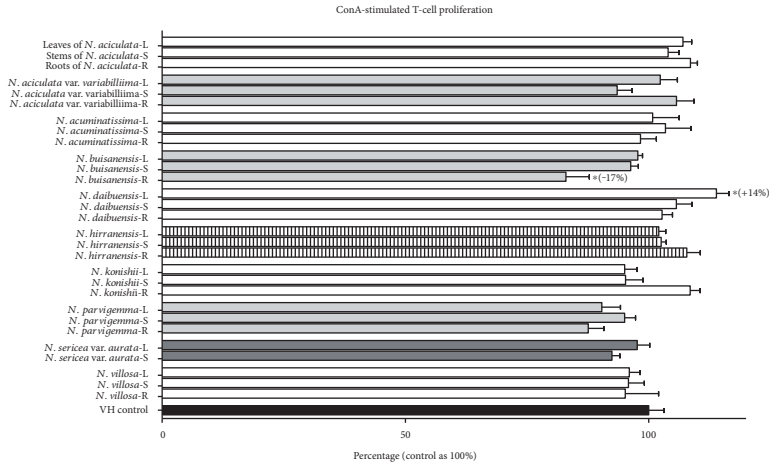
2.8. Intracellular Cytokine Staining by Flow Cytometry Analysis. CD4 T cells were cultured in a 48-well plate and treated with *N.* species for 36 h. For analysis of intracellular cytokine production, the cells then treated with GolgiStop (0.6 mL/mL; BD Biosciences) for 10 h prior to being harvested for antibody staining. The CD4 T cells then were fixed and permeabilized using Fixation and Perm/Wash buffers (BD Biosciences) before intracellular IFN-γ and IL-4 staining by PE-conjugated anti-mouse IFN-γ and IL-4 mAb (clone XMG1.2 and 11B11; Biolegend). Ten thousand of CD4 T cells were acquired on a BD LSR II flow cytometer (BD Biosciences). The mean fluorescence intensity (MFI) of IFN-γ in total CD4 T cells was quantified by gating CD4 T cells and then analyzed using FlowJo software (Treestar, Inc., CA).

2.9. Statistical Analysis. All the data were analyzed using a GraphPad software Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Each treatment group was measured in quadruplicate and the data were presented as the mean $\pm$ standard error (SE). Data were analyzed using a one-way analysis of variance (ANOVA) for multiple comparisons and Dunnett's two-tailed *t*-test was used to compare the results for the treatment groups with vehicle control group. $p < 0.05$ was defined as statistically significant.

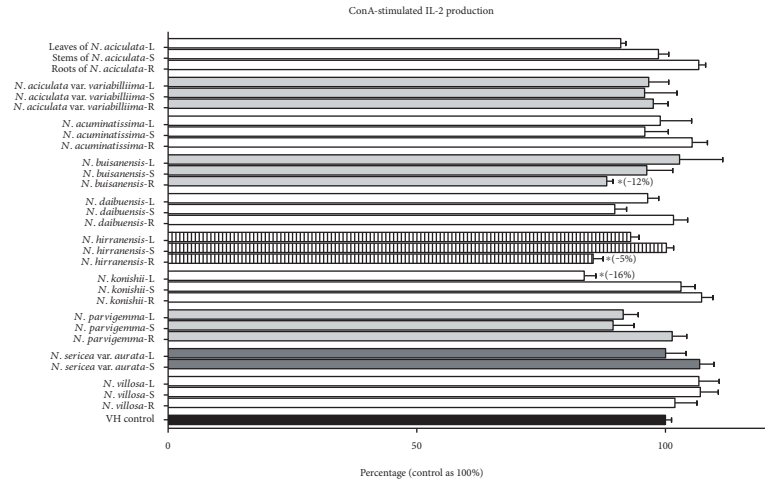
3. Results

3.1. The Effects of Crude Extracts of Neolitsea (N.) Species on Cell Viability and IL-2 Production by Murine Primary Splenocytes In Vitro. To evaluate the effects of *N.* species on splenocyte viability, we first investigated the direct cytotoxicity of *N.* species in vitro. Most of these crude extracts at the concentration of 10 μg/mL did not significantly affect the cell viability compared to vehicle control (VH was referred as 100%), except for the roots of the *N. buisanensis* which reduced the cell viability by 17% (Figure 1(a) and Supplemental Table 1 available online at https://doi.org/10.1155/2017/3529859). As IL-2 plays important roles in T-cell clonal expansion and activation, the supernatants of the treated groups were collected to determine the IL-2 levels. Most of the crude extracts of *N.* species did not alter IL-2 production, except for the roots of the *N. hiiranensis* and the leaves of the *N. konishii* which suppressed IL-2 production by 15% and 16% (at the concentration of 10 μg/mL), respectively (Figure 1(b) and Supplemental Table 1). Collectively, these data showed that the root part of *N. buisanensis* is relatively toxic to primary immune cells compared to other extracts. It should be circumspectly considered for further development of the immunomodulatory ingredients from this plant. Moreover, the roots of the *N. hiiranensis* and the leaves of the *N. konishii* suppressed IL-2, revealing that these extracts may affect the maturation and early activation of T helper cells.

3.2. Neolitsea Crude Extracts Differentially Modulated Th1 and Th2 Cytokines Production. We next investigated the effects of crude extracts of *N.* species on ConA-stimulated cytokine production by splenocytes (Figure 2 and Supplemental Table 1). IFN-γ and IL-4, which belong to the Th1 and Th2 signature cytokines, were determined to study the



(a)



(b)

Figure 1: The effects of Taiwanese *Neolitsea* plants on T-cell viability and activation. Splenocytes (5×10^6) were treated with vehicle control (VH, 0.05% DMSO) and/or various crude extracts of *Neolitsea* plants (10 μg/mL) followed by ConA treatment for 48 h. (a) The cell viability was determined using an MTT assay and the level of (b) IL-2 in the supernatants was quantified by ELISA. The viability and IL-2 level of the VH-treated group was shown as 100%. L, S, and R were represented as leaf, stem, and root parts of different plants. The effects of *N. aciculata*, *N. aciculata* var. *variabillima*, *N. acuminatissima*, *N. buisanensis*, *N. daibuensis*, *N. hiiranensis*, *N. konishii*, *N. parvigemma*, *N. sericea* var. *aurata*, and *N. villosa* on (a) cell viability and (b) IL-2 production were shown from top to bottom, respectively. Data were expressed as the mean ± SE of triplicated cultures. Results were pooled from three independent experiments. $^*p < 0.05$, compared to the VH group.

effects of plant-derived extracts (10 μg/mL) on Th1/Th2 immune responses. *N. aciculata* did not affect the production of IFN-γ and IL-4 except that the stem extracts of *N. aciculata* inhibited both IFN-γ and IL-4 by approximately 20% compared to VH control. These data suggested that the stem of *N. aciculata* inhibited both Th1 and Th2 functionality. The leaves and roots of *N. aciculata* var. *variabillima* (gray bar) significantly suppressed IFN-γ production by 37–39%, while the IL-4 was not altered. Interestingly, the stem of *N. aciculata* var. *variabillima* not only inhibited IFN-γ (26%) but also inhibited IL-4 (17%). These data indicated that Th1 cells were more sensitive to be suppressed by components of *N. aciculata* var. *variabillima*, and the leaf and root parts differentially modulated Th1 functionality. Different parts of *N. acuminatissima* (white bar) inhibited both IFN-γ and IL-4 cytokine productions by 28–59% and 32–43%, respectively. It was speculated that *N. acuminatissima* may affect the development of total Th cells in response to ConA stimulation. The leaves of *N. buisanensis* (gray bar) did not affect the IFN-γ but reduced IL-4 indicating the differential modulatory effects on Th2 functionality. *N. hiiranensis* attenuated IFN-γ by 17%–90% and inhibited IL-4 by 22–39%. It is suggested that the whole extract of *N. hiiranensis* modulated both Th1 and Th2 activities (slash gray bar). The leaves and roots of *N. konishii* mainly attenuated IFN-γ by 39–55% while IL-4 was not affected. The leaf and stem parts of *N. parvigemma* inhibited Th1 and Th2 cytokine production by 22–39% (gray bar). The leaves and stems of *N. sericea* var. *aurata* inhibited both IFN-γ and IL-4 (22–28% inhibition rate). Finally, *N. villosa* extracts attenuated IFN-γ by 17–76%, but IL-4 was not altered. Taken together, the above results showed that crude extracts of *N.* species differentially modulated T helper cell functionality. The leaf extracts of *N. acuminatissima*, *N. hiiranensis*, and *N. parvigemma* and *N.*

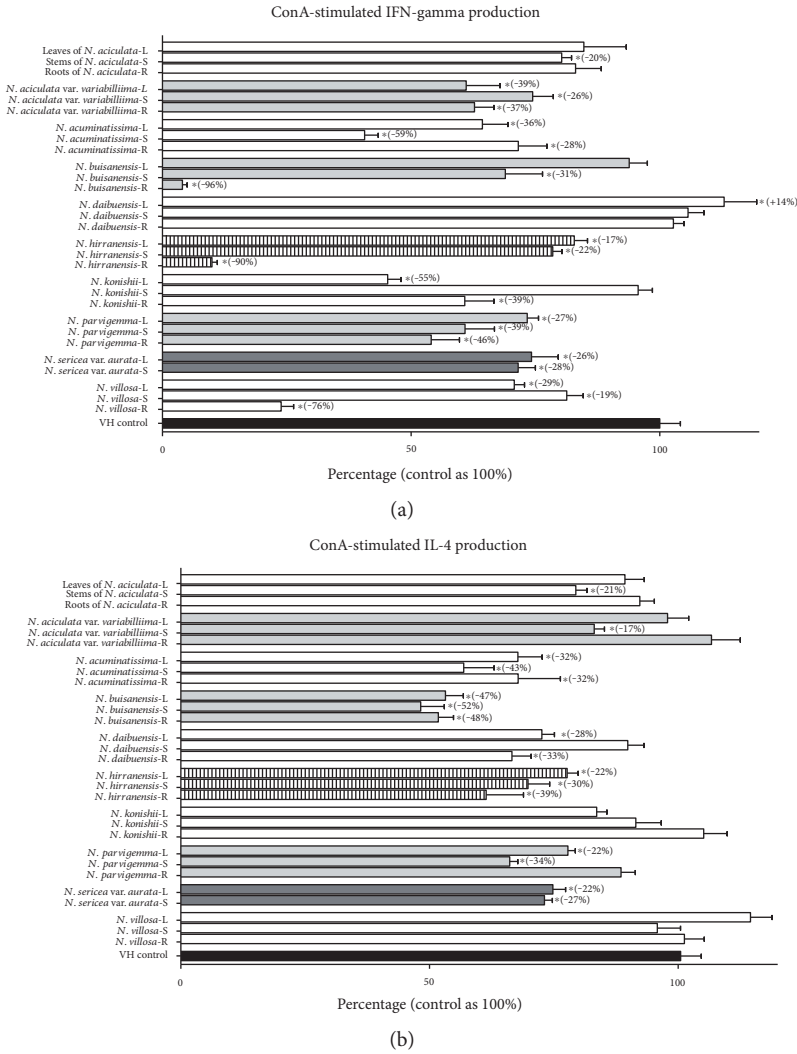


Figure 2: The effects of Taiwanese *Neolitsea* plants on Th1/Th2 cytokine production. Splenocytes (5×10^6) were treated with vehicle control (VH, 0.05% DMSO) and/or various crude extracts of *Neolitsea* plants (10 μg/mL) followed by ConA stimulation for 48 h. The supernatants were collected for measuring the concentration of cytokines by ELISA. The cytokine level of the VH-treated group was shown as 100%. L, S, and R were represented as leaf, stem, and root parts of different plants. The effects of *N. aciculata*, *N. aciculata* var. *variabillima*, *N. acuminatissima*, *N. buisanensis*, *N. daibuensis*, *N. hiiranensis*, *N. konishii*, *N. parvigemma*, *N. sericea* var. *aurata*, and *N. villosa* on (a) IFN-γ and (b) IL-4 were shown from top to bottom, respectively. Data were expressed as the mean ± SE of triplicated cultures. Results were pooled from three independent experiments. $^*p < 0.05$, compared to the VH group.

sericea var. *aurata* suppressed both Th1 and Th2 functionality. *N. aciculata* var. *variabillima*, *N. konishii*, and *N. villosa* mainly inhibited IFN-γ production. In contrast, *N. buisanensis* and *N. daibuensis* attenuated IL-4 production.

3.3. The Differential Immunomodulatory Effects of Leaf and Root Extracts of Selected Neolitsea Species on Th1/Th2 Functionality in Dose-Dependent Manners. According to our abovementioned results, several leaf extracts of *Neolitsea* species may differentially regulate the functionality of Th1 and Th2 cells by modulating the production of IFN-γ and IL-4 cytokines. We next further evaluated the concentration-dependent effects of these particular leaf extracts on ConA-stimulated splenocytes. The effects of leaf extracts of *N. aciculata*, *N. aciculata* var. *variabillima*, *N. daibuensis*, *N. hiiranensis*, *N. konishii*, and *N. villosa* on the cell viability and Th1/Th2 cytokine secretions were shown in Table 2. *N. aciculata* at the concentration of 5–50 μg/mL did not alter the viability as well as IL-4 production. In contrast, *N. aciculata* concentration dependently inhibited IFN-γ (25–50 μg/mL) while IL-2 was suppressed at the concentration of 50 μg/mL, indicating that leaf extracts of *N. aciculata* mainly affect Th1 functionality from 25 μg/mL (Table 2). *N. aciculata* var. *variabillima* (5–50 μg/mL) dramatically attenuated IFN-γ production while IL-2 was suppressed at the concentration of 25–50 μg/mL. *N. aciculata* var. *variabillima* (50 μg/mL) significantly inhibited cell viability and all tested cytokines demonstrating the cytotoxic effects of this plant at high concentration (Table 2). *N. daibuensis* (10–25 μg/mL) attenuated IL-4 production while Th1 cytokines were not altered, demonstrating its differential effects on Th2 functionality (Table 2). *N. hiiranensis* did not affect cell viability and

Table 2: The effects of *N.* species leaf extracts on Th1/Th2 cytokine production by ConA-stimulated splenocytes.

Plants	Concentration (μg/mL)	Cell viability MTT (O.D.)	Th1 cytokines IL-2 (ng/mL)	Th1 cytokines IFN-γ (ng/mL)	Th2 cytokines IL-4 (pg/mL)
	Control	0.17 ± 0.01	0.06 ± 0.04	Not detectable	Not detectable
N. aciculata	VH	0.52 ± 0.05	7.61 ± 0.42	119.30 ± 6.28	173.70 ± 8.07
	5	0.57 ± 0.04	7.23 ± 0.47	116.40 ± 0.91	174.10 ± 9.39
	10	0.59 ± 0.04	6.93 ± 0.39	121.30 ± 6.50	167.40 ± 8.63
	25	0.52 ± 0.04	6.40 ± 0.40	60.71 ± 6.23*	195.50 ± 19.73
	50	0.41 ± 0.05	5.42 ± 0.27*	42.15 ± 1.55*	210.80 ± 16.73
N. aciculata var. *variabillima*	VH	0.39 ± 0.02	10.86 ± 0.90	91.71 ± 2.51	222.00 ± 33.97
	5	0.44 ± 0.02	10.34 ± 0.95	74.44 ± 6.36*	246.20 ± 19.19
	10	0.40 ± 0.03	9.74 ± 0.86	62.35 ± 6.32*	208.40 ± 19.92
	25	0.34 ± 0.01	7.45 ± 0.35*	43.94 ± 3.21*	161.80 ± 17.45
	50	0.27 ± 0.03*	3.44 ± 0.52*	10.58 ± 3.38*	74.62 ± 9.46*
N. acuminatissima	VH	0.64 ± 0.03	15.53 ± 0.30	98.21 ± 2.68	50.87 ± 4.75
	5	0.67 ± 0.03	15.31 ± 0.35	90.15 ± 2.80	40.28 ± 1.20*
	10	0.67 ± 0.03	14.46 ± 0.54	41.33 ± 1.52*	28.12 ± 2.90*
	25	0.71 ± 0.02	11.80 ± 1.17*	39.61 ± 3.91*	19.61 ± 4.20*
	50	0.62 ± 0.02	9.08 ± 1.75*	22.45 ± 0.44*	12.63 ± 1.98*
N. buisanensis	VH	0.67 ± 0.02	19.22 ± 0.88	444.20 ± 10.79	44.90 ± 1.89
	5	0.67 ± 0.03	21.74 ± 2.60	407.70 ± 23.01	55.90 ± 9.88
	10	0.65 ± 0.02	21.05 ± 2.20	394.70 ± 16.88	21.40 ± 2.00*
	25	0.64 ± 0.03	20.88 ± 2.55	339.70 ± 27.08*	21.20 ± 6.40*
	50	0.64 ± 0.02	18.87 ± 2.68	294.00 ± 5.38*	11.40 ± 3.16*
N. daibuensis	VH	0.60 ± 0.02	8.60 ± 0.18	157.30 ± 10.25	92.32 ± 5.13
	5	0.63 ± 0.01	8.20 ± 0.23	179.20 ± 7.68	83.36 ± 3.57
	10	0.66 ± 0.02	8.02 ± 0.15	164.90 ± 5.46	64.89 ± 3.33*
	25	0.66 ± 0.01	7.94 ± 0.09	151.60 ± 6.44	61.88 ± 4.07*
	50	0.52 ± 0.03*	8.08 ± 0.23	123.90 ± 5.13*	46.47 ± 2.46*
N. hiiranensis	VH	0.72 ± 0.01	10.60 ± 0.24	110.20 ± 2.97	112.00 ± 3.91
	5	0.72 ± 0.01	12.09 ± 0.36	103.50 ± 8.71	93.28 ± 2.90*
	10	0.72 ± 0.01	12.09 ± 0.16	84.78 ± 7.32*	87.92 ± 5.67*
	25	0.75 ± 0.01	11.95 ± 0.57	80.71 ± 6.52*	83.18 ± 4.46*
	50	0.80 ± 0.03	9.80 ± 0.19	59.68 ± 11.64*	60.32 ± 4.95*
N. konishii	VH	0.72 ± 0.00	17.07 ± 0.32	94.47 ± 5.90	127.90 ± 8.11
	5	0.74 ± 0.01	14.99 ± 0.43*	56.30 ± 7.77*	132.50 ± 9.40
	10	0.74 ± 0.03	14.10 ± 0.60*	42.55 ± 3.01*	114.60 ± 5.03
	25	0.67 ± 0.01	12.19 ± 0.33*	38.55 ± 4.37*	120.70 ± 2.99
	50	0.35 ± 0.01*	10.39 ± 0.38*	27.30 ± 3.88*	116.80 ± 5.19
N. parvigemma	VH	0.35 ± 0.02	9.30 ± 1.18	196.70 ± 14.12	243.80 ± 8.27
	5	0.32 ± 0.03	8.42 ± 0.25	178.40 ± 15.70	218.90 ± 7.82
	10	0.33 ± 0.01	8.29 ± 0.23	146.50 ± 4.94*	189.70 ± 3.67*
	25	0.30 ± 0.02	8.35 ± 0.26	140.60 ± 9.96*	172.40 ± 4.48*
	50	0.30 ± 0.02	8.35 ± 0.15	119.60 ± 5.20*	157.00 ± 4.89*
N. sericea var. *aurata*	VH	0.65 ± 0.03	21.21 ± 1.22	393.30 ± 16.52	147.80 ± 11.74
	5	0.66 ± 0.02	22.86 ± 0.43	314.30 ± 12.97*	109.50 ± 3.70*
	10	0.67 ± 0.02	23.98 ± 2.31	307.50 ± 11.61*	87.00 ± 4.91*
	25	0.67 ± 0.01	21.76 ± 0.95	215.60 ± 16.29*	72.83 ± 3.16*
	50	0.61 ± 0.07	20.26 ± 0.33	199.80 ± 26.79*	69.50 ± 4.17*

Table 2: Continued.

Plants	Concentration (μg/mL)	Cell viability MTT (O.D.)	Th1 cytokines IL-2 (ng/mL)	Th1 cytokines IFN-γ (ng/mL)	Th2 cytokines IL-4 (pg/mL)
N. villosa	VH	0.47 ± 0.03	7.95 ± 0.54	87.60 ± 4.38	78.08 ± 2.72
	5	0.42 ± 0.01	8.28 ± 0.11	77.24 ± 7.08	81.61 ± 3.81
	10	0.44 ± 0.01	8.21 ± 0.65	61.96 ± 1.89*	95.23 ± 2.68
	25	0.52 ± 0.02	8.21 ± 0.44	50.67 ± 8.33*	90.93 ± 2.91
	50	0.55 ± 0.02	7.54 ± 0.28	29.95 ± 4.15*	73.51 ± 3.18

Data were expressed as the mean ± SE of quadruplicate cultures. Results were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group.

