Food Chemistry 141 (2013) 1104–1113

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Anti-inflammatory effects of 27 selected terpenoid compounds tested through modulating Th1/Th2 cytokine secretion profiles using murine primary splenocytes

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ARTICLE INFO

Article history:
Received 22 December 2012
Received in revised form 27 February 2013
Accepted 18 April 2013
Available online 25 April 2013

Keywords:
Anti-inflammatory effects
Mouse primary splenocytes
Terpenoid compounds
Th1/Th2 cytokines

ABSTRACT

This study investigated 27 selected terpenoid compounds, including 8 monoterpenoids, 7 sesqui-terpenoids, 3 di-terpenoids, 8 tri-terpenoids, and 1 tetra-terpenoid, for their Th1/Th2 immunomodulatory potential using mouse primary splenocytes. Changes in Th1 cytokines, including interleukin (IL)-2 and interferon (IFN)-γ, and Th2 cytokines, including IL-4, IL-5 and IL-10, secreted by terpenoid-treated splenocytes were measured using the ELISA method. The results showed that triptolide, a diterpenoid, was most cytotoxic, reflecting an IC_{50} value of 46 nM. Eucalyptol, limonene, linalool, thymol, parthenolide, andrographolide, 18β-glycyrrhetinic acid, lupeol, ursolic acid and β-sitosterol showed a strong Th2-inclination and anti-inflammation potential *in vitro*. In addition, (−)-trans-caryophyllene, oridonin, triptolide, diosgenin, betulinic acid, escin, and β-sitosterol treatments significantly inhibited both IL-2 (Th1) and IL-10 (Th2) cytokine production at the same time, suggesting that these terpenoid compounds have an anti-inflammation potential through the inhibition of T-cell immune responses. Diosgenin treatments significantly increased IFN-γ secretion levels using mouse splenocytes, suggesting that diosgenin may be useful in treating a viral infection through the stimulation of IFN-γ production. Menthone, farnesol and oridonin treatments did not markedly increase IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that menthone, farnesol and oridonin may have a relative Th1-inclination property, compared to the other selected terpenoid compounds. The relative Th1-inclination property of menthone, farnesol and oridonin may be applied to improve Th2-skewed allergic diseases.

1. Introduction

Among phytochemicals, terpenoids have five-carbon isoprene units ($(C_5H_8)n$, $n = 1, 2, 3, 4, 5, 6, 8, >8$), also called isoprenoids, which will be further modified in a number of ways. This is a group of compounds that exhibit a diverse biological relevance and have attracted more attention in recent years (Wagner & Elmadfa, 2003). Most terpenoids have multi-cyclic structures that can be viewed as modified terpenes, wherein methyl groups are moved or removed, or oxygen atoms added, differing from each other in functional groups and also in their basic chemical carbon skeletons. Based on the number of building blocks, terpenoids are classified into hemi-terpenoids (5 carbons, 5C), mono-terpenoids (10C), sesqui-terpenoids (15C), di-terpenoids (20C), sester-terpenoids (25C), tri-terpenoids (30C), tetra-terpenoids (40C) and poly-terpenoids that have a larger number of isoprene units (>45C). More than 40,000 terpenoid compounds have been found, existing broadly in fruits and vegetables (Thoppil & Bishayee, 2011). In bacteria and yeasts, terpenoid compounds are basic metabolites (Thoppil & Bishayee, 2011) however, these compounds are important secondary metabolites synthesized from mevalonate or isopentenyl pyrophosphate precursors in plants, serving as plant hormones, e.g. gibberellins, abscisic acid, photosynthetic pigments, phytols, carotenoids, electron carriers, ubiquitin and structural constituents in cell membranes and phytosterols hormones (e.g. gibberellins and abscisic acid), photosynthetic pigments (e.g. phytols and carotenoids), electron carriers (e.g. ubiquitin) and structural constituents in cell membranes (e.g. phytosterols) (McGarvey & Croteau, 1995). Certain terpenoids in plants that are C10, C15, and C20 families can exert communicative and defensive responses, serving as attractants to pollinators, plant toxins, or plant antibiotics to inhibit the spread of plant pathogens (McGarvey & Croteau, 1995; Wagner & Elmadfa, 2003). Terpenoid compounds have been reported to be beneficial to the human body for anti-inflammatory, anti-oxidant, anti-cancer and anti-biotic effects (Wagner & Elmadfa, 2003). However, the effects of terpenoid compounds on cytokine secretion profiles are still scarcely reported. Considering the beneficial health effects of terpenoids, it is relevant to determine their action on cytokine secretions by immune cells.

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http://dx.doi.org/10.1016/j.foodchem.2013.04.044

There are two major $CD4^+$ T lymphocytes, T-helper type 1 (Th1) and Th2 lymphocytes that produce some common cytokine products; others are mutually exclusive (Wong et al., 2001). Th1 cells secrete the interferon (IFN)-γ, tumour necrosis factor (TNF)-α/β, interleukin (IL)-2, IL-3, IL-10, and granulocyte–macrophage colony-stimulating factor (GM-CSF) to help against intracellular infections caused by bacteria, viruses and parasites. In contrast, Th2 lymphocytes produce IL-3, IL-4, IL-5, IL-6, IL-10, and GM-CSF to augment immune responses that help against extracellular pathogens (Abbas, Murphy, & Sher, 1996; Mahajan & Mehta, 2011). It is recognized that Th1-cell-secreted cytokines, such as TNF-α, enhance inflammatory responses *in vivo*, however Th2-cell-secreted cytokines, particularly IL-10 that are also produced by T regulatory cells (Th3 cells), macrophages and some B cells, inhibit the synthesis of other cytokines and macrophage functions during the late inflammation phase (Lin & Li, 2010), that potentially inhibits the secretion of Th1 cells, which are recognized as anti-inflammatory cytokines. Th1 and Th2 cytokine secretion profiles may reflect the Th1/Th2 immune balance in the host (Wong et al., 2001). Th1-skewed immune responses in hosts may cause pro-inflammatory autoimmune diseases such as, type 1 diabetes and rheumatoid arthritis. However, Th2-skewed immune responses *in vivo* may induce allergic diseases (Wong et al., 2001). To maintain an accurate Th1/Th2 immune balance, certain foods and medicines may avoid many immuno-deficiency driven diseases (Wong et al., 2001). Consequently, regulation of Th1/Th2 cytokine expression in immune cells, using potential food components, may prevent immune disorder diseases. It is relevant to systematically establish Th1/Th2 cytokine expression profiles of immune cells influenced by active components in foods and herbal plants beneficial to health.

To evaluate the Th1/Th2 immunomodulatory potential of food components *in vitro*, the mouse primary splenocyte culture model is suitable and has been widely adopted (Liao & Lin, 2012; Lin & Lin, 2011a, 2011b; Lin & Tang, 2007, 2008). Mouse primary splenocytes are composed mainly of 41.5% B cells and 47.1% T cells and may contain about 10% antigen-presenting cells (APCs) and other cells that may perfectly reflect systemic immune responses or local lymphoid tissues *in vivo*. Changes in Th1/Th2 cytokine secretion profiles or ratios may indicate the Th1/Th2 immunomodulatory properties of test compounds.

Terpenoid compounds have been reported to be beneficial to the human body for anti-inflammation, anti-oxidation, anti-biotic and anti-cancer effects. However, the influence of terpenoids on Th1/Th2 cytokine secretion profiles remains unclear. To determine whether terpenoid compounds have immunomodulatory effects on the Th1/Th2 immune balance, 27 terpenoid compounds referred to in the literature were selected for study using murine primary splenocytes from female BALB/c mice.

