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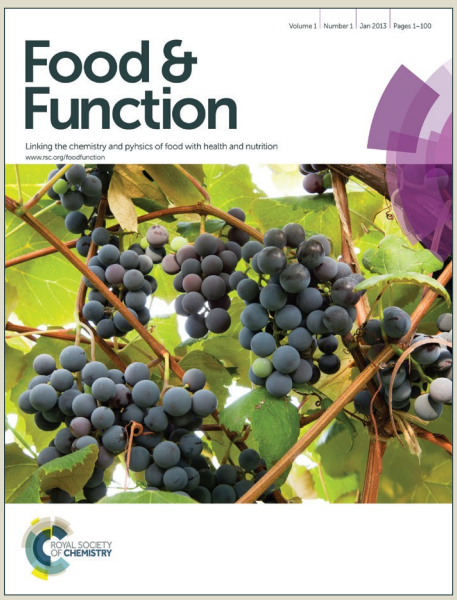


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# Inhibitory effects of wild bitter melon leaf extract on *Propionibacterium acnes*-induced skin inflammation in mouse and cytokine production *in vitro*

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Short title: Anti-acne activity of wild bitter melon leaf

## Abstract

*Propionibacterium acnes* is a key pathogen involved in acne inflammation. Wild bitter melon (WBM, *Momordica charantia* L. var. *abbreviate* Seringe), is consumed as both a vegetable and as folk medicine in Taiwan. We examined the inhibitory activity of the total phenolic extract (TPE) of WBM leaf on *P. acnes*-induced inflammatory responses *in vivo* and *in vitro*. Our data showed that TPE significantly attenuated *P. acnes*-induced ear swelling in mice along with microabscess. Flow cytometry analysis revealed that TPE treatment significantly decreased the migration of neutrophils and IL-1β+ populations *in vivo*. In *P. acnes*-stimulated human monocytic THP-1 cells, TPE suppressed the mRNA levels and production of interleukin (IL)-8, IL-1β, and tumor necrosis factor (TNF)-α *in vitro*. In addition, TPE suppressed *P. acnes*-induced matrix metalloproteinase-9 levels. TPE blocked nuclear factor-κB (NF-κB) activation and inactivated mitogen-activated protein kinases (MAPK), these actions may partially account for its inhibitory effect on cytokine production. The quantitative HPLC analysis revealed gallic, chlorogenic, caffeic, ferulic, and cinnamic acids, myricetin, quercetin, luteolin, apigenin, and thymol in TPE. All these phenolics significantly suppressed *P. acnes*-induced IL-8 production i*n vitro*. Our results suggest that WBM leaf extract effectively inhibit *P. acnes*-induced inflammatory responses and may be useful to relieve the inflammation of acne.

**Key words:** wild bitter melon leaf, *Propionibacterium acnes*, anti-inflammation

## Introduction

Acne is one of the most common skin diseases. The pathogenesis of acne vulgaris is complex and incompletely understood, although it is believed that inflammation is a key component.[1] *Propionibacterium acnes*, a gram-positive anaerobic bacterium species, may play a major role in the initiation of the inflammatory reaction.[2] *P. acnes* triggers pro-inflammatory cytokine release from inflammatory cells via activation of toll-like receptor 2, which in turn activates an intracellular signaling cascade resulting in the release of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-8, and IL-1β.[3] Of these cytokines, IL-8 is the major inflammatory mediator and a strong chemotactic factor for neutrophils, basophils, and T cells. IL-8 has been implicated in mounting an inflammatory response in acne lesions.[4] Therefore, suppression of *P. acnes*-induced inflammatory response is a prime target for the treatment of acne vulgaris. Moreover, aberrant remodeling of the extracellular matrix occurs in acne lesions, and its breakdown is mainly accomplished by matrix metalloproteinases (MMPs). *P. acnes*-induced MMP-9 has been shown to be involved in acne pathogenesis.[5] Modulation of MMPs seems to be a useful therapeutic option in the treatment of inflammatory conditions in which excess MMP activity has been implicated.[6]

Wild bitter melon (WBM; *Momordica charantia* L.var. *abbreviata* Seringe), also

called wild bitter melon, is a wild variety of bitter melon (*Momordica charantia*). In Taiwan, WBM fruits are consumed as vegetables and have been used as a folk remedy for a variety of diseases, particularly diabetes. WBM fruit extract and its components have been shown to possess numerous pharmacological actions including the antioxidant and anti-inflammatory activities, and hepatoprotection against alcoholic fatty liver.[7-9] Besides its fruit being consumed, the young shoots and leaves of WBM are traditionally eaten as greens by the indigenous peoples of Taiwan. The young tender leaves of *M. charantia* are also eaten as a vegetable in the Philippines and in Indonesia.[10] Leaf extracts of *M. charantia* have been demonstrated to possess broad-spectrum antimicrobial and potent antioxidant activities.[11, 12] Recently, it was shown that WBM leaf extract possesses significant antioxidant, cyto-protective and anti-melanogenic activities.[13] However, scientific literature concerning the biological properties of the WBM leaf remains limited.

This study is aimed at exploring the suppressive effects of a polyphenol-enriched extract of WBM leaves on *P. acnes*-induced inflammatory responses *in vivo* and *in vitro*. Furthermore, the molecular mechanism of the *in vitro* anti-inflammatory activity was addressed. Thus, this study provides new knowledge regarding the anti-acne potential of WBM leaf extract.

## Materials and methods

### Preparation of total phenolic extract of WBM leaves

A cultivar of WBM (Hualien No.1) was grown in the Hualien District Agricultural Research and Extension Station, Hualien, Taiwan. WBM leaves were collected and then a voucher specimen was deposited in the Department of Human Development and Family Studies, National Taiwan Normal University. After cleaning with water, the leaves of WBM were air-dried. The polyphenol-enriched extract of WBM leaves was prepared using a procedure described by Kumar et al., [14] with some modification. In brief, five gram of dried leaves was mixed with 50 mL of methanol/HCl (100:1, v/v); the mixture was then centrifuged at 5,000g; and supernatant was evaporated to dryness under reduced pressure (45-50 °C). The residue was re-dissolved in 25 mL of water/ethanol (80:20, v/v) and extracted 4 times with 25 mL of ethyl acetate. The organic fractions were combined, dried for 30-40 min with anhydrous sodium sulfate, and filtered through a Whatman-40 filter, and evaporated to dryness under vacuum (45-50 °C) to obtain a total phenolic extract (TPE) of WBM leaves. The TPE was reconstituted in dimethyl sulfoxide (DMSO) for the subsequent experiments. The yield of TPE was 19.2%.

### *P. acnes*-induced inflammation *in vivo*

Eight-week-old male ICR mice were purchased from the Animal Center of

College of Medicine, National Taiwan University, Taipei, Taiwan. All animal experiments were approved by the Animal Care Committee of the National Taiwan Normal University. Mice were fed with chow diet and water *ad libitum*. To examine the anti-inflammatory effect of TPE and luteolin (as a control) *in vivo*, an intradermal injection model was employed.[8] In the preliminary test, 10 μL of TPE (up to 0.5mg/site) or luteolin (up to 0.05mg/site) was intra-dermally injected into mice ears. No noticeable skin irritation occurred (data not shown). Hence, TPE (0.5 mg/site) and luteolin (0.05 mg/site) were used for the following experiments. For measuring inhibitory effect of TPE and luteolin on ear edema, mice were randomly grouped (n = 5 per group). *P. acnes* ($6 \times 10^7$ CFU per 10 μL in phosphate-buffered saline, PBS) was intradermally injected into the left ear of ICR mice. Right ears received an equal amount (10 μL) of PBS. Ten microliters of TPE or luteolin in 5% DMSO in PBS was injected into the same location of both ears right after *P. acnes* or PBS injection. Twenty-four hours after bacterial injection, the increase in ear thickness was measured using a micro-caliper (Mitutoyo, Kanagawa, Japan). Mice were then sacrificed with carbon dioxide asphyxiation. Each 4 mm punch ear disk was taken immediately and weighed. The extent of edema was evaluated by the weight difference between the left and the right ear disks. The increase in ear thickness and weight of the *P. acnes*-injected ear was calculated and expressed as percentage of the PBS-injected

control. For histological observation, the paraffin embedded ears were vertically cut into cross-sections through the ear central cartilage. The cross-sections were stained with hematoxylin and eosin (H&E) and then viewed on a microscope.

