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ORIGINAL ARTICLE

Marantodes pumilum (Blume) Kuntze Inhibited Secretion of Lipopolysaccharide- and Monosodium Urate Crystal-stimulated Cytokines and Plasma Prostaglandin E_2

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ABSTRACT

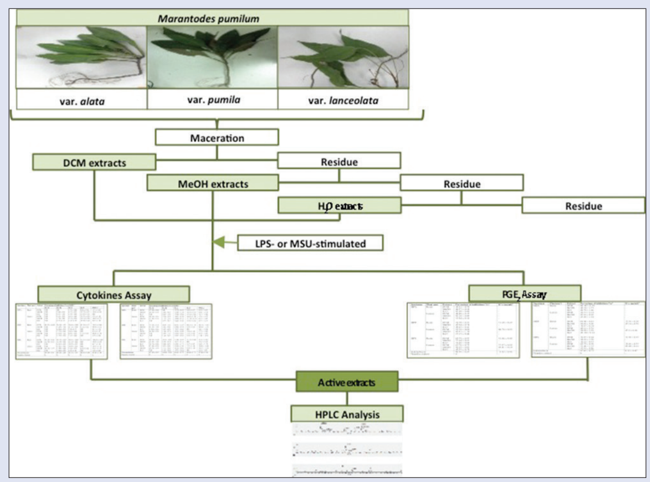
Background: *Marantodes pumilum* is traditionally used for dysentery, gonorrhea, and sickness in the bones. Previous studies revealed its antibacterial and xanthine oxidase inhibitory activities. **Objective:** To evaluate the inhibitory effects of three *M. pumilum* varieties on the secretion of lipopolysaccharide (LPS)- and monosodium urate crystal (MSU)-induced cytokines and plasma prostaglandin E_2 (PGE_2) *in vitro*. **Materials and Methods:** The leaves and roots of *M. pumilum* var. *alata* (MPA), *M. pumilum* var. *pumila* (MPP), and *M. pumilum* var. *lanceolata* (MPL) were successively extracted with dichloromethane (DCM), methanol, and water. Human peripheral blood mononuclear cells and ELISA technique were used for the cytokine assay, whereas human plasma and radioimmunoassay technique were used in the PGE_2 assay. Flavonoids content was determined using a reversed-phase high-performance liquid chromatography. **Results:** DCM extract of MPL roots showed the highest inhibition of LPS-stimulated cytokine secretion with IC_{50} values of 29.87, 7.62, 5.84, 25.33, and 5.40 μg/mL for interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, respectively; while that of plasma PGE_2 secretion was given by DCM extract of MPP roots (IC_{50} 31.10 μg/mL). Similarly, the DCM extract of MPL roots demonstrated the highest inhibition against MSU-stimulated IL-1α, IL-1β, IL-6, IL-8, TNF-α, and PGE_2 secretion with IC_{50} values of 11.2, 8.92, 12.29, 49.51, 9.60, and 31.58 μg/mL, respectively. Apigenin in DCM extracts of MPL (0.051 mg/g) and MPP (0.064 mg/g) roots could be responsible for the strong inhibitory activity against IL-1β, IL-6, TNF-α, and PGE_2. **Conclusion:** The results suggested that DCM extracts of MPL and MPP roots are potential anti-inflammatory agents by inhibiting the secretion of LPS- and MSU-stimulated pro-inflammatory cytokines and PGE_2.

Key words: Lipopolysaccharide, *Marantodes pumilum*, monosodium urate crystals, pro-inflammatory cytokines, prostaglandin E_2

SUMMARY

- Amongst 18 tested extracts, DCM extracts of MPL and MPP roots remarkably inhibited LPS- and MSU-stimulated pro-inflammatory cytokines and PGE_2 secretion
- Phytochemical analysis was performed for the active extracts using RP-HPLC system
- The presence of flavonoids particularly apigenin could be responsible for the anti-inflammatory activity.

Abbreviations used: BSA: Bovine serum albumin, COX-2: Cyclooxygenase-2, CPM: Count per minute, DAMP: Danger-associated molecular pattern, DCM: Dichloromethane, DMSO: Dimethyl sulfoxide, ELISA: Enzyme-linked immunosorbent assay, FBS: Fetal bovine serum, H_2O: Water, HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, HMC-1: Human mast cell-1, HMGB1: High-mobility group box 1, ICAM: Intercellular adhesion molecule, IFN: Interferon, IgG: Immunoglobulin G, IKK: IκB kinase, IL: Interleukin, iNOS: Inducible nitric oxide synthase, LPS: Lipopolysaccharide, MeOH: Methanol, MPA: *Marantodes pumilum* var. *alata,* MPL: *Marantodes pumilum* var. *lanceolata,* MPP: *Marantodes pumilum* var. *pumila,* MSU: Monosodium urate, MTT: Methylthiazole tetrazolium, NF-κB: Nuclear factor-kappa B, NLR: NOD-like receptor, NLRP3: NLR family pyrin domain containing protein 3, NO: Nitric oxide, NOD: Nucleotide-binding oligomerization domain, NSAID: Nonsteroidal anti-inflammatory drug, PAMP: Pathogen-associated molecular pattern, PBMC: Peripheral blood mononuclear cell, PBS: Phosphate buffered saline, PGE_2: Prostaglandin E_2, PMACI: Phorbol-12-myristate 13-acetate and calcium ionosphere A23187, PRR: Pathogen recognition receptor, PTFE: Polytetrafluoroethylene, RIA: Radioimmunoassay, RIG: Retinoic acid-inducible gene I, RLR: RIG I-like receptor, RP-HPLC: Reversed-phase high-performance liquid chromatography, RPMI-1640: Roswell Park Memorial Institute-1640, TLR: Toll-like receptor, TNF: Tumor necrosis factor, VCAM: Vascular cell adhesion molecule.