2. Materials and methods

2.1. Selected terpenoid compounds

Twenty-seven terpenoid compounds, including 8 mono-terpenoids (S-(−)-β-citronellol, eucalyptol, geraniol, R-(+)-limonene, (−)-linalool, menthone, (+)-α-pinene, thymol), 7 sesqui-terpenoids ((−)-α-bisabolol, (−)-trans-caryophyllene, (−)-caryophyllene oxide, farnesol, α-humulene, nerolidol, parthenolide), 3 di-terpenoids (andrographolide, oridonin, triptolide), 8 tri-terpenoids (α-amyrin, betulinic acid, diosgenin, escin, 18β-glycyrrhetinic acid, lupeol, ursolic acid, β-sitosterol), and 1 tetra-terpenoid (lycopene) were purchased at the highest available purity from Sigma Co. (St. Louis, MO, USA). The purities of the terpenoid compounds used in this study were all higher than 95%. Molecular formulas and the known effects of the 27 selected terpenoid compounds are shown in Table 1.

2.2. Preparation of selected terpenoid compounds

All selected terpenoid compounds, except (−)-trans-caryophyllene, were dissolved in dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) to prepare stock solutions at appropriate concentrations (0.2–100 mM) and sterilized using a filter (Millipore, Billerica, MA, USA) with a 0.22 μm pore size. The terpenoid (−)-trans-caryophyllene was dissolved in 95% ethyl alcohol (ECHO, Taiwan) to prepare a stock solution with a concentration of 50 mM and sterilized using a 0.22 μm pore size filter (Millipore). All stock solutions were stored at −80 °C for future use.

2.3. Primary splenocytes isolation

The animal use protocol, listed below, was reviewed and approved by the Institutional Animal care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC. The female BALB/c mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC and maintained in the Department of Food Science and Biotechnology at the National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet). After the mice were acclimatized for 2 weeks, the animals were weighed, anaesthetized with diethyl ether and immediately bled using a retro-orbital venous plexus puncture to collect blood. Immediately after the blood collection, the animals were sacrificed using CO_2 inhalation for primary splenocyte culture studies. The splenocytes were prepared by aseptically removing the spleens from the BALB/c mice. Spleens were homogenized in TCM (tissue culture medium, a defined commercial serum replacement, Protide Pharmaceuticals, Inc., Lake Zurich, IL, USA) medium (a mixture of 10 ml of TCM, 500 ml of RPMI 1640 medium, Hyclone, Logan, UT, USA), and 2.5 ml of an antibiotic–antimycotic solution (100 × PSA) containing 10,000 U/ml of penicillin, 10,000 μg/ml of streptomycin, and 25 μg/ml of amphotericin B in 0.85% saline (Biological Industries, Israel). Single spleen cells were collected and treated by lysing the red blood cells with a RBC lysis buffer (0.017 M Trizma base (Sigma–Aldrich Co., St. Louis, MO, USA), 0.144 M ammonium chloride (Sigma–Aldrich Co.), pH 7.4, 0.22 μm filtered). Splenocytes were isolated from each animal and adjusted to 1×10^7 cells/ml in TCM medium with a hemocytometer using the trypan blue (Sigma, MO, USA) dye exclusion method.

2.4. Determination of cell viability (MTT assay) of selected terpenoid compounds

To evaluate the possible cytotoxic effects of selected terpenoid compounds, the cell viabilities of splenocytes treated with individual terpenoid compounds, at different concentrations, were determined using the 3-(4,5-dimethylthiazol-2-diphenyl)-2,5-tetrazolium bromide (MTT) assay. All terpenoid compound stock solutions were aseptically diluted into working solutions, using TCM medium before use. The splenocytes (50 μl/well) were cultured in 96-well plates in the absence and presence of the samples (50 μl/well), at different concentrations, and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 72 h. The lipopolysaccharide (LPS) (Sigma–Aldrich Co., L2654, St. Louis, MO, USA), a B cell mitogen, at the concentration of 2.5 μg/ml, was selected as a positive control in each experiment. After incubation, aliquots of 10 μl (5 mg/ml) MTT ((Sigma, MO, USA) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.22 μm filtered)) were added to each well in the 96-well plate. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for another 4 h. After

Table 1
Molecular formulas and known effects of 27 selected terpenoid compounds in this study.

Terpenoid compounds	Molecular formula	Major plant sources	Known possible effects
Mono-terpenoids			
Citronellol	$C_{10}H_{20}O$	*Cymbopogon winterianus*	Anti-inflammatory effects
Eucalyptol	$C_{10}H_{18}O$	*Illicium anisatum*	Anti-inflammatory effects
Geraniol	$C_{10}H_{18}O$	*Lavandula angustifolia Mill; Cymbopogon winterianus*	Anti-inflammatory effects
Limonene	$C_{10}H_{16}$	*Citrus junos Tanaka*	Anti-cancer; anti-inflammatory effects; Anti-allergy
Linalool	$C_{10}H_{18}O$	*Ocimum sanctum; Bursera aloexylon*	Anti-inflammatory effects
Menthone	$C_{10}H_{18}O$	*Menthax piperita*	Anti-allergy; anti-inflammatory effects
(+)-α-Pinene	$C_{10}H_{16}$	*Bupleurum fruticescens*	Anti-inflammatory effects; anti-asthma
Thymol	$C_{10}H_{14}O$	*Lippia multiflora*	Anti-oxidation; anti-microbial agent; anti-inflammatory effects
Sesqui-terpenoids			
(−)-α-Bisabol	$C_{15}H_{26}O$	*Matricaria chamomilla*	Anti-ulcerative, anti-oxidative and anti-inflammatory effects
β-Caryophyllene	$C_{15}H_{24}$	*Bupleurum fruticescens*	Anti-inflammatory effects
(−)-Caryophyllene oxide	$C_{15}H_{24}O$	*Annona squamosa*	Anti-inflammatory effects
Farnesol	$C_{15}H_{26}O$	*Zea mays*	Anti-metabolic disorders; Anti-tumor; Anti-oxidation; Anti-inflammatory effects
α-Humulene	$C_{15}H_{24}$	*Zingiber zerumbet*	Anti-asthmatic inflammation; Anti-inflammatory effects
Nerolidol	$C_{15}H_{26}O$	*Accharis dracunculifolia*	Anti-ulcerative effects
Parthenolide	$C_{15}H_{20}O_3$	*Tanacetum parthenium*	Anti-cancer and anti-inflammatory effects; Inhibits IL-4 mRNA and protein expression
Di-terpenoids			
Andrographolide	$C_{20}H_{30}O_5$	*Andrographis paniculate*	Anti-inflammatory effects in airways
Oridonin	$C_{20}H_{28}O_6$	*Rabdosia rubescens*	Enhances $CD4^+/CD25^+$ regulatory T cells differentiation; Regulates Th1/Th2 immune balance
Triptolide	$C_{20}H_{24}O_6$	*Tripterygium wilfordii*	Anti-inflammatory effects in airways; anti-autoimmune diseases; anti-cancer through the inhibition of Bcl-2 expression
Tri-terpenoids			
α-Amyrin	$C_{30}H_{50}O$	*Diospyros kaki*	Anti-inflammatory effects in airways; anti-type 2 diabetes
Betulinic acid	$C_{30}H_{48}O_3$	*Forsythiae Fructus*	Anti-asthma
Diosgenin	$C_{27}H_{42}O_3$	*Trigonella foenum graecum*	Anti-diabetic complications; anti-inflammatory effects; anti-allergic diseases
Escin	$C_{54}H_{86}O_{24}$	*Aesculus hippocastanum*	Anti-asthma
18β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$	*Glycyrrhizza glabra L.*	Anti-inflammatory and anti-oxidative effects
Lupeol	$C_{30}H_{50}O$	*Lonchocarpus araripensis*	Anti-inflammatory effects in airways
Ursolic acid	$C_{30}H_{48}O_3$	*Ocimum sanctum; Forsythiae fructus*	Anti-inflammatory effects; anti-asthma; anti-arthritis by increasing Th2, but inhibiting Th1 cytokine secretion
β-Sitosterol	$C_{29}H_{50}O$	*Moringa oleifera Lam.*	Anti-inflammatory effects in airways
Tetra-terpenoid			
Lycopene	$C_{40}H_{56}$	*Solanum lycopersicum*	Anti-asthma