**Isolation of inflammatory cells from ear tissues and flow cytometric analysis**

In a separate experiment, mice were intradermally injected in the ear with *P. acnes*/TPE, *P. acnes*/luteolin, *P. acnes* alone, or PBS alone as described above. Twelve hours after the initial injection, the ears were excised (n=5). Ears were carefully split into dorsal and ventral halves using forceps and then crushed through a 70-μm cell strainer (BD Biosciences) into the RPMI medium to disperse cells. Each sample was then brought to a final volume of 4 mL with RPMI medium. Cells were washed with PBS and filtered again through a 40-μm cell strainer (BD Biosciences). Single- cell suspensions were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ly6G, a neutrophil marker (BioLegend, San Diego, CA, USA), and peridinin chlorophyll protein (PerCP)-conjugated anti-mouse CD45, a common leukocyte marker (BioLegend), in the FACS buffer (PBS containing 0.5% bovine serum albumin and 0.09% sodium azide) for 30 min and washed three times with FACS buffer. For intracellular cytokine analysis, cells were washed, surface stained as above, fixed, and permeabilized for intracellular staining of allophycocyanin (APC)-conjugated anti-IL-1β (eBioscience, San Diego, CA, USA) as instructed by the

manufacturer. Samples were analyzed with FacsCantoII (BD Biosciences) using FACS Diva software, and the data were analyzed using FlowJo software.

**Determination of the viability of THP-1 cells**

The human monocytic THP-1 cell line (BCRC 60430) was obtained from the Bioresource Collection and Research Center and maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere with 5% $CO_2$. Cell viability of THP-1 cells was assessed using the AlamarBlue Assay (Invitrogen, Carlsbad, CA, USA). Cells ($1\times10^6$ cells/ml) were cultured in 96-well culture plates in medium and treated with various concentrations of tested samples. After 24 h of incubation, 20 μL of AlamarBlue® reagent was added to each well. After 2 h of incubation, the optical density (OD) of the resulting medium was measured. Measurements of reduction of AlamarBlue® were taken as the absorbance values at 570 and 600 nm, using a Synergy HT multidetection microplate reader (Bio-Tek, Nevada, USA).

**Measurement of cytokine production in human monocytic THP-1 cells**

The strain of *P. acnes* (BCRC10723, isolated from facial acne) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). *P. acnes* was cultured in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) with 1%

glucose. The bacteria were cultured in an anaerobic atmosphere using BBL GasPak systems (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). To prepare the *P. acnes* suspension for the sequential stimulation of cells, the log-phase bacterial *P. acnes* culture was harvested, washed three times with PBS, and then centrifuged at 10,000g for 5 min. After two additional washes using PBS, the *P. acnes* pellet was re-suspended in RPMI medium. THP-1 cells ($1\times10^6$ cells/mL) were seeded in 24-well plates with serum-free medium, and were stimulated with live *P. acnes* (7.5 $\times10^7$ CFU/mL; multiplicity of infection (M.O.I.) = 75) alone or in combination with different concentrations of TPE and luteolin (as a control) for a 24-h incubation. Cell-free supernatants were collected, and concentrations of TNF-α, IL-1β, and IL-8 were analyzed with respective enzyme immunoassay kits (Invitrogen, Carlsbad, CA, USA).

**RNA isolation and quantitative real-time polymerase chain reaction (PCR)**

Total RNA was isolated using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. 2 μg of total RNA of each sample was reverse-transcribed with 1 μL of reverse transcriptase (Promega, Madison, WI, USA) using an oligo (dT) primer according to the manufacturer's instructions. Following primers were used for IL-1β cDNA amplification: 5'-AAGCTGAGGAAGATGCTG-3' (forward) and 5'-ATCTACACTCTCCAGCTG-3'

(reverse); for IL-8 gene: 5'-TGCCAAGGAGTGCTAAAG-3' (forward) and 5'-CTCCACAACCCTCTGCAC-3' (reverse); for TNF-α gene: 5'-TCTTCTGCCTGCACTTTGG-3' (forward) and 5'-ATCTCTCAGCTCCACGCCATTG-3' (reverse); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as a housekeeping gene) cDNA amplification: 5'-GTGAAGGTCGGAGTCAACG-3' (forward) and 5'-TGAGGTCAATGAAGGGGTC-3'(reverse). The primers amplified a 157 bp fragment of the IL-8 cDNA, a 300 bp fragment of the IL-1β cDNA, a 224 bp fragment of the TNF-α cDNA, and a 113 bp fragment of the GAPDH cDNA. Real-time PCRs were conducted in an iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA, USA) using iQ$^{TM}$ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Thermal cycling conditions for all assays were initial denaturation at 95 ℃ for 3 min and 40 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. Melting analysis was performed by denaturing at 95℃ for 1 minute and cooling to 55℃ for 1 minute followed by heating at the rate of 0.5℃/cycle with holding 10 s from 55℃ to 95℃. The relative amounts of the PCR products were analyzed by iQ™5 optical system software, vers. 2.1. The messenger (m)RNA level of each sample for each gene was normalized to that of the GAPDH mRNA.

**Detection of MMP-9 expression and MAPK phosphorylation by Western blot**

**analysis**

Human monocytic THP-1 cells were seeded at $2 \times 10^6$ cells/mL in 6-cm dishes and were stimulated with viable *P. acnes* (M.O.I.= 75) alone or co-incubated with various concentrations of tested samples. Cells were harvested and washed with PBS, after 2 h and 24 h of treatments for the measurement of MAPK phosphorylation and MMP-9 level, respectively. Whole cell lysates were prepared in a lysis buffer (Cell Signaling, Beverly, MA, USA) containing 10 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates were sonicated and cleared by centrifugation at 4°C, 14,000 rpm for 10 min. Protein concentrations were determined by DC protein assay (Bio Rad). Aliquots of the lysates (each containing 30 μg of protein) were boiled for 15 min and electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Following SDS- polyacrylamide gel electrophoresis, proteins were transferred to PVDF membranes. Membranes were blocked by incubation in gelatin-NET buffer at room temperature, and then incubated with 1:1000 dilution of primary antibodies of MMP-9 (Epitomics, Burlingame, CA, USA), MAPK, phosphor-MAPK (Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (Sigma-Aldrich), followed by horseradish peroxidase-conjugated secondary antibody according to the manufacturer's instructions. The immunoreactive proteins were detected using the enhanced ECL chemiluminescence Western Blotting Detection System (ChemeDoc

XRS, Bio-Rad). Signal strengths were quantified using densitometric program (Image Lab, Bio-Rad).

**Determination of NF-κB activation**

To determine the effect of TPE of WBM on *P. acnes*-induced activation of NF-κB in THP-1 cells, human monocytic THP-1 cells ($3 \times 10^6$ cells/mL) cultured in serum-free medium were stimulated with *P. acnes* (M.O.I.= 75) alone or in combination with tested samples. After 16 h incubation, determination of free p65 in nuclear extracts was performed according to the manufacturer's protocol (NF-κB/p65 ActivELISA kit, Imgenex; San Diego, CA, USA). Briefly, the cytoplasmic fraction was collected in the supernatant of whole-cell lysates after centrifugation at 12,000*g* for 30 s at 4 °C. The nuclear pellet was re-suspended in 100 μL nuclear lysis buffer at 4 °C for 30 min, and the suspension was centrifuged at 12,000*g* for 10 min at 4°C. The supernatant containing the nuclear fraction was subjected to an enzyme-linked immunosorbent assay (ELISA). ELISA plates were pre-coated with anti-p65 antibody, and the presence of p65 was detected by the addition of a second anti-p65 antibody followed by alkaline phosphatase-conjugated secondary antibody and colorimetric analysis at 405 nm.

**Determination of total phenolic content**

The amount of total phenolics in TPE was evaluated using spectrophotometric

analysis with Folin-Ciocalteu reagent. Briefly, Folin-Ciocalteu phenol reagent was added to the reconstituted samples and held for 3 min. Then 2 mL of 10% (w/v) sodium carbonate solution was added and allowed to stand at room temperature for 30 min. The absorbance at 765 nm was measured. The total phenolic content was calculated by a standard curve prepared with gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per gram of solid of extract.