Table 3: The effects of *N.* species root extracts on Th1/Th2 cytokine production by ConA-stimulated splenocytes.

Plants	Concentration (μg/mL)	Cell viability MTT (O.D.)	Th1 cytokines IL-2 (ng/mL)	Th1 cytokines IFN-γ (ng/mL)	Th2 cytokines IL-4 (pg/mL)
	Control	0.06 ± 0.00	0.14 ± 0.12	Not detectable	Not detectable
N. aciculata var. *variabillima*	VH	0.40 ± 0.01	10.15 ± 0.71	66.21 ± 6.82	166.50 ± 5.30
	5	0.43 ± 0.02	9.31 ± 0.31	50.21 ± 7.96	171.00 ± 18.73
	10	0.41 ± 0.02	10.32 ± 0.22	40.42 ± 3.31*	210.20 ± 18.04
	25	0.37 ± 0.03	8.02 ± 0.11*	21.15 ± 2.55*	141.20 ± 7.61
	50	0.29 ± 0.02*	5.90 ± 0.31*	8.379 ± 2.25*	86.02 ± 6.78*
N. konishii	VH	0.68 ± 0.00	12.91 ± 0.09	99.63 ± 8.09	102.40 ± 1.41
	5	0.69 ± 0.01	13.01 ± 0.13	64.55 ± 4.33*	106.70 ± 4.83
	10	0.72 ± 0.01	13.21 ± 0.14	48.06 ± 6.32*	107.60 ± 4.82
	25	0.71 ± 0.03	13.05 ± 0.10	25.46 ± 2.70*	106.20 ± 7.55
	50	0.64 ± 0.01	12.45 ± 0.21	7.93 ± 1.34*	102.00 ± 8.80

Data were expressed as the mean ± SE of quadruplicate cultures. Results were representative of four independent experiments. *$p < 0.05$ was significant compared to the VH group.

IL-2 production. Interestingly, both Th1 and Th2 cytokines were attenuated by *N. hiiranensis* leaf extracts (Table 2). *N. konishii* dramatically suppressed IFN-γ at the concentration of 5–25 μg/mL while the cell viability was not altered. In addition, IL-2 production was suppressed from 5 to 25 μg/mL. These data indicated the potential effects of *N. konishii* on Th1 functionality (Table 2). Interestingly, *N. villosa* at the concentration of 25 μg/mL slightly increased cell viability and IL-4 production while IFN-γ was significantly suppressed. This data demonstrated the differential modulatory effects of *N. villosa* on Th1/Th2 balance (Table 2). As the leaf extracts of *N. aciculata* var. *variabillima* and *N. konishii* mainly inhibited IFN-γ production, we further determined the effects of leaf extracts on IL-12 production and their root extracts on the functionality of T cells. The leaves of *N. aciculata* var. *variabillima* did not affect IL-12 production; however, *N. konishii* dramatically attenuated IL-12 secretion at 25 μg/mL, revealing that leaf extracts of *N. konishii* suppressed IFN-γ production through the downregulation of upstream IL-12 production by splenic dendritic cells (Supplemental Fig.1). The root extracts of *N. aciculata* var. *variabillima* attenuated IL-2 and IFN-γ production. The root extracts of *N. konishii* dramatically suppressed IFN-γ at the concentration of 5–50 μg/mL. These results demonstrated that both *N. aciculata* var. *variabillima* and *N. konishii* significantly suppressed Th1 functionality (Table 3).

3.4. The Direct Immunomodulatory Effects of Leaf Extracts of Selected Neolitsea Species on Th1/Th2 Functionality. Based on the above results, *N. aciculata* var. *variabillima* and *N. konishii* mainly modulated Th1 functionality in ConA-stimulated splenocytes. We next analyzed the direct immunomodulatory effects of these two leaf extracts on CD4 T cells under stimulation of CD3 and CD28. CD3 is a major component of the T-cell receptor (TCR) complex, as well as CD28, which is a costimulatory molecule. The activation of both CD3 and CD28 will induce T-cell proliferation and cytokine production [37]. In addition, the production of IL-10 and IL-17 were determined to study the effects of these leaf extracts on the functionality of regulatory T cells and Th17 cells. The leaves extracts of *N. aciculata* var. *variabillima* significantly suppressed IFN-γ, IL-10, and IL-17 production at the concentration of 5–25 μg/mL in CD3/CD28 stimulated CD4 T cells (Figures 3(a), 3(c), and 3(d)), while IL-4 were slightly altered at the concentration of 25 μg/mL (Figure 3(b)). The leaf extracts of *N. konishii* mainly attenuated IFN-γ and IL-10 productions at the concentration of 5–25 μg/mL (Figure 4), while IL-17 was not affected (Figure 4). We also performed the intracellular staining to detect the protein levels of IFN-γ and IL-4 in CD4 T cells. Interesting, the leaf extracts of *N. aciculata* var. *variabillima* decreased the percentage of IFN-γ^+ in CD4 T cells from 42% (VH) to 32%, but the level of mean fluorescence intensity of

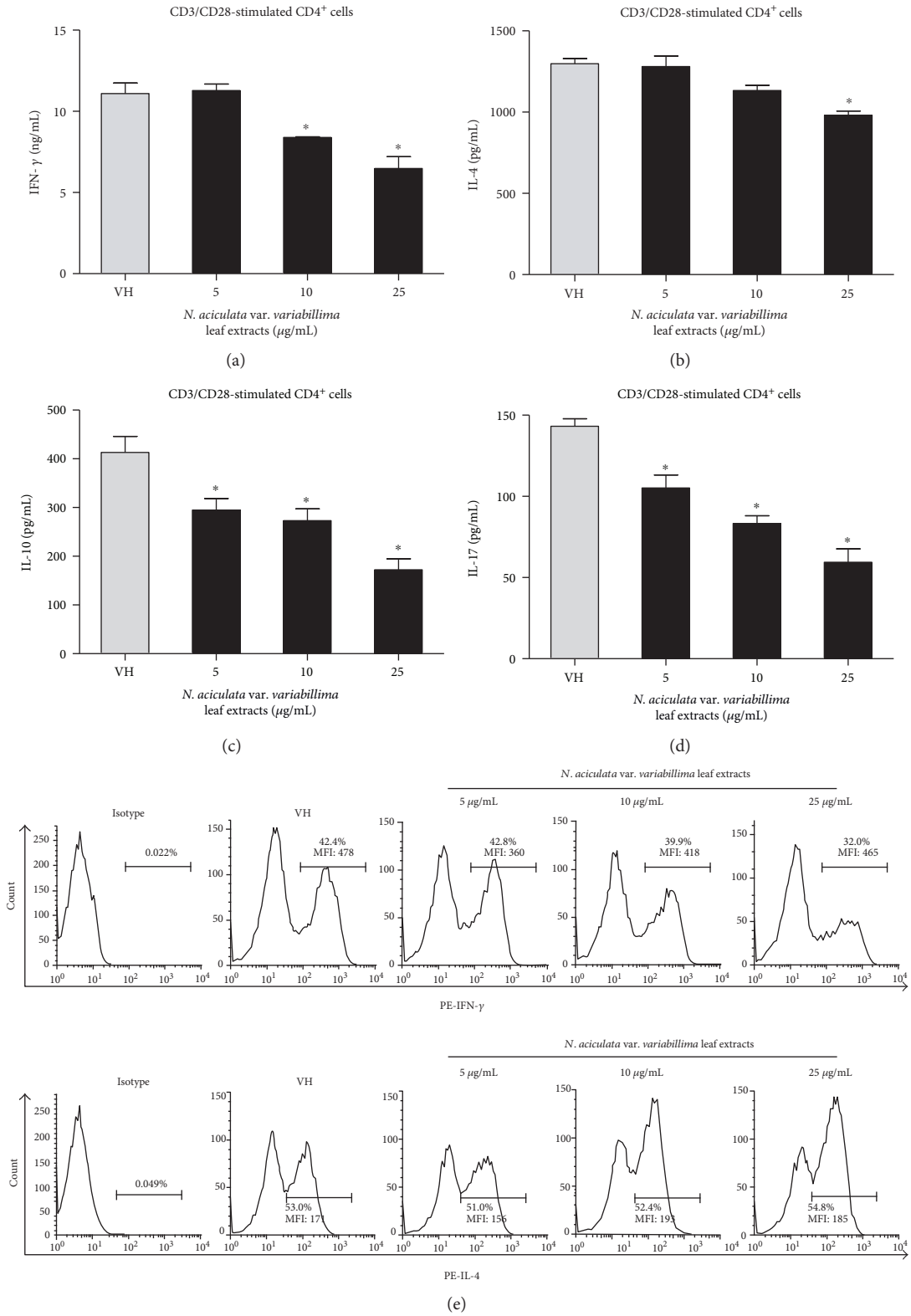


Figure 3: The effects of leaf extracts of *N. aciculata* var. *variabillima* on T-cell functionality. The enriched CD4 T cells (5×10^5 cells/mL) were stimulated with anti-CD3 and anti-CD28 (1 μg/mL) in the absence or in the presence of the leaf extracts of *N. aciculata* var. *variabillima* (5–25 μg/mL) for 48 h. (a)–(d) The concentration of IFN-γ, IL-10, IL-4, and IL-17 in the supernatants was measured by ELISA. Data were expressed as the mean ± SE of quadruplicate cultures. $^*p < 0.05$ was significant compared to the VH group. (e) The representative histogram of intracellular cytokine staining. Total percentage and the level of mean fluorescence intensity (MFI) of IFN-γ^+ and IL-4^+ cells in $CD4^+$ T cells were shown. Results were representative of two independent experiments.

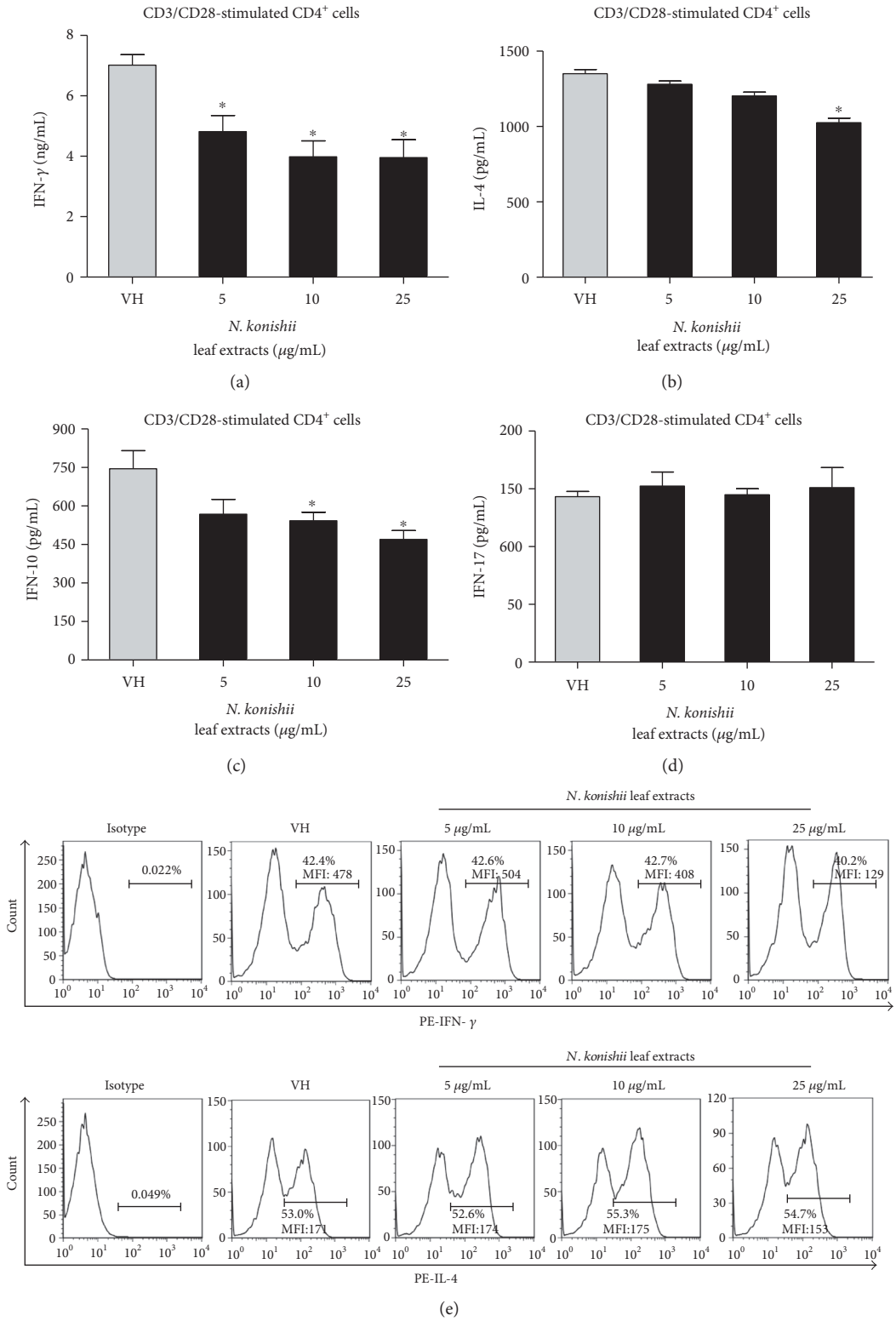


Figure 4: The effects of leaf extracts of *N. konishii* on T-cell functionality. The enriched CD4 T cells (5×10^5 cells/mL) were stimulated with anti-CD3 and anti-CD28 (1 μg/mL) in the absence or the presence of the leaf extracts of *N. konishii* (5–25 μg/mL) for 48 h. (a)–(d) The concentration of IFN-γ, IL-10, IL-4, and IL-17 in the supernatants was measured by ELISA. Data were expressed as the mean ± SE of quadruplicate cultures. $^*p < 0.05$ was significant compared to the VH group. (e) The representative histogram of intracellular cytokine staining. Total percentage and the level of mean fluorescence intensity (MFI) of IFN-γ^+ and IL-4^+ cells in $CD4^+$ T cells were shown. Results were representative of two independent experiments.

IFN-γ was not significantly altered in IFN-γ^+ cells. *N. aciculata* var. *variabillima* did not affect the percentage of IL-4^+ cells in CD4 T cells nor the protein levels of IL-4 in CD4 T cells, suggesting that the leaves of *N. aciculata* var. *variabillima* decreased the proportion of IFN-γ^+ in CD4 T cells. By contrast, the leaf extracts of *N. konishii* attenuated the protein level of IFN-γ in CD4 T cells while the proportion of IFN-γ^+ cells were not changed.

4. Discussion

Natural products isolated from traditional medicinal plants have therapeutic effects in the prevention and treatment of various immune disorders. *Neolitsea* species exhibit extensive bioactivities and have been used as traditional herbal medicines in oriental countries. However, a comparative study of immunomodulatory properties of different *Neolitsea* species on immunocompetent cells such as T helper cells has not been demonstrated to date. Our previous reported study found that the leaf extracts of *N. hiiranensis* significantly inhibited IL-12, IFN-γ, and IL-2 cytokine productions as well as the serum levels of OVA-primed antigen-specific IgM and IgG_{2a} in vivo [27]. In the present study, we evaluated the immunomodulatory properties of ten Taiwanese *Neolitsea* plants on T helper cells. Our results showed that most crude extracts of Taiwanese *Neolitsea* species decreased IFN-γ production at concentrations below the IC_{50} by mitogen-stimulated splenocytes, and the immunomodulatory activities of *Neolitsea* extracts, especially leaf extracts, were mainly on the suppression of Th1 immunity.

Several reports showed that *Neolitsea* species and its derived secondary compounds, including sesquiterpenoids, triterpenoids, alkaloids, and steroids, possess several bioactivities including anti-inflammatory activities [18, 20]. *Neolitsea aciculata* essential oil (NAE) attenuated the *Propionibacterium acnes*-induced secretion of tumor necrosis factor-alpha (TNF-alpha) and interleukin-8 (IL-8) productions in human cell lines, revealing its anti-inflammatory effects [38]. Six furanogermacrane sesquiterpenes isolated from the stems of *N. parvigemma*, including deacetylzeylanidine, linderalactone, neolitrane, pseudoneolinderane, zeylanidine, and zeylanicine, have been shown to possess anti-inflammatory activities; among them, pseudoneolinderane and linderalactone have the ability to inhibit the superoxide anion generation by human neutrophils in response to formyl-l-methionyl-l-leucyl-l-phenylalanine/cytochalasin B (fMLP/CB) with IC_{50} values of 3.21 and 8.48 μg/mL, respectively [39]. Daibucarboline A, isolinderalactone, 7-omethylnaringenin, and prunetin isolated from the roots of *N. daibuensis* exhibited moderate inhibition of inducible nitric oxide synthase (iNOS) [40]. Hiiranlactone B and hiiranlactone D, isolated from the leaves of *N. hiiranensis* were classified as sesquiterpenes and possessed anti-inflammatory and immunomodulatory effects [27, 34]. Thaliporphine, the alkaloid of the bark extracts of *N. konishii*, demonstrated vasoconstricting effects by promoting Ca^{2+} entry [25] and possessed antioxidant, anti-inflammatory, and antiapoptotic activity to prevent cardiovascular system disorder in guinea pigs [41]. In addition to the anti-inflammatory effects, the ingredients from *Neolitsea* species possess several bioactivities. Flavone glycosides, 2′-*p*-coumaroylafzelin and 2′,3′-di-*O*-(*p*-coumaroyl)afzelin, have been isolated from *N. aciculata* to demonstrate the antimelanogenesis activities [42]. Neolitacumone A–C and 2,6-dimethoxy-*p*-benzoquinone from the stem barks of *N. acuminatissima* displayed significant inhibitory activities against Hep 2.2.15 cells [43]. Isolinderalactone, a sesquiterpenes from the roots of *N. villosa*, exhibited antitumor activity [44]. Our recent study reported that *N. hiiranensis*-derived caryophyllene oxide inhibited several aspects of adaptive immune responses, including T-cell differentiation, IFN-γ production, and Th1-associated genes [27]. These studies suggested that the immunomodulatory effects of *Neolitsea* plants are worth further investigation; in addition, the present study aimed to clear how other Taiwanese *Neolitsea* species modulate the functionality of Th cells by ConA-stimulated splenocytes.

Th cells play pivotal roles in the acquired immunity. They are categorized into several subsets based on the cytokine production after stimulation [5]. The main roles of the type 1 Th cells (Th1) are against intracellular bacteria and protozoa. They are predominantly induced by interleukin-12 (IL-12) and are fully activated by IL-2 and IFN-γ. Induction of mitotic activity and cytokine production are associated with Th cell functionality. IFN-γ, an upstream cytokine of Th1 cells, regulates inflammatory immune response, promotes Th1 cell differentiation, enhances MHC class II expression on antigen-presenting cells, and possesses protective immune responses against cancer formation [4, 45–48]. Saha et al. reported that *Viscum album*-derived Qu Spez significantly stimulated IFN-γ secretion showing its ability to modulate the immune system and to suppress tumor regression by regulation of dendritic cells [49]. Th1 cells secreted IFN-γ to activate cell-mediated immune responses that several immune cells are involved such as macrophages, cytotoxic T cells, and natural killer cells [4, 45, 46, 50]. Th2 cells are against extracellular multicellular parasites by releasing interleukin-4 (IL-4), IL-5, and IL-13 cytokines [51]. Furthermore, the interaction of dendritic cells with activated B cells may help dendritic cells to acquire unique abilities to promote polarization of Th2 [50]. The imbalance of Th1/Th2 may lead to immunological diseases, such as rheumatoid arthritis, type-1 diabetes, multiple sclerosis, and asthma [52]. Discovering the selective immunomodulators on different subsets of Th cells may be beneficial for the treatment of immune disorders.