incubation, the plates were centrifuged at 400*g* for 10 min and the culture medium discarded. The plates were carefully washed with PBS buffer three times. Aliquots of 100 μl dimethyl sulfoxide (DMSO) were added to each well and oscillated for 30 min to extract formed insoluble formazan. The absorbance (A) was measured at 550 nm on a plate reader (ELISA reader, ASYS Hitech, GmbH, Austria). The cell viability was described as the survival rate (%) compared to the mean absorbency of the control. The cell viability (%) in each biological determination was calculated using the equation: cell viability (%) = $[(A_{sample}-A_{blank})/(A_{control}-A_{blank})] \times 100$. The non-cytotoxic doses of individual terpenoid compounds were selected to conduct cytokine secretion assessments. In this study, each stock solution was appropriately diluted using TCM medium. The organic solvent concentrations, such as DMSO and 95% ethyl alcohol, in the medium were therefore lower than 1%. The lower concentrations of organic solvents (<1%) in the medium may not cause cytotoxicity and influence the cell viability (Wahlkvist et al., 2008).

2.5. Effects of selected terpenoid compounds on cytokine secretions by murine primary splenocytes

To evaluate the effects of individual terpenoid compounds on cytokine secretions by immune cells, the splenocytes (0.50 ml/well, 1×10^7 cells/ml in TCM medium) in the absence or presence of terpenoid compounds (0.50 ml/well) were cultured at their optimal non-cytotoxic concentrations. The LPS, an endotoxin, at the concentration of 2.5 μg/ml, was selected as a positive control in each experiment. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 48 h. The plates were centrifuged at 200*g* for 10 min. The supernatants in cell cultures were collected and stored at −80 °C for cytokine assays.

2.6. Measurement of Th1/Th2 cytokine secretion levels in splenocyte cultures by an enzyme-linked immunosorbent assay (ELISA)

Both Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-5, IL-10) cytokine secretion levels were determined using sandwich ELISA kits. The cytokine concentrations were assayed according to the cytokine ELISA protocol from the manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN, USA). The sensitivity of these cytokine assays was 15.6 pg/ml. Briefly, 100 μl of 1:180 diluted (with PBS) anti-mouse captured antibodies were added to 96-microwell plate wells (Nunc) and incubated overnight at 4 °C. After incubation, the plates were washed three times with a ELISA wash buffer (0.05% Tween 20 in PBS, pH 7.4). To block the non-specific binding, 200 μl of the block buffer (1% bovine serum albumin (BSA) and 0.05% NaN_3 in PBS) were added to each well. The plates were incubated at room temperature for 1 h. After incubation, the plates were washed three times with the ELISA wash buffer. Volumes of 100 μl of test sample or standard in reagent diluent (0.1% BSA in Tris-buffered saline (20 mM Trizma base, 150 mM NaCl, pH 7.4, 0.22 μm filtered)) were added to the 96-microwell plate wells and the plates were incubated at room temperature for 2 h. A seven point (in duplicate) standard curve using 2-fold serial dilutions in reagent diluent were conducted. After incubation, the plates were washed three times with the ELISA wash buffer. One hundred microlitres of the

Table 2
Effects of 27 selected terpenoid compounds on cell viabilities of splenocytes from female BALB/c mice.[a,b]

Cell viability (%)							
	Conc. (μM)	**0**	**0.5**	**5**	**50**	**500**	**IC_{50}**
	S-(−)-β-Citronellol	100 ± 0a	99.9 ± 2.8a	105 ± 22a	93.8 ± 14.4a	73.3 ± 21.4b	>500 μM
	eucalyptol	100 ± 0	91.4 ± 19.5	104 ± 17	88.3 ± 19.1	96 ± 14.4	>500 μM
Mono-terpenoids	Geraniol	100 ± 0a	94.5 ± 21.5a	94.6 ± 10.6a	86.2 ± 18.4a	60.7 ± 13.6b	>500 μM
	R-(+)-limonene	100 ± 0	95.4 ± 31.3	85 ± 14.4	104 ± 26	92.4 ± 20.6	>500 μM
	(−)-Linalool	100 ± 0a	101 ± 20a	88.8 ± 24.0a	93.3 ± 26.0a	67.2 ± 20.9b	>500 μM
	menthone	100 ± 0	101 ± 21	100 ± 21	97.7 ± 20.9	92.4 ± 17	>500 μM
	(+)-α-Pinene	100 ± 0	120 ± 47	101 ± 40	125 ± 51	85.7 ± 38.6	>500 μM
	Thymol	100 ± 0a	96.9 ± 14.5a	75.3 ± 16.4b	83.4 ± 12.7b	35.2 ± 19.0c	362 μM
	(−)-Caryophyllene oxide	100 ± 0a	97.7 ± 30.7a	96.1 ± 25.8a	94.2 ± 33.0a	33.7 ± 18.5b	379 μM
	Farnesol	100 ± 0a	93.8 ± 27.1a	85.9 ± 13.0a	53.3 ± 27.3b	38.2 ± 26.1b	148 μM
	Nerolidol	100 ± 0a	96.5 ± 36.6a	89.3 ± 25.3a	98.6 ± 32.3a	39.1 ± 22.4b	418 μM
Sesqui-terpenoids	**Conc. (μM)**	**0**	**0.25**	**2.5**	**25**	**250**	
	(−)-α-Bisabolol	100 ± 0a	93.4 ± 29.5a	89.2 ± 15.1a	87.6 ± 24.8a	61.2 ± 39.8b	>250 μM
	(−)-Trans-caryophyllene	100 ± 0a	84.6 ± 20.1a	95.4 ± 14.4a	98.7 ± 20.8a	68 ± 19.9b	>250 μM
	α-Humulene	100 ± 0	103 ± 24	102 ± 22	104 ± 23	78.7 ± 42.6	>250 μM
	Conc. (μM)	**0**	**0.01**	**0.1**	**1**	**10**	
	Parthenolide	100 ± 0a	100 ± 31a	90.7 ± 28.2a	96.6 ± 21.9a	28.6 ± 26.7b	7.2 μM
	Andrographolide	100 ± 0a	114 ± 42a	113 ± 31a	85.5 ± 19.2a	45.5 ± 49.6b	9.0 μM
Di-terpenoids	**Conc .(μM)**	**0**	**0.1**		**10**	**100**	
	Oridonin	100 ± 0	97.5 ± 32.9		90 ± 60.9	115 ± 72	>100 μM
	Conc. (nM)	**0**	**0.1**	**1**	**10**	**100**	
	Triptolide	100 ± 0a	77.3 ± 11.7b	84.5 ± 19.5ab	77.9 ± 27.8b	32.8 ± 15.0c	46 nM
	Conc. (μM)	**0**	**0.01**	**0.1**	**1**	**10**	
	β-sitosterol	100 ± 0a	91.6 ± 18.9a	89.6 ± 11.5a	99.8 ± 19.4a	63.1 ± 15.8b	>100 μM
	Conc. (μM)	**0**	**0.02**	**0.2**	**2**	**20**	
	α-Amyrin	100 ± 0a	104 ± 19a	102 ± 16.5a	111 ± 16a	36.2 ± 13.9b	17 μM
Tri-terpenoids	**Conc (μM)**	**0**	**0.05**	**0.5**	**5**	**50**	
	Lupeol	100 ± 0a	101 ± 23a	94.8 ± 25.4a	113 ± 44a	41.7 ± 27.1b	45 μM
	Conc. (μM)	**0**	**0.1**		**10**	**100**	
	Betulinic acid	100 ± 0a	83.8 ± 22.9b		89.1 ± 15.1ab	63.8 ± 10.3c	>100μM
	Conc. (μM)	**0**	**0.25**	**2.5**	**25**	**250**	
	Diosgenin	100 ± 0ab	121 ± 23a	107 ± 25ab	102 ± 11ab	92.3 ± 27.6b	n.d.
	18β-Glycyrrhetinic acid	100 ± 0a	127 ± 40a	89.3 ± 20.4a	94.3 ± 26.6a	37 ± 32.9b	199 μM
	Ursolic acid	100 ± 0a	84.4 ± 16.9a	87.1 ± 29.7a	44.3 ± 20.6b		22 μM
	Conc. (μM)	**0**	**0.5**	**5**	**50**	**500**	
	Escin	100 ± 0ab	94.9 ± 32.3ab	69.6 ± 27.8b	118 ± 85a	9.28 ± 8.4c	335 μM
Tetra-terpenoid	**Conc. (μM)**	**0**	**0.002**	**0.02**	**0.2**	**2**	
	Lycopene	100 ± 0a	115 ± 9a	108 ± 13a	119 ± 27.0a	63.1 ± 27.3b	>2 μM