**Phenolic Constituent Determination by HPLC**

Phenolic constituents of TPE were determined using a HPLC method as described by Kubola et al.[12] with some modification. Then standard phenolics were used as standards for HPLC determination of individual phenolics. Gallic acid, chlorogenic acid, caffeic acid, ferulic aicd, cinnamic acid, myricetin, quercetin, apigenin, thymol, and carvacrol were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Luteolin purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetic acid, methanol, and water were HPLC grade.

Ten milligram of TPE was dissolved in 1 mL of methanol, and the solution was filtered through a 0.2-μm phobic PTFE syringe filter (Millipore, MA, USA). HPLC analysis was performed using HPLC pumps (Ecom LCP 4100,Czech Rep.) equipped with a UV detector (Ecom LCD 2084, Czech Rep.) and chromatographic separations were performed on a LiChrospher® 100 RP-18E (5μm) HPLC column (125×4 mm

i.d., Merck Millpore, Darmstadt, Germany).

The mobile phase was a mixture of solvent A (water/methanol, 98:2), and solvent B (methanol/acetic acid, 98:2) according to a linear gradient elution. The following gradient was used: 0-10 min, from 80% A and 20% B to 50% A and 50% B; 10-25 min, from 50% A and 50% B to 30% A and 70% B; and 25-30 min, from 30% A and 70% B to 80% A and 20% B with a flow rate of 0.8 mL/min. The absorbance of elutes was measured at 280 nm, near the maximum absorption of most phenols. The injection volume was 20 μL and all samples were analyzed in triplicate. Identification of the individual compounds was based on the comparison of the retention times of unknown peaks to those of reference authentic standards. Chromatographic processing was done using the Peak-ABC Chromatography Data Handling System.

**Statistical analysis**

All data are presented as means ± SD. Statistical analyses were performed using the SPSS 19.0 statistical package (Chicago, IL, USA). The Mann–Whitney U-test was used to compare differences between the vehicle and treatments. A p value of $< 0.05$ was considered statistically significant.

## Results

### Effect of TPE on *P. acnes*-induced skin inflammation in mouse

Prior to the determination of anti-inflammatory effect of TPE and luteolin *in vivo*, an intradermal injection test was performed to evaluate their skin irritation effect. Intradermal administration of TPE (0.5 mg/site) alone or luteolin alone (0.05 mg/site) produced no apparent irritation, such as ear swelling, redness, and cutaneous erythema, and no significant infiltration of neutrophils (data not shown). To examine the *in vivo* anti-inflammatory effect of TPE, mouse ears were intradermally injected with viable *P. acnes* for one day. Infiltrated neutrophils were observed at an H&E-stained cross-section of the *P. acnes*-injected ear (Fig. 1A). Co-injection of TPE and luteolin significantly reduced *P. acnes*-induced ear swelling measured by ear thickness (Fig. 1A) and ear biopsy weight (Fig. 1A). TPE's control over the cellular infiltration in a *P. acnes*-induced inflammatory process was confirmed by the quantification of total leukocytes (CD45+) and IL-1β+ populations in the inflamed ear tissue (Figs. 1B and 1C). A predominantly neutrophil (CD45+Ly6G+) infiltration was seen during infection, with significantly elevated intracellular expression level of IL-1β after 12 h bacterial injection (Fig. 1B). Flow cytometric analysis also showed a reduction of neutrophil numbers and IL-1β+ populations in the TPE-treated group as compared to the vehicle group (Fig. 1C). TPE treatment attenuated the microabscess response to *P.*

*acnes* as compared to vehicle group (Fig. 1). These data provide evidence that epicutaneous application of TPE effectively suppressed *P. acnes*-induced inflammation *in vivo*.

**Effects of TPE on *P. acnes*-induced cytokine production *in vitro***

In our preliminary study, luteolin (up to 20 μM) had no significant cytotoxicity toward THP-1 cells. At concentrations of 5 and 10 μM, treatment of luteolin significantly inhibited IL-8 production by *P. acnes*-treated THP-1 cells (data not shown). Therefore, luteolin at a concentration of 10 μM was used for the subsequent experiments as a control. TPE (up to 50 μg/mL) had no significant cytotoxicity toward THP-1 cells and did not affect the secretion of IL-8, IL-1β or TNF-α by THP-1 cells in the absence of *P. acnes* (data not shown).

Following treatment with *P. acnes*, the secretion of pro-inflammatory cytokines of THP-1 cells was significantly increased (Fig. 2A). Treatment of TPE significantly suppressed the *P. acnes*-induced IL-8, IL-1β and TNF-α production (Fig. 2A). We further analyzed the mRNA levels of pro-inflammatory cytokines by quantitative real-time PCR. As shown in Figure 2B, TPE suppressed the gene expressions of IL-8, IL-1β, and TNF-α in *P. acnes*-stimulated THP-1 cells.

**Effects of TPE on *P. acnes*-induced MMP-9 levels *in vitro***

*P. acnes*-induced MMP-9 expression was shown to be involved in acne

pathogenesis.[5] The effects of TPE and luteolin on *P. acnes*-induced MMP-9 levels were examined. Results of the Western blot analysis are shown in Figure 3. TPE reatments led to significant inhibition of the MMP-9 level.

**TPE inhibited MAPK phosphorylation and *NF-κB activation* in *P. acnes*-stimulated THP-1 cells**

To elucidate the underlying anti-inflammatory mechanism, we evaluated the inflammation-related signaling cascades, MAPK including extracellular signal-related kinase (ERK), p38-mitogen-activated kinase (p38), and c-Jun N-terminal kinase (JNK). The levels of phosphorylated p38, ERK, and JNK, were measured by Western blot. *P. acnes* treatment of THP-1 cells induced a phosphorylation of MAPK peaking at 2h (Fig. 4A). In the subsequent experiments, the levels of phosphorylated p38, JNK, and ERK were evaluated after 2 h of stimulation. The results showed that TPE treatment decreased *P. acnes*-induced phosphorylation of p38, ERK, and JNK (Fig. 4B).

The transcription factor NF-κB regulates the expression of a wide variety of pro-inflammatory genes. Activation of p65 was determined by ELISA, and the results showed that the NF-κB p65 translocation in THP-1 cells was increasing in a time-dependent manner. The activation of NF-κB showed a statistically significant increase after 8 h of exposure to *P. acnes* and up to 2-fold increase after 16 h of

stimulation (Fig. 5A). Treatment of TPE significantly attenuated the NF-κB p65 translocation in *P. acnes*-stimulated THP-1 cells (Fig. 5B).

**Total phenolic content and phenolic constituents of TPE**

The total phenolic content of TPE was 58.7 ± 1.8 mg GAE/g dry weight (DW). The examination of HPLC chromatograms revealed the presence of several phenolic compounds in TPE (Fig. 6). HPLC profiles of eleven standard phenolic compounds could be obtained. There were still several unidentified HPLC peaks. The possible identified phenolic compounds of TPE were gallic acid (0.03 mg/g), chlorogenic acid (1.36 mg/g), caffeic acid (0.05 mg/g), ferulic acid (0.05 mg/g), cinnamic acid (0.05 mg/g), myricetin (0.12 mg/g), quercetin (0.26 mg/g), luteolin (0.01 mg/g), apigenin (0.68 mg/g), and thymol (5.88 mg/g).

**Effects of phenolic constituents of TPE on *P. acnes*-induced IL-8 production *in vitro***

The effect of phenolics on THP-1 cell viability was determined. Cells cultured with gallic acid (20 μM), chlorogenic acid (10 μM), caffeic acid (10 μM), ferulic acid (10 μM), cinnamic acid (50 μM), myricetin (50 μM), quercetin (10 μM), apigenin (50 μM), and thymol (50 μM) for 24h did not affect the cell viability (data not shown). When *P. acnes*-stimulated THP-1 cells were treated with different concentrations of phenolics, the inhibition of IL-8 production was detected significantly (Fig. 6B).