In our presented data, most of the leaf extracts of *Neolitsea* species have low cytotoxicity. The leaf extracts of *N. aciculata* and *N. villosa* possessed differential immunomodulatory effects on Th1/Th2 balance. *N. daibuensis* leaf extracts slightly attenuated IL-4 production; in contrast, *N. aciculata* var. *variabillima* and *N. hiiranensis* leaf extracts attenuated both Th1 and Th2 cytokines. *N. konishii* leaf extracts dramatically suppressed IFN-γ cytokine. The leaf extracts of *N. aciculata* var. *variabillima* and *N. konishii* differentially affect the functionality of different subsets of Th cells. Collectively, these *Neolitsea* species demonstrated selective immunomodulatory effects and the underlying mechanisms are needed for further study. Although there are a variety of immunosuppressive drugs such as cyclosporine A, tacrolimus, daclizumab, and basiliximab to prevent the rejection of transplanted organs and tissues,

autoimmune diseases, and inflammatory disorders [53], these drugs could cause systemic immunosuppression which greatly increases the risks of tumor formation and infections [54]. Hence, development of new therapeutic and preventive immunomodulators from medicinal plants to manage immune disorders is of great importance. There are many studies devoted to discover new immunomodulatory therapeutic compounds from natural plants with low toxicity [55–57]. *p*-Coumaryl alcohol-gamma-*O*-methyl ether (CAME) isolated from *Alpinia galanga* was selectively and substantially suppressed in IFN-γ production in Th cells [11]. Farrerol, a new type of 2,3-dihydro-flavonoid isolated from the leaves of *Rhododendron dauricum* L, markedly suppressed concanavalin A- induced lymphocyte proliferation, Th1 and Th2 cytokine production, and differentiation of T helper cell populations [46]. Physalin H isolated from *Physalis angulata* exhibited an immunosuppressive activity on T-cell activation and proliferation by modulation of Th1/Th2 immune balance [58]. There are 14 compounds from leaves of *N. hiiranensis* that exhibited anti-inflammatory activity to suppress the generation of superoxide anion from neutrophils [34]. Furthermore, four major metabolites of the leaves of *N. hiiranensis*, including elemane type, caryophyllene type, aromadendrene type, eudesmane type, and germacrane dilactone type, are the main effective anti-inflammatory constituents [34]. Caryophyllene oxide, a sesquiterpene compound from the leaves of *N. hiiranensis*, inhibited the influx of neutrophil into the inflammatory site and the activation of NF-κB pathway [59]. We recently reported that *N. hiiranensis*-derived terpenoids, including hiiranlactone D, *trans*-phytol, and β-caryophyllene oxide, attenuated antigen-specific T helper 1 immunity [27]. Here, the leaf extracts of *Neolitsea* plants showed potential immunomodulatory activities on T-cell functionality. To further study the potential, immunomodulatory compounds from these plants will help to discover new immunomodulators.

5. Conclusions

The study demonstrated that most of the crude extracts of Taiwanese *Neolitsea* species, especially leaf extracts, were not toxic to primary splenocytes, but they are capable of decreasing IFN-γ production without affecting IL-2 production by T cells. The selective Th1 immunomodulatory effects of the *Neolitsea* extracts indicate that the phytochemicals in these extracts have potential to be further evaluated and developed as immunomodulatory agents.

Conflicts of Interest

The authors declare no conflicts of interest. The authors are responsible for the content of this manuscript.

Acknowledgments

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Research Article

Therapeutic Effects of Methanol Extract from *Euphorbia kansui* Radix on Imiquimod-Induced Psoriasis

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The roots of *Euphorbia kansui*, which belong to the family Euphorbiaceae, have been used as a traditional medicine for the treatment of various diseases such as diabetes, ascites, and leukemia. Recently, it was reported that the methylene chloride fraction of *E. kansui* radix (EKC) regulated the differentiation of Th17 cells and alleviated the symptoms of Th17-related inflammatory bowel disease. Imiquimod (IMQ), a TLR7/8 agonist, has been used to induce psoriasis in a mouse model. In this study, we evaluated the effect of EKC in an IMQ-induced psoriasis model. EKC effectively inhibited the production of interleukin-17A and interferon-γ in vitro. On this basis, EKC was administered to an animal model of psoriasis. Acanthosis and the infiltration of inflammatory cells into the dermis were significantly reduced by EKC. EKC also inhibited the expression of IL-17A, IL-22, IL-23, IL-12, and RAR-related orphan receptor gamma t (RORγt) in the spleen, skin-draining lymph nodes, and the skin. Additionally, EKC inhibited the activity of dendritic cells but not that of keratinocytes. In conclusion, EKC ameliorated the symptoms of psoriasis through inhibition of Th17 differentiation and activation of dendritic cells. These effects are expected to be beneficial in the treatment and prevention of psoriasis.

1. Introduction

Psoriasis is recognized as the most common type of chronic inflammatory dermatosis, which is caused by a disorder of the immune system in which T cells play a primary role [1]. Psoriatic inflammation was initially considered to be mediated by T-helper 1 (Th1) cells that produced interferon-γ (IFN-γ) [2]. However, research increasingly indicates that T-helper 17 (Th17) cells, which produce interleukin-17 (IL-17) and interleukin-22 (IL-22), are critical in the pathogenesis of psoriasis [3, 4]. Th17 cells are important in the host defense against specific extracellular bacteria, and they have been associated with various autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis [5–8]. In the skin, after plasmacytoid dendritic cells (pDCs) in the epidermis have been activated by a trigger (stress, microorganisms, or DNA mutation), they secrete a high level of IFN-α, which activates dermal DC and initiates T cell-mediated immunity. Activated dendritic cells produce IL-12 and IL-23, which induce the differentiation of naive T cells into Th17 cells. The cytokines and activated Th17 cells advance the pathogenesis of psoriasis [9]. The pathogenic symptoms of psoriasis are characterized by hyperplasia of epidermal keratinocytes, scaling, and infiltration of neutrophils and lymphocytes [10]. Many immune-derived cytokines, including IL-23, IL-17A, IL-22, TNF-α, and IFN-γ, are involved in disease development [11, 12]. Various studies of patients with psoriasis have demonstrated that the amelioration of psoriasis was associated with reduced Th17 response. It was reported that ustekinumab, a monoclonal antibody (mAb) to IL-12/23 p40, significantly reduced IL-23p19 and IL-12/23p40 gene expression compared with

baseline levels in the lesions of psoriatic patients [13, 14]. In addition, secukinumab and ixekizumab, specific mAbs for human IL-17A, are in phase III clinical development for the treatment of plaque psoriasis [15–19].

Imiquimod (IMQ) has been approved for the treatment of genital warts and actinic keratosis but occasionally leads to the development of psoriasis in humans [20–24]. IMQ is a potent agonist of TLR7/8 and facilitates local and acquired immune responses [25]. Experimental data showed that IMQ-induced dermatitis in mice closely resembled human psoriatic lesions, not only in the histological characteristics but also in the development of lesions [26]. In addition, IMQ application induced the epidermal expression of IL-23, IL-17A, and IL-17F, as well as increased their expression in splenic Th17 cells [27–29]. This experimental method has therefore allowed the elucidation of new therapies for psoriasis. Recent studies have established the mechanism of pathogenesis and the molecular targets for the treatment of psoriasis through an IMQ-induced mouse model [30–32].

The roots of *Euphorbia kansui* (Kansu), which are plants belonging to the Euphorbiaceae family, have been used as a traditional medicine throughout the Far East. Many herbs in this family have been traditionally utilized to treat various diseases such as edema, ascites, and asthma. Approximately 100 chemical compounds have been isolated and identified from *E. kansui.* Diterpenoids (e.g., kansuinine A and B) and triterpenoids (e.g., euphol) are the main chemical constituents responsible for the biological effects, which include antiviral, antiproliferative, and immunomodulatory activities [33]. The ethanol extract of *E. kansui* has the ability to activate lymphocytes, which enhanced its capacity to remove virus-infected cells [34]. Terpenoid compounds derived from the roots of *E. kansui* showed a significant inhibition of proliferative activity of embryonic cells and intestinal epithelioid cells [35, 36]. It was reported that ingenane-type diterpenes from Kansu modulated IFN-γ production by regulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [37].

Th17 cell activity was reduced by the activation of extracellular signal-regulated protein kinase (ERK) and increased by the phosphorylation of Stat3. A topical application of euphol, a triterpenoid isolated from the roots of *E. kansui,* significantly inhibited TPA-induced ear edema through activation of ERK [38]. Moreover, kansuinine A and B, chemical compounds from *E. kansui*, have an inhibitory effect on IL-6-induced Stat3 activation [39]. Previous studies have identified that ethanol extracts of *E. kansui* radix inhibited Th17 cell differentiation and increased the division of regulatory T cells (Treg cells) in Th17-driving conditions (unpublished data). In addition, the methylene chloride fraction of *E. kansui* radix extracted with methanol (EKC) repressed DSS-induced colitis (unpublished data). These regulatory effects on Th17 and related factors led us to investigate the in vivo effect of Kansu extract on the development of psoriasis.

This study was designed to investigate the effect of EKC in an IMQ-induced psoriasis model and to explore underlying mechanism on its therapeutic efficacy.

2. Materials and Methods

2.1. Mice. Female Balb/c mice were purchased from Orient Bio (Sungnam, Korea). The mice were aged 6–9 weeks for all experiments. Animals were maintained in a specific pathogen-free environment under the following controlled conditions: temperature, $21 \pm 3^{\circ}$C; relative humidity, $50 \pm 10\%$; and illumination, 10 h light and 14 h darkness. All studies and procedures were conducted in accordance with the National Institutes of Health Guide for the care and use of laboratory animals, and the protocol was approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center of Chung-Ang University.

2.2. Materials. The materials used in this study included Aldara cream (5% imiquimod, Dong-Ah Pharmaceutical, Seoul, Korea) and human IL-17A recombinant protein (ebioscience, San Diego, USA). The roots of *E. kansui* were obtained from a commercial herbal drug market (Jaesung Medicinal Herbal Drug Market, Seoul, Korea). The voucher specimens were stored at the Pharmacognosy Laboratory of the College of Pharmacy, Dankook University, Korea. The dried roots of *E. kansui* were pulverized and extracted with 90% aqueous methanol. The methanolic extract was dissolved in water and partitioned with *n*-hexane and methylene chloride. The methylene chloride fraction of the methanol extract from *E. kansui* radix was referred to by the abbreviation EKC.

2.3. Purification of $CD4^+$ T Cells and Th17/Th1 Differentiation. The spleens were extracted from Balb/c mice and naive $CD4^+$ T cells were purified by using a magnetic cell sorting system (MACS® separation, Miltenyi Biotech, Bergisch Gladbach, Germany). The cells were cultured in RPMI 1640 culture media with 10% heat-inactivated FBS (Cellgro, Herndon, VA, USA), 100 U/mL penicillin (Cellgro), 0.1 mg/mL streptomycin (Cellgro), 2 mM L-glutamine (Cellgro), and 0.05 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, USA) at 37°C in a 5% CO_2-humidified incubator. Plate-bound anti-CD3 antibody (1 μg/mL, ebioscience) and anti-CD28 antibody (1 μg/mL, ebioscience) were used to stimulate T cells. Recombinant mouse IL-6 (25 ng/mL, BD, San Jose, CA, USA) and TGF-β (2.5 ng/mL, BD) were used for Th17 differentiation, and IL-2 (10 ng/mL, BD), IL-12 (5 ng/mL, Biosource, Camerillo, CA, USA), and anti-IL-4 (5 μg/mL) were added to induce differentiation into Th1 cells.

2.4. Induction or Evaluation of Imiquimod-Induced Psoriasis. Balb/c mice were separated into six groups each containing five animals. To induce skin inflammation in the mice using the inflammation response modifier drug imiquimod (IMQ), five of the groups received a consecutive daily topical dose (62.5 mg) of commercially available 5% imiquimod cream (3.215 mg of active compound) on shaved back skin for 7 days. The noninduced control group was treated similarly with a vehicle cream (Vaseline, Unilever, London, UK). For oral administration, EKC was dissolved in Dimethylacetamide (DMA) and diluted in tap water to achieve a final DMA concentration < 5%. EKC was daily

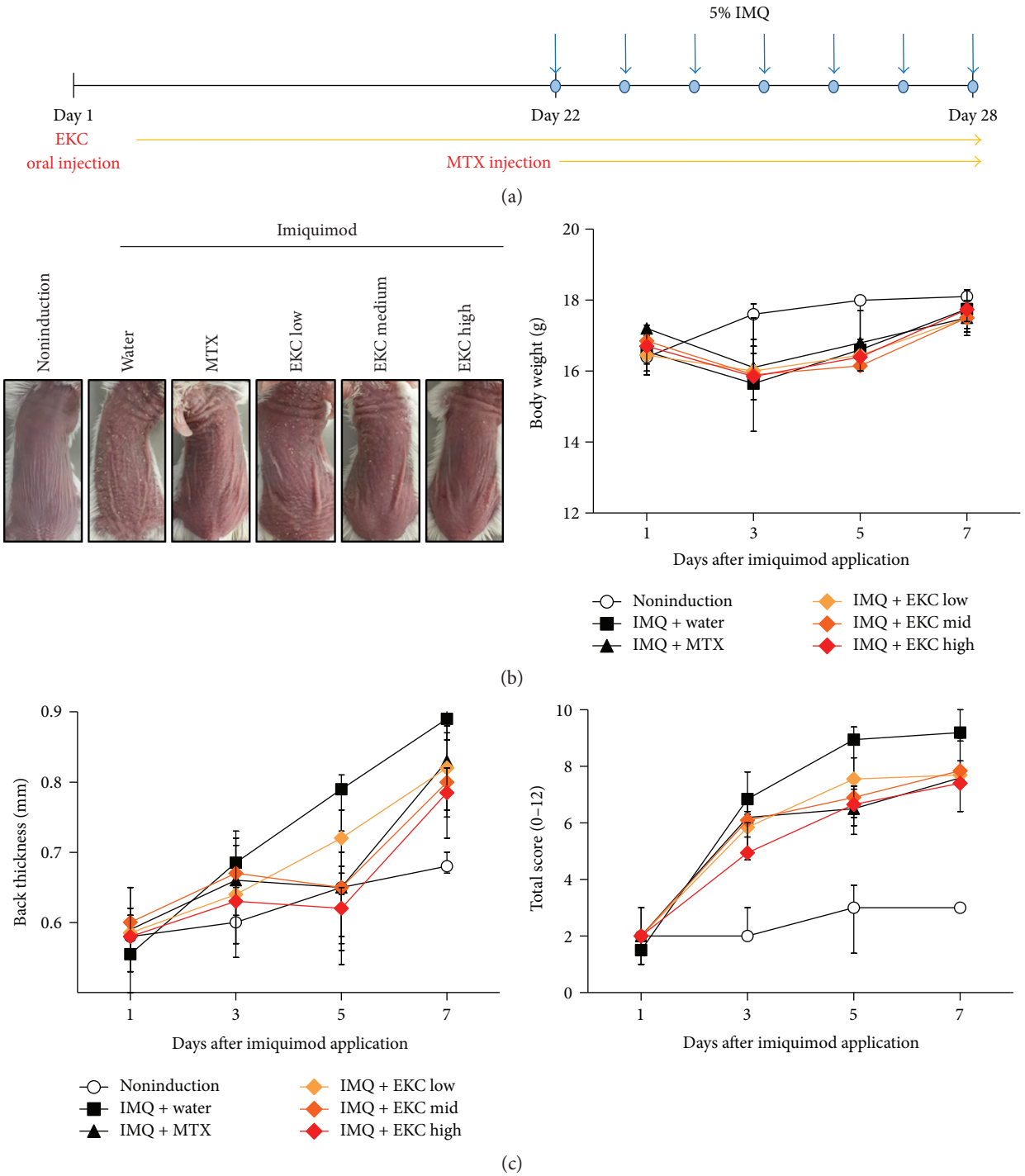


Figure 1: Continued.

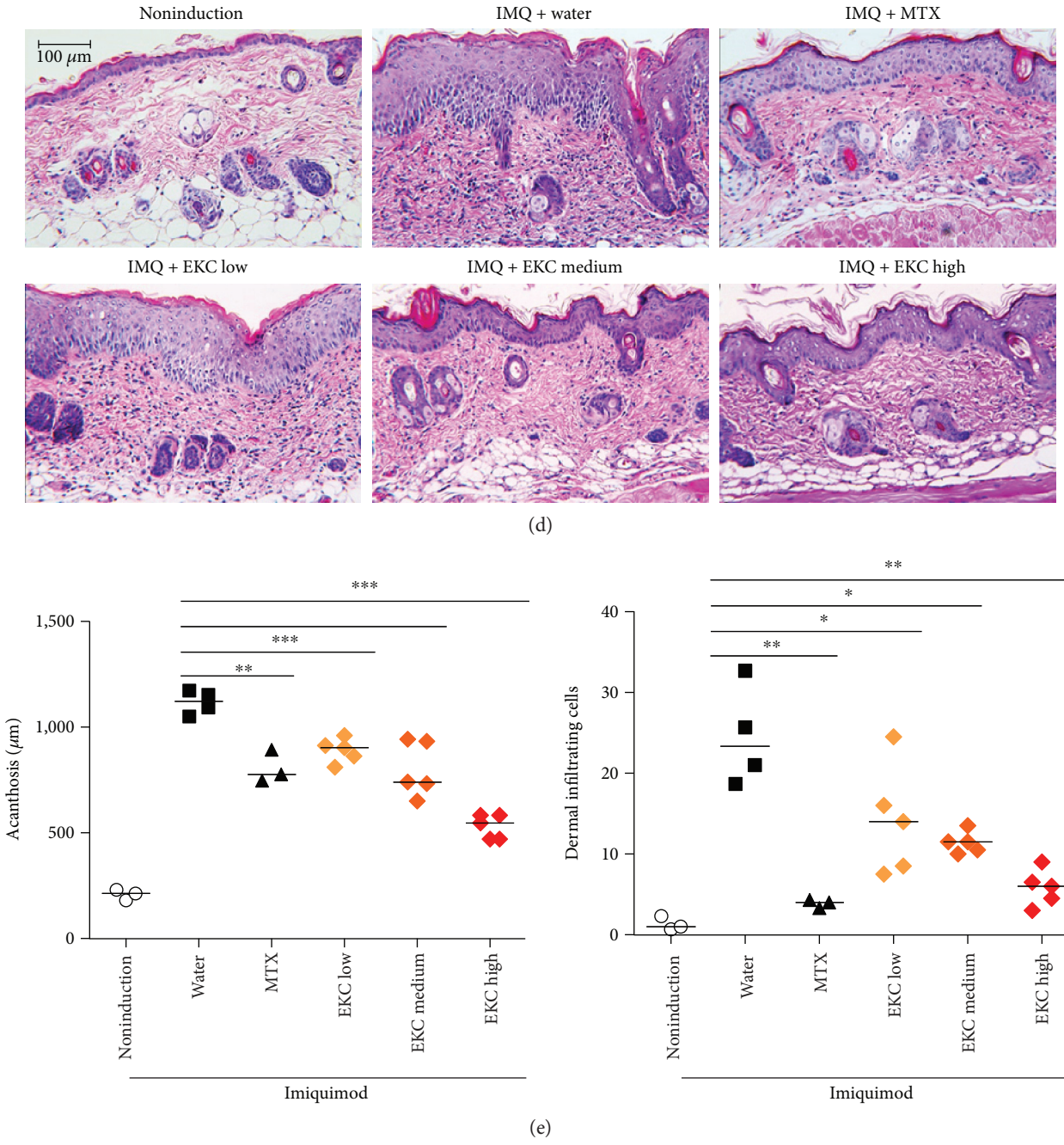


Figure 1: Experimental procedure and effects of the oral administration of EKC on dermatitis lesions. (a) Experimental overview. Day 1 was defined as the first administration of EKC (20 mg/kg, 100 mg/kg, and 200 mg/kg). After 22 days, imiquimod cream was applied to the shaved back skin of the mice. Methotrexate (MTX) was used as positive control. (b) Imiquimod was applied daily to Balb/c mice. After 7 days, pictures of mice were taken and the phenotypical symptoms of the mouse back skin were observed. The variation in body weight was measured for 7 days. (c) Dermatitis scores (back thickness, redness, and scaling) were evaluated every other day from day 22 to day 28. Back thickness and total score (erythema plus thickness plus scaling) are presented as mean ± SD ($n = 5$). (d) H&E staining of skin tissue treated with MTX or different doses of EKC (original magnification, ×200). (e) Acanthosis was evaluated by measuring the length of the epidermal cell layers. Three sections per mouse sample were analyzed. The dermal infiltrating cells were counted from three random sections of each sample. Bars represent the median. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared with imiquimod + water group.

administered from 22 days before IMQ application to the final of the study, and methotrexate was used as a positive control (Figure 1(a)). The psoriasis-induced groups were IMQ + water, IMQ + MTX (1 mg/kg methotrexate; Yuhan corporation, Seoul, Korea), and IMQ + EKC low (20 mg/kg), middle (100 mg/kg), and high (200 mg/kg). Back thickness was measured using digital thickness gauge (Bluebird, Seoul, Korea). A scoring system based on the clinical Psoriasis Area and Severity Index (PASI) was used to score the severity of the skin inflammation. Erythema, thickness, and scaling were

scored independently on a scale from 0 to 4 as follows: 0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked. The cumulative score served as a measure of the severity of inflammation (scale 0–12).

2.5. Histological Analysis. For the histological examinations, a skin sample with a diameter of 3 mm was removed from the back skin of psoriasis-induced mice on the final day of the administration schedule and fixed in 10% phosphate-buffered formalin (pH 7.2). The biopsies were embedded in paraffin, cut, and stained with hematoxylin and eosin for the evaluation of acanthosis and dermal infiltrating cells. The staining was analyzed by using a microscope and observing three sections from each mouse.