[a] Values are means ± SD (n = 9 biological determinations).
[b] Values within same row not sharing a common small letter are significantly different ($P < 0.05$) from each other assayed by one-way ANOVA, followed by Duncan's new multiple range test.

detection antibody (biotinylated goat anti-mouse monoclonal antibody at 1:180 dilution in reagent diluent) were then added to each well. The plates were incubated at room temperature for 2 h. After incubation, the plates were washed three times with the ELISA wash buffer. One hundred microlitres of the diluted Streptavidin–HRP (horseradish peroxidase) solution were added to each well. The plates were incubated at room temperature for 20 min. After incubation, the plates were washed three times with the ELISA wash buffer. One hundred microlitres of the substrate solution (tetramethylbenzidine; TMB) were pipetted into the 96-microwell plate wells. The plates were incubated at room temperature for 20 min for colour development. Fifty microlitres of stop solution (2 N H_2SO_4) were added to each well to stop the reaction. The absorbance was measured at 450 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Eugendorf, Austria). The cytokine levels were determined using the seven point standard curves. The limit of detection (LOD) of the ELISA kits used in this study was <15.6 pg/ml.

2.7. Statistical analysis

The data is expressed as a mean ± SD and analyzed statistically using ANOVA, if justified by the statistical probability ($P < 0.05$), followed by Duncan's New Multiple Range test. The differences between the control and other treatments were considered statistically significant if $P < 0.05$.

3. Results

This study compared the effects of terpenoid compounds on Th1/Th2 cytokine secretion profiles using murine primary splenocytes. Therefore, 27 terpenoid compounds were selected to assay

their possible cytotoxicities and Th1/Th2 immunomodulatory effects on mouse primary splenocytes.

3.1. Effects of 27 selected terpenoid compounds on cell viabilities of murine primary splenocytes

To avoid an excessive cytotoxicity at high doses, appropriate concentrations of individual terpenoid compounds were used to assess their effects on the cell viability of primary splenocytes. The remaining cell viability was determined using the MTT assay, showing that terpenoid compound treatment had differential cytotoxicities on the splenocytes (Table 2). Importantly, LPS, a B-cell mitogen, significantly ($P < 0.05$) increased cell proliferation in primary splenocytes in each experiment, reflecting the *in vitro* splenocyte culture model was suitable (data not shown). Among the selected terpenoid compounds, triptolide, a di-terpenoid, was the most cytotoxic, reflecting the half maximal (50%) inhibitory concentration (IC_{50}) to be 46 nM. However, the IC_{50} of nerolidol, a sesqui-terpenoid, was 418 μM and the most selected mono-terpenoid compounds were >500 μM, showing a comparative low cytotoxicity compared to that of triptolide. Our results suggested that the cytotoxicity of terpenoids should be circumspectly considered, when these compounds are used for health benefits. The IC_{50} of selected terpenoid compounds can be approximately measured based on the data in Table 2. To avoid an excessive cytotoxicity at high doses, appropriate concentrations (much smaller than IC_{50} or not significantly different from the control) of selected terpenoid compounds were adopted to further assess their effects on cytokine secretions using murine primary splenocytes. The adopted optimal concentrations of selected terpenoid compounds are listed in Tables 3–6.

3.2. Effects of 8 selected mono-terpenoid compounds on Th1/Th2 cytokine secretions using murine primary splenocytes

To unravel the effects of terpenoid compound treatments on Th1/Th2 cytokine secretions, 8 selected terpenoid compounds, at the indicated non-cytotoxic concentrations, were added to the splenocyte cultures for 48 h. Table 3 shows the effects of the selected mono-terpenoid compounds on Th1/Th2 cytokine secretions. The results showed that citronellol, eucalyptol, geraniol, linalool, menthone, pinene and thymol treatments significantly ($P < 0.05$) decreased IL-2 (Th1) secretion levels. Interestingly, menthone treatments significantly ($P < 0.05$) increased IFN-γ (Th1) secretion levels, although IFN-γ secretion levels in most of the selected mono-terpenoid compound treatments were lower than the LOD of the ELISA kit (<15.6 pg/ml). Moreover, IL-4 (Th2) and IL-5 (Th2) secretion levels in all selected mono-terpenoid compound treatments were also lower than the LOD of the ELISA kits (<15.6 pg/ml). Importantly, eucalyptol, limonene, linalool and thymol treatments significantly ($P < 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios although all selected mono-terpe-

Table 3
Effects of selected mono-terpenoid compounds on Th1/Th2 cytokine secretions by splenocytes from female BALB/c mice.[a,b,c,d]