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INTRODUCTION

Inflammation is a pathophysiological response of tissue to injury, infection, or destruction. This is important to protect tissue and leads to the healing of damaged tissue to its preinjury state.[1] During the recognition of inflammation, cytokines are one of the first signals induced by immune response, particularly innate immune response, via pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and retinoic acid-inducible gene I-like receptors, which differentiate pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) from normal host protein.[2] PAMPs include various bacterial cell wall components such as lipopolysaccharide (LPS), lipopeptides, peptidoglycans, and teichoic acid that initiate the infectious pathogen-induced inflammation. LPS is well known as the best studied model on innate immunity in order of its role as the prototypic activator of those response. On the other hand, DAMPs include protein DAMPs such as high-mobility group box 1 (HMGB1) and hyaluronan fragments, and nonprotein DAMPs such as uric acid.[3,4] High level of uric acid (>7 mg/dL) leads to crystallization of monosodium urate that causes gouty inflammation. During inflammatory response, a variety of soluble factors are involved in leukocyte recruitment; for example, some derivatives of arachidonic acid including prostaglandins, leukotrienes, and lipoxins.[5] The main therapeutic treatment for inflammation is by administration of steroids and nonsteroid anti-inflammatory drugs that may cause adverse effects such as gastrointestinal toxicity, renal toxicity, or gastrointestinal bleeding. Thus, there is a need to identify alternative anti-inflammatory agents from natural resources that can be used for the treatment of inflammatory disorders.[6]

Marantodes pumilum (Blume) kuntze (Primulaceae) is previously named as *Labisia pumila* (Blume) Fern.-Vill or locally known as Kacip Fatimah in Malaysia.[7] It is a popular herb that has been used by many generations of the Malay women as a traditional medicine for reproductive-related conditions, including to induce and facilitate childbirth and to help women regain strength after giving birth.[8,9] The preparations have also been used for flatulence, dysentery, dysmenorrhea, gonorrhea, and sickness in the bones.[8-10] Three varieties of *M. pumilum* are mainly found in Malaysia, namely, *M. pumilum* (Blume) Kuntze var. *alata* (Scheff.) Mez, var. *pumila*, and var. *lanceolata* (Scheff.) Mez, and these can be differentiated based on the characteristics of petiole and leaf-shape, microscopic anatomy, as well as phytochemically.[11-13] The popularity of traditional herbal products containing *M. pumilum* has tempted many researchers to investigate the phytochemistry and pharmacological actions of this plant.

Several phytochemical compounds have been characterized, including phenolics (e.g., gallic acid, caffeic acid, pyrogallol, benzoic acid, cinnamic acid, and methyl gallate);[14-16] flavonoids (e.g., quercetin, myricetin, kaempferol, naringin, and rutin);[15] flavanols (e.g., catechin and epigallocatechin),[14] and isoflavonoids (e.g., daidzein and genistein).[17] Strong antioxidative compounds such as β-carotene and ascorbic acid were also found in *M. pumilum*.[14,15] Triterpenoid saponins, alkenyl compounds, and benzoquinone derivatives were also isolated.[18] These compounds have been found to be responsible for many biological activities such as antibacterial, antioxidant, anti-inflammatory, and anticancer.[19] Previous scientific studies have also reported various activities of *M. pumilum* extracts including antioxidant,[20] antibacterial,[17] antifungal,[15] anticarcinogenic,[21] xanthine oxidase inhibition,[22] and anti-inflammatory.[23]

Karimi *et al*. reported that *M. pumilum* inhibited nitric oxide (NO) release in RAW 264.7 cells induced with LPS and interferon-γ.[23] In addition, *M. pumilum* has been used traditionally for bacterial infection such as dysentery and gonorrhea that could initiate inflammatory response. The plant has also been used for bone sickness including gout.[9,11] However, to the best of our knowledge, the effect of *M. pumilum* on bacterial and gouty inflammation in pro-inflammatory cytokines and prostaglandin E_2 (PGE_2) secretion remained to be investigated. The rationale of using LPS and monosodium urate crystals (MSU) to induce inflammation was to mimic the bacterial infection and gouty inflammation pathways, respectively. Thus, this study was aimed to determine the effect of extracts of *M. pumilum* varieties on the inhibition of LPS- and MSU-stimulated cytokines and plasma PGE_2 secretion *in vitro*. The work will provide some insights for the development of a natural anti-inflammatory agent and an evidence-based herbal supplement.

MATERIALS AND METHODS

Materials

All chemicals and organic solvents used in this work were of analytical grade. Solvents were purchased from Merck (Darmstadt, Hesse, Germany). High-performance liquid chromatography (HPLC)-grade methanol (MeOH) and acetonitrile were obtained from Merck (Darmstadt, Hesse, Germany) while HPLC-grade orthophosphoric acid was obtained from Fisher Scientific (Loughborough, Leicestershire, UK). HPLC-grade gallic acid (99%), caffeic acid (98%), myricetin (96%), quercetin (98%), apigenin (95%), and kaempferol (97%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone was supplied from Hospital Canselor Tuanku Muhriz of Universiti Kebangsaan Malaysia. Lymphoprep was obtained from Axis-Shield PoC AS (Oslo, Norway), while unlabeled PGE_2, anti-PGE_2, LPS from *Salmonella abortus equii*, uric acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue (methyl thiazol tetrazolium [MTT]), 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), fetal bovine serum (FBS), penicillin-streptomycin solution, Roswell Park Memorial Institute (RPMI)-1640 medium containing L-glutamine, dextran from *Leuconostoc mesenteroides*, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS) was purchased from MP Biomedicals (USA) while bovine serum albumin (BSA) and activated charcoal were purchased from Merck (Darmstadt, Hesse, Germany). IL-8 ELISA kits were purchased from Abnova, Germany, while all of the other kits were purchased from Cayman, USA. Radiolabeled PGE_2 ([^{3}H]-PGE_2, 50 μCi) and liquid scintillation cocktail were purchased from Perkin Elmer (Massachusetts, USA). The leaves and roots of three varieties of *M. pumilum* were collected from Hutan Gunung Bujang Melaka, Kampar, Perak, Malaysia. The plants were authenticated by Emeritus Professor Dato' Dr. Abdul Latiff Mohamad. Voucher specimens of *M. pumilum* var. *alata* (herbarium number: UKMB 30006/SM 2622), *M. pumilum* var. *pumila* (MPP, UKMB 30007/SM s.n), and *M. pumilum* var. *lanceolata* (MPL, UKMB 30008/SM s.n) were deposited in the Herbarium of Universiti Kebangsaan Malaysia.

Preparation of plant extracts

The leaves and roots of plant materials were separated, air-dried, and ground. Each dried powder was individually extracted with dichloromethane (DCM) and MeOH by sequential exhaustive maceration. The final residue was then extracted under reflux with distilled water (H_2O) for 2 h at 80°C. The organic filtrate was collected and concentrated under reduced vacuum pressure, while the H_2O extract was freeze-dried. The percentage yield of extract was calculated with respect to its air-dried powder [Table 1]. The extract was dissolved in dimethyl sulfoxide (DMSO; the final concentration of DMSO was not exceeded 0.5% in medium).