2.6. Cell Culture. JAWSII cells, a mouse dendritic cell line, were cultured in RPMI 1640 medium (Cellgro) supplemented with FBS (10%, heat-inactivated; Cellgro), penicillin (100 U/mL; Cellgro), streptomycin (0.1 mg/mL; Cellgro), L-glutamine (2 mM; Cellgro), 2-mercaptoethanol (0.05 μM; Sigma), and mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF; 5 ng/mL, R&D Systems, Minneapolis, MN, USA) at 37°C in a 5% CO_2-humidified incubator. The cells were seeded at 1×10^6 cells/well in a 24-well plate and stimulated for 24 h with conditioned medium containing 1 μg/mL imiquimod, with or without EKC (5 or 10 μg/mL). The cell supernatants were collected to measure IL-12p40 and IL-23p19. HaCaT human keratinocyte cell lines were cultured in DMEM (Cellgro) supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin at 37°C in a 5% CO_2-humidified incubator. The cells were seeded at 1×10^6 cells/well in 24-well plates and treated with human IL-17A (100 ng/mL, ebioscience) and EKC (5 or 10 μg/mL) for 24 h. After treatment, the cells were harvested for RNA extraction.

2.7. Sample Preparation for ELISA. $CD4^+$ T cells (1×10^6) were plated in 24-well plates in Th17- and Th1-driving conditions and treated with 5 or 10 μg/mL EKC. The supernatants were harvested at days 1, 2, and 3. The concentration of IL-17A, TNF-α, IFN-γ, and IL-12p40 in each sample was detected by ELISA. Splenocytes, axillary lymph node (ALN) cells, and brachial lymph node (BLN) cells from mice were plated on flat-bottom 24-well plates (1×10^6 cells/well) in the presence of plate-bound anti-CD3 Ab, and the supernatant was collected after 24 h and 48 h. The concentration of IL-17A, IL-12p40, TNF-α, and IFN-γ in each sample was detected by ELISA using Ab pairs. Skin biopsies from back skin were collected at the end of the experimental day. The samples were homogenized and extracted using T-PER tissue protein extraction reagent (Thermo Fisher Scientific, San Jose, CA, USA) in the presence of a protease inhibitor cocktail (Thermo Fisher Scientific) and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The protein extracts were centrifuged at 10,000*g* for 5 min. The protein concentration of the skin extracts was estimated and quantified using BCA protein assay reagents (Thermo Fisher Scientific). For the measurement of total serum IgG1 and cytokines, blood specimens were obtained from the retro-orbital sinus on day 28. The serum was separated and stored at −80°C until use.

2.8. RNA Isolation and Real-Time RT-PCR. Splenocytes, ALN cells, and BLN cells (1×10^6) from mice were activated ex vivo by incubation with anti-CD3 (1 μg/mL, BD) for 2 days, and total RNA was isolated from each sample using TRIzol (Invitrogen, Carlsbad, CA, USA). Skin samples were homogenized and centrifuged at 12,000*g* for 10 min, and the total RNA was extracted from the biopsies of the back skin using TRIzol reagent. RNA was transcribed to cDNA at 42°C for 1 h in a total reaction volume of 25 μL, which contained 5× RT buffer, 10 mM dNTPs (200 units), MMLV-RT (Moloney murine leukemia virus reverse transcriptase), and 100 pmol oligo-dT primer. The cDNA was then used for quantitative real-time PCR with 2× iQTM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) to determine the mRNA levels of IL-17A, IL-22, IL-12p40, IL-23p19, RORγt, human IL-8, human IL-36γ, human CCL20, and GAPDH. To confirm PCR specificity, the PCR products were subjected to melting curve analysis. The comparative threshold method was used to calculate the relative amount of mRNA in the experimental samples compared with the control samples. Gene expression was normalized to the expression of GAPDH.

2.9. Flow Cytometry Analysis. Splenocytes were collected in FACS buffer and stained with the following antibodies: Thy1.2 FITC, CD4 APC, CD8 APC, $\gamma\delta$ TCR PE-Cyanine5, CD19 PE, CD11b FITC, F4/80 PE, and CD11c FITC (BD and ebioscience). The stained cells were analyzed using a FACSCalibur flow cytometer and Cell Quest analysis software (BD Bioscience).

2.10. Western Blot. Skin samples from the back lesions of mice were cut into pieces, snap-frozen in liquid nitrogen, and stored at −80°C until use. The samples were homogenized in T-PER buffer (Thermo Fisher Scientific) in the presence of a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membranes. The blots were blocked and incubated at 4°C overnight with antibodies against p-IκB and β-actin (Cell signaling, Beverly, MA, USA). The membranes were then incubated with anti-rabbit IgG or anti-mouse IgG HRP-linked antibodies (cell signaling). Chemiluminescence was measured by using the chemiDoc system (Bio-rad) and analyzed with Quantity One software (Bio-Rad).

2.11. Cell Viability Assay. JAWSII cells (2×10^5) were plated in a 96-well multiplate, EKC was added, and the cells were cultured for 24 h. After culture, 10 μL MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] solution (5 mg/mL, Sigma-Aldrich) was added to each well and incubated at 37°C for 2 h. One hundred microliters of solubilization solution (0.04 N HCl in isopropanol) was added to each well. The plate was evaluated at 570 nm wavelength using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA).

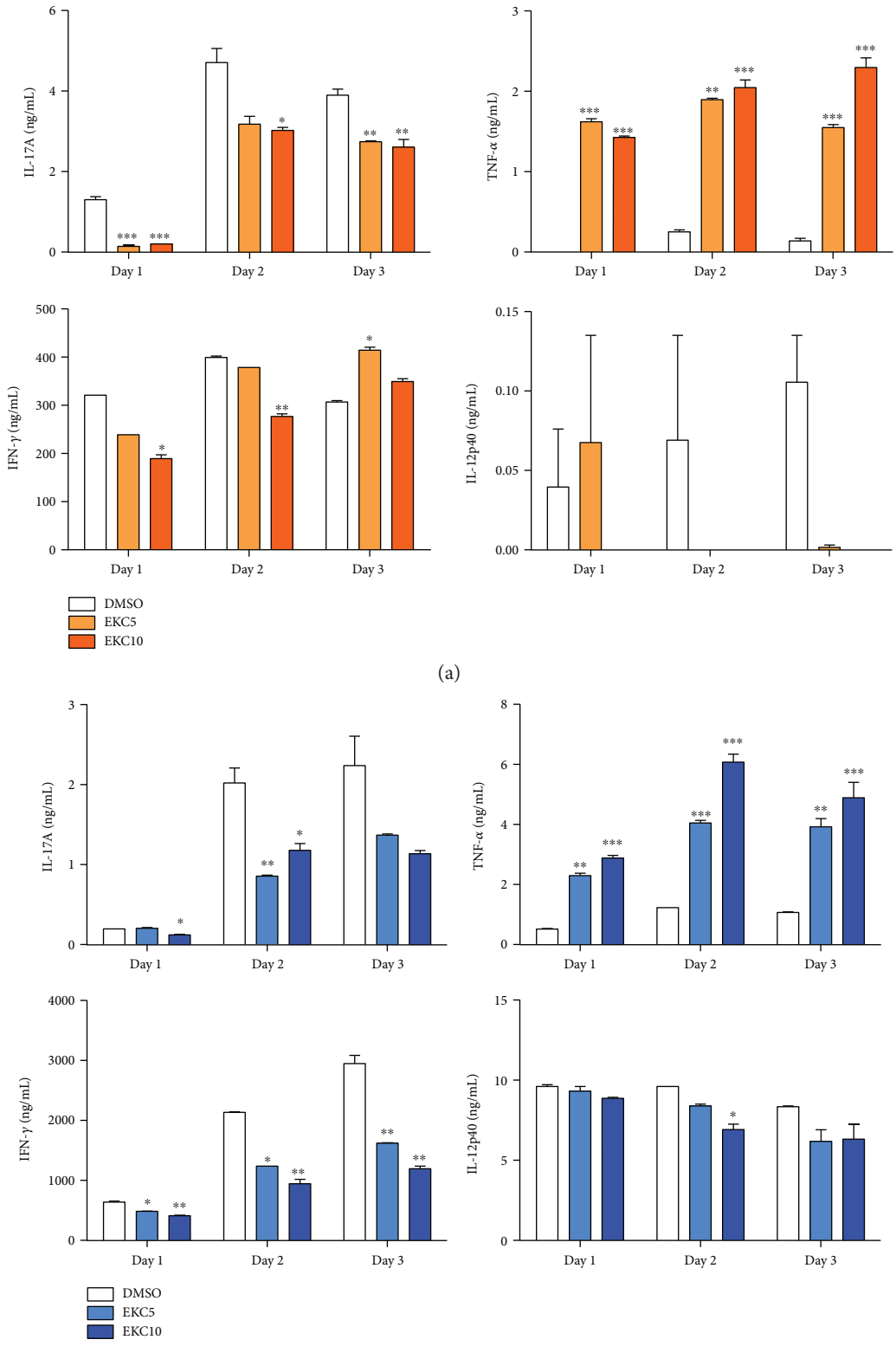


Figure 2: Decreased secretion of IL-17A and IFN-γ in both Th17- and Th1-driving conditions by EKC. (a) $CD4^+$ T cells were purified from Balb/c mice and activated with 1 μg/mL of plate-bound anti-CD3/CD28 antibodies in the presence of 25 ng/mL IL-6 and 2.5 ng/mL TGF-β, with or without EKC. Production of IL-17A, IFN-γ, TNF-α, and IL-12p40 was detected by ELISA. (b) Isolated $CD4^+$ T cells were stimulated with plate-bound anti-CD3/CD28 antibodies with or without *E. kansui* under Th1-driving cytokine conditions (10 ng/mL IL-2, 5 ng/mL IL-12, and 5 μg/mL anti-IL-4) for 3 days. The concentration of cytokines in the supernatant was detected by ELISA. Values represent the mean ± SD. ($^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared with the DMSO-treated group).

Table 1: Statistical analysis of the total score.

Day	Noninduction Mean ± SD	IMQ + water Mean ± SD	IMQ + MTX Mean ± SD	IMQ + EKC low Mean ± SD	IMQ + EKC mid Mean ± SD	IMQ + EKC high Mean ± SD
1	2.00 ± 0.00	1.50 ± 0.58	2.00 ± 0.00	1.75 ± 0.50	2.25 ± 0.50	2.00 ± 0.82
3	2.33 ± 0.58***	6.98 ± 0.60###	5.73 ± 0.90	5.88 ± 0.35*	6.25 ± 0.45	5.20 ± 0.54**
5	2.73 ± 1.22**	8.75 ± 0.73##	6.70 ± 0.53**	7.33 ± 1.18	6.80 ± 0.43**	6.55 ± 0.73**
7	3.00 ± 0.00***	9.33 ± 0.50###	7.73 ± 0.42**	7.95 ± 0.72*	7.53 ± 0.76*	7.40 ± 0.08**

*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ compared with imiquimod + water group. ##$p < 0.01$; ###$p < 0.001$ compared with noninduction group.

2.12. Statistical Analysis. Data were expressed as the mean ± SD, and statistical significance was analyzed by using Student's t-test. Different levels of statistical significance were denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

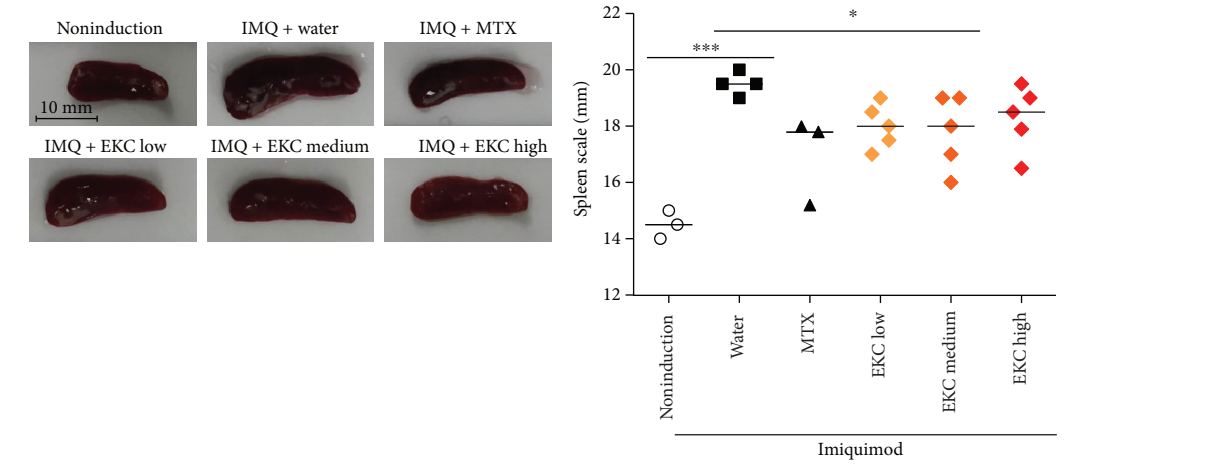
3. Results

3.1. Methylene Chloride Fraction of E. kansui Radix Suppresses Th17-Specific Cytokines. To determine the effect of EKC on Th17 and Th1 polarization, $CD4^+$ T cells were treated with EKC under Th17- and Th1-driving conditions. After 3 days, the supernatant was harvested and analyzed by ELISA. In Th17-driving conditions, the production of IL-17A was significantly decreased and IFN-γ secretion was slightly decreased by EKC treatment. In contrast to the results for IL-17A and IFN-γ secretion, TNF-α secretion significantly increased (Figure 2(a)). In Th1-driving conditions, the EKC-treated sample showed consistent results with Th17-driving conditions (Figure 2(b)). IL-12p40 was not detected in Th17-driving conditions, but a decreasing trend was observed in the presence of EKC in Th1-driving conditions (Figure 2(b)).

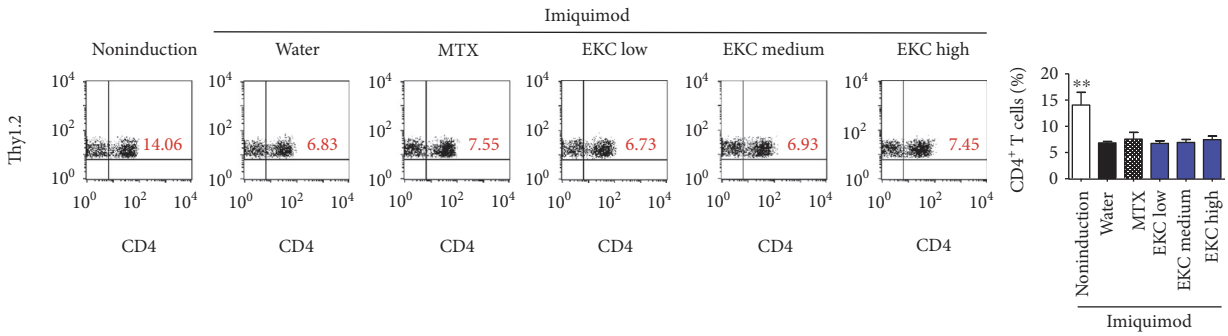
3.2. EKC Prevents Epidermal Hyperplasia and Infiltration of Inflammatory Cells in IMQ-Induced Psoriasis. In the pathogenesis of psoriasis, the immune response of Th17 cells is recognized as a critical factor. To investigate whether there was a beneficial effect on psoriasis as well, EKC was orally administered to mouse models. EKC was administered daily for 3 weeks at three escalating doses (20, 100, and 200 mg/kg). After 21 days, the animals were challenged topically on the back skin with IMQ according to the schedule summarized in Figure 1(a). The phenotype of psoriasis (back thickness, redness, and scaling) was observed throughout the 7-day period of IMQ application. Mice that were orally EKC displayed reduced thickness at day 5, less culminated redness at day 3, and sparser scales (data not shown). The total scores for all groups in the experiment are depicted in Figure 1(b). Significant difference in the disease severity was observed in EKC and MTX groups compared with IMQ + water group (Figure 1(b) and Table 1). The body weight of the mice that received 20, 100, and 200 mg/kg EKC did not differ significantly from that of the noninduced control group (Figure 1(c)). Similar to the clinical score, the histological analyses of skin samples at day 7 of IMQ application revealed that EKC treatment alleviated acanthosis and infiltration of inflammatory cells in a dose-dependent manner. MTX decreased this psoriatic symptoms (Figures 1(d) and 1(e)). Thus, MTX treatment and EKC administration ameliorated dermatitis in IMQ-induced skin in both clinical and pathological measures.

3.3. IMQ-Induced Splenomegaly Is Slightly Decreased by EKC Treatment, and the Population of T Cells in the Spleen Is Not Affected by EKC. IMQ treatment resulted in a significant enlargement of the spleen (Figure 3(a)), and the number of cells increased in dermatitis-induced groups compared to that in the noninduced control (data not shown). The administration of low and medium concentrations of EKC and MTX slightly suppressed the observed increase (Figure 3(a)). In addition, the cellular composition of the spleen was determined by flow cytometry. IMQ treatment induced a decrease in the percentage of $CD4^+$ and $CD8^+$ T cells. However, no obvious differences were observed in the EKC treatment groups compared to those reported with IMQ. However, $CD8^+$ T cells were slightly restored by MTX application (Figures 3(b) and 3(c)). The percentage of $\gamma\delta$ T cells somewhat increased in IMQ-treated mice, while administration of EKC and MTX did not change this percentage (Figure 3(d)). In contrast to that of T cells, the percentage of B cells, dendritic cells, and macrophages significantly increased in mice after the topical treatment with IMQ. In particular, the IMQ-induced increase in dendritic cell population decreased when EKC was administered at medium and high concentrations. On the contrary, the elevated population of macrophage was reduced by MTX, whereas the effect of EKC was insignificant (Figures 3(e), 3(f), and 3(g)). Overall, IMQ caused systemic effects on the cellular composition of the spleen. EKC was likely to regulate the population of dendritic cells altered by IMQ-induced psoriasis, with a tendency in regulating that of macrophages but not that of T cells and B cells.

3.4. EKC Inhibits Th17 Cell Differentiation in the Lymphoid Organs of the Psoriatic Model. To determine the functionality of the spleen cells, splenocytes were activated ex vivo by anti-CD3 for 2 days and Th17-associated factors were analyzed. First, the supernatant was collected and cytokine levels were detected. IMQ treatment remarkably enhanced the production of IL-17A and TNF-α, which is the signature of Th17 cytokines; however, administration of medium and high concentrations of EKC repressed this increase on days 1 and 2 (Figures 4(a) and 4(b)). On day 2, secretion of Th1 cytokines, such as IL-12p40 and IFN-γ, was higher than that in the vehicle cream application group (Figures 4(c) and 4(d)), whereas EKC significantly suppressed the release of IL-12p40 (day 1) and IFN-γ (day 2) (Figures 4(c) and 4(d)). The intake of the positive control of (MTX) or EKC decreased the production



(a)



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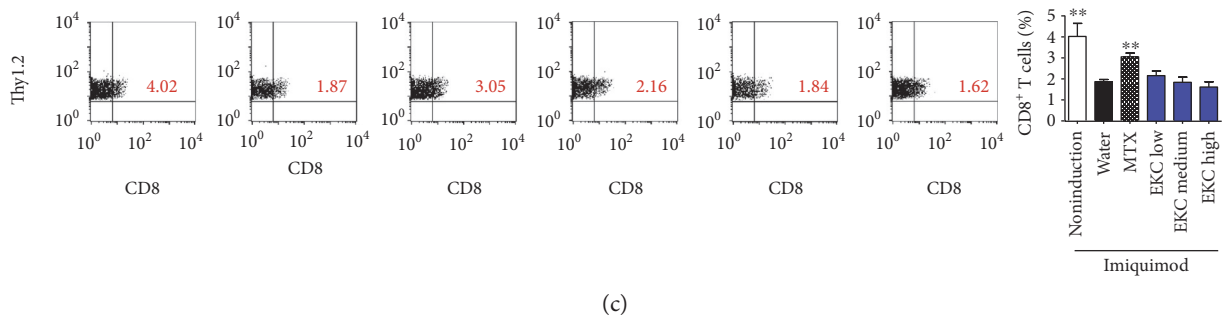

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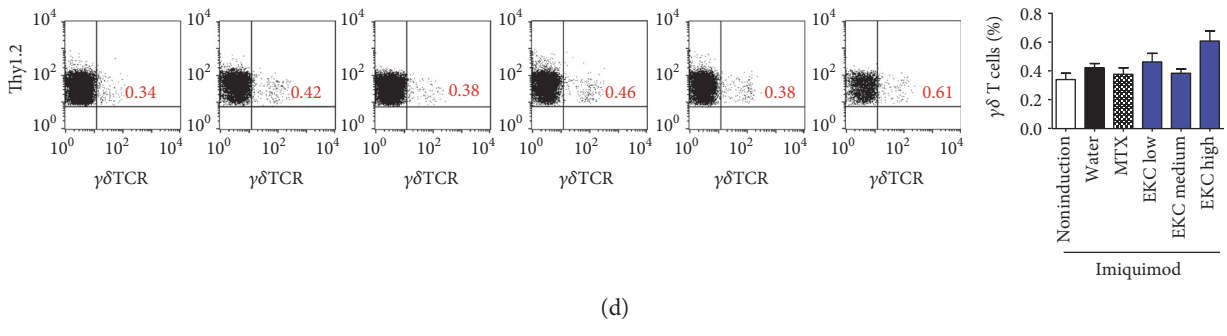


(d)

Figure 3: Continued.