Mono-terpenoid compounds	Conc.	Th1 cytokines (pg/ml)		Th2 cytokines (pg/ml)		Th2/Th1 (pg/pg)	
		IL-2	IFN-γ	IL-4	IL-5	IL-10	IL-10/IL-2
S-(−)-β-Citronellol (μM)	0	46 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03
	0.5	25.4 ± 8.3b	5.89 ± 9.24	ND	5 ± 5.5	72 ± 16.2	3.16 ± 1.33
	5	28.7 ± 12.4ab	7 ± 9.54	ND	8.4 ± 9.3	77.4 ± 18.5	3.25 ± 1.78
	50	30.2 ± 11.7ab	6.7 ± 5.32	ND	ND	68.2 ± 16.4	2.95 ± 2.46
Eucalyptol (μM)	0	46 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.5	26.8 ± 6.5b	ND	ND	ND	83.1 ± 21.7	3.29 ± 1.37a
	5	27.1 ± 7.1b	10.5 ± 11.6	ND	ND	72 ± 20.4	2.8 ± 0.89ab
	50	27.6 ± 9.8b	5.12 ± 6.86	ND	ND	72.5 ± 17.4	2.96 ± 1.33ab
Geraniol (μM)	0	46 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03
	0.5	26.9 ± 11.8b	8.48 ± 4.48	ND	ND	75.4 ± 22.8	3.54 ± 2.24
	5	30.5 ± 8.6ab	15.6 ± 4.3	ND	ND	77.8 ± 15.5	2.75 ± 0.98
	50	29.2 ± 10.6ab	10.7 ± 6.6	ND	ND	76.5 ± 19.6	2.91 ± 1.15
R-(+)-limonene (μM)	0	46 ± 20.5	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.5	29.2 ± 8.2	11.4 ± 10.7	ND	4.49 ± 6.71	75.4 ± 16.3	2.89 ± 1.16ab
	5	28.9 ± 9.8	10.6 ± 11.2	ND	4.01 ± 5.16	82.8 ± 29.8	3.74 ± 2.10a
	50	28.4 ± 13.3	7.33 ± 9.13	ND	ND	79.4 ± 19.4	3.06 ± 1.18ab
(−)-Linalool (μM)	0	46 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.5	30.1 ± 10.2ab	8.18 ± 7.05	3.88 ± 4.34	ND	78.2 ± 14.9	3.29 ± 1.37a
	5	27.1 ± 10.2b	9.05 ± 8.5	ND	ND	85.3 ± 20.6	2.8 ± 0.89ab
	50	26.1 ± 9.6b	11.1 ± 8.8	ND	ND	71.9 ± 17.1	2.96 ± 1.33ab
Menthone (μM)	0	23.4 ± 3.0a	4.27 ± 4.52b	2.59 ± 2.61	8.87 ± 6.54	73.4 ± 10.1	3.15 ± 0.82
	0.5	8.53 ± 9.38b	31.5 ± 28.3a	2.67 ± 3.62	ND	71.3 ± 14.3	4.25 ± 2.22
	5	8.07 ± 8.93b	31.7 ± 20.0a	ND	ND	66 ± 8.1	2.25 ± 1.99
	50	8.02 ± 9.06b	16.3 ± 7.4b	ND	3.7 ± 2.9	73 ± 9.1	2.74 ± 2.39
(+)-α-Pinene (μM)	0	46 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03
	0.5	29.8 ± 10.1ab	5.72 ± 6.33	ND	4.42 ± 5.04	82.2 ± 25.1	3.01 ± 1.19
	5	30.4 ± 8.9ab	8.47 ± 9.71	ND	ND	77.2 ± 26.3	2.6 ± 0.82
	50	27.1 ± 8.3b	7.72 ± 8.52	ND	ND	76.2 ± 19.9	3.02 ± 1.09
Thymol (μM)	0	46 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.05	24.9 ± 6.6b	9.06 ± 10.4	ND	ND	70.8 ± 12.6	3.01 ± 0.93ab
	0.5	25 ± 4.8b	7.79 ± 9.68	ND	ND	72.4 ± 11.6	3 ± 0.88ab
	5	23.7 ± 7.7b	9.18 ± 8.34	ND	ND	76 ± 14.3	3.69 ± 1.99a

[a] Values are means ± SD ($n = 6$ biological determinations).
[b] Values within same column under same compound treatment not sharing a common small letter are significantly different ($P < 0.05$) from each other assayed by one-way ANOVA, followed by Duncan's new multiple range test.
[c] The limit of detection (LOD) of these kits used in this study was <15.6 pg/ml.
[d] ND, not detectable.

Table 4
Effects of selected sesqui-terpenoid compounds on Th1/Th2 cytokine secretions by splenocytes from female BALB/c mice.[a,b,c,d]

Sesqui-terpenoid compounds	Conc.	Th1 cytokines (pg/ml)		Th2 cytokines (pg/ml)		Th2/Th1 (pg/pg)	
		IL-2	IFN-γ	IL-4	IL-5	IL-10	IL-10/IL-2
(−)-α-Bisabolol (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.25	25.3 ± 8.6b	21.1 ± 18.5	ND	ND	62.2 ± 23.0b	2.56 ± 0.91
	2.5	29.0 ± 8.1b	13.9 ± 9.7	2.39 ± 2.62	ND	63.4 ± 17.6b	2.41 ± 1.28
	25	27.1 ± 6.6b	18.1 ± 11.8	ND	ND	60.0 ± 13.4b	2.39 ± 1.03
(−)-Trans-caryophyllene (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.25	27.4 ± 7.3b	7.55 ± 7.36	ND	ND	66.1 ± 12.6ab	2.19 ± 0.19
	2.5	32.3 ± 12.6ab	11.9 ± 9.3	2.49 ± 2.73	ND	59.0 ± 20.9b	1.74 ± 1.12
	25	27.6 ± 4.5b	8.96 ± 8.81	ND	ND	74.5 ± 20.7ab	2.71 ± 0.68
(−)-Caryophyllene oxide (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03
	0.5	26.9 ± 11.8b	8.48 ± 4.48	ND	6.96 ± 9.41	72.6 ± 16.0	3.17 ± 1.47
	5	30.5 ± 8.6b	15.6 ± 4.3	ND	ND	70.2 ± 12.1	2.59 ± 1.01
	50	29.2 ± 10.6b	10.7 ± 6.6	ND	ND	68.5 ± 13.0	2.97 ± 0.94
Farnesol (μM)	0	46.0 ± 20.5	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.05	34.8 ± 17.4	8.62 ± 9.62	ND	ND	58.5 ± 20.7b	1.80 ± 1.21
	0.5	37.6 ± 19.6	7.63 ± 6.29	ND	4.81 ± 6.54	62.4 ± 19.4ab	1.84 ± 0.61
	5	38.3 ± 18.4	9.39 ± 10.4	ND	ND	61.4 ± 22.6ab	1.72 ± 0.59
α-Humulene (μM)	0	46.0 ± 20.5	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.25	30.0 ± 6.9	20.1 ± 15.5	ND	ND	59.9 ± 14.5b	2.02 ± 0.43
	2.5	29.6 ± 8.7	17.6 ± 19.2	ND	ND	63.9 ± 18.5ab	2.20 ± 0.52
	25	29.5 ± 13.9	18.7 ± 20.8	ND	ND	73.1 ± 25.9ab	2.68 ± 1.08
Nerolidol (μM)	0	46.0 ± 20.5	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03
	0.5	26.2 ± 5.0	15.6 ± 16.8	ND	ND	80.5 ± 32.3	3.32 ± 1.95
	5	35.1 ± 22.8	18.2 ± 22.6	ND	5.40 ± 6.70	75.2 ± 27.3	2.86 ± 2.29
	50	34.3 ± 23.5	13.3 ± 15.1	ND	6.20 ± 7.00	73.0 ± 26.6	2.80 ± 2.04
Parthenolide (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.01	30.5 ± 10.6ab	6.21 ± 7.37	ND	ND	67.7 ± 10.2	2.51 ± 1.10ab
	0.1	29.0 ± 10.6b	7.32 ± 8.82	2.21 ± 2.45	ND	66.1 ± 13.2	2.48 ± 0.89ab
	1	21.8 ± 5.6b	ND	3.90 ± 3.83	ND	76.6 ± 15.0	3.81 ± 1.75a

[a] Values are means ± SD (n = 6 biological determinations).
[b] Values within same column under same compound treatment not sharing a common small letter are significantly different ($P < 0.05$) from each other assayed by one-way ANOVA, followed by Duncan's new multiple range test.
[c] The LOD of these kits used in this study was about <15.6 pg/ml.
[d] ND, not detectable.