Study subjects

Human blood was obtained from healthy volunteers (n = 3, ≥18 years old) who fulfilled the inclusion criteria of nonsmoker, fasted overnight, and had not taken any medicines or supplements. The experimental protocol for the cytokines and PGE_2 assays was approved by the Human Ethical Committee of Universiti Kebangsaan Malaysia with an approval number of UKM 1.5.3.5/244/NF-040-2011 and UKM

Table 1: Percentage yield of extracts

Specimens	Plant parts	Percentage yield (% w/w)[a]		
		DCM	MeOH	H_2O
MPA	Roots	0.85	8.08	6.00
	Leaves	1.80	2.23	2.74
MPP	Roots	1.59	4.63	2.58
	Leaves	11.38	1.09	4.56
MPL	Roots	3.16	7.21	5.16
	Leaves	3.30	8.70	1.03

[a]Percentage yield was calculated based on dry weight. DCM: Dichloromethane; MeOH: Methanol; H_2O: Water; MPA: *Marantodes pumilum* var. *alata*; MPP: *Marantodes* var. *pumila*; MPL: *Marantodes* var. *lanceolata*

1.5.3.5/244/NF-016-2013, respectively. The protocol was in accordance with the principles outlined in the Declaration of Helsinki.[24]

Cytokine assay

Cell preparation and viability test

Cell preparation procedure was based on the method of Boyum with slight modification.[25] Briefly, fresh heparinized venous blood from healthy volunteers was diluted in 1:1 ratio with RPMI-1640 medium containing L-glutamine. Peripheral blood mononuclear cells (PBMCs) were separated from the blood using Lymphoprep gradient centrifugation at 600 ×*g* for 20 min.[26] The cells were washed twice with RPMI-1640 medium, resuspended in a complete RPMI-1640 medium buffered with HEPES to pH 7.4, and supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS (hereafter referred to as a complete medium). The cells were counted using a hemocytometer under a light microscope and then adjusted up to 5×10^5 mL^{-1}. Cell viability was determined by MTT assay as described by Mosmann with slight modification by using DMSO to dissolve the formazan crystals.[27,28] The PBMCs (100 μL) were incubated at 37°C with 5% CO_2 for 27 h with 100 μL of extracts (50 and 100 μg/mL) or dexamethasone (0.5 and 5 μg/mL) or complete medium with 0.5% DMSO as a negative control. The concentration of extracts used in MTT assays was based on the previous study by Karimi *et al.*,[23] who performed NO assay on RAW 264.7 cells. After 27 h, 20 μL of MTT (5 mg/mL in sterile PBS) was added to each well and incubated again for 4 h. The supernatant was carefully discarded. The formazan blue crystals formed by cells were dissolved in 100 μL of DMSO (100%) and the absorbance was measured at a wavelength of 570 nm using a microplate reader (Multiskan Go, Thermo Fisher Scientific, USA).

Determination of cytokine level

PBMCs (100 μl) were preincubated at 37°C with 5% CO_2 for 3 h with 100 μL of either extracts as test samples or dexamethasone as a positive control with final concentrations being 50 and 5 μg/mL, respectively, while 0.5% DMSO in complete medium was used as a negative control. After 3 h, 20 μl of LPS (1 μg/mL) was added to induce cytokine release from cells and the mixture was incubated based on the type of cytokines, i.e., 12 h for tumor necrosis factor (TNF)-α and interleukin (IL)-1β, 20 h for IL-6, and 24 h for IL-1α and IL-8. Meanwhile, for MSU-induced inflammation, cells were incubated with 20 μL of MSU crystal suspension (200 μg/mL) for 24 h for all cytokines.[29-31] After incubation, cells were centrifuged at 300 × *g* and 4°C for 10 min to obtain cell-free supernatants and the resultants were stored at − 80°C until analysis. The concentration of cytokines was tested using appropriate ELISA kits for human cytokines according to manufacturer's instruction. The cytokine level was measured using a microplate reader (BioTek, USA). Limit of detection of these kits were 2.0 ρg/mL for IL-8, 7.8 ρg/mL for IL-6, and 3.9 ρg/mL for all the others. The cytokine secretion levels were compared with the negative control that was considered as 100% cytokine secretion. The percentage inhibition (% I) was calculated using equation (1):

$$\% \, I = \left(1 - \frac{[\text{Concentration of cytokine or PGE}_2 \text{ in sample or positive control}]}{[\text{concentration of cytokine or PGE}_2 \text{ in negative control}]} \right) \times 100\,\% \quad (1)$$

The IC_{50} values were determined for the active extracts in five concentrations ranging from 3.125 to 50 μg/mL.

Prostaglandin E_2 assay

Plasma preparation

Fresh heparinized blood from healthy volunteers were incubated at 37°C containing 5% CO_2 for 24 h with LPS or MSU after preincubation for 30 min in the presence or absence of extracts (50 μg/mL) or indomethacin (10 μg/mL) as a positive control or 0.5% DMSO in radioimmunoassay (RIA) buffer (PBS [0.01 M; pH 7.4] containing BSA [0.1%] and sodium azide [0.1%]) as a negative control. After incubation, the blood was centrifuged at 3000 ×*g* for 15 min at 4°C and the plasma supernatant was removed carefully and immediately used for the assay.[32]

Radioimmunoassay of plasma prostaglandin E_2 secretion

The assay was carried out according to the modified method of Saadawi *et al.* and Patrignani *et al.*[32,33] A reaction mixture containing 100 μL of plasma, 100 μL of anti-PGE_2 (diluted with ratio of 1:50,000), and 100 μL of [^{3}H]-PGE_2 (0.1 μCi/ml) was incubated at 4°C for 24 h. After incubation, 200 μL of dextran-coated charcoal was added to the mixture and incubated again for 10 min at 4°C. After centrifugation at 3000 ×*g* for 15 min at 4°C, 300 μL of supernatant was added to 3 mL liquid scintillation cocktail in Pico Pro Vial™ (Perkin Elmer, Massachusetts, USA). The radioactivity was measured using a liquid scintillation analyzer (Packard Tri-Carb, Models B3110TR, Hamburg, Germany). The average count per min value of standards and samples was calculated by subtracting the value of antibody-antigen binding in the samples (B) with nonspecific binding (Nc; [^{3}H]-PGE_2 and RIA buffer) together with the total binding between antibody and antigen (Bo; [^{3}H]-PGE_2, anti-PGE_2, and RIA buffer). The normalized percentage bound (%B/Bo) was then calculated using equation (2).

$$\% \frac{B}{Bo} = \left(\frac{B - Nc}{Bo - Nc} \right) \times 100\,\% \quad (2)$$

The %B/Bo values were plotted using semi-logarithmic graph against the corresponding concentration of standard PGE_2 in picogram. The %B/Bo values of serial dilutions of standard PGE_2 with concentrations ranging from 2.45 to 400 ρg/0.1 mL were used to obtain a standard curve plot. The concentration of PGE_2 (ρg/0.1 mL) in each sample was determined by interpolating the %B/Bo values using standard curve. Percentage inhibition was calculated by using equation (1). The IC_{50} values were determined for the active extracts and indomethacin at five concentrations ranging from 3.125 to 50 μg/mL and from 0.625 to 10 μg/mL, respectively.