## Discussion

Besides being as a vegetable, the crushed leaves of *M. charantia* are applied to the body as a remedy for skin diseases and burns in Malaysia and India.[10] Although *M. charantia* leaves have been traditionally used in treatments for skin diseases, the potential effects of WBM leaf for relieving inflammatory acne lesions has not yet been fully explored. We previously reported that ethyl acetate (EA) extract of WBM fruit and its bioactive components inhibited *P. acnes*-induced pro-inflammatory cytokine releases *in vitro*.[8] Phytol (a diterpene) and lutein (a carotenoid) present in WBM fruits showing the activation of peroxisome proliferator-activated receptor (PPAR) α or γ may contribute, at least in part, to the anti-inflammatory activity.[8] Polyphenols are considered to be possibly effective in treating certain dermatological conditions, however, available evidence concerning their benefit effect on acne vulgaris is limited.[15] Luteolin has been demonstrated to be a potent anti-inflammatory agent,[16, 17] However, little is known about its effect on *P. acnes*-induced inflammatory reactions. To prove that the anti-inflammatory activities of a polyphenol-enriched extract of WBM leaves and luteolin were observed *in vivo*, an intra-dermal injection of *P. acnes* into mice ear was employed. Nakatsuji et al.[18] reported that an intradermal injection of living *P. acnes* into the mouse ear leads to CD11b+ macrophages infiltration at *P. acnes*-injected site and induces an increase in granulomatous response.

As shown in Fig. 1A, the injection of *P. acnes* in the ears of mice resulted in significant ear swelling within 24 hours and a strong inflammatory response histologically comprised primarily of neutrophils. To test further *in vivo* effect of TPE on the *P. acnes*-induced skin inflammation, we examined infiltrated cells and intracellular cytokine expression by flow cytometry (Figs. 1B and 1C). TPE effectively ameliorated the *P. acnes*-induced edema formation, migration of neutrophils and IL-1β+ populations (Fig. 1). Acne is characterized by neutrophilic infiltration of the skin.[19] Neutrophil activation results in increased the expression of inflammatory mediators. Hence, a reduction of neutrophilic inflammatory response to *P. acnes* by TPE may mediate its anti-inflammatory properties.

*P. acnes* may contribute to the development of inflammatory lesions by releasing chemotactic substances, and stimulating the secretion IL-1β, TNF-α, IL-8, and IL-12 by monocytic cells.[20] Indeed, the high levels of IL-1β were observed in human acne lesion and in mouse skin lesion induced by *P. acnes* and in *P. acnes*-exposed human monocytes.[19] However, the molecular mechanisms of TPE involved in *P. acnes*-exposed cells of human monocytic lineage remain largely unknown. We therefore applied *P. acnes*-induced inflammatory reactions in human monocytic THP-1 cells *in vitro*. Our results showed that TPE significantly reduced *P. acnes*-induced IL-8, IL-1β, and TNF-α production *in vitro* (Fig. 2A). To determine

whether TPE attenuate *P. acnes*-induced cytokine production at either translational or transcriptional levels, mRNA levels of pro-inflammatory cytokine genes induced by *P. acnes* were measured by real-time PCR. The up-regulated mRNA levels of IL-8, IL-1β, and TNF-α were inhibited by TPE treatment (Fig. 2B), indicating that TPE inhibit the expression of IL-8, IL-1β, and TNF-α at the transcriptional level.

The genes of MMPs are expressed in response to stimulation by many pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6.[21] In most tissues, the constitutive expression of MMPs is low and their over-expression at the sites of inflammation is considered to be critical in extracellular matrix degradation.[22] *P. acnes*-induced MMPs were considered to contribute to the inflammatory process and cause tissue destruction, leading to scar formation.[5] We previously found that EA extract of WBM fruit reduced *P. acnes*-induced MMP-9 levels in THP-1 cells.[8] In this study, TPE also showed an inhibitory effect on the MMP-9 levels in *P. acnes*-treated THP-1 cells (Fig. 3). The balanced control of MMP expression and MMP activity is essential to prevent permanent dermal damage or scarring after acne inflammation. Tetracycline derivatives have been reported to have an anti-acne effect due to anti-MMP action in addition to their antimicrobial activity against *P. acnes*.[23] Our results showed that TPE has an inhibitory effect on *P. acnes*-induced MMP-9, implying their potential role for preventing extracellular matrix degradation in acne

lesions.

MAPK signaling pathways leading to increased expression of MMP-9 have already been studied in different TNF-α-stimulated cells.[24] Seelinger et al.[16] reported that luteolin suppresses MAPK pathway in lipopolysaccharides (LPS)-stimulated RAW 264.7 cells. These previous findings led us to examine the effect of TPE and luteolin on these signaling pathways. We found that *P. acnes* activated MAPKs phosphorylation in THP-1 cells (Fig. 4). Treatment with TPE inhibited the phosphorylation of p38, JNK and ERK induced by *P. acnes* (Fig. 4B). The transcription factor NF-κB is the most critical transcriptional regulator of inflammation. The promoters of cytokines, chemokines and MMP-9 have a NF-κB-binding site that is required for their transcriptional induction.[25] NF-κB activation is regulated by MAPKs. We therefore examined the effect of TPE on NF-κB activation. Stimulation of THP-1 cells with *P. acnes* promoted the nuclear translocation of p65 NF-κB (Fig. 5) and TPE inhibited *P. acnes*-induced nuclear translocation of p65 NF-κB after 16 h of incubation (Fig. 5B). Luteolin has been shown to regulate MAPK and NF-κB signaling that inhibits TNF-α-induced IL-8 production in human colon epithelial cells.[25] Also, luteolin was found to significantly inhibit LPS-induced TNF-α, NO and superoxide production of microglia by inhibiting JNK phosphorylation and activation of AP-1.[26] Herein, our results suggested that TPE

and luteolin decreased *P. acnes*-induced cytokine and MMP-9 levels through attenuation of MAPK and NF-κB activation *in vitro*.

*M. charantia* fruit has been considered to be good sources of phenolic compounds which possess potent antioxidant activity.[10] *M. charantia* leaves have numerous phytochemicals such as momordicine, kuguacins, and phenolic compounds.[10] However, there is little report concerning the bioactive components contained in WBM leaf or its fruits. In our previous experiments, we found that the total phenolic content of WBM fruit extracts ranged from 26 to 34 mg GAE/g DW. In this study, the total phenolic content of TPE (58.7 GAE/g DW) is higher than that of WBM fruit extract. Budrat and Shotipruk reported that the total phenolic content of *M. charantia* fruit extract obtained by subcritical water extraction ranged from 10 to 48 mg GAE/g DW.[27] Therefore, WBM leaf is a potentially good source of natural phenolics. Several phenolic compounds, including gallic acid, caffeic acid, catechin, rutin, gentistic acid, and *o*-coumaric acid are determined in *M. charantia* leaves by HPLC analysis.[12, 28] In this study, it was possible to identify ten phenolic compounds in WBM leaves, including gallic acid, chlorogenic acid, caffeic acid, ferulic acid, cinnamic acid, myricetin, quercetin, luteolin, apigenin, and thymol (Fig. 6A). Since IL-8 is a potent chemoattractant involved in the recruitment of neutrophils which is found to occur in acne-related lesions,[3, 4] we evaluated the effects of phenolics present

in TPE on *P. acnes*-induced IL-8 production. The levels of IL-8 are significantly reduced in the phenolics-treated cells compared to those in the vehicle-treated cells (Fig. 6B). These data suggest that all ten phenolics may contribute to the anti-inflammatory activity of WBM leaf against *P. acnes* and may represent a new therapeutic opportunity in acne vulgaris.

We further determined the total triterpene saponin content of TPE using the colorimetric method of Xiang et al.[29] with slight modifications. The 173.3 ± 6.2 mg/g of total triterpene saponin contents of TPE was quantified by using a standard calibration curve of protopanaxadiol (Sigma-Aldrich). Therefore, it is possible that the anti-inflammatory activity observed with TPE may be attributable to its phenolics and triterpenoid components. Whether these other compounds also contribute to the anti-inflammatory activity of the WBM leaf needs further investigation. Hence, further work will be focused on the isolation and characterization of bioactive components with anti-acne potential from WBM leaf extract.