Table 5
Effects of selected di-terpenoid compounds on Th1/Th2 cytokine secretions by splenocytes from female BALB/c mice.[a,b,c,d]

Di-terpenoid compounds	Conc.	Th1 cytokines (pg/ml)		Th2 cytokines (pg/ml)		Th2/Th1 (pg/pg)	
		IL-2	IFN-γ	IL-4	IL-5	IL-10	IL-10/IL-2
Andrographolide (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.01	27.7 ± 6.4b	10.0 ± 13.7	ND	4.86 ± 5.47	75.2 ± 13.2	2.78 ± 1.01ab
	0.1	28.4 ± 6.9b	15.9 ± 22.0	ND	5.09 ± 6.47	75.8 ± 16.2	2.83 ± 1.08ab
	1	26.5 ± 7.0b	ND	ND	ND	93.8 ± 14.0	3.78 ± 1.35a
Oridonin (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.1	26.3 ± 8.4b	ND	1.43 ± 2.22	ND	67.3 ± 8.7b	2.70 ± 0.63
	10	3.14 ± 5.22c	ND	ND	ND	2.77 ± 6.07c	ND
	100	ND	6.59 ± 7.5	ND	5.90 ± 6.7	4.31 ± 6.72c	ND
Triptolide (nM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.01	26.6 ± 8.9b	ND	ND	8.47 ± 10.1	68.3 ± 11.0b	2.95 ± 1.62
	0.1	30.3 ± 1.1b	ND	ND	7.15 ± 8.09	70.4 ± 12.2b	2.28 ± 0.46
	1	21.7 ± 5.2b	ND	2.73 ± 3.15	ND	59.0 ± 10.7b	2.82 ± 0.62

[a] Values are means ± SD (n = 6 biological determinations).
[b] Values within same column under same compound treatment not sharing a common small letter are significantly different ($P < 0.05$) from each other assayed by one-way ANOVA, followed by Duncan's new multiple range test.
[c] The LOD of these kits used in this study was <15.6 pg/ml.
[d] ND: not detectable.

noid compounds did not significantly ($P > 0.05$) influence IL-4 (Th2), IL-5 (Th2), and IL-10 (Th2) secretion levels. In addition, the other mono-terpenoid compounds selected in this study slightly ($P > 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios. Our results indicated that the selected mono-terpenoid compounds, particularly eucalyptol, limonene, linalool and thymol, had a strong Th2-inclination potential, by increasing IL-10/IL-2 (Th2/Th1) cytokine secretion ratios but decreasing IL-2 (Th1) secretion levels. That is to say, treatments with selected mono-terpenoid compounds increased comparative amounts of IL-10. IL-10 is a Th2 cytokine and a cytokine synthesis inhibitory factor that may play a role in anti-inflammation by inhibiting pro-inflammatory Th1 cytokine production. Our results suggested that most mono-terpenoid compounds, especially eucalyptol, limonene, lin-

Table 6
Effects of selected tri-terpenoid and tetra-terpenoid compounds on cytokine secretions by splenocytes from female BALB/c mice.[a,b,c,d]

	Conc.	Th1 cytokines (pg/ml)		Th2 cytokines (pg/ml)		Th2/Th1 (pg/pg)	
		IL-2	IFN-γ	IL-4	IL-5	IL-10	IL-10/IL-2
Tri-terpenoid compounds	0	46.0 ± 20.5	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.02	30.8 ± 8.7	10.8 ± 8.1	ND	ND	65.6 ± 15.6ab	2.22 ± 0.67
α-Amyrin (μM)	0.2	32.4 ± 8.8	14.5 ± 11.1	ND	ND	64.7 ± 13.4b	2.08 ± 0.59
	2	29.8 ± 7.7	13.8 ± 13.2	2.59 ± 2.90	ND	72.7 ± 17.8ab	2.55 ± 0.81
Betulinic acid (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.01	25.5 ± 8.1b	5.80 ± 6.67	ND	6.81 ± 7.50	64.2 ± 11.4b	2.84 ± 1.35
	0.1	31.5 ± 15.6a,b	ND	ND	5.05 ± 6.41	70.9 ± 10.9ab	2.70 ± 1.27
	1	34.0 ± 10.2a,b	12.7 ± 12.9	ND	10.7 ± 13.8	73.4 ± 17.1ab	2.37 ± 1.04
Diosgenin (μM)	0	23.4 ± 3.0a	4.27 ± 4.52b	2.59 ± 2.61	8.87 ± 6.54	73.4 ± 10.1a	3.15 ± 0.82
	0.25	14.7 ± 4.2b	29.6 ± 28.5ab	7.18 ± 8.48	3.20 ± 3.55	60.6 ± 2.0ab	2.82 ± 2.54
	2.5	11.1 ± 1.5b	32.1 ± 26.8a	3.64 ± 4.99	4.77 ± 5.36	65.7 ± 14.3ab	5.01 ± 2.61
	25	13.5 ± 3.6b	20.4 ± 16.2ab	ND	5.04 ± 4.46	51.1 ± 10.5b	3.91 ± 0.76
Escin (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03
	0.5	28.4 ± 4.8b	9.93 ± 9.13	ND	ND	75.7 ± 16.9a	2.70 ± 0.61
	5	28.6 ± 6.3b	ND	ND	ND	81.4 ± 23.8a	2.88 ± 0.75
	50	3.5 ± 5.1c	ND	ND	ND	12.8 ± 13.5b	1.80 ± 0.83
18β-Glycyrrhetinic acid (μM)	0	46.0 ± 20.5	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.25	31.3 ± 13.8	18.2 ± 19.3	ND	ND	67.8 ± 19.1	2.54 ± 1.24ab
	2.5	31.5 ± 13.5	15.2 ± 16.0	ND	ND	68.4 ± 23.0	2.52 ± 1.57ab
	25	25.9 ± 16.0	6.05 ± 9.27	ND	4.54 ± 5.35	86.1 ± 36.4	4.10 ± 2.20a
Lupeol (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.05	25.7 ± 4.7b	7.83 ± 9.78	ND	ND	73.5 ± 16.7	2.94 ± 0.81b
	0.5	25.5 ± 9.4b	7.18 ± 11.62	ND	ND	74.4 ± 25.0	3.26 ± 1.63ab
	5	18.0 ± 6.6b	6.40 ± 9.18	ND	8.2 ± 7.5	81.9 ± 18.1	5.05 ± 2.19a
Ursolic acid (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03b
	0.025	25.3 ± 4.5b	7.39 ± 7.39	ND	8.34 ± 7.54	68.0 ± 11.4	2.78 ± 0.76b
	0.25	0.25 ± 5.0b	ND	ND	8.11 ± 6.35	67.4 ± 17.8	3.24 ± 1.25a
	2.5	24.5 ± 8.7b	ND	ND	6.88 ± 10.9	68.9 ± 13.4	3.01 ± 0.81a
β-Sitosterol (μM)	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3a	1.75 ± 1.03b
	0.01	23.5 ± 7.0b	ND	ND	ND	73.0 ± 13.2bc	3.41 ± 1.51a
	0.1	25.7 ± 5.2b	4.49 ± 4.95	ND	ND	65.0 ± 12.8c	2.62 ± 0.72ab
	1	27.7 ± 11.1b	ND	ND	9.21 ± 13.6	64.5 ± 9.1c	2.55 ± 0.79ab
Tetra-terpenoid	0	46.0 ± 20.5a	8.84 ± 12.2	ND	12.8 ± 14.3	85.8 ± 15.3	1.75 ± 1.03
	0.002	29.3 ± 9.0b	10.7 ± 10.3	ND	13.4 ± 14.6	72.1 ± 14.8	2.75 ± 1.25
Lycopene (μM)	0.02	30.0 ± 6.8b	ND	ND	ND	73.6 ± 10.5	2.61 ± 0.91
	0.2	29.1 ± 4.4b	ND	ND	17.5 ± 19.9	73.3 ± 15.9	2.54 ± 0.54

[a] Values are means ± SD (n = 6 biological determinations).
[b] Values within same column under same compound treatment not sharing a common small letter are significantly different ($P < 0.05$) from each other assayed by one-way ANOVA, followed by Duncan's new multiple range test.
[c] The LOD of these kits used in this study was <15.6 pg/ml.
[d] ND, not detectable.

alool, and thymol, may have a strong Th2-inclination and anti-inflammation potential *in vitro*. Interestingly, menthone treatments did not increase IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that menthone may have a relative Th1-inclination among selected mono-terpenoid compounds.