High-performance liquid chromatography analysis

Phytochemical analysis was performed for the most active extracts, i.e., DCM root extracts of MPL and MPP, using an RP-HPLC based on the method described by Karimi *et al.* to quantify the amount of quercetin and apigenin.[15] The extracts solution (100 mg/mL) and a mixture of reference standard solution containing gallic acid, caffeic acid, myricetin, quercetin, apigenin, and kaempferol (1 mg/mL) were prepared in HPLC-grade MeOH, sonicated for 5 min, and filtered through 0.45 μm Millipore Millex PTFE membrane (Maidstone, Kent, UK) before analysis using the following validated HPLC conditions: XBridge C-18 column (250 mm × 4.6 mm, 5 μm; Waters, Dublin, Ireland), photodiode array detector (Waters 2998 with Waters Prep Degasser; Waters, Dublin, Ireland) at a wavelength of 330 nm, flow rate of 0.6 mL/min, mobile phase consisted of acetonitrile (A) and acidified H_2O with 0.1% orthophosphoric acid (B) and eluted by a linear gradient of 15%–85% A and 85%–15% B (0–55 min), and isocratic composition of 85% A and 15% B (55–60 min). The column was equilibrated for 20 min before the next injection. Injection volume of each solution was 10 μL.

Statistical analysis

All of the data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was carried out in duplicate determinations from three independent experiments ($n = 3$) and presented as mean ± standard error of mean. Data were analyzed using one-way analysis of variance and *post hoc* Tukey's test for multiple comparisons and $P \leq 0.05$ was considered statistically significant. The IC_{50} values were determined using GraphPad Prism 5 software.

RESULTS

Cell viability of human peripheral blood mononuclear cells

The concentration of 50 μg/mL of all *M. pumilum* extracts and 5 μg/mL of dexamethasone gave >90% cell viability [Figure 1]. Thus, the respective concentrations were used as the highest concentration in this experiment.

Effect of *Marantodes pumilum* extracts on cytokine secretion in lipopolysaccharide-stimulated human peripheral blood mononuclear cells

Of 18 extracts tested, three extracts were strongly active against the cytokine assay [Table 2]. DCM extract of MPL roots had the highest inhibitory activity for five cytokine secretions (IL-1α, IL-1β, IL-6, IL-8, and TNF-α) with values of 99.20, 99.91, 99.65, 51.65, and 100.04%, respectively, followed by MPP roots that inhibited secretion of IL-1α (88.87%), IL-6 (99.68%), and TNF-α (70.29%), while MeOH extract of MPL leaves inhibited only TNF-α (51.15%). The extracts showed inhibition of cytokine secretion in a concentration-dependent manner. Interestingly, DCM extract of MPL roots gave significantly higher ($P \leq 0.05$) inhibitory activity against IL-1α, IL-1β, IL-6, and TNF-α compared to dexamethasone, while that of IL-8 was comparable to dexamethasone ($P > 0.05$). Moreover, DCM extract of MPP roots also had significantly higher ($P \leq 0.05$) inhibitory activity against IL-1α and IL-6 and comparable to dexamethasone ($P > 0.05$) against TNF-α secretion.

As presented in Table 3, DCM extract of MPL roots was found to have the strongest inhibitory activity with IC_{50} values of 29.87, 7.62, 5.84, 25.33, and 5.40 μg/mL for IL-1α, IL-1β, IL-6, IL-8, and TNF-α, respectively, followed by DCM extract of MPP roots but only for IL-1α (15.86 μg/mL), IL-6 (5.87 μg/mL), and TNF-α (19.92 μg/mL). All active extracts had significantly lower activity compared to dexamethasone (IC_{50} values of 0.90, 0.03, 0.89, 1.94, and 0.08 μg/mL for IL-1α, IL-1β, IL-6, IL-8, and TNF-α, respectively; $P \leq 0.001$).

Effect of *Marantodes pumilum* extracts on plasma prostaglandin E_2 secretion in lipopolysaccharide -stimulated human whole blood

At the concentration of 50 μg/mL, the DCM extract of MPP roots showed the highest inhibition (72.78%) compared to indomethacin (90.56%) [Table 4]. The results also showed that the DCM extracts of MPL roots, MPL leaves, and MPP leaves, as well as MeOH extract of MPL leaves were active with inhibitory values of 65.79, 63.76, 53.19, and 50.07%, respectively. The IC_{50} determination revealed that the DCM extract of MPP roots inhibited the secretion of plasma PGE_2 in a concentration-dependent manner with an IC_{50} value of 31.1 μg/mL followed by DCM extracts of MPL roots (33.66 μg/mL), MPL leaves (37.27 μg/mL), and MPP leaves (48.79 μg/mL) and MeOH extract of MPL leaves (49.81 μg/mL). All extracts had significantly higher IC_{50} values than indomethacin ($IC_{50} = 0.52$ μg/mL; $P \leq 0.001$).

Effect of *Marantodes pumilum* extracts on cytokine secretion in monosodium urate crystals-stimulated human peripheral blood mononuclear cells

In this study, two DCM extracts of MPP and MPL roots (50 μg/mL) actively inhibited cytokine secretion [Table 5]. DCM extract of MPL roots significantly inhibited the secretion of IL-1α (94.96%), IL-1β (99.91%), IL-6 (97.04%), and TNF-α (97.65%) compared to dexamethasone ($P \leq 0.05$), but inhibition of IL-8 (51.74%) was comparable to dexamethasone at