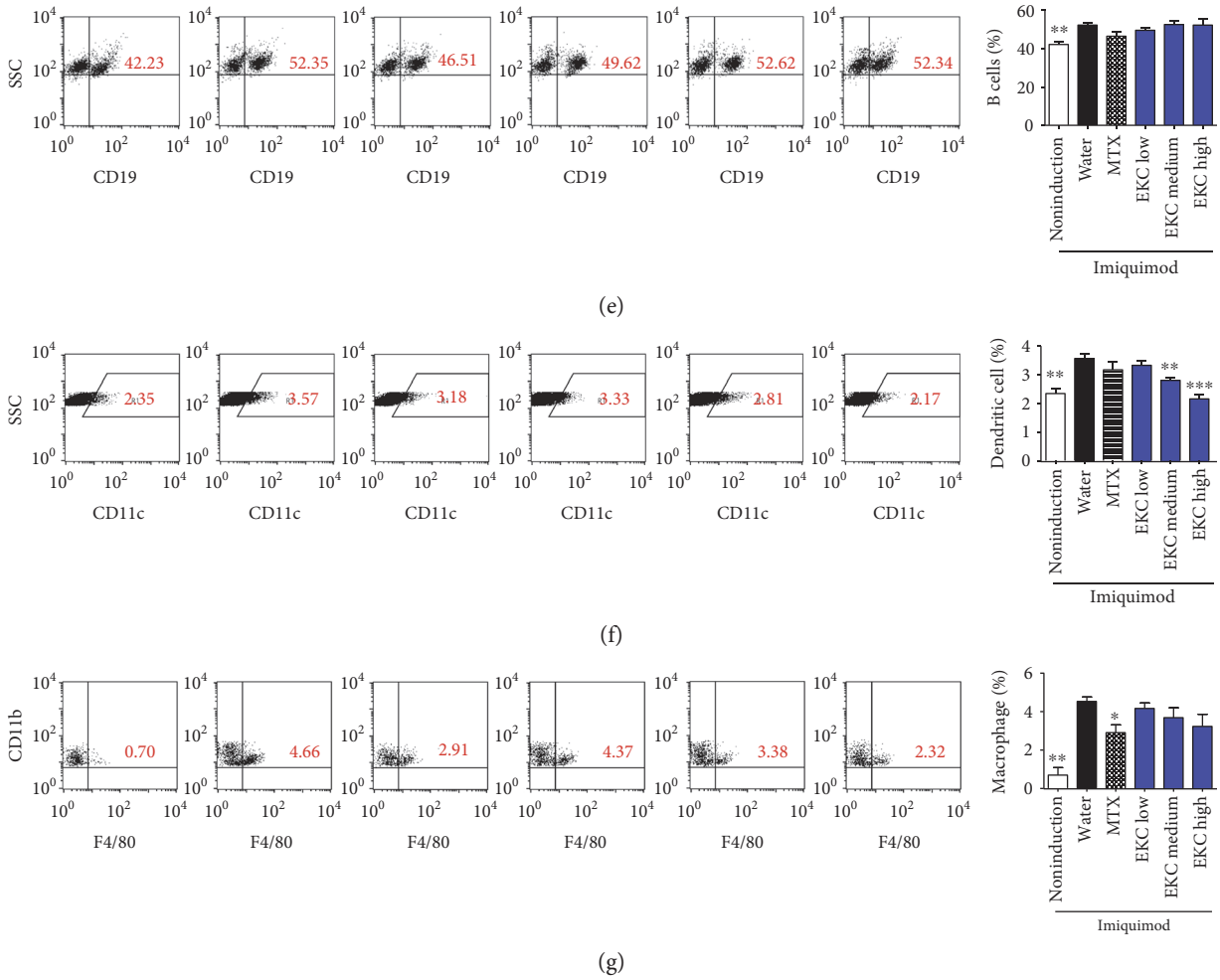


FIGURE 3: Effects of EKC on the spleen and analysis of cellular composition. (a) Photographs of the spleen on day 28. Each spleen was measured, and the data presented are the median ($n = 5$). (b–g) Mice were treated with IMQ or vehicle cream for 7 days consecutively and sacrificed. Splenocytes were analyzed for the percentage of T cells ((b) $Thy1.2^+CD4^+$; (c) $Thy1.2^+CD8^+$; (d) $Th1.2^+\gamma\delta$ TCR^+; (e) B cells [$CD19^+$]; (f) dendritic cells [$CD11c^+$]; and (g) macrophages [$F4/80^+CD11b^+$]) by flow cytometry. Numbers indicate the mean percentage of cells present within a quadrant or gate ($n = 5$ mice/group). Graphs are presented as the mean ± SD ($n = 5$). $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared with imiquimod + water group.

of the two Th17 (IL-17A and TNF-α) and two Th1 (IL-12p40 and IFN-γ) cytokines (Figures 4(a), 4(b), 4(c), and 4(d)). To investigate the alteration of molecular levels, total RNA was extracted from anti-CD3-stimulated splenocytes and the gene levels were analyzed using real-time PCR. Similar to the results reported for protein secretion, an increase in mRNA levels of genes related to Th17 and Th1 responses in psoriasis IL-17A, IL-22, IL-23p19, RORγt, and IL-12p40 was observed in IMQ-treated group (Figure 4(e)). At three analyzed concentrations of EKC and MTX groups, the expression levels of the Th17- and Th1-related cytokine genes were efficiently downregulated (Figure 4(e)). Moreover, the gene level of a Th17-specific transcription factor, RORγt, was diminished by EKC administration (Figure 4(e)).

It is known that a lymphoid organ near the local inflammatory sites generally participates in antigen presentation, lymphocyte differentiation, and proliferation, to accomplish the rapid and effective elimination of antigen. For these reasons, the effects of EKC were examined on axillary lymph nodes and brachial lymph nodes during IMQ-induced skin inflammation.

In the axillary lymph node, the increase in inflammatory cytokines, especially TNF-α and IFN-γ, was suppressed by oral administration of EKC (Figure 5(a)). The expression levels of Th17-associated genes were significantly inhibited by EKC administration (Figure 5(b)). After EKC treatment, the production of IL-17A, TNF-α, and IFN-γ in brachial lymph node cells was also lower than that in the IMQ-only treatment group (Figure 5(c)). As expected, IL-17A, IL-22, and RORγt levels were also depressed by EKC (Figure 5(d)). Thus, these results showed that EKC negatively regulated Th17 differentiation in the IMQ-induced psoriasis model.

3.5. EKC Lowers Elevated Inflammatory Cytokines in Skin Lesion and Alleviates Systemic Immune Activation. To clarify whether EKC effectively suppressed the function of Th17 cell in skin lesion, similar to the effects observed in lymphoid

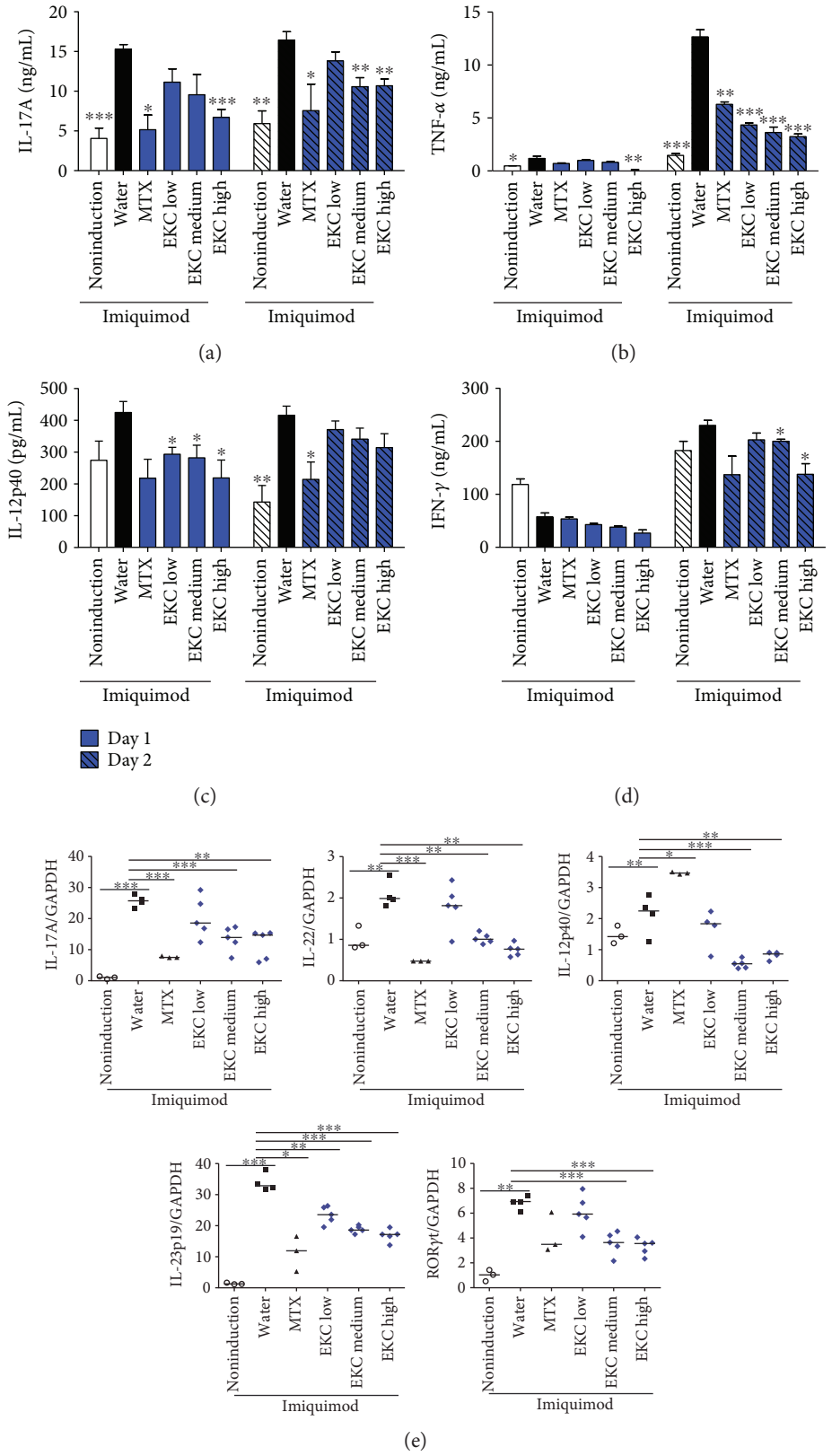
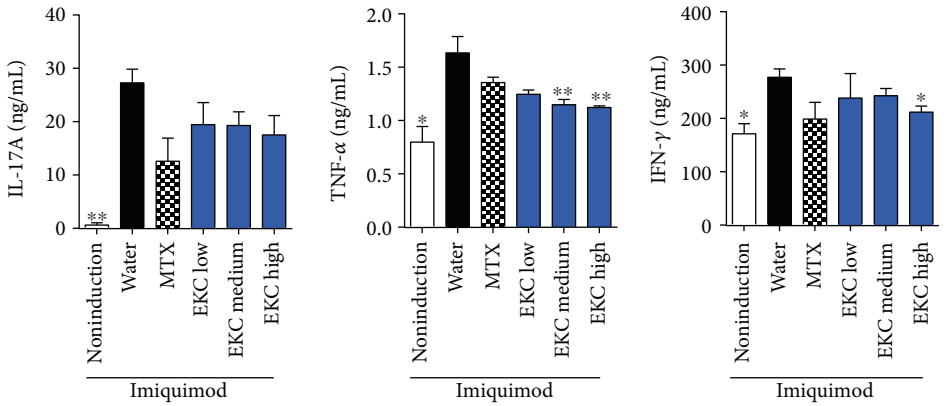
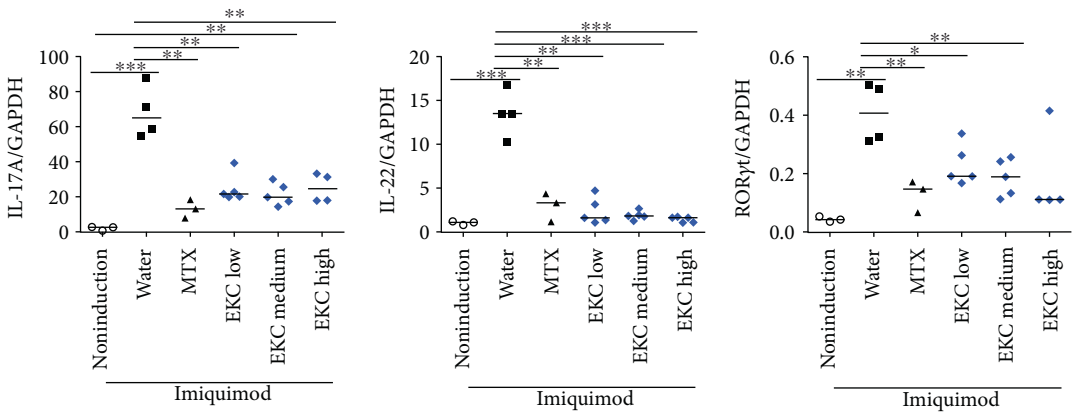


Figure 4: Effects of oral administration of EKC in splenocytes. (a–d) Spleen cells were purified from mice on day 28, stimulated with anti-CD3 Ab for 24 h and 48 h, and the cytokine levels were measured by ELISA. Values represent the mean ± SD. (e) Spleen cells were purified from mice on day 28 and stimulated with anti-CD3 Ab for 2 days. Total mRNA was extracted, and the levels of IL-17A, IL-22, IL-23p19, IL-12p40, and RORγt were evaluated by real-time PCR. Values represent the median. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared with the imiquimod + water group.



(a)



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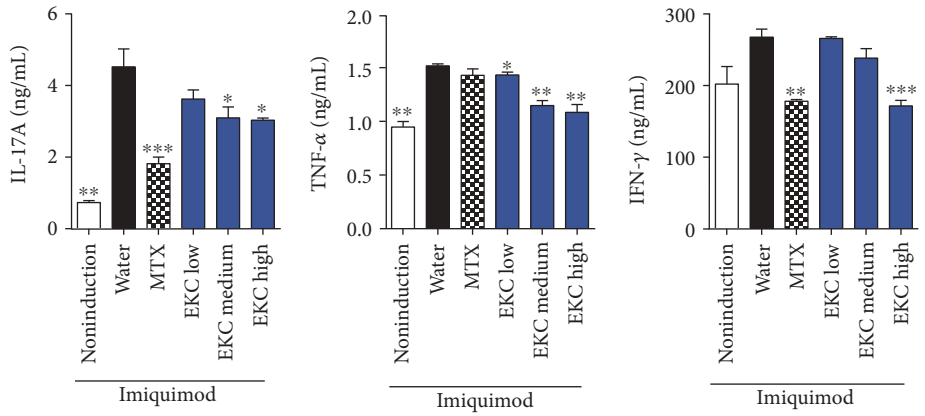

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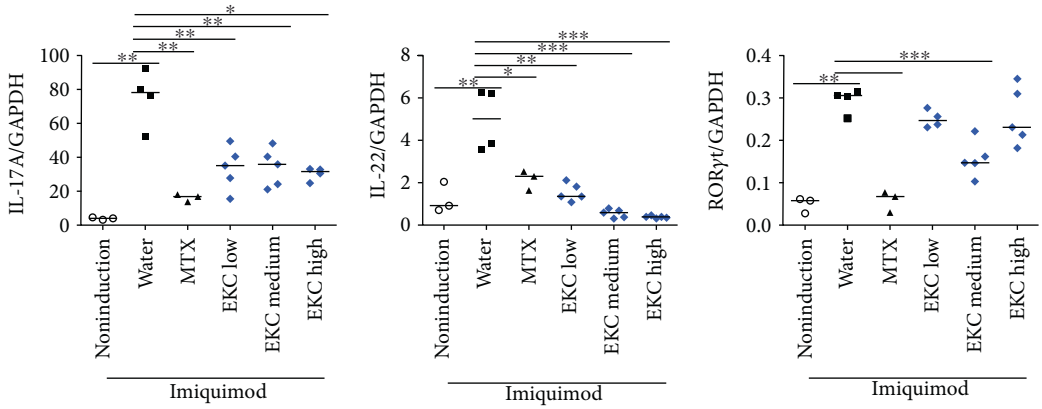

(d)

Figure 5: Effects of oral administration of EKC in axillary lymph nodes and brachial lymph nodes of psoriasis-induced mice. (a) At the end of the experimental day, mice were sacrificed and the axillary lymph node was isolated. The axillary lymph node cells were stimulated with anti-CD3 Ab for 2 days, and the supernatant was harvested. The cytokine levels were measured by ELISA. Values represent the mean ± SD. (b) The axillary lymph node cells were stimulated with plate-bound anti-CD3 Ab for 2 days. Total mRNA was extracted, and the levels of IL-17A, IL-22, and RORγt were measured by real-time PCR. Values represent the median. (c) At the end of the experimental day, mice were sacrificed and the axillary lymph node was isolated. The axillary lymph node cells were stimulated with anti-CD3 Ab for 2 days, and the supernatant was harvested. Cytokine levels were measured by ELISA. (d) The brachial lymph node cells were stimulated with plate-bound anti-CD3 Ab for 2 days. Total mRNA was extracted, and the levels of IL-17A, IL-22, and RORγt were measured by real-time PCR. Values represent the median. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared with the imiquimod + water group.

organs, Th17-related factors were analyzed in IMQ-treated skin. Compared with the noninduced control group, the production of IL-17A and IL-12p40 was exceptionally increased in skin samples from IMQ-treated mice. In contrast, IL-17A was significantly decreased in MTX and EKC groups. The high concentration of EKC group displayed lower IFN-γ and IL-12p40 production than the IMQ + water group did (Figure 6(a)). Additionally, the cytokine genes supporting Th17 and Th1 differentiation, IL-23p19 and IL-12p40, were significantly diminished, and the Th17 signature transcription factor, RORγt, was downregulated by EKC (Figure 6(b)).

Increased levels of IL-17A in the skin induce phosphorylation of IκB in keratinocytes, which activates NF-κB signaling. Therefore, the expression levels of p-IκB-α were evaluated in a Western blot of skin tissue. MTX treatment decreased expression of p-IκB. In the EKC-treated group, the phosphorylation of IκB was impeded in a dose-dependent manner (Figure 6(c)).

In addition, serum cytokine levels were detected by ELISA. IMQ induced elevation of IL-17A and IL-12p40, whereas MTX and EKC inhibited these cytokine secretions in a dose-dependent manner (Figure 6(d)). As an expanded population of B cells was observed in the IMQ-treated group (Figure 3(e)), the IgG level in the serum was measured. As shown in Figure 6(e), total IgG1 levels in IMQ-treated mice were higher than those in the noninduced control group ($p > 0.05$), whereas MTX application and EKC administration significantly decreased the IgG1 levels in a dose-dependent manner.

3.6. EKC Regulates the Activity of Dendritic Cells and Does Not Affect Keratinocyte. To verify whether EKC affects T cell activation and differentiation only at the onset of psoriasis, the effect of EKC on dendritic cells and keratinocytes was investigated.

In IMQ-induced psoriasis model, dendritic cells are stimulated by TLR7/8 ligand (IMQ) and produce IL-12 and IL-23, which lead to accelerate Th1 and T17 cell differentiation, respectively [40]. Thus, JAWS II cells, murine dendritic cell lines, were stimulated by IMQ with or without EKC. The imiquimod had no cytotoxicity and was founded to be suitable for cell treatment (Figure 7(a)). The IMQ-stimulated cells significantly increased secretion of IL-12p40 and IL-23p19. The levels of these cytokines decreased in the EKC-treated cells (Figure 7(b)).

Additionally, aberrant activation and proliferation of the keratinocytes were caused by Th17 signature cytokines in psoriatic skin [41]. Under these conditions, keratinocytes produced cytokines and chemokines, such as IL-8, IL-36γ, and CCL20, which recruit inflammatory cells and exacerbate skin inflammation [42–44]. EKC did not affect the viability of the human keratinocyte HaCaT cell line (Figure 7(c)). Cells were challenged by IL-17A, with or without EKC, and total RNA was extracted. Although the levels of IL-8, IL-36γ, and CCL20 were increased by IL-17A (no significant changes except for IL-36γ), they were not modulated by EKC (Figure 7(d)).

Consequently, it was concluded that EKC affected the IMQ-induced activation of dendritic cells but not Th17-stimulated keratinocytes.