*M. charantia* fruit supplementation prevents high-fat diet-induced neuroinflammation, systemic stress and inflammation.[30] Nagarani et al. recently reported that ethanolic extract of *M. charantia* leaf exhibits anti-inflammatory activity and significantly reduces the rat paw edema induced by carrageenan.[31] A recent study suggests that WBM fruit supplementation ameliorates system

inflammation responses in LPS-induced sepsis mice by reducing spleenic pro-inflammatory cytokine concentrations.[32] However, the effect of WBM leaf extracts on systemic inflammation is unclear and worthy of further investigation.

## Conclusions

In summary, the *in vivo* curative activity of TPE strongly implies great potential for the polyphenolics-enriched fraction obtained from WBM leaf as an agent for acne vulgaris treatment. TPE is able to down-regulate the expression of *P. acnes*-induced pro-inflammatory cytokines implicated in cutaneous inflammation, confirming its potential interest in inflammatory acne. The modulations of neutrophilic inflammatory responses and MAPK and NF-κB signaling pathway may mediate the anti-inflammatory effects of polyphenol-enriched extract of WBM leaf.

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## AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

## References

1 M. D. Farrar and E. Ingham, *Clin. Dermatol.*, 2004, 22, 380–384.

2 A. Koreck, A. Pivarcsi, A. Dobozy and L. Kemény, *Dermatol.*, 2003, 20, 96–105.

3 J. Kim, *Dermatol.*, 2005, 211, 193–198.

4 N. R. Trivedi, K. L. Gilliland, W. Zhao, W. Liu and D.M. Thiboutot, *J. Invest. Dermatol.*, 2006, 126, 1071–1079.

5 H. R. Jalian, P. T. Liu, M. Kanchanapoomi, J. N. Phan, A. J. Legaspi and J. Kim, *J. Invest. Dermatol.*, 2008, 128, 2777–2782.

6 A. Khodadadi, M. H. Pipelzadeh, N. Aghel, M. Esmaeilian and I. Zali , *Iran J. Basic Med. Sci.*, 2011, 14, 340–348.

7 Y. L. Lu, T. H. Liu, J. H. Chyuan, K. T. Cheng, W. L. Liang and W. C. Hou, *Bot. Stud.*, 2012, 53, 207–214.

8 C. Hsu, T. H. Tsai, Y. Y. Li, W. H. Wu, C. J. Huang and P. J. Tsai, *Food Chem.*, 2012, 135, 976–984.

9 K. H. Lu, H. C. Tseng, C. T. Liu, C. J. Huang, J. H. Chyuan and L. Y. Sheen LY, *Food Funct.*, 2014, 5, 1027-1037.

10 T. K. Lim, in *Edible medicinal and non-medicinal plants*, ed. T. K. Lim, Springer, New York, 2012, vol. 2, pp. 331–368.

11 M. R. Khan and A. D. Omoloso, *Korean J. Pharmacogn.*, 1998, 29, 155–158.

12 J. Kubola and S. Siriamornpun S, *Food Chem.*, 2008, 110, 881–890.

13 T. H. Tsai, C. J. Huang, W. H. Wu, W. C. Huang, J. H. Chyuan and P. J. Tsai, *Bot. Stud.*, 2014, 55, 78–88.

14 R. Kumar, S. Balaji, R. Sripriya, N. Nithya, T. S. Uma and P. K. Sehgal, *J. Agric. Food Chem.*, 2010, 58, 1518–1522.

15 W. Tuong, L. Walker and R. K. Sivamani, *J. Dermatolog. Treat*., 2014, DOI:10.3109/09546634.2014.991675.

16 G. Seelinger, I. Merfort and C. M. Schempp, *Planta Med*., 2008, 74, 1667–1677.

17 A. Miniati, Z. Weng, B. Zhang, A. Therianou, M. Vasiadi, E. Nicolaidou, A. J. Stratigos, C. Antoniou C and T. C. Theoharides, *Clin. Exp. Dermatol.*, 2014, 39, 54–57.

18 T. Nakatsuji, M. C. Kao, J. Y. Fang, C. C. Zouboulis, L. F. Zhang, R. L. Gallo, C. M. Huang, *J. Invest. Dermatol.*, 2009, 129, 2480–2488.

19 M. Kistowska, S. Gehrke, D. Jankovic, K. Kerl, A. Fettelschoss, L. Feldmeyer, G. Fenini, A. Kolios, A. Navarini, R. Ganceviciene, J. Schauber, E. Contassot and L. E. French, *J. Invest. Dermatol.*, 2014, 134, 677–685.

20 I. Kurokawa, F. W. Danby, Q. Ju, X. Wang, L. F. Xiang, L. Xia, W. Chen, I. Nagy, M. Picardo, D. H. Suh, R. Ganceviciene, S. Schagen, F. Tsatsou and C. C. Zouboulis, *Exp. Dermatol.*, 2009, 18, 821–832.

21 Y. C. Chou, J. R. Sheu, C. L. Chung, C. Y. Chen, F. L. Lin, M. J. Hsu, Y. H. Kuo and G. Hsiao, *Chem. Biol. Interact.*, 2010, 184, 403–412.

22 W. C. Parks, C. L. Wilson, Y. S. Lopez-Boado, *Nat. Rev. Immunol.*, 2004, 4, 617–629.

23 J. Y. Choi, M. S. Piao, J. B. Lee, J. S. Oh, I. G. Kim and S. C. Lee, *J. Invest. Dermatol.*, 2008, 128, 846–854.

24 M. Cohen, A. Meisser, L. Haenggeli and P. Bischof , *Mol. Hum. Reprod.*, 2006, 12, 225–232.

25 A. Kumar, Y. Takada, A. M. Boriek and B. B. Aggarwal, *J. Mol. Med.*, 2004, 82, 434–448.

26 M. H. Pan, C. S. Lai and C. T. Ho, *Food Funct.*, 2010, 1, 15–31.

27 P. Budrat and A. Shotipruk, *Chiang Mai J. Sci.*, 2008, 35, 123–130.

28 M. Zhang, N. S. Hettiarachchy, R. Horax, P. Y. Chen and F. Kenneth, *J. Food Sci.*, 2009, 74, C441–C448.

29 Z. B. Xiang, C. H. Tang, G. Chen and Y. S. Shi, *Nat. Prod. Res. Dev.*, 2001, 13, 23–26.

30 P. V. Nerurkar, L. M. Johns, L. M. Buesa, G. Kipyakwai, E. Volper, R. Sato, P. Shah, D. Feher, P. G. Williams and V. R. Nerurkar, *J. Neuroinflamm.*, 2011, 8, 64, doi: 10.1186/1742-2094-8-64.

31 G. Nagarani, A. Abirami and P. Siddhuraju, *Food Sci. Hum. Wellness,* 2014, 3, 36–46.

32 C. Y. Chao, P. J. Sung, W. H. Wang and Y. H. Kuo, *Molecules,* 2014, 19, 12777-12788.

## Captions to illustrations

**Figure 1.** *In vivo* inhibitory effects of total phenolic extract (TPE) of WBM leaf on *P. acnes*-induced skin inflammation. (A) Ear biopsy of ICR mice which were intra-dermally injected with PBS vehicle, TPE, *P. acnes*, TPE+*P. acnes*, or luteolin + *P. acnes* were observed after hematoxylin and eosin staining. Scale bars represent 200 μm. Infiltrated neutrophils were observed at an H&E-stained cross-section of the *P. acnes*-injected ear (x1000 magnification panel). The inhibitory effects of TPE and luteolin on *P. acnes*-induced ear edema in mice were evaluated by measuring the ear thickness and ear biopsy weight. (B) At various times after bacterial injection, flow cytometric analysis of the inflammatory cells harvested from *P. acnes*-infected ear tissues. Cell suspensions were incubated with anti-CD45/PerCP, anti-Ly6G/FITC and anti- IL-1β/APC and analyzed by flow cytometry. (C) The changes in *P. acnes*-induced infiltrated CD45+ leukocytes and Ly6G+ neutrophils and intracellular IL-1β expression levels by co-injection with TPE after 12 h stimulation. Each column shows the mean ± SD. ** and *** denote significant difference from vehicle (*P. acnes* alone) at $p< 0.01$ (**) and $p< 0.001$ (***).