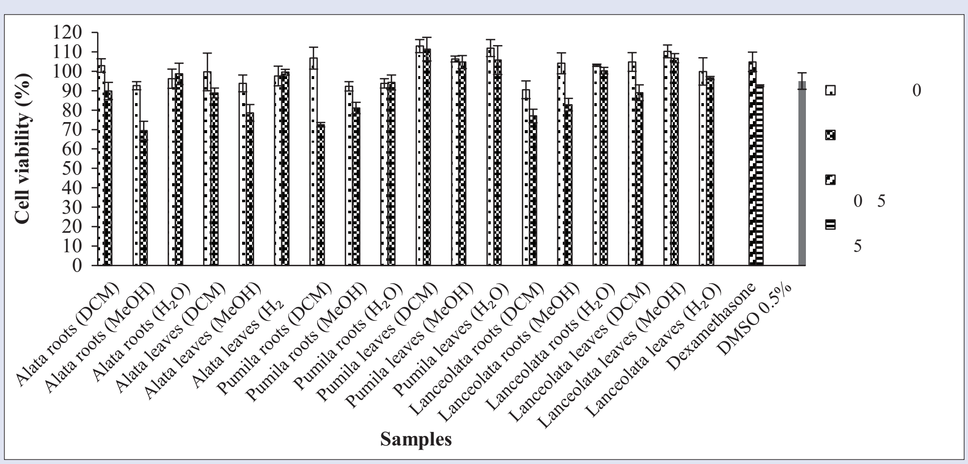

Figure 1: Viability of peripheral blood mononuclear cells after 27 h of exposure to extracts of *Marantodes pumilum*, dexamethasone, and 0.5% of dimethyl sulfoxide. Data are presented as mean ± standard error of mean ($n = 3$)

concentration of 5 μg/mL ($P > 0.05$). The activity was followed by MPP roots that inhibited the secretion of IL-1α (73.96%), IL-6 (80.91%), and TNF-α (64.74%) and was statistically comparable ($P > 0.05$) for IL-1α and TNF-α and significantly higher ($P \leq 0.05$) for IL-6 than dexamethasone.

Determination of IC_{50} value of active extracts demonstrated that inhibitory activity was in a concentration-dependent manner. DCM extract of MPL roots strongly inhibited IL-1α, IL-1β, IL-6, and TNF-α with IC_{50} values of 11.2, 8.92, 12.29, and 9.60 μg/mL, respectively. However, as presented in Table 6, all extracts possessed significantly lower IC_{50} values compared to dexamethasone (IC_{50} values of 0.02, 0.71, 0.46, 0.68, and 0.15 μg/mL for IL-1α, IL-1β, IL-6, IL-8, and TNF-α, respectively; $P \leq 0.001$).

Effect of *Marantodes pumilum* extracts on plasma prostaglandin E_2 secretion in monosodium urate crystals-stimulated human whole blood

Only six extracts (50 μg/mL) were found to actively inhibited plasma PGE_2 secretion [Table 7]. DCM extract of MPL roots had the highest inhibitory activity with a value of 72.87%, followed by DCM extracts of MPP roots (66.08%) and MPL leaves (65.72%) and MeOH extracts of MPP roots (54.45%), MPL leaves (52.08%), and MPP leaves (50.28%). However, all active extracts gave significantly lower activity ($P \leq 0.05$) compared to indomethacin (10 μg/mL), with an inhibition value of 97.45%. The lowest IC_{50} value was obtained for DCM extract of MPL roots (31.58 μg/mL), followed by DCM extract of MPP roots (33.01 μg/mL) and DCM extract of MPL leaves (35.26 μg/mL). All extracts showed a concentration-dependent inhibition; however, the activity was significantly lower ($P \leq 0.001$) compared to indomethacin (IC_{50} = 0.35 μg/mL).

High-performance liquid chromatography analysis

Of 18 extracts tested for bioassays, only DCM extracts of MPP and MPL roots showed higher inhibitory activities for five cytokines and PGE_2 secretion against LPS and MSU stimulation compared to the other extracts. From this study, the DCM extract of MPP roots was found to contain quercetin (1.27 mg/g) and apigenin (0.064 mg/g). However, only apigenin (0.051 mg/g) was detected in the DCM extract of MPL roots [Figure 2].

DISCUSSION

The viability of human PBMCs was assessed by MTT assay based on the conversion of MTT to purple-colored formazan by mitochondrial

Table 2: Percentage of inhibitory activities of *Marantodes pumilum* extracts on cytokine secretion in lipopolysaccharide-stimulated human peripheral blood mononuclear cells

Specimen	Plant part	Extract	Percentage of inhibition (%)±SEM				
			IL-1α	IL-1β	IL-6	IL-8	TNF-α
MPA	Roots	DCM	ND	ND	2.15±0.48	8.06±0.26	1.19±0.69
		MeOH	ND	2.40±0.18	4.75±0.53	12.72±0.27	19.95±0.55
		H_2O	ND	0.61±1.03	5.78±0.44	21.04±0.32	ND
	Leaves	DCM	ND	2.41±2.52	6.81±0.42	12.27±0.29	3.53±1.49
		MeOH	ND	1.38±2.82	2.21±0.38	19.21±0.20	10.92±0.73
		H_2O	ND	0.77±0.15	5.16±0.54	10.44±0.20	0.87±1.90
MPP	Roots	DCM	88.87±0.04[b]	34.80±1.22	99.68±0.00[b]	42.18±0.17	70.29±0.06[a]
		MeOH	ND	7.259±1.02	23.12±0.64	6.22±0.22	49.90±0.12
		H_2O	ND	4.004±0.12	7.48±0.37	ND	0.13±3.30
	Leaves	DCM	ND	3.315±0.62	5.06±0.68	ND	0.34±1.07
		MeOH	ND	6.418±1.22	8.60±0.66	7.41±0.23	48.08±1.54
		H_2O	ND	1.719±0.72	7.13±0.40	ND	2.78±2.04
MPL	Roots	DCM	99.20±0.01[b]	99.76±0.02[b]	99.65±0.00[b]	51.65±0.25[a]	100.04±0.00[b]
		MeOH	14.30±0.32	16.59±0.22	12.32±0.40	ND	39.95±0.39
		H_2O	ND	4.013±0.52	10.25±0.31	ND	4.17±1.59
	Leaves	DCM	ND	4.701±0.42	10.69±0.27	ND	16.57±1.63
		MeOH	28.30±1.79	3.589±0.42	7.24±0.70	4.68±0.24	51.15±1.27
		H_2O	ND	2.894±0.92	9.50±1.40	ND	1.89±0.69
Dexamethasone			69.68±0.51	85.00±2.40	65.32±3.37	60.68±0.68	83.48±1.04
Negative control			0	0	0	0	0

Data are presented as mean±SEM (n=3). Data were analysed by using one-way ANOVA followed by *post hoc* Tukey. Concentration of extracts was 50 μg/mL, while dexamethasone was 5 μg/mL. Percentage inhibition >2.5% was significant at $P \leq 0.05$ when compared with negative control. [a]$P > 0.05$ was considered not significantly different compared to dexamethasone; [b]$P \leq 0.05$. Percentage inhibition was significantly higher than dexamethasone. SEM: Standard error of mean; DCM: Dichloromethane; MeOH: Methanol; H_2O: Water; MPA: *Marantodes pumilum* var. *alata*; MPP: *Marantodes* var. *pumila*; MPL: *Marantodes* var. *lanceolata*; TNF: Tumor necrosis factor; IL: Interleukin; ANOVA: Analysis of variance; ND: Inhibition was not detected