3.3. Effects of 7 selected sesqui-terpenoid compounds on Th1/Th2 cytokine secretions using murine primary splenocytes

Table 4 shows the effects of selected sesqui-terpenoid compounds on Th1/Th2 cytokine secretions. The results showed that trans-caryophyllene, caryophyllene oxide, and parthenolide treatments significantly ($P < 0.05$) decreased IL-2 (Th1) secretion levels. IFN-γ, IL-4 and IL-5 secretion levels in most selected sesqui-terpenoid compound treatments were lower than the LOD of the ELISA kits and were not significantly affected by all selected sesqui-terpenoid compounds. However, α-bisabolol, trans-caryophyllene, farnesol and α-humulene treatments significantly ($P < 0.05$) decreased IL-10 (Th2) secretion levels. Importantly, parthenolide treatments significantly ($P < 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that parthenolide may have a Th2-inclination and anti-inflammation potential *in vitro*. In addition, the other sesqui-terpenoid compounds, except farnesol, selected in this study, slightly ($P > 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting a mild anti-inflammatory potential. Moreover, (−)-trans-caryophyllene treatments significantly ($P < 0.05$) inhibited both IL-2 (Th1) and IL-10 (Th2) cytokine production at the same time, suggesting that (−)-trans-caryophyllene may have an anti-inflammatory potential through the inhibition of T-cell immune responses. Interestingly, farnesol treatments did not increase IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that farnesol may have a relative Th1-inclination among the selected sesqui-terpenoid compounds. Our results suggested that parthenolide may have a strong Th2-inclination and anti-inflammatory potential *in vitro*, but farnesol may have a relative Th1-inclination compared to the other selected sesqui-terpenoid compounds.

3.4. Effects of 3 selected di-terpenoid compounds on Th1/Th2 cytokine secretions using murine primary splenocytes

Table 5 shows the effects of selected di-terpenoid compounds on Th1/Th2 cytokine secretions. The results showed that all selected di-terpenoid compounds andrographolide, oridonin and tri-

ptolide significantly ($P < 0.05$) decreased IL-2 (Th1) secretion levels. IFN-γ, IL-4 and IL-5 secretion levels in most selected di-terpenoid compound treatments were lower than the LOD of the ELISA kits and were not significantly affected by all selected di-terpenoid compounds. However, oridonin and triptolide treatments also significantly ($P < 0.05$) decreased IL-10 (Th2) secretion levels. Importantly, andrographolide treatments significantly ($P < 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that andrographolide may have a Th2-inclination and anti-inflammatory potential *in vitro*. In addition, triptolide slightly ($P > 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting a mild anti-inflammatory potential. Moreover, oridonin and triptolide treatments significantly ($P < 0.05$) inhibited both IL-2 (Th1) and IL-10 (Th2) cytokine production at the same time, suggesting that oridonin and triptolide might have anti-inflammatory potential by inhibiting T-cell immune responses. Our results suggested that andrographolide may have a strong Th2-inclination and anti-inflammatory potential *in vitro* and triptolide may also have a mild and similar potential. Oridonin and triptolide might have anti-inflammatory potential through the inhibition of T-cell immune responses. Interestingly, oridonin treatments did not increase IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that oridonin may have a relative Th1-inclination among the selected di-terpenoid compounds.

3.5. Effects of 8 selected tri-terpenoid and 1 tetra-terpenoid compounds on Th1/Th2 cytokine secretions using murine primary splenocytes

Table 6 shows the effects of selected tri-terpenoid and tetra-terpenoid compounds on Th1/Th2 cytokine secretions. Among the tri-terpenoid compounds, the results showed that betulinic acid, diosgenin, escin, lupeol, ursolic acid and β-sitosterol significantly ($P < 0.05$) decreased IL-2 (Th1) secretion levels. IL-4 and IL-5 secretion levels in most of the selected tri-terpenoid compound treatments were lower than the LOD of the ELISA kits and were not significantly affected by all of the selected tri-terpenoid compounds. Interestingly, diosgenin treatments significantly ($P < 0.05$) increased IFN-γ (Th1) secretion levels although the IFN-γ secretion levels in most of the selected tri-terpenoid compound treatments were lower than the LOD of the ELISA kit. However, α-amyrin, betulinic acid, diosgenin, escin and β-sitosterol treatments also significantly ($P < 0.05$) decreased IL-10 (Th2) secretion levels. Importantly, 18β-glycyrrhetinic acid, lupeol, ursolic acid and β-sitosterol treatments significantly ($P < 0.05$) increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that 18β-glycyrrhetinic acid, lupeol, ursolic acid, and β-sitosterol may have a Th2-inclination and anti-inflammatory potential *in vitro*. In addition, diosgenin, betulinic acid, escin and β-sitosterol treatments significantly ($P < 0.05$) inhibited both IL-2 (Th1) and IL-10 (Th2) cytokine production at the same time, suggesting that betulinic acid, escin and β-sitosterol may have anti-inflammatory potential through the inhibition of T-cell immune responses. Our results suggested that 18β-glycyrrhetinic acid, lupeol, ursolic acid and β-sitosterol may exert their anti-inflammatory effects by modulating the immune balance towards a Th2-polarization. However, betulinic acid, escin, and β-sitosterol may have an anti-inflammatory potential through the inhibition of T-cell immune responses. Among the selected tri-terpenoid compounds, β-sitosterol seems to possess these two different immunomodulatory properties.

As to the tetra-terpenoid compound, the results showed that lycopene significantly ($P < 0.05$) decreased IL-2 (Th1) secretion levels (Table 6). IFN-γ, IL-4 and IL-5 secretion levels in lycopene-treated splenocyte cultures were lower than the LOD of the ELISA kits and were not significantly affected by lycopene. However, lycopene treatments slightly, but not significantly ($P > 0.05$), increased IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that lycopene may have a mild Th2-inclination and anti-inflammatory potential *in vitro*.