**Figure 2.** Total phenolic extract (TPE) of WBM leaf and luteolin inhibited *P.*

*acnes*-induced production (A) and mRNA expression (B) of IL-8, TNF-α, and IL-1β by human monocytic THP-1 cells. Cells were co-incubated with DMSO (as vehicle) or the indicated concentration of sample and *P. acnes* (M.O.I. = 75) for 24 h and 16 h for the determinations of concentration and mRNA expression of cytokine, respectively. A control experiment without *P. acnes* treatment was conducted in parallel. Each column shows the mean ± SD. *, **, and *** denote significant difference from vehicle (*P. acnes* alone) at $p< 0.05$ (*), $p< 0.01$ (**), and $p< 0.001$ (***).

**Figure 3.** Inhibitory effect of total phenolic extract (TPE) of WBM leaf and luteolin on *P. acnes*-induced matrix metalloproteinase (MMP)-9 expression by THP-1 cells. Cells were co-incubated with DMSO (as the vehicle) or the indicated concentration of samples and *P. acnes* (M.O.I. = 75) for 24 hr. A control experiment without *P. acnes* treatment was conducted in parallel. The relative density of each band after normalization for β-actin was shown under each immunoblot as a fold-change compared with non-*P. acnes*-treated control. Each column shows the mean ± SD. *** denotes significant difference from vehicle (*P. acnes* alone) at $p<0.001$.

**Figure 4.** Treatment of *P. acnes* significantly increased the levels of activated MAPK in THP-1 cells. THP-1 cells were incubated with *P. acnes* (M.O.I. = 75) for 8 hr

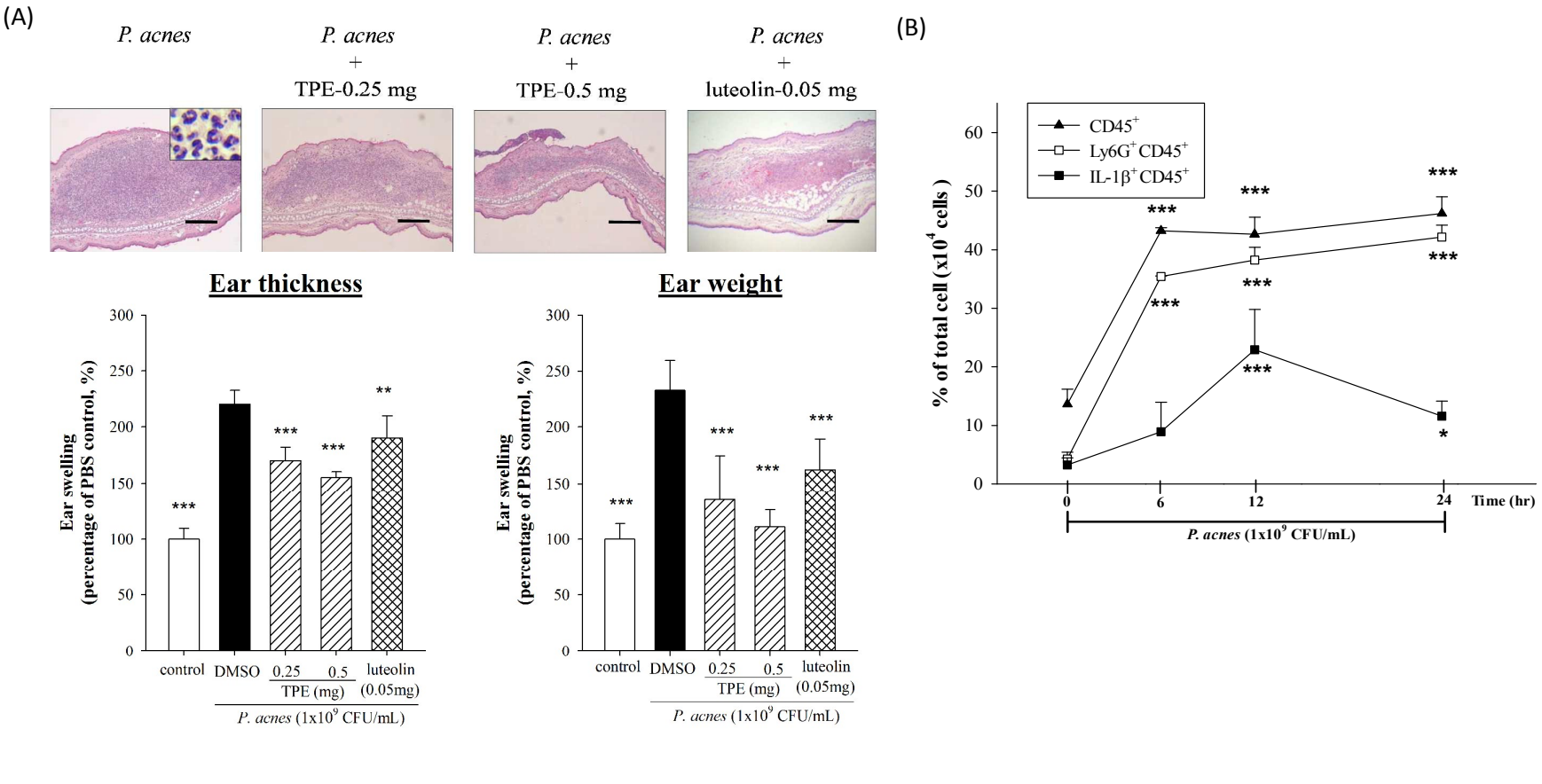
(A). Total phenolic extract (TPE) of WBM leaf and luteolin inhibited *P. acnes*-induced p38, ERK, and JNK activation in THP-1 cells (B). THP-1 cells were incubated 2 h without *P. acnes* (control), with *P. acnes* alone (DMSO vehicle), and with *P. acnes* in the presence of indicated concentration of samples. The levels of phosphorylated p38, ERK, and JNK were determined by Western blot. Each column shows the mean ± SD. *, ** and *** denote significant difference from vehicle (*P. acnes* alone) at $p< 0.05$ (*), $p< 0.01$ (**) and $p< 0.001$ (***).

**Figure 5.** NF-κB p65 activation of *P. acnes*-stimulated THP-1 cells for the indicated periods of time (A). Data are presented as the mean ± SD. * $p <0.05$ and *** $p <0.001$ compared to time zero (baseline). In Fig. 5B, total phenolic extract (TPE) of WBM leaf and luteolin suppressed *P. acnes*-induced nuclear factor-κB (NF-κB) activation in THP-1 cells. THP-1 cells were incubated 16 h without *P. acnes* (control), with *P. acnes* alone (DMSO vehicle), and with *P. acnes* in the presence of indicated concentration of samples. Data are presented as the mean ± SD. *** denotes significant difference from DMSO vehicle (*P. acnes* alone) at $p< 0.001$.

**Figure 6.** HPLC chromatograms of phenolic compounds in total phenolic extract (TPE) of WBM leaf (A). Detection was at 280nm. Peak: (1) gallic acid, (2)

chlorogenic acid, (3) caffeic acid, (4) ferulic aid, (5) cinnamic acid, (6) myricetin, (7) quercetin, (8) luteolin, (9) apigenin, (10) carvacrol, and (11) thymol; (*) un-identified. Retention times (min) of phenolic peaks of TPE were shown (A). In Fig. 6B, phenolic compounds inhibited *P. acnes*-induced IL-8 production by human monocytic THP-1 cells. Cells were co-incubated with DMSO (as vehicle) or the indicated concentration of sample and *P. acnes* (M.O.I. = 75) for 24 h for the determinations of IL-8. A control experiment without *P. acnes* treatment was conducted in parallel. Each column shows the mean ± SD. *$p < 0.001$, significant difference from vehicle (*P. acnes* alone).