Table 3: The IC_{50} values (μg/mL) of *Marantodes pumilum* extracts on cytokine secretion in lipopolysaccharide-stimulated human peripheral blood mononuclear cells

Specimen	Plant part	Extract	IC_{50} values (μg/mL)±SEM				
			IL-1α	IL-1β	IL-6	IL-8	TNF-α
MPP	Roots	DCM	15.86±0.88	-	5.87±0.61	-	19.92±1.44
MPL	Roots	DCM	29.87±1.49	7.62±0.37	5.84±0.25	25.33±0.57	5.40±0.32
MPL	Leaves	MeOH	-	-	-	-	42.24±2.97
Dexamethasone			0.90±0.16	0.03±0.02	0.89±0.08	1.94±1.38	0.08±0.00

Data are presented as mean±SEM (n=3); Data were analyzed using one-way ANOVA followed by *post hoc* Tukey. -: Not determined as none of tested concentration exceeded 50% inhibition. All IC_{50} values of extracts were statistically different compared to dexamethasone ($P \leq 0.001$). SEM: Standard error of mean; DCM: Dichloromethane; MeOH: Methanol; MPP: *Marantodes* var. *pumila*; MPL: *Marantodes* var. *lanceolata*; TNF: Tumor necrosis factor; IL: Interleukin; ANOVA: Analysis of variance

Table 4: Percentage of inhibition of *Marantodes pumilum* extracts and the IC_{50} values of active extracts on plasma prostaglandin E_2 secretion in lipopolysaccharide-stimulated human whole blood

Specimen	Plant part	Extract	Percentage of inhibition (%)	IC_{50} (µg/mL)
MPA	Roots	DCM	45.61±0.70	-
		MeOH	46.27±0.58	-
		H_2O	43.99±0.46	-
	Leaves	DCM	40.88±0.35	-
		MeOH	42.28±0.49	-
		H_2O	31.24±0.36	-
MPP	Roots	DCM	72.78±0.31	31.10±0.23
		MeOH	43.24±0.78	-
		H_2O	49.43±0.41	-
	Leaves	DCM	53.19±0.60	48.79±0.23
		MeOH	45.41±1.30	-
		H_2O	21.15±0.44	-
MPL	Roots	DCM	65.79±0.97	33.66±0.26
		MeOH	37.94±0.53	-
		H_2O	32.48±0.88	-
	Leaves	DCM	63.76±0.72	37.27±0.25
		MeOH	50.07±0.05	49.81±0.18
		H_2O	45.97±0.78	-
Indomethacin			90.56±0.27	0.52±0.01
Negative control			0	-

Data are presented as mean±SEM (*n*=3). Data were analysed by using one-way ANOVA followed by *post hoc* Tukey. Concentration of extracts was 50 µg/mL; while indomethacin was 10 µg/mL. -: Not determined as none of tested concentration exceeded 50% inhibition. Percentage inhibition >2.5% was significant at $P \leq 0.05$ when compared with negative control; $^{a}P \geq 0.05$ was considered not significant compared with indomethacin (positive control). All IC_{50} values of extracts were statistically different compared to indomethacin ($P \leq 0.001$). SEM: Standard error of mean; DCM: Dichloromethane; MeOH: Methanol; H_2O: Water; MPA: *Marantodes pumilum* var. *alata*; MPP: *Marantodes* var. *pumila*; MPL: *Marantodes* var. *lanceolata*; ANOVA: Analysis of variance; ND: Inhibition was not detected

dehydrogenases from viable cells. The resultant was quantified spectrophotometrically at a wavelength of 570 nm and was proportional to the number of living cells present.[27,28] MTT assay has been enormously used as a quantitative, sensitive, and reliable assay to monitor viable cell.[34] The percentage of living cell >90% was indicating that the extracts and dexamethasone had no significant effect on viability of cells after 27 h of exposure. This experiment was crucial to show that the inhibition of cytokine secretion was not due to cell death.

Pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α, are produced in monocytes, neutrophils, and macrophages when stimulated by LPS or MSU.[3] TNF-α plays an important role in autoimmune diseases associated with the acceleration of inflammatory responses. IL-1 is involved in the control of inflammatory responses, mostly in cell proliferation and differentiation, and in pyrexia. IL-6, together with other pro-inflammatory cytokines such as IL-1 and TNF-α, induces many inflammatory conditions and plays an important role in the acute response by activating lymphocytes to increase antibody production, cell differentiation into plasma cells, and immunoglobulin G production. Meanwhile, IL-8 acts chemotactically by recruiting neutrophils at the inflammatory sites.[35] MSU is a very important factor for gouty inflammation as a pro-inflammatory stimulus by stimulating cells via TLR signaling.[36] MSU can interact with almost all of the synovial cell types including neutrophils, monocytes/macrophages, and fibroblast-like synoviocytes. In monocytes, microcrystals stimulate the synthesis of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, TNF-α, and PGE_2.[37] A previous study on MSU-induced cell activation showed that MSU deposition in the joints was often caused by leukocyte infiltration that leads to inflammation. A long-term inflammation would result in trophy, deformation of joints, and thickening of synovial walls.[38] Thus, inhibiting these pro-inflammatory cytokines would indicate potential anti-inflammatory properties.

NLR family pyrin domain containing 3 (NLRP3) induces inflammatory responses and cell death in response to PAMPs or DAMPs. Basically,

Table 5: Percentage of inhibitory activities of *Marantodes pumilum* extracts on cytokine secretion in monosodium urate crystals-stimulated human peripheral blood mononuclear cells