4. Discussion

E. kansui, an herb of the family of Euphorbiaceae, has been used to treat various diseases related to excessive inflammation [45, 46]. Previous studies have identified that *E. kansui* decreased the differentiation of Th17 cells and relieved

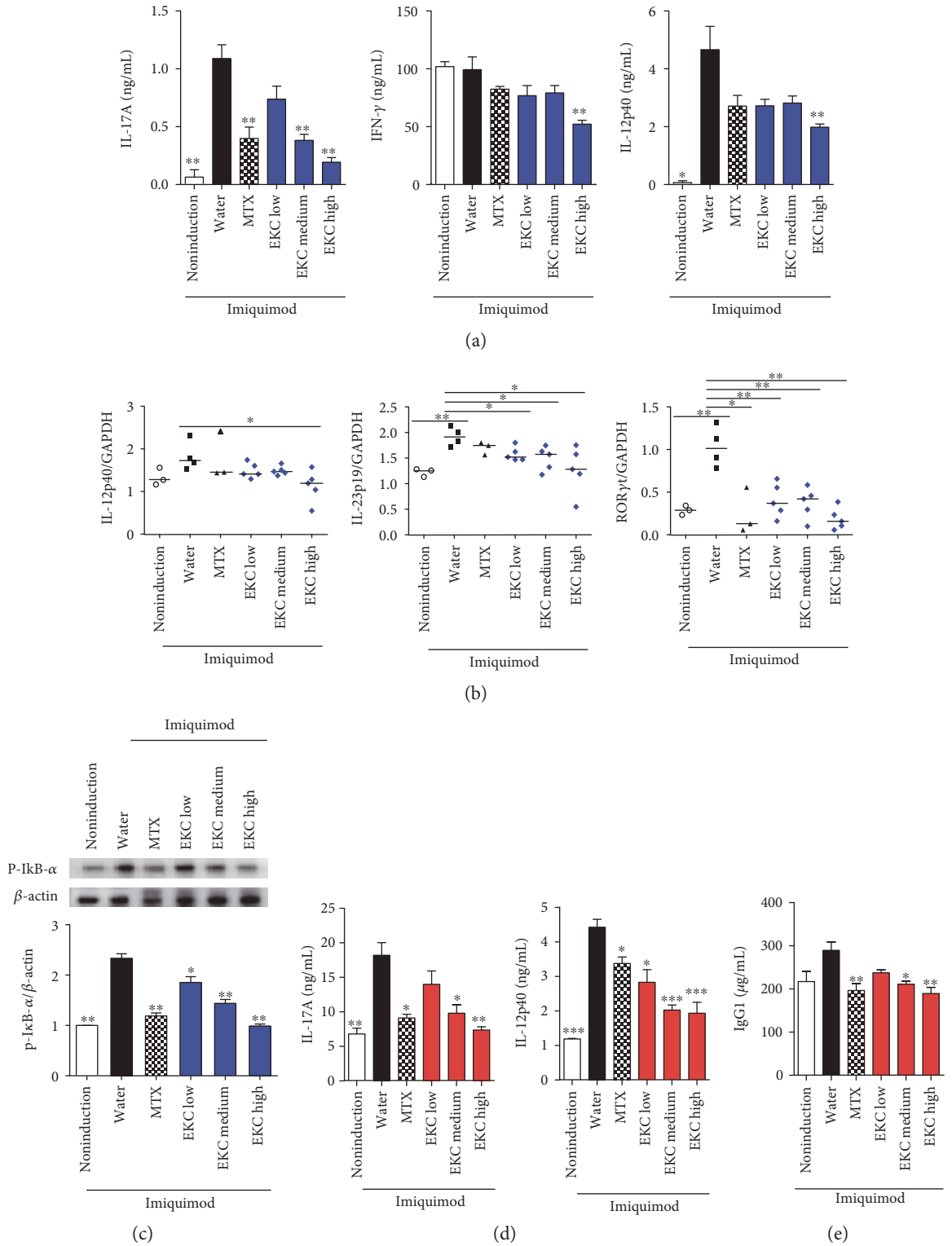

FIGURE 6: Inhibition of Th17 associated factors in inflammatory skin lesions and serum by EKC. (a) Mice were sacrificed, and protein was extracted from the back skin of each mouse. Cytokine levels were measured by ELISA. Values represent the mean ± SD. (b) Total mRNA was extracted from the skin using TRIzol, and the levels of IL-23p19 and IL-12p40 were measured by real-time PCR. Values represent the median. (c) The skin samples were prepared by homogenization with T-PER protein extraction buffer. p-IκB-α expression levels were determined by Western blot analysis. The intensity of β-actin staining in each sample was used as a loading control. (d) The concentration of IL-17A and IL-12p40 collected in serum on day 28 was determined by ELISA. Values represent the mean ± SD. (e) Concentration of IgG1 in the collected serum was measured by ELISA. Values represent the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared with the IMQ + water group.

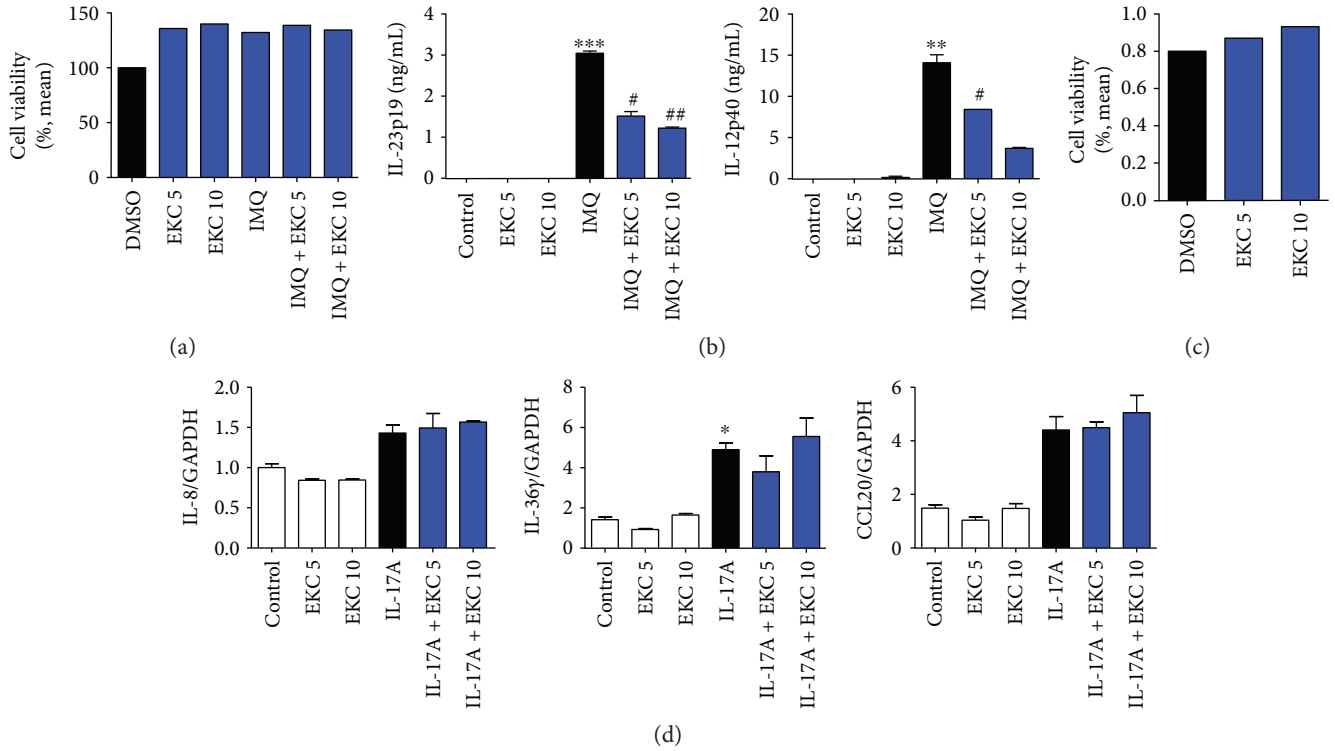

Figure 7: Reduced production of IL-12 and IL-23 in IMQ-stimulated dendritic cells by EKC. (a) JAWSII cells were plated at 2×10^5 cells/well in a 96-well plate. EKC (5 μg/mL and 10 μg/mL) dissolved in DMSO (final concentration of 0.05%) was added to the cells, which were then cultured for 24 h with or without IMQ (1 μg/mL). Cytotoxicity was analyzed by MTT assay. (b) JAWSII cells (1×10^6) were plated in a 24-well multiplate, cultivated with EKC (5 μg/mL and 10 μg/mL), and treated with or without IMQ (1 μg/mL). After 18 h, total RNA was isolated from each sample and reverse-transcribed cDNA was analyzed by quantitative real-time PCR (qPCR). Values represent the mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control (0.05% DMSO); $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared with the IMQ group. (c) HaCaT cells were plated at 2×10^5 cells/well in a 96-well plate. EKC (5 μg/mL and 10 μg/mL) dissolved in DMSO (final concentration of 0.05%) was added, and the cells were cultured for 24 h. Cytotoxicity was analyzed by MTT assay. (d) HaCaT cells (1×10^6) were plated in a 24-well multiplate, cultivated with EKC (5 μg/mL and 10 μg/mL), and treated with or without human IL-17A (100 ng/mL). After 18 h, total RNA was isolated from each sample and reverse-transcribed cDNA was analyzed by quantitative real-time PCR (qPCR). Values represent the mean ± SD. $^{*}p < 0.05$ compared with control (0.05% DMSO).

inflammatory bowel disease, a Th17-associated autoimmune disease (unpublished data).

In this study, it was investigated whether psoriasis, a known autoimmune disease with pathogenesis focused on Th17 cells, was affected by *E. kansui*. The topical application of imiquimod activated plasmacytoid dendritic cells, triggered downstream Th1 and Th17 cell-mediated adaptive immunities, and resulted in mice with similar lesions to human psoriasis [26]. This quick and cost-effective model furthered the elucidation of pathogenic mechanisms and the evaluation of new treatments for psoriasis [47, 48].

Recent studies have suggested that regulatory B cells, $\gamma\delta$ T cells (dendritic epidermal T cells [DETCs]), and innate immune cells perform an essential role in the pathogenesis of psoriasis and that regulation of these cells was also important [49–52]. However, the IL-17A/IL-23 axis was still emphasized as an important factor in the treatment of psoriasis [15, 26].

Treatment with the methylene chloride fraction of *E. kansui* radix (EKC) reduced IL-17A and IFN-γ significantly in Th17- and Th1-driving conditions. Although the in vitro data indicated that EKC increased TNF-α production, the clinical data from patients with psoriasis demonstrated a spectacular improvement with an IL-17 blockade rather than increased TNF [53]. Furthermore, recent studies have reported that TNF-alpha inhibitors therapy exacerbated psoriasis or induced new onset of psoriatic skin lesions in some cases [54, 55]. Thus, the curtailed symptoms in the psoriasis model may be attributed to the effective reduction of IL-17 by EKC; IL-17 is considered a major factor in the treatment of psoriasis.

The severity of inflammation (erythema, scaling, and thickness) was alleviated by EKC administration in a dose-dependent manner. The histological analysis demonstrated that acanthosis in the EKC group was more improved than that in the MTX group whereas the infiltration of inflammatory cells in the MTX group was similar to that observed after the highest dose of EKC administration. The relief of symptoms was associated with the suppression of Th17 and Th1 differentiation.

In the spleen, similar to a previous study, the percentage of T cell population ($CD4^+$ and $CD8^+$ T cells) was decreased by IMQ [26]. However, when considering the total number of splenocytes, the number of T cells did not change (data

not shown). In addition, elevated percentages of splenic $CD4^+$ IL-17A$^+$ IFN-γ^- cells and $CD8^+$ IFN-γ^+ cells were observed in IMQ-treated mice. In contrast, $CD4^+$ IFN-γ^+ double positive cells were almost absent [26]. It was estimated that the reduction of IL-17A, IL-22, TNF-α, and ROR-γt compared to that in the IMQ-treated group occurred through the inhibition of Th17 cells ($CD4^+$ IL-17A$^+$ IFN^- cells) by EKC and that the reduction of IFN occurred through the inhibition of $CD8^+$ IFN^+ cells. The $\gamma\delta$ T cells in the skin-draining lymph node of psoriasis produce approximately 10 times more IL-17A and IL-22 than CD4 T cells [48, 56]. Also, these cells express the Th17-specific transcription factor, RORγt [57]. The reduction of cytokines and RORγt expression in the draining lymph node was considered to be related to the effect of EKC on $\gamma\delta$ T cells. However, the effect of EKC on $\gamma\delta$ T cells requires further study.

IL-23 and IL-12 are cytokines with critical roles in the differentiation and growth of Th17 and Th1 in psoriasis [58, 59]. IMQ increases the activation and population of dendritic cells and macrophages [60, 61]. EKC not only reduced dendritic cell populations in the spleen but also decreased IL-12 and IL-23 expression in the spleen and skin. Moreover, the in vitro experiments have shown that the activation of dendritic cells was suppressed by EKC. These results suggested that EKC inhibited dendritic cell activity independently of Th17 inhibition.

IMQ treatment was shown to increase serum IL-17A as well as skin inflammation, which led to systemic immune activation [62]. When EKC was administered, the level of IL-17A and IL-12p40 in both skin tissue and blood was decreased. Th17 cells induce expansion of cognate B cells, immunoglobulin class switching, and the increase in the number of B cells. Moreover, Th1 cells have a character promoting higher IgG2 whereas Th17 cells support higher IgG1 levels [63]. As the B cell population was increased in the spleen, blood IgG1 was investigated and the IgG level was found to be decreased in a dose-dependent manner by EKC treatment. In contrast, the number of B cells in splenocytes was not diminished by EKC administration. According to these results, the decrease in IgG1 seems to be related to the prevention of Th17-mediated class switching by inhibiting Th17 cells rather than directly involved in B cells.

In order to determine if the suppression of IκB phosphorylation in skin resulted from a decrease in IL-17A or EKC affected keratinocyte activation, keratinocyte cell lines were activated by IL-17A, with or without EKC. The results elucidated that EKC did not affect keratinocytes. These results have drawn that suppression of EKC-induced Th17 resulted in relief of psoriasis through interruption of the phosphorylation of IκB in skin.

In conclusion, this study revealed that reduced inflammation in the EKC-treated group was caused by the inhibition of Th17 but not keratinocytes. EKC suppressed not only the differentiation of Th17 but also the activation of dendritic cells. EKC is expected to be suitable for the treatment of patients with early and intermediate stages of psoriasis. Additionally, the effect of single compounds extracted from EKC should also be assessed as potential therapies for psoriasis.

Conflicts of Interest

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Glycomacropeptide Attenuates Inflammation, Pruritus, and Th2 Response Associated with Atopic Dermatitis Induced by 2,4-Dinitrochlorobenzene in Rat

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Atopic dermatitis (AD) is one of the most common skin diseases, whose incidence is increasing in industrialized countries. The epicutaneous application of a hapten, such as 2,4-dinitrochlorobenzene (DNCB), evokes an experimental murine AD-like reaction. Glycomacropeptide (GMP) is a dairy bioactive peptide derived from hydrolysis of κ-casein by chymosin action. It has anti-inflammatory, prebiotic, and immunomodulatory effects. The present study was aimed to investigate the effect of GMP administration on DNCB-induced AD in rats. The severity of inflammatory process, pruritus, production of cytokines, and total immunoglobulin E (IgE) content were measured, and the histopathological features were analyzed. GMP reduced the intensity of inflammatory process and edema of DNCB-induced dermatitis, with a significant decrease in eosinophils recruitment and mast cells hyperplasia. In addition GMP suppressed the serum levels of total IgE and IL-4, IL-5, and IL-13 expression in AD-lesions. Besides, the levels of IL-10 were significantly increased. Remarkably, GMP administration before AD-induction abolished pruritus in dermatitis-like reactions in the rats. Taken together, these results indicate that GMP has an inhibitory effect on AD by downregulating Th2 dominant immune response, suggesting GMP as a potential effective alternative therapy for the prevention and management of AD.

1. Introduction

Atopic dermatitis (AD) is a chronic and relapsing skin disease that is characterized by skin inflammation and pruritus. It is one of the most common skin diseases, affecting about 15–30% of children and 2–10% of adults worldwide, with an increasing prevalence rate in industrialized countries [1]. Although it is not a life-threatening disease, AD has a significant impact on patients' quality of life and on economy of health services. Besides, AD is often the first manifestation of allergic disease, as most patients with AD will further develop another atopic disorder, such as allergic rhinitis or asthma [2].

The precise etiology of AD is not yet determined, but one possibility is a deregulation of adaptive and innate immune response raised by environmental and genetic factors [3]. In AD patients, genetic conditions, external stimuli, or scratching episodes disrupt barrier skin that facilitates allergen penetration and activation of keratinocytes to produce thymic stromal lymphopoietin that triggers dendritic cells to induce a Th2-cell mediated response [4]. In the acute phase of disease, infiltrated CD4+ T cells in skin lesions predominantly secrete IL-4, IL-5, and IL-13. These Th2 cytokines orchestrate a skin inflammation characterized by eosinophil recruitment and mast cells hyperplasia. Besides, IL-4 induces immunoglobulin (Ig)E isotype switching in B

cells, increasing serum IgE levels which is associated with the pathogenesis of the disease [5]. In the chronic phase of the AD, Th1 cells appear and secrete interferon-gamma (IFN-γ) that is mainly associated with epidermal hyperplasia [1]. Therefore, the imbalance in the rate of Th1 and Th2 cells, or in Treg cells that maintain immune homeostasis locally, has special consideration in AD [6].

Animal models for human diseases are very important to analyze the mechanisms involved in the onset and development of pathologies and to establish treatment strategies for the disease [7]. Mice model has been widely used for the detailed study of AD and for the development of rapid trials of possible therapies for the disease [8]. Dermatitis model induced by skin repeated application of haptens causes histopathological, immunological, and clinical features similar to human AD [7, 9]. Although most of AD-models by hapten repeated application are developed in mice, thickness of the cornea layer and chemical permeability of skin in mouse are greater than rat and human, so rat skin suffers AD-like injuries less severe than mouse and more similar to human [10–12].

Many kinds of bioactive peptides that might prevent lifestyle-related diseases are released from food proteins after enzymatic digestion. Glycomacropeptide (GMP) is an active biopeptide derived from milk κ-casein that is released to the whey during cheese-making process by the action of chymosin [13]. It is composed of 64 amino acids extensively glycosylated with units of N-acetylneuraminic (sialic) acid that confers several nutraceutical and biological properties [14]. GMP has an excellent safe record and is not immunogenic [15]. As component of the whey, it is included in infant food formulas as a source of amino acids; besides, it is added to nutritional formulas for phenylketonuria patients due to the lack of phenylalanine [16]. Recently, GMP has deserved much interest for its proposed prebiotic, anti-inflammatory, and immunoregulatory properties. It has anti-inflammatory activity in rat models of colitis and ileitis induced by trinitrobenzene-sulphonic acid [17–19] and prevents extensive damage in colon in a model of colonic damage induced by dimethyl hydrazine [20]. Both GMP effects are mediated by the regulation of lymphocytes differentiation. Recent studies carried out in our laboratory show the prophylactic effect of orally administered GMP on the development of immune response associated with allergic sensitization, protecting animals from the severity of urticarial reaction and systemic anaphylaxis induced by allergens. This effect is related to changes in gut microbiota composition, upregulation of TGF-β and downregulation of IL-13 production by splenocytes, reduction in allergen-specific IgE production, and mast cells inhibition [21, 22]. GMP has also immunoregulatory activity in allergic asthma models, as it effectively suppresses blood and lung eosinophilia, goblet cell hyperplasia, and collagen deposition in airways. Beneficial effect of GMP in asthma is associated with downregulation of IL-5 and IL-13 and upregulation of IL-10 expression in asthmatic lung tissue [23].

The aim of this study was to evaluate whether oral GMP administration, previously or once pathology was established, can influence the development of AD. Firstly, we characterized a rat model of dermatitis by systemic sensitization followed by hapten repeated application. We further examined the effect of GMP in skin inflammation, pruritus, as well as Th2-immune response associated with AD to determine its potential prophylactic and therapeutic activity.

2. Material and Methods

2.1. Animals. Male Wistar rats (150–180 g) obtained from the Laboratory Animal Service of the Autonomous University of Aguascalientes were used throughout the study. Rats were housed under controlled conditions of temperature (22–24°C) and illumination (12 h light cycle) and fed with Rodent Laboratory Chow 5001 (Purina, Mexico City, Mexico) and tap water ad libitum. All experiments were carried out with strict adherence to ethical guidelines approved by the Institutional Normative Welfare Standards.