**Figure 1.**



(C)

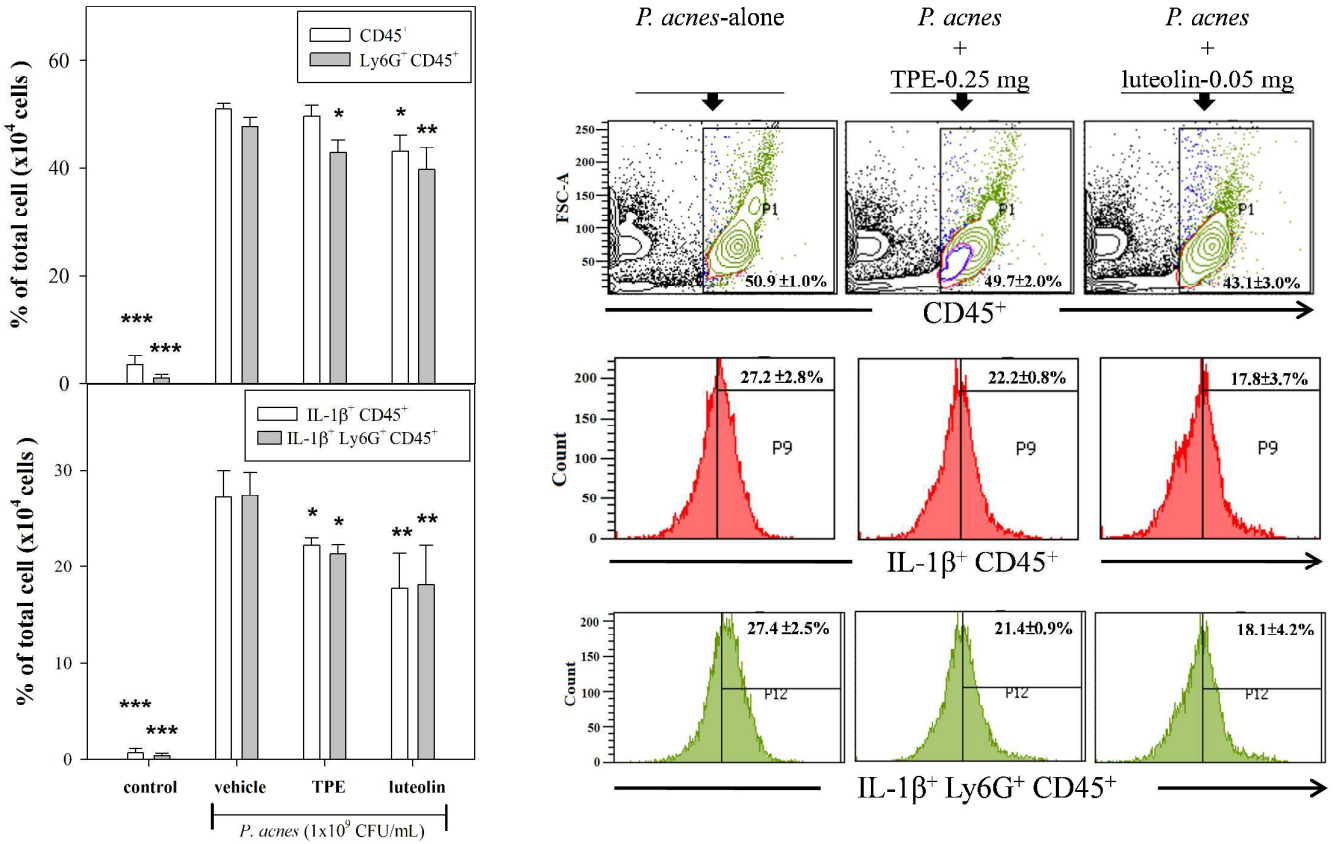


**Figure 2.**

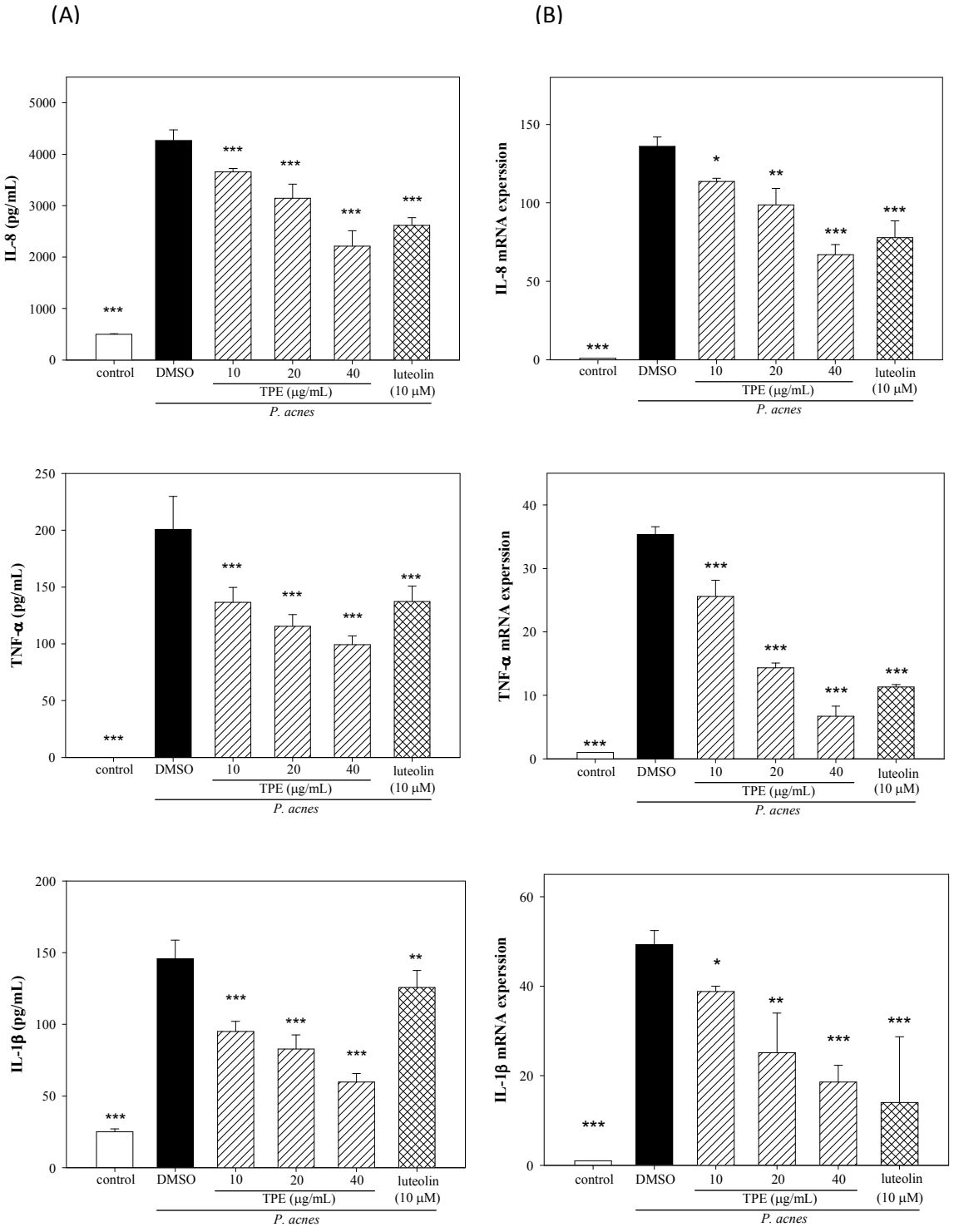
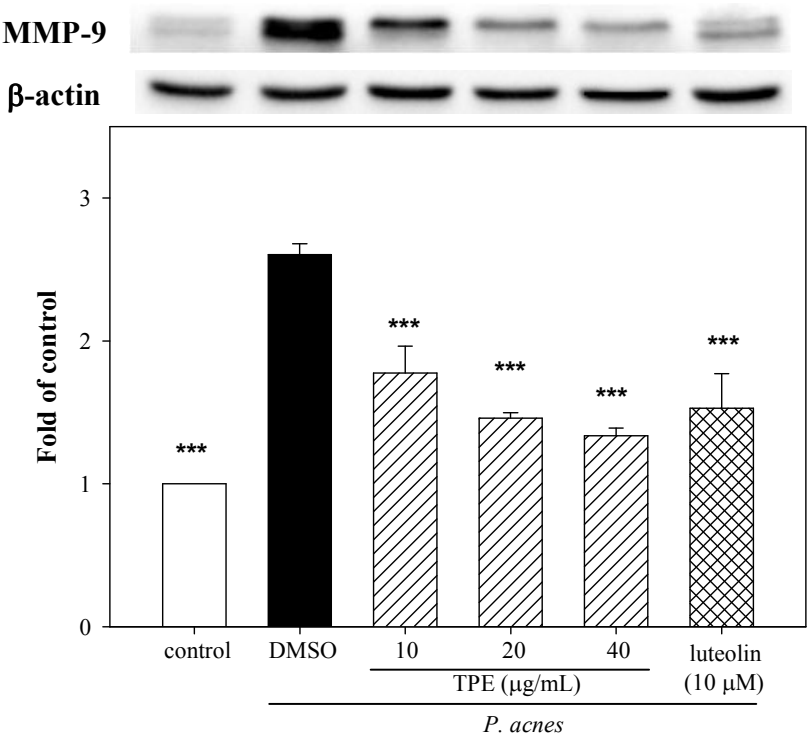