Specimen	Plant part	Extract	Percentage of inhibition (%)±SEM				
			IL-1α	IL-1β	IL-6	IL-8	TNF-α
MPA	Roots	DCM	23.86±0.06	ND	13.74±0.52	9.89±0.37	6.78±0.21
		MeOH	24.66±0.36	2.40±0.18	13.04±1.24	14.70±0.15	0.40±0.18
		H_2O	ND	0.62±1.03	17.84±0.84	13.34±2.24	7.13±0.04
	Leaves	DCM	28.26±2.16	2.38±2.52	14.91±0.18	14.99±1.28	3.76±0.33
		MeOH	21.96±0.36	1.35±2.83	14.40±0.12	13. 10±0.76	0.55±0.07
		H_2O	ND	0.77±0.15	5.448±0.10	13.96±0.34	0.73±0.17
MPP	Roots	DCM	73.96±0.36	44.4±1.18	80.91±1.07[b]	40.10±1.58	64.74±0.93[a]
		MeOH	41.56±1.96	5.82±0.41	9.002±0.74	6.95±0.25	40.44±2.31
		H_2O	8.496±0.66	3.05±0.85	7.917±0.68	ND	2.61±0.20
	Leaves	DCM	46.46±0.26	1.88±0.74	10.05±0.48	3.80±1.68	3.79±0.02
		MeOH	ND	6.89±1.72	9.550±0.05	2.76±0.64	4.39±0.07
		H_2O	ND	1.24±0.23	9.923±0.92	0.99±3.72	1. 00±0.58
MPL	Roots	DCM	94.96±0.96[b]	99.91±0.01[b]	97.04±0.47[b]	51.74±0.99[a]	97.65±0.02[b]
		MeOH	25.86±1.06	6.94±0.34	10.71±0.11	ND	37.15±0.55
		H_2O	14.76±0.06	5.08±0.23	6.581±1.71	2.37±2.07	2.42±1.74
	Leaves	DCM	47.46±0.26	4.23±0.01	41.06±1.26	7.23±1.50	34.08±0.45
		MeOH	20.36±5.36	3.11±0.02	10.26±0.02	0.99±1.58	35.16±0.33
		H_2O	ND	1.02±0.04	1.973±0.64	ND	2.64±078
Dexamethasone			87.45±0.83	54.99±2.98	66.63±2.72	60.46±1.71	67.48±2.36
Negative control			0	0	0	0	0

Data are presented as mean±SEM (*n*=3). Data were analyzed using one-way ANOVA followed by *post hoc* Tukey. Concentration of extracts was 50 µg/mL, while dexamethasone was 5 µg/mL. Percentage inhibition >2.5% was significant at $P \leq 0.05$ when compared with negative control. $^{a}P>0.05$ was considered not significantly different compared to dexamethasone; $^{b}P \leq 0.05$, percentage inhibition was significantly higher than dexamethasone. SEM: Standard error of mean; DCM: Dichloromethane; MeOH: Methanol; H_2O: Water; MPA: *Marantodes pumilum* var. *alata*; MPP: *Marantodes* var. *pumila*; MPL: *Marantodes* var. *lanceolata*; TNF: Tumor necrosis factor; IL: Interleukin; ANOVA: Analysis of variance; ND: Inhibition was not detected

Table 6: The IC_{50} values (µg/mL) of *Marantodes pumilum* extracts on cytokine secretion in monosodium urate crystals-stimulated human peripheral blood mononuclear cells

Specimen	Plant part	Extract	IC_{50} values (µg/mL)±SEM				
			IL-1α	IL-1β	IL-6	IL-8	TNF-α
MPP	Roots	DCM	19.83±0.13	-	22.81±0.72	-	31.67±0.93
MPL	Roots	DCM	11.2±0.47	8.92±0.21	12.29±0.30	49.51±1.87	9.60±0.15
Dexamethasone			0.02±0.002	0.71±0.24	0.46±0.03	0.68±0.14	0.15±0.01

Data are presented as mean±SEM (*n*=3). Data were analyzed using one-way ANOVA followed by *post hoc* Tukey. -: Not determined as none of tested concentration exceeded 50% inhibition. All IC_{50} values of extracts were statistically different compared to dexamethasone ($P \leq 0.001$). SEM: Standard error of mean; DCM: Dichloromethane; MPP: *Marantodes* var. *pumil*a; MPL: *Marantodes* var. *lanceolata*; TNF: Tumor necrosis factor; IL: Interleukin; ANOVA: Analysis of variance

Table 7: Percentage of inhibition of *Marantodes pumilum* extracts and the IC_{50} values of active extracts on plasma prostaglandin E_2 secretion in monosodium urate crystals-stimulated human whole blood

Specimen	Plant part	Extract	Percentage of inhibition (%)	IC_{50} (µg/mL)
MPA	Roots	DCM	46.46±0.20	-
		MeOH	45.74±1.71	-
		H_2O	45.78±1.66	-
	Leaves	DCM	22.51±2.14	-
		MeOH	41.16±0.03	-
		H_2O	47.49±1.54	-
MPP	Roots	DCM	66.08±0.81	33.01±0.59
		MeOH	54.45±0.96	47.14±0.55
		H_2O	34.87±0.24	-
	Leaves	DCM	49.33±5.53	-
		MeOH	50.28±3.29	47.2±0.58
		H_2O	43.60±0.14	-
MPL	Roots	DCM	72.87±0.44	31.58±0.57
		MeOH	34.93±1.38	-
		H_2O	14.22±0.37	-
	Leaves	DCM	65.72±0.70	35.26±0.57
		MeOH	52.08±2.25	45.44±0.61
		H_2O	37.57±0.86	-
Indomethacin			97.45±0.73	0.35±0.47
Negative control			0	-

Data are presented as mean±SEM (*n*=3). Data were analyzed using one-way ANOVA followed by *post hoc* Tukey. Concentration of extracts was 50 µg/mL, while indomethacin was 10 µg/mL. -: Not determined as none of tested concentration exceeded 50% inhibition. Percentage inhibition >2.5% was significant at $P \leq 0.05$ when compared with negative control. $^{a}P \geq 0.05$ was considered not significant compared with indomethacin (positive control). All IC_{50} values of extracts were statistically different compared to indomethacin ($P \leq 0.001$). SEM: Standard error of mean; DCM: Dichloromethane; MeOH: Methanol; H_2O: Water; MPA: *Marantodes pumilum* var. *alata*; MPP: *Marantodes* var. *pumila*; MPL: *Marantodes* var. *lanceolata*; ANOVA: Analysis of variance; ND: Inhibition was not detected

MSU can activate NLRP3 inflammasome in gout condition. The activation of NLRP3 converts pro-caspase 1 into caspase-1, which then catalyzes the cleavage of pro-IL-1β into IL-1β. Moreover, MSU also leads to activation of nuclear factor-kappa B (NF-κB) which upregulates the secretion of inflammatory mediators.[2-4]

NF-κB is a major transcription factor that plays an important role in gene regulations in inflammation responses by controlling the expression of genes encoding the pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-8, and TNF-α), adhesion molecules (e.g., intercellular adhesion molecule, vascular cell adhesion molecule, and E-selectin), inducible enzymes (e.g., cyclooxygenase-2 [COX-2] and inducible nitric oxide synthase [iNOS]), growth factors, certain acute phase proteins, and immune receptors.[39,40] Cell activation with LPS and MSU regulates cytoplasmic levels of NF-κB by forming a complex with its inhibitors, the IκBs (IκB-α and IκB-β), that are phosphorylated and degraded via IκB kinases. Therefore, the inhibition of this regulatory enzyme is considered as an important point in inflammatory response in terms of inhibition of pro-inflammatory cytokine secretion.[41] Moreover, activation of NF-κB also mediates the expression of rapid response genes in the inflammatory response to injury, including iNOS and COX-2.