4. Discussion

This study examined 27 selected terpenoid compounds, including 8 monoterpenoids, 7 sesqui-terpenoids, 3 di-terpenoids, 8 tri-terpenoids, and 1 tetra-terpenoid subjected to a bio-assay using mouse primary splenocytes (Table 1). Our results found that terpenoid compounds have differential cytotoxic effects on immune cells. Among the 27 selected terpenoid compounds, triptolide, a diterpenoid, showed to be most cytotoxic, as reflected by its IC_{50} value (46 nM). However, the other selected terpenoid compounds showed a comparative low cytotoxicity compared to triptolide. Triptolide, which is a di-terpenoid from *Tripterygium wilfordii*, has been implicated in anti-inflammation in airways, anti-autoimmune diseases and anti-cancer (Table 1) (Mo et al., 2010; Qiu & Kao, 2003; Tao et al., 2008; Zhang & Ma, 2010). Most of the terpenoids were found to have the potential to induce apoptosis in cancer cells (Thoppil & Bishayee, 2011) and anti-inflammatory effects by inhibiting the nuclear factor (NF)-κB-related signalling pathways (de las Heras & Hortelano, 2009). This study further proved the strong cytotoxic effect of triptolide on immune cells. The cytotoxicities of these terpenoids should circumspectly be considered when using these compounds for health benefits (Table 2). Most terpenoids are lipophilic substances that may target cell membranes, controlling the chemical infiltration into the membrane, mobility and cell signalling, or modulating the mevalonate pathway to induce cell death (Zore, Thakre, Jadhav, & Karuppayil, 2011). Based on the cytotoxicities of the selected terpenoid compounds, it seems that the higher the carbon number, the higher the cytotoxicity of the terpenoids towards immune cells (Table 2), suggesting that the higher molecular weight terpenoids may induce cell membrane instability and death more easily. In addition, the position of the oxygen on the terpenoid carbon number, may cause differential cytotoxic effects (Zhou, Yang, Ding, Li, & Miao, 2012). According to our experiments, the relationship between the chemical structure and cytotoxicity of terpenoid compounds could not be totally deduced and clarified (Table 2). This study suggests the use of appropriate concentrations of selected terpenoid compounds for splenocyte cultures (Tables 3–6). However, the cytotoxicity of terpenoid compounds towards different cells, may involve different and complicated mechanisms. More data is needed to clarify the cytotoxicity of terpenoid compounds.

To evaluate the effects of terpenoids on Th1/Th2 cytokine secretions, selected terpenoid compounds at the indicated non-cytotoxic concentrations were added to mouse splenocyte cultures for 48 h. Changes in IL-2, IFN-γ, IL-4, IL-5 and IL-10 secretion levels were determined. To date, it is clear that Th1 cells secret pro-inflammatory cytokines, such as IFN-γ, IL-2 and IL-12, however Th2 cells produce anti-inflammatory cytokines, such as IL-4, IL-5, IL-6, and IL-10 (Huang, Zhang, & Zhang, 2009). In the present study, most IFN-γ, IL-4, and IL-5 secretion levels in terpenoid compounds in treated splenocyte cultures were lower than the LOD of the ELISA kits and were not significantly influenced by the selected terpenoid compounds. Consequently, IL-2 and IL-10 were selected as Th1 and Th2 cytokine indicators. Most importantly, 4 monoterpenoid compounds, eucalyptol, limonene, linalool, and thymol (Table 3), 1 sesqui-terpenoid compound parthenolide (Table 4), 1 di-terpenoid compound andrographolide (Table 5), 4 tri-terpenoid compounds, 18β-glycyrrhetinic acid, lupeol, ursolic acid, and β-sitosterol (Table 6), showed a strong Th2-inclination and anti-inflammatory potential *in vitro*. In addition, (−)-trans-caryophyllene, oridonin, triptolide, diosgenin, betulinic acid, escin, and

β-sitosterol treatments significantly ($P < 0.05$) inhibited both IL-2 (Th1) and IL-10 (Th2) cytokine production at the same time, suggesting that these terpenoid compounds also have an anti-inflammatory potential through the inhibition of T-cell immune responses (Tables 4–6). Interestingly, β-sitosterol possessed these two different immunomodulatory properties, suggesting a promising potential for immunomodulation in the future (Mahajan & Mehta, 2011). Undoubtedly, most terpenoid compounds are reported to have an anti-inflammatory potential (Table 1). Our results are identical to previous studies and further suggest that certain terpenoid compounds may exert their anti-inflammatory effects through the modulation of Th1/Th2 cytokine secretion profiles or inhibition of T-cell immune responses.

Most terpenoids seem to have apparent Th2-inclination properties. However, diosgenin treatments significantly ($P < 0.05$) increased IFN-γ (Th1) secretion levels by mouse primary splenocytes, suggesting diosgenin may be useful to treat virus infections through the stimulation of IFN-γ production which in turn interferes with the virus replication. Importantly, our results disclosed a new immunomodulatory function of diosgenin to known possible effects (Table 1) (Jung et al., 2010; Uemura et al., 2011). Interestingly, menthone, farnesol and oridonin treatments did not increase IL-10/IL-2 (Th2/Th1) cytokine secretion ratios, suggesting that menthone, farnesol and oridonin may have a relative Th1-inclination property among the selected mono-, sesqui- and di-terpenoid compounds (Tables 3 and 4). The relative Th1-inclination property of menthone, farnesol and oridonin may be applied to improve Th2-skewed allergic diseases. Methanol has been found to exert anti-allergic effects; our results are identical to previous published studies (Inoue, Sugimoto, Masuda, & Kamei, 2001). Farnesol has been found to attenuate 1,2-dimethylhydrazine induced oxidative stress, inflammation and apoptotic responses in the colon of Wistar rats and to improve metabolic abnormalities in mice (Goto et al., 2011; Khan & Sultana, 2011); however, the present study further unveiled its immunomodulatory potential through the modulation of Th1/Th2 cytokine secretions. It has been found that oridonin can modulate the differentiation of rat splenocyte's $CD4^+/CD25^+$ regulatory T cells and regulate the Th1/Th2 immune balance by increasing heme oxygenases (HO)-1 mRNA and protein expression (Hu, Du, Li, & Liu, 2008). We hypothesized that active terpenoid compounds might modulate the differentiation of Th cells; however, more experiments should be performed in the future to clarify their possible mechanisms.

There are still some limitations to this study. It is recognized that the representative marker cytokine for Th1 is IFN-γ, and for Th2 is IL-4. Most of the selected terpenoid compounds could not stimulate IFN-γ and IL-4 secretion; therefore IL-2 was selected as a Th1 indicator and IL-10 as a Th2 indicator. IL-2 is a common T cell growth factor, although it is primarily produced by effector T cells (i.e. Th1 cells). IL-10 is mainly produced by Th2 cells, macrophages or other APCs. Using the single ratio of IL-10/IL-2, to reflect the status of Th2/Th1 immunomodulatory potential by splenocytes, might not be absolutely correct. This study brought some achievements, however experiments using B or T cell-depleted splenocytes should be repeated in the future to verify our hypothesis.

5. Conclusions

This study evaluated 27 selected terpenoid compounds for their Th1/Th2 immunomodulatory potential using mouse primary splenocytes. The results indicated that triptolide, a diterpenoid, exhibited the highest cytotoxicity with a IC_{50} value of 46 nM. Eucalyptol, limonene, linalool, thymol, parthenolide, andrographolide, 18β-glycyrrhetinic acid, lupeol, ursolic acid and β-sitosterol showed a strong Th2-inclination and anti-inflammatory potential *in vitro*. (−)-trans-caryophyllene, oridonin, triptolide, diosgenin, betulinic acid, escin, and β-sitosterol treatments significantly inhibited both IL-2 (Th1) and IL-10 (Th2) cytokine production at the same time, suggesting that these terpenoid compounds have an anti-inflammatory potential through the inhibition of T-cell immune responses. Diosgenin treatments significantly increased IFN-γ secretion levels using mouse splenocytes, suggesting diosgenin may be useful to treat virus infections through the stimulation of IFN-γ production. Menthone, farnesol and oridonin treatments did not markedly increase IL-10/IL-2 (Th2/Th1) cytokine secretion ratios compared to the other selected terpenoid compounds, suggesting that menthone, farnesol and oridonin may have a relative Th1-inclination property among the selected terpenoid compounds. The relative Th1-inclination property of menthone, farnesol and oridonin may be applied to improve Th2-skewed allergic diseases.

Acknowledgement

This study was kindly supported by a research Grant NSC101-2313-B-005-042-MY3 from the National Science Council, Taipei, Taiwan, ROC.

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