2.2. Protocol for Induction of Experimental Atopic Dermatitis. Ear cutaneous reaction was induced by repeated applications of 2,4-dinitrochlorobenzene (DNCB; Sigma, St. Louis, MO, USA) after systemic sensitization, as previously described [24]. Briefly, animals were sensitized at day 0, with an intramuscular injection of 1 mg of dinitrophenyl-bovine serum albumin (DNP-BSA) precipitated in 7.8 mg of aluminum hydroxide gel ($Al(OH)_3$; Thermo Scientific, Waltham, MA, USA) in 1 mL of saline solution. Simultaneously, and as an adjuvant, 0.5 mL of *Bordetella pertussis* vaccine (Zuvirac, Mexico City, Mexico) containing $10–15 \times 10^9$ heat-killed bacilli/mL was injected subcutaneously. On days 14, 16, 18, 20, 22, and 36, animals were resensitized with a topical application of 60 μL of 1.5% w/v DNCB prepared in acetone-olive oil (A-OO) solution (4 : 1) to both sides of the right ear lobe of the rats. Control group was only injected with adjuvants and topically applied with A-OO solution (Figure 1).

2.3. Experimental Design. For characterization of dermatitis model, rats were randomly assigned to two different groups (5 rats per group): control and DNCB sensitized. For analysis of GMP effect, rats were randomly assigned to five different groups (8 rats per group): control, not sensitized and water administered before AD-induction; DNCB-P, DNCB sensitized and water administered before AD-induction; GMP-P, DNCB sensitized and GMP administered before AD-induction; DNCB-T, DNCB sensitized and water administered after AD-induction; and GMP-T, DNCB sensitized and GMP administered after AD-induction. GMP (Lacprodan® cGMP-10; a gift from Arla Foods Amba, Viby, Denmark) was orally administered to animals at 500 mg/kg/day dissolved in tap water. Oral intake of GMP was started from 3 days before sensitization to day 36 as prophylaxis (GMP-P) and from day 23 to day 36 when employed in a therapeutic manner, that is, once AD was established (GMP-T). Control, DNCB-P, and DNCB-T groups were administered orally with tap water during corresponding times (Figure 1). An esophageal catheter was used to deliver GMP solution or water. All animals were sacrificed with an overdose of ether at day 37, and blood and ear samples were obtained.

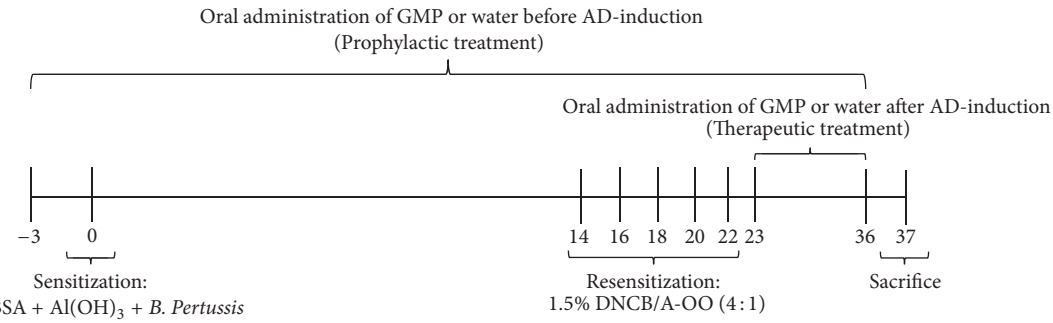

Figure 1: Schematic diagram of experimental dermatitis induction protocol and GMP administration. Rats were sensitized on day 0 with injection of DNP-BSA mixed with $Al(OH)_3$ gel and simultaneously with *B. pertussis* vaccine. Animals were resensitized with topical application of DNCB in A-OO on days 14, 16, 18, 20, 22, and 36. Control group was injected with the adjuvants but without DNP-BSA and applied topically with A-OO mixture. GMP or water was administered, daily and orally, from 3 days before AD-induction or from day 23 after AD-induction, and until day 36 to analyze the prophylactic or therapeutic effect, respectively. Animals were sacrificed at day 37.

2.4. Evaluation of Ear Cutaneous Inflammatory Reaction and Edema. Cutaneous reaction was evaluated by ear swelling induced by the challenge with DNCB. Ear thickness was measured using a dial thickness gauge (Milomex, Ltd., Bedfordshire, UK) at 0, 1, 6, and 24 h after DNCB application on day 36. Ear swelling was calculated based in the increase of ear thickness as RT-LT, where RT and LT represent the thickness of the right and left ear, respectively, at the corresponding time point. At day 37 animals were sacrificed, the ears were excised from the base, and identical portions of the middle of the ears were removed using a metallic punch. The tissue samples were individually weighted on an analytical balance (Precisa XT220A, Dietikon, Switzerland). Edema was calculated based on the increase of ear weight as RW-LW, where RW and LW represent the weight of the fragment of the right and left ear, respectively.

2.5. Evaluation of Scratching Behavior. The total number of scratching events was counted during 10 minutes immediately after the application of DNCB on days 16, 22, and 36. For that purpose, rats were placed into an acrylic cage divided into eight compartments. Their behavior was recorded using a digital video camera (Samsung HMX-W350, New Jersey, USA). Videos were watched by two observers and the number of scratching events was counted. One scratching event or episode was defined as a series of one or more scratching movements by the hind paw directed toward the application site and ended when the rat either licked its hind paw or placed it back on the floor [25].

2.6. Histological Analysis. Upper portions of the right ears of each rat were fixed in 10% neutral formalin, embedded in paraffin, and sectioned into 5 μm slices. Slices were stained with hematoxylin and eosin for evaluation of eosinophils infiltration and with toluidine blue for evaluation of mast cells number. After microscopic fields were photographed, the numbers of stained eosinophils and mast cells were counted in random areas (40,000 μm^2) with an AxioPlan Carl Zeiss microscope (Oberkochen, Germany) at 400x magnification. Three slides were stained per rat and three fields were examined per slide. Morphometric assessment was performed using AxioVision Rel 4.8 software by two observers who were not aware of the group of rats from which the samples originated.

2.7. Determination of Total IgE. Serum samples prepared from blood obtained on day 37 were stored at −70°C until used to IgE determination. Total IgE level in serum was quantified using a rat IgE ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.8. RNA Purification and Semiquantitative or Real-Time Quantitative PCR (qRT-PCR). Total RNA was isolated from the lower ear tissue using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Purified RNA was quantified with a NanoDrop 2000 Spectrophotometer (Thermo Scientific) with the A260/280 ratio. Only samples with ratio >1.8 were employed for cDNA synthesis. Reverse transcriptions of 2 μg of RNA were performed with the RETROscript® Reverse Transcription kit (Thermo Scientific). Semiquantitative PCR was performed with 1 μL of 1 : 10 diluted cDNA product, 5 μL of PCR Master Mix 2x (Thermo Scientific), and 1 μL of forward and reverse primers at 5 μM each (listed on Table 1); all reactions were completed with nuclease-free water to 10 μL. PCR conditions were as follows: initial denaturing at 95°C for 3 min, with 25, 30, or 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 10 sec, and later for all reactions a final extension of 72°C for 3 min was included. Amplicons were separated in 2% agarose gels containing GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) as recommended by the manufacturer, in TBE 1x (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). Gels were visualized under UV light in a MiniBis Pro documentation system (DNR Bio-Imaging Systems, Jerusalem, ISR). For RT-PCR, 2 μL of diluted cDNA reaction was used as template for the detection of IL-4, IL-5, IL-13, IL-10, and β-actin with the GoTaq® qPCR Master Mix (Promega) in an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). Relative quantification was determined with $\Delta\Delta$Ct method using β-actin as housekeeping gene for normalization.

Table 1: Oligonucleotides for gene expression quantification.

Gene	Oligonucleotides	Accession number
IL-4	Fw: CACCTTGCTGTCACCCTGTT Rv: ACATCTCGGTGCATGGAGTC	NM_201270.1
IL-5	Fw: CAGTGGTGAAAGAGACCTTG Rv: GTATGTCTAGCCCCTGAAAG	NM_021834.1
IL-13	Fw: ATCGAGGAGCTGAGCAACAT Rv: ATCCGAGGCCTTTTGGTTAC	NM_053828.1
IFN-γ	Fw: GCCTAGAAAGTCTGAAGAAC Rv: GAGATAATCTGGCTCTCAAG	NM_138880.2
IL-10	Fw: CACCTTGCTGTCACCCTGTT Rv: ACATCTCGGTGCATGGAGTC	NM_012854.2
β-Actin	Fw: GTCGTACCACTGGCATTGTG Rv: GCTGTGGTGGTGAAGCTGTA	NM_031144.3

2.9. Data Analysis. Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by Student's t-test. Ear thickness data were analyzed by multicomparative Bonferroni test. Significance was set at $p < 0.05$.

3. Results

3.1. Characterization of Dermatitis Evoked by Repeated Challenges with DNCB after Systemic Sensitization. First, rats were systemically sensitized with DNP-BSA and later challenged 6 times by painting the right ear with DNCB/A-OO solution. As shown in Figures 2(a)-2(b), repeated impregnation with DNCB solution caused potent inflammatory changes in the ear skin, such as the thickening of both dermis and epidermis, edema, and the accumulation of eosinophils and mast cells. The number of eosinophils and mast cells in dermis of rats from DNCB group increased by 12.6- and 2.3-fold (Figure 2(c)). The ear thickness, measured as an indicator of skin inflammation [11], increased after each application of DNCB. On day 36, the ear thickness picket at 1 h after DNCB painting and maintained significantly greater than control rats at 6 and 24 h (Figure 2(d)). On day 37, edema in DNCB group was 98-fold higher than that in control rats (Figure 2(e)). Scratching toward the ear receiving DNCB application was observed from day 16. Scratching occurred immediately after the application of DNCB, with its frequency decreasing as time passed, and no scratching was observed at 1 h and thereafter. The scratching events counted for the first 10 min, as shown in Figure 2(f), significantly increased at day 16 and were almost equal at day 22, with a slight decrease at day 36. Total RNA was extracted from the skin lesions excised 24 h after the sixth DNCB challenge and the expression of inflammatory cytokines was examined. As shown in Figure 2(g), the IFN-γ, IL-5, and IL-13 mRNA expression in skin of control rats was very weak, but it was potentiated in DNCB group. Furthermore, although the expression of IL-4 mRNAs in skin was undetectable in control rats, DNCB-treatment induced their expression in dermatitis lesion.

3.2. Oral GMP Administration Diminishes Inflammatory Process in Dermatitis. First we investigated whether oral intake of GMP might modify the development of the inflammatory response associated with dermatitis. So, ear thickness was measured after DNCB-repeated applications. On day 36, before the sixth DNCB application (0 h), DNCB-P and DNCB-T animals reported an increase of 0.15 and 0.23 mm over control animals. But animals administered with GMP reduced in 95.6 and 54.55% the thickness induced by the previous five DNCB applications when used in a prophylactic or therapeutic manner, respectively. One hour after the last DNCB application, ear thickness presented a peak of 0.41 and 0.48 mm in the ears of DNCB-P and DNCB-T animals, which was sustained at 6 h and presented a slight decrease at 24 h. However, when animals were GMP administered before AD-induction the inflammatory process was reduced in 99.4, 93.98, and 85.89% at 1, 6, and 24 h after challenge, and if they received GMP after AD-induction the ear inflammation was diminished in 47.16, 49.41, and 34.06% (Figure 3(a)).

Another way to assess changes in the inflammatory process is to evaluate the ear edema as the increment in ear weight. As shown in Figure 3(b), when animals were repeatedly challenged with DNCB the ear edema was 6.64 (DNCB-P) and 8.05 (DNCB-T) higher than in control animals. However, when animals were GMP administered before AD-induction there was a decrease of 97.03% on ear DNCB-induced edema. Besides, animals that received GMP once dermatitis was established showed a decrease of 39.87% on edema when compared to untreated group (DNCB-T).

3.3. Scratching Behavior Is Inhibited by GMP-Prophylaxis. Pruritus is one of the major symptoms of AD and impacts quality of life of patients in a significant manner [26]. Control animals did not show any scratching event in the right ear during 10 min immediately after the application of A-OO mixture (data not shown). The chronological profile of scratching behavior in DNCB challenged rats, treated or not with GMP, is shown in Figure 4. In DNCB-P and DNCB-T rats the number of scratching events remained almost constant during 10 min after DNCB topical application at days

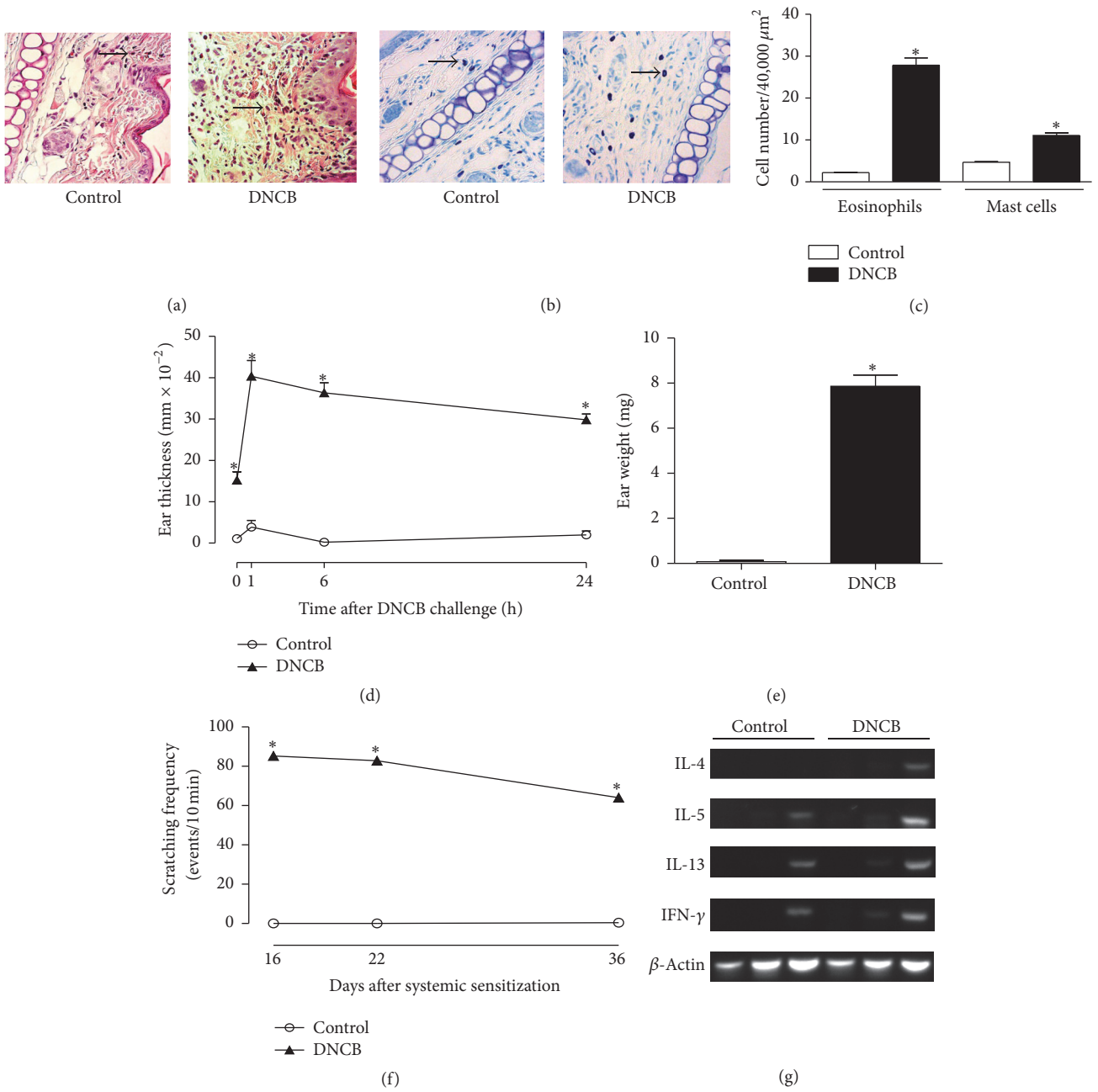

Figure 2: Characteristics of dermatitis-like reaction in rats challenged with DNCB after systemic sensitization. Histopathological features of the ears of control and DNCB challenged rats, 24 h after the sixth challenge, stained with (a) hematoxylin and eosin and (b) toluidine blue. Arrows indicated (a) eosinophils and (b) mast cells. (c) Eosinophils and mast cells were counted in dermis with a microscope at a magnification of 400x. (d) Ear thickness was measured at 0, 1, 6, and 24 h after last DNCB challenge. (e) To measure ear edema, equal areas from ears were punched and weighed 24 h after last challenge. (f) Scratching frequency was measured during the first 10 min after DNCB application and reported at days 16, 22, and 36. (g) Inflammatory cytokine mRNA expression in the skin lesion 24 h after the last DNCB challenge. Values represent mean ± SEM; $N = 5$ rats. $^{*}p < 0.001$ versus control at each time point.

16, 22, and 36, with an average of 36.87 and 41.71 scratching events. Oral GMP administration before AD-induction resulted in a significant and dramatic inhibition of more than 99% in the number of scratching episodes of DNCB-applied animals during the same days, pruritus being almost completely abolished. In contrast, there were no differences in scratching behavior between GMP-T and DNCB-T groups, indicating that GMP has no effect on pruritus when it was administered once dermatitis was established.

3.4. GMP Administration Reduces the Infiltration of Inflammatory Cells into DNCB-Induced Skin Lesions. Cellular changes

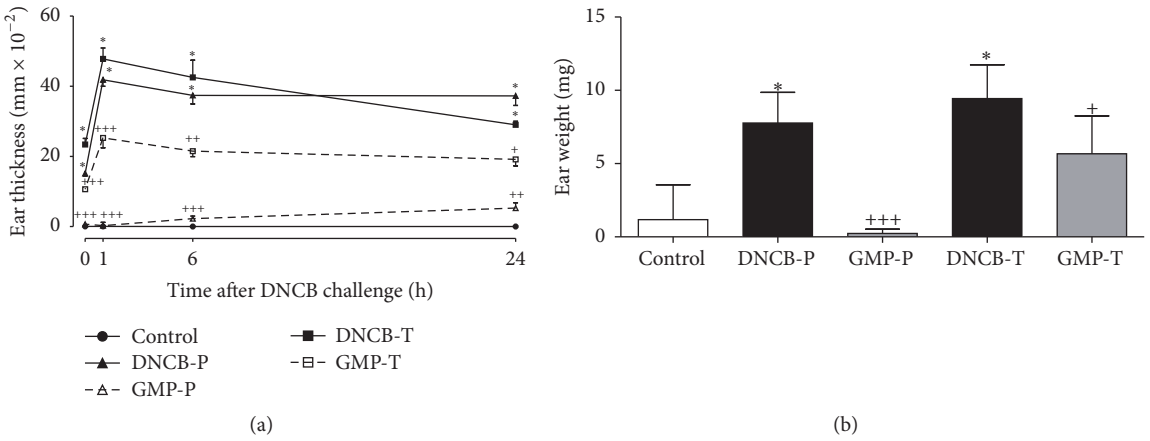

Figure 3: Effect of GMP administration on inflammatory process. (a) Ear thickness value is represented by the difference between right and left ear, at day 36. (b) Edema is represented by the difference between right ear weight and left ear weight at day 37. Data are presented as mean ± SEM, $N = 8$. Control, not sensitized and water administered before AD-induction; DNCB-P, DNCB sensitized and water administered before AD-induction; GMP-P, DNCB sensitized and GMP administered before AD-induction; DNCB-T, DNCB sensitized and water administered after AD-induction; and GMP-T, DNCB sensitized and GMP administered after AD-induction; $^{*}p < 0.0001$ versus control; $^{+}p < 0.02$; $^{++}p < 0.002$; $^{+++}p < 0.0001$ versus the respective DNCB without GMP administration at each time point.

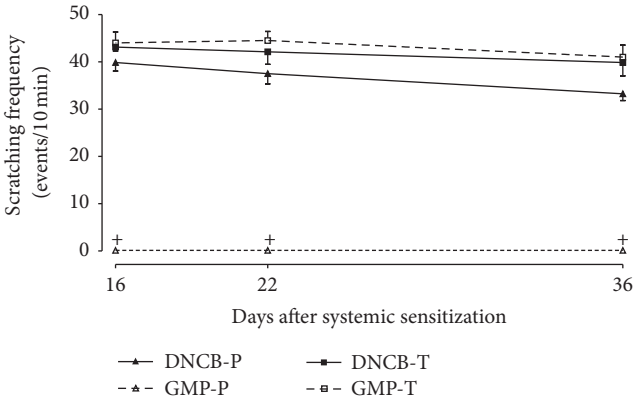

Figure 4: Effect of GMP administration on scratching frequency. Events of scratching were measured after DNCB challenge during 10 minutes. Data are presented as mean ± SEM, $N = 8$. DNCB-P, DNCB sensitized and water administered before AD-induction; GMP-P, DNCB sensitized and GMP administered before AD-induction; DNCB-T, DNCB sensitized and water administered after AD-induction; and GMP-T, DNCB sensitized and GMP administered after AD-induction; $^{+}p < 0.0001$ versus DNCB-P at each time point. GMP-T versus DNCB-T was ns.

in dermatitis skin include marked infiltration of eosinophils and mast cells hyperplasia [27]. Histological analysis revealed that topical DNCB elicited the infiltration of inflammatory cells into ear skin lesion but GMP administration attenuated the amount of infiltrated inflammatory cells (Figures 5(a)-5(b)). Morphometric assessment showed that the number of eosinophils in ears with DNCB applications was 13.12 and 12.89 times higher than in control group, for DNCB-P and DNCB-T rats. Whereas in animals administrated with GMP, amount of eosinophils in dermis was reduced in 94.47% when GMP was used as prophylaxis or 78.71% when it was administered in a therapeutic manner (Figure 5(c)). On the other hand, the amount of mast cells in the dermis of DNCB untreated animals was 2-fold compared to control animals. However, mast cells number was remarkably lowered in 61.51% by GMP administration before AD-induction and in 39.59% by GMP administration after AD-induction (Figure 5(d)).