PGE_2 is an eicosanoid that is biosynthesized from arachidonic acid precursor by the action of COX-2 enzyme and PGE_2 synthase in endothelial cells. PGE_2 plays a crucial role as a pro-inflammatory mediator in inflammation. Therefore, an inhibitor of the PGE_2 secretion may be effective as a therapeutic agent for inflammation.[1]

The stimulation of pro-inflammatory stimuli such as LPS or MSU has been known to be responsible in COX-2 expression in various cells, leading to excessive production of PGE_2.[42,43] Thus, the inhibition of COX-2 expression, known to be regulated by NF-κB, would be expected to result in the inhibition of PGE_2 secretion and potentially cause anti-inflammatory action.[43,44]

From this study, only DCM extracts of MPL and MPP roots gave strong inhibitory activities in both LPS- and/or MSU-induced inflammation of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and PGE_2 secretion. The inhibitory activities of DCM extract of MPL roots (50 µg/mL) were even higher or comparable to dexamethasone (5 µg/mL). However, the IC_{50} values were significantly lower than dexamethasone. It is suggested that this extract would be highly potential as an anti-inflammatory. Interestingly, previous study by Mamat *et al.*[22] reported that *M. pumilum* possessed xanthine oxidase inhibitory activity that is related to gouty inflammation. Thus, it strengthens the postulation that this plant might be potentially useful for antigout treatment that can reduce uric acid level as well as inflammatory response.

When only two varieties of MPP and MPL were biologically active, it suggested that the active components and amounts affecting the anti-inflammatory activity were different in all species and plant parts. The presence of phenolics and flavonoids as reported by previous studies might be responsible for the activity.[14,15] Flavonoids are ubiquitous phenolic compounds that have been recognized as potential anti-inflammatory, antioxidant, and anticancer agents. It was reported that flavonoids inhibit the activity and gene expression of various pro-inflammatory mediators as well as up- or down-regulate the transcription factors in inflammatory pathway.[45] Previous studies reported that flavonoids such as quercetin, kaempferol, and apigenin exhibited the inhibition of NF-κB signaling.[46] Quercetin was found to inhibit LPS-stimulated IκB phosphorylation in PBMCs and significantly inhibit TNF-α production and gene expression in concentration-dependent manner. Moreover, quercetin was reported to attenuate the MSU-induced inflammation in rats by decreasing the recruitment of leukocytes, cytokines, and chemokines levels in rats.[46,47] Quercetin and kaempferol also showed potential inhibitory activity via gene expression and secretion of IL-1β or IL-6 in phorbol-12-myristate 13-acetate and calcium ionophore A23187-stimulated human mast cell-1, while myricetin inhibited IL-6 and TNF-α, but not IL-1β and IL-8.[48] Park *et al.*[49] revealed that quercetin and kaempferol scavenged

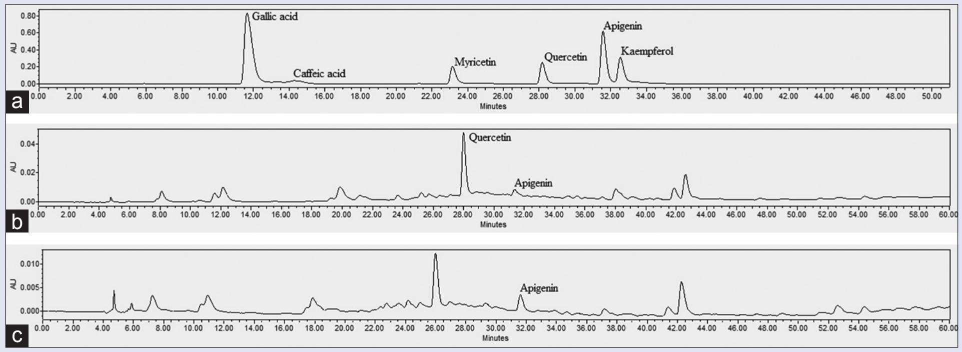

Figure 2: High-performance liquid chromatography chromatograms of (a) a mixture of gallic acid, caffeic acid, myricetin, quercetin, apigenin, and kaempferol standards; (b) dichloromethane extract of *Marantodes pumilum* var. *pumila* roots showing peaks corresponding to quercetin (Rt 27.997 min) and apigenin (Rt 31.354 min); and (c) dichloromethane extract of *Marantodes pumilum* var. *lanceolata* roots showing a peak corresponding to apigenin (Rt 31.065 min)

reactivity of NO and inhibited iNOS in LPS-stimulated RAW264.7 cells, as well as decreased iNOS and COX-2 protein level in activated Chang liver cells. Several flavonoids including quercetin, galangin, apigenin, and naringin have been reported to decrease PGE_2 release in the macrophage cell line J774A.1.[50] Apigenin was reported to inhibit the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in LPS-stimulated PBMCs.[51] The molecular mechanism involves in anti-inflammatory activity of apigenin is suggested to be the inhibition of iNOS, COX-2, IL-6, IL-1β, and TNF-α gene expression and amelioration of p38-MAPK, JNK, and ERK phosphorylation.[45] In short, flavonoids may inhibit pro-inflammatory cytokine secretion and COX-2 expression, as well as PGE_2 secretion. The HPLC analysis in this study substantiated the fact that strong inhibition of DCM extract of MPL roots against IL-1α, IL-1β, IL-6, IL-8, TNF-α, and PGE_2 could be mainly due to the presence of apigenin as previously reported.[52]

CONCLUSION

This investigation demonstrated that extracts of *M. pumilum*, especially DCM extract of MPL and MPP roots, possessed inhibitory activity of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and PGE_2 in LPS- and MSU-stimulated inflammation. The presence of flavonoids such as apigenin might contribute to the activity. The findings suggested that *M. pumilum* has an anti-inflammatory potential against microbial infection and gouty inflammation due to its ability to inhibit inflammatory mediators stimulated by LPS and MSU, respectively. To the best of our knowledge, this is the first report of inhibitory activity of three varieties of *M. pumilum* in LPS- and MSU-induced cytokines and PGE_2 secretion. The results may provide useful data for further investigation of pharmacological activity of *M. pumilum*, especially for antigout activity.

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Conflicts of interest

There are no conflicts of interest.

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