**Figure 3.**



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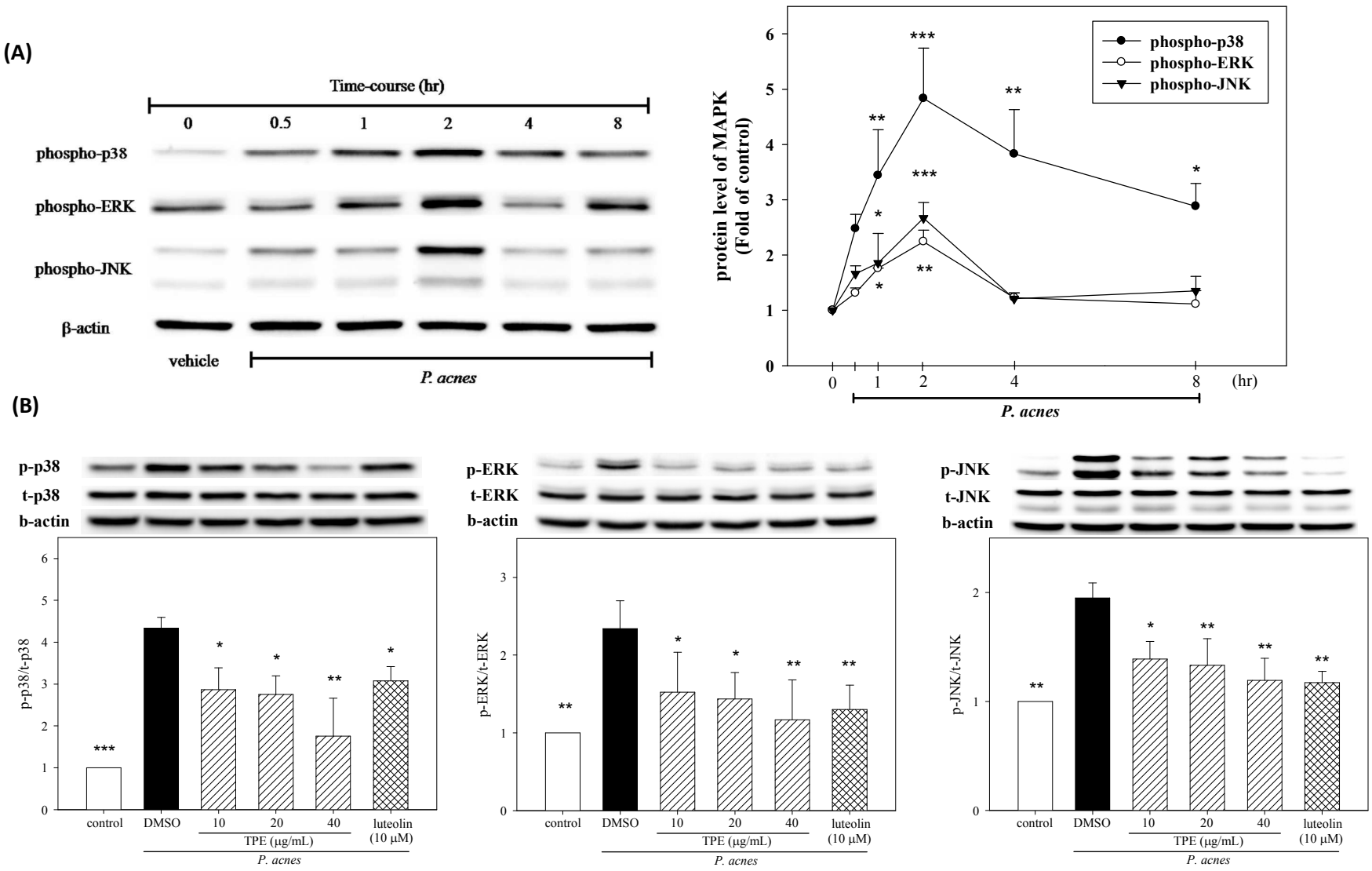

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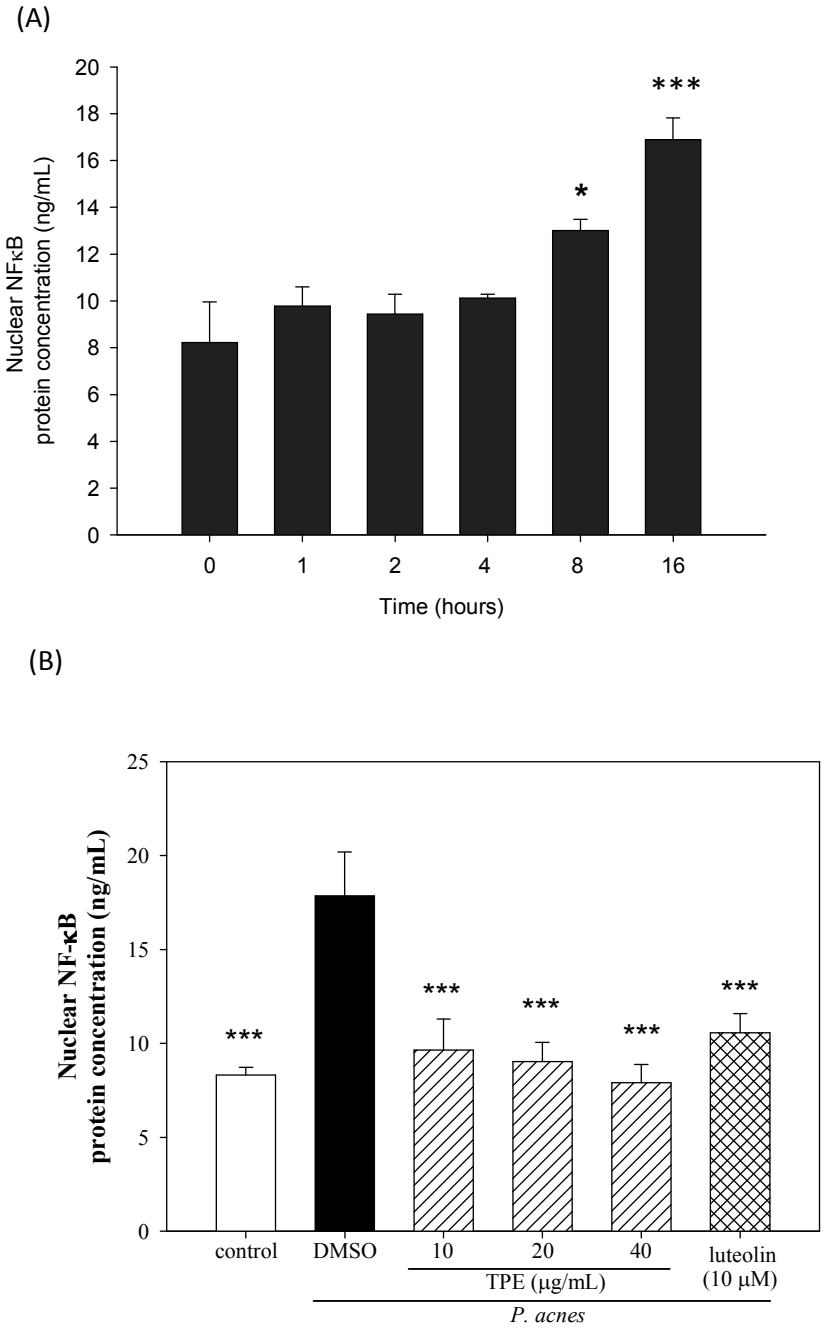

**Figure 6.**