3.5. Influence of GMP on Serum Levels of IgE. It is known that dermatitis is characterized by high levels of serum total IgE [28]. Therefore, we investigated whether GMP suppresses IgE in serum. After the sixth DNCB application, serum samples were collected and total IgE levels were measured by ELISA. In rats receiving topical DNCB total IgE levels were 3.7-fold higher than in control group. Prophylaxis with GMP significantly reduced in 86.53% total serum IgE levels as compared with nontreated animals (DNCB-P). When GMP was administered in a therapeutic manner the decrease in IgE level was 63.68% (Figure 6).

3.6. Effect of GMP Administration on IL-4, IL-5, IL-13, and IL-10 Expression in Dermatitis Skin Lesion. Inflammation in AD is mediated by an initial Th2 phase, which is orchestrated by IL-4, IL-5, and IL-13 cytokines and is related to IgE production and eosinophilia [1]. To address the question whether GMP administration might modulate this Th2 inflammatory response in dermatitis, we examined mRNA changes of IL-4, IL-5, and IL-13 by qRT-PCR in injured skin tissue. We found that IL-4, IL-5, and IL-13 mRNA were 11.24-, 3.93-, and 12.50-fold higher, on average, in DNCB groups. Interestingly, GMP administration before AD-induction decreased in 83.56, 96.5, and 88.38% the expression of IL-4, IL-5, and IL-13 in dermatitis skin. When GMP was administered after AD-induction the decrease of these Th2-inflammatory cytokines, although

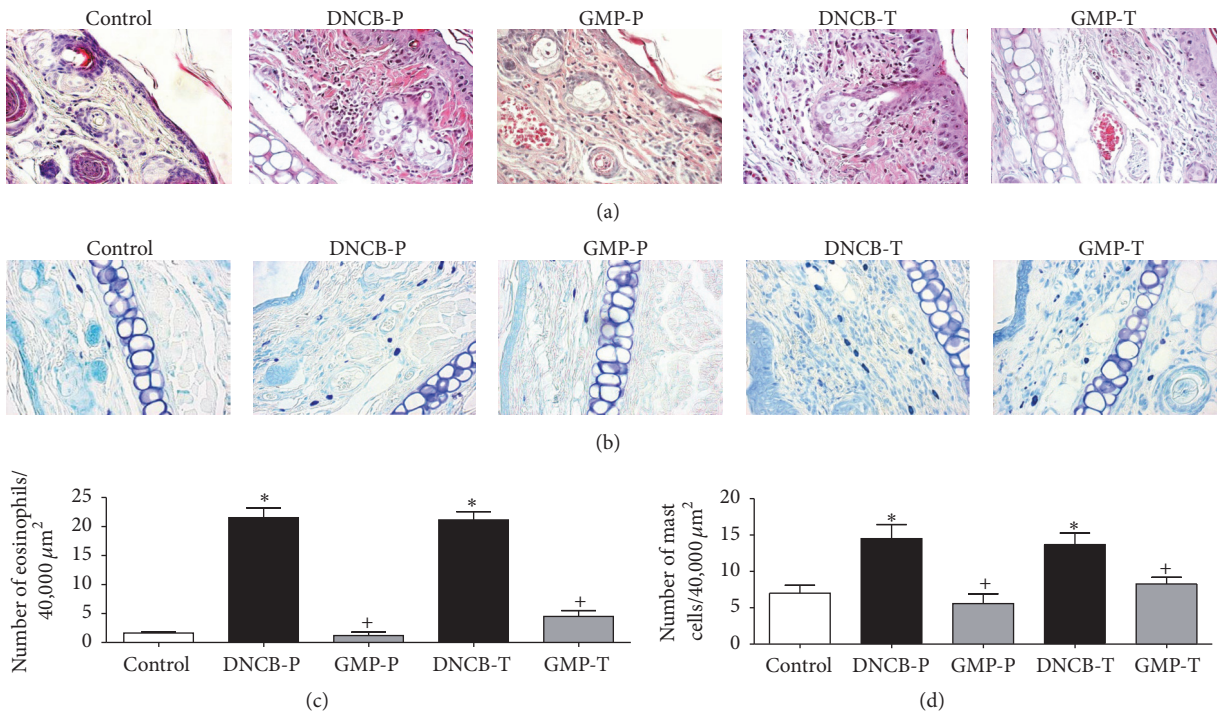

Figure 5: Effect of GMP on inflammatory cell infiltration. Sections of right ears were stained with (a) hematoxylin and eosin to identify eosinophils and (b) blue toluidine for mast cells. Quantitative analysis of (c) eosinophils and (d) mast cells per 40,000 μm^2 of dermis was developed with a microscope at magnification of 400x. Data are presented as mean ± SEM, $N = 8$. Control, not sensitized and water administered before AD-induction; DNCB-P, DNCB sensitized and water administered before AD-induction; GMP-P, DNCB sensitized and GMP administered before AD-induction; DNCB-T, DNCB sensitized and water administered after AD-induction; and GMP-T, DNCB sensitized and GMP administered after AD-induction; $^*p < 0.0001$ versus control; $^+p < 0.0001$ versus the respective DNCB without GMP administration.

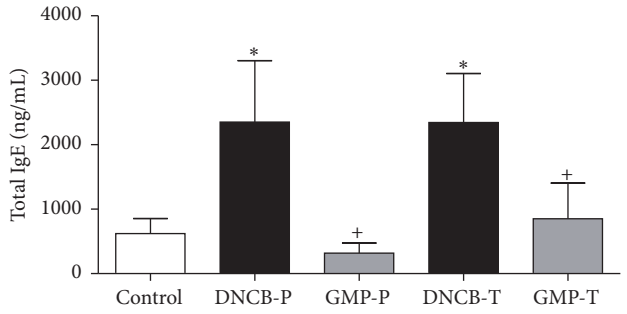

Figure 6: Effect of GMP on total serum IgE. Serum was collected 24 h after last challenge with DNCB. IgE level was measured by ELISA. Data are presented as mean ± SEM, $N = 8$; control, not sensitized and water administered before AD-induction; DNCB-P, DNCB sensitized and water administered before AD-induction; GMP-P, DNCB sensitized and GMP administered before AD-induction; DNCB-T, DNCB sensitized and water administered after AD-induction; and GMP-T, DNCB sensitized and GMP administered after AD-induction; $^*p < 0.001$ versus control; $^+p < 0.001$ versus the respective DNCB without GMP administration.

not so marked, was still significant in order of 57.05, 65.89, and 63.3% lower than nontreated animals (Figures 7(a)–7(c)).

Besides, we analyzed mRNA changes on IL-10, one of the most important anti-inflammatory cytokines which downregulates the immune system minimizing tissue damage during inflammation [29]. As shown in Figure 7(d), the expression of IL-10 was significantly higher in DNCB challenged than in control animals, but it was clearly potentiated by GMP administration. IL-10 expression was 4.68-fold higher in DNCB challenged animals when receiving GMP as prophylaxis and 2.44-fold higher when it was administered in a therapeutic manner.

4. Discussion

AD is one of the most common skin inflammatory disorders [30] and its early onset in childhood often triggers the atopic march, which leads to the consequent development of asthma and allergic rhinitis [31]. The anti-inflammatory therapy of AD includes topical corticosteroids and calcineurin inhibitors; however resolution is often temporary and long-term usage can be associated with significant adverse effects [32, 33]. Due to the deleterious effect of AD on the quality of life of patients and the significant economic impact in health systems, new therapies that prevent or act on the immunological mechanisms involved in AD and with minimal side effects are required.

In this study we investigated whether GMP attenuates the severity of AD-like lesions induced by DNCB in rat. We chose rat as animal model because thickness of the corneal

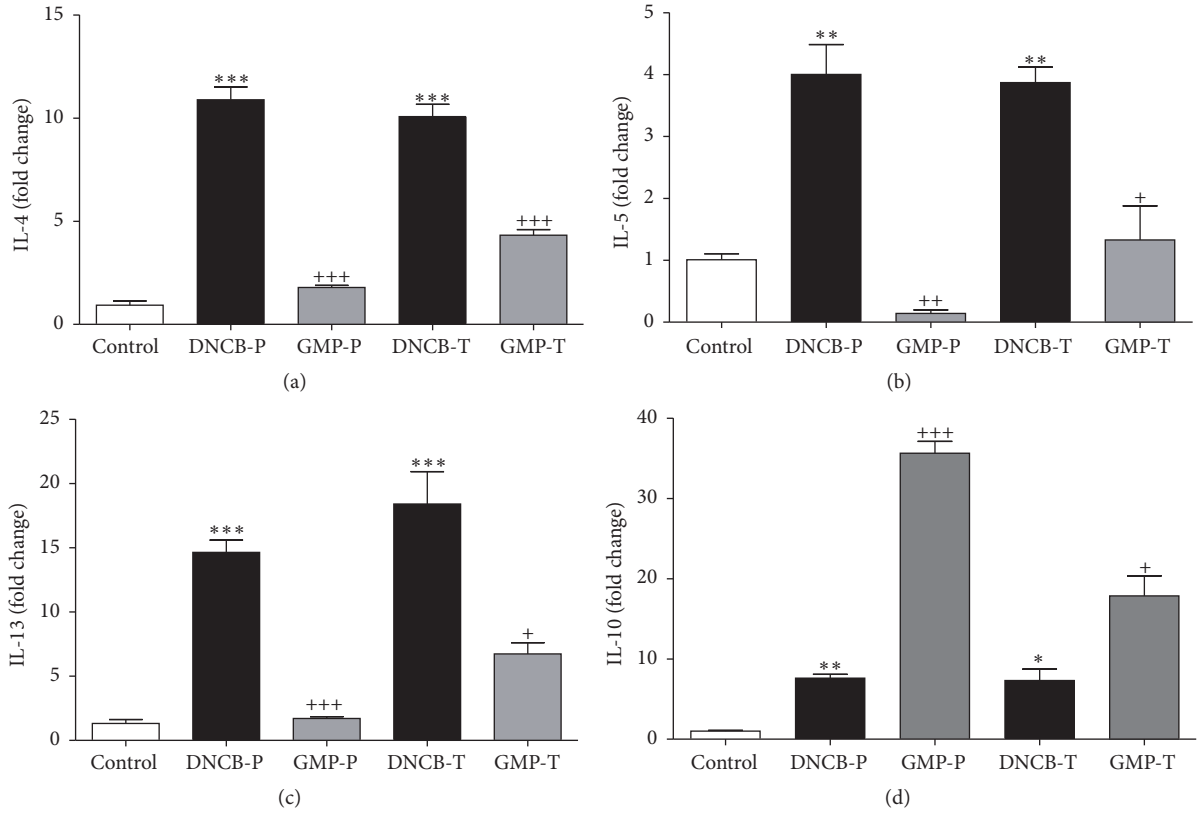

Figure 7: Effect of GMP on mRNA expression for IL-4, IL-5, IL-13, and IL-10 in ear tissue. Changes in (a) IL-4, (b) IL-5, (c) IL-13, and (d) IL-10 mRNA expression relative to β-actin were measured. Skin tissue was obtained at day 37 from control and DNCB sensitized rats, administered or not with GMP. Three rats from each experimental group were analyzed. Skin samples from each rat were analyzed in triplicate for qPCR. Each value represents the mean ± SE. Control, not sensitized and water administered before AD-induction; DNCB-P, DNCB sensitized and water administered before AD-induction; GMP-P, DNCB sensitized and GMP administered before AD-induction; DNCB-T, DNCB sensitized and water administered after AD-induction; and GMP-T, DNCB sensitized and GMP administered after AD-induction; $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ versus control; $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.0001$ versus the respective DNCB without GMP administration.

layer and chemical permeability of skin rat is more similar to human than mice [10–12]. So, we firstly characterized an experimental model of AD in rat based on a reported protocol of epicutaneous DNCB sensitization in mice [24]. The rat model demonstrates immunological dysregulation, such as IgE hyperproduction in serum and elevated IL-4, IL-5, IL-13, and IFN-γ expression in skin injuries. It also shows hypertrophy of epidermis, intracellular edema, and infiltration of inflammatory cells, such as eosinophils and mast cells, which are histopathological features of AD [1]. Besides, DNCB applications induce a scratching behavior toward the affected area that denotes the appearance of pruritus, one of the most characteristic AD symptoms [26]. So, in our rat model AD-like lesions have histopathological, immunological, and clinical features of human lesions.

GMP is a bioactive peptide that has been demonstrated to prevent allergic sensitization and attenuate the severity of urticarial reaction, anaphylaxis, and asthmatic airway inflammation and remodeling [21, 23]. It is already incorporated in nutritional products and is safe and not immunogenic [15–17]. In the present study, we demonstrated that oral administration of GMP in a prophylactic or therapeutic manner induces a significant reduction in the development of AD by strongly reducing skin inflammation, eosinophils, and mast cells number in dermis and total IgE levels. Besides, GMP administration targets the Th2-inflammatory response, as it decreases IL-4, IL-5, and IL-13 but increases IL-10 expression in AD-like skin lesions. Prophylaxis with GMP also impacts on pruritus, as it suppresses scratching episodes associated with disease. So, we demonstrate that oral intake of GMP before or after AD establishment modulates immune response and pathophysiology in experimental AD.

The epidermis of AD patients is characterized by significant skin barrier disruption which activates keratinocytes to develop an extreme Th2-dominant response that strengthens IgE production [34]. Thus, IgE level in the serum is correlated with the severity of AD [5]. In our experimental model of AD, high levels of total IgE were quantified in serum. GMP administration before or after AD-induction significantly reduces serum total IgE levels. When GMP was administered before AD-induction IgE levels were lower than in control animals, suggesting that in this condition GMP

administration can suppress serum total IgE. It is known that IgE released from B cells binds to mast cells. Allergens induce mast cells degranulation through IgE-FcεRI complex and the release of several biological mediators involved in skin inflammation [35]. So, a lessened level of IgE is in line with the reduction of edema and skin inflammation of AD-lesions observed in animals with GMP administration. Previously, it has been demonstrated that GMP inhibits mast cells activation by allergens [22] and we observed a reduced number of mast cells in dermis of GMP-treated animals, so the reduction in edema and skin inflammation as a consequence of GMP administration might also be mediated by alterations in mast cells number and function.

One of the central causes of the AD is the dysregulated Th1 and Th2 response that induces the characteristic Th2-dominant skin allergic inflammation [36]. In this Th2 response, the involvement of IL-4, IL-5, and IL-13 is crucial in humans [37]. In transgenic mice that overproduce IL-4, IL-5, and IL-13, investigators have demonstrated a positive correlation between the onset and progression of AD-like disease and the expression of these Th2 cytokines [38]. In our experimental model of AD the expression of IL-4, IL-5, and IL-13 was increased in skin lesions. It is known that IL-5 plays an important role in eosinophil differentiation, activation, proliferation, and chemotaxis [39, 40]. The number of eosinophils and levels of IL-5 have previously been shown to be elevated in injured skin of patients with AD [5, 41]. We show that GMP administration before or after AD-induction induces a significant reduction in IL-5 expression in AD-lesions, which is correlated with the decrease in the number of eosinophils infiltrated in dermis. On the other hand, transgenic mice overexpressing epidermal IL-4 or IL-13 spontaneously developed signs and symptoms associated with AD, including elevated IgE levels [42, 43]. So, reduced levels of IL-4 and IL-13 in skin of animals treated with GMP in a prophylactic or therapeutic manner are in concordance with the decrease in total IgE. The downregulation of the Th2-dominant skin inflammation by GMP administration may be associated with the increased expression of IL-10, a known regulatory cytokine. It has been reported that IL-10 inhibits both the proliferation and the cytokine synthesis of CD4+ Th2 cells [44]. Recently, the role of IL-10 in the control of AD development and maintenance has been highlighted by the fact that polymorphisms in the IL-10 gene could represent a genetic marker for AD in childhood [45]. As Th2 cytokines destabilize cutaneous barrier function [46, 47] and IFN-γ is crucial in dermal thickening and in the progression to chronic AD skin lesions [1], the study of the effect of GMP administration on the recovery of skin barrier integrity and on levels of IFN-γ expression is the aim of our current research.

Pruritus is a clinical manifestation of AD [26] that causes a great deterioration in patient's quality of life [48]. Besides, scratching worsens the dermatitis, increasing lesions in skin and thereby aggravating pruritus [49]. Thus, proper treatment of pruritus is the critical part of therapeutic approach to AD. Our rats with dermatitis showed an intense pruritus after DNCB application, but prophylaxis with GMP totally abolished the scratching episodes of the rats. A wide range of itch-inducing stimuli generated within the skin are able to trigger pruritus. Among them, histamine is recently considered relevant, as combined H1R/H4R antagonists therapy is successfully addressing pruritus in AD [50]. The decrease in IgE levels and mast cells number observed in animals administered with GMP before AD-induction, together with the reported inhibitory action of GMP on mast cells activation by allergen [22], might cause a decrease in histamine levels in skin, impacting on itching. Besides, it has been reported that transgenic mice expressing IL-13 in skin develops intense pruritus [43]. Dupilumab, a monoclonal antibody that binds to IL-4Rα and blocks both IL-4 and IL-13 signaling pathways, induces a reduction in the pruritus score of patients with moderate to severe AD [51, 52]. These data indicate a role of IL-4 and IL-13 in triggering pruritus. Thus, antipruritic action of GMP-prophylaxis might be also mediated by the reduction of IL-4 and IL-13 expression in skin. However, due to the wide range of stimuli able to trigger pruritus in AD we cannot exclude a possible effect of GMP on other itching-inducing element.

GMP exerted a clearly superior therapeutic effect when it was given before AD-induction than when administered once AD-lesions were established. This is a common observation with GMP, because when it is used as anti-inflammatory therapy in experimental colitis its effect is greater when used as prophylaxis [17]. We recently demonstrated that GMP administration before allergen sensitization induces a significant increase in the amount of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in the gut of sensitized animals [22]. In this regard, data about the effect of probiotics in the prevention and treatment of AD remain elusive, with negative and positive results, but evidencing that their positive effects depend on factors such as the type of probiotic strain, method of administration, onset time, duration of exposure, and dosage [53]. Particularly *Lactobacillus* and *Bifidobacterium* as therapy in AD show a promissory effect on prevention of pediatric AD, while there is less convincing information about their effects when used in a therapeutic manner [54], which is in concordance with our results. It is important to highlight that even after AD-induction most of the beneficial effects of GMP were retained, with exception of antipruritic effect. This may be due to the lesser reduction in IgE levels, mast cells number, and IL-4 and IL-13 expression in animals administered with GMP once AD was established. The remaining levels of these immune elements might be sufficient to maintain pruritus in the animals. However, we must consider that patients with AD may benefit from anti-inflammatory and Th2-downregulation properties of GMP used in a therapeutic manner.

In conclusion, the present study shows that GMP possesses prophylactic and therapeutic effects in the development of AD. GMP effectively suppresses skin inflammation, eosinophils recruitment, and mast cells hyperplasia in dermis, as well as total IgE in serum. Beneficial effect of GMP is associated with downregulation of IL-4, IL-5, and IL-13 expression together with upregulation of IL-10. Prophylactic administration of GMP also abolished pruritus. This study provides the first experimental basis for the potential use of GMP in the prevention and therapy of AD.

Disclosure

Cervantes-García is a CONACYT research fellow in Autonomous University of Aguascalientes.

Competing Interests

The authors confirm that there are no competing interests.

Authors' Contributions

All authors read the paper and participated in revising it for intellectual content and style. The authors participated as follows: for experimental design: Eva Salinas and Daniel Cervantes-García; for acquisition of data: Fabiola Carolina Muñoz, Maritza Montserrat Cervantes, Daniel Cervantes-García, and Mariela Jiménez; for analysis and interpretation of data: Eva Salinas, Daniel Cervantes-García, Fabiola Carolina Muñoz, and Javier Ventura-Juárez; for drafting of the paper: Eva Salinas, Daniel Cervantes-García, Fabiola Carolina Muñoz, and Mariela Jiménez.

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