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ORIGINAL RESEARCH

Inhibitory effects of compounds from *Phyllanthus amarus* on nitric oxide production, lymphocyte proliferation, and cytokine release from phagocytes

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Abstract: Standardized extract of *Phyllanthus amarus* has previously been shown to have a strong inhibitory effect on phagocytic activity of human neutrophils. The current study was carried out to evaluate the effects of constituents of the extract of *P. amarus* on nitric oxide (NO) production as well as lymphocyte proliferation and cytokine release from phagocytes. Three compounds, ethyl 8-hydroxy-8-methyl-tridecanoate, 7β,19α dihydroxy-urs-12-ene, and 1,7,8-trihydroxy-2-naphtaldehyde, together with seven known compounds were isolated from the whole plant of *P. amarus*. The isolated compounds and reference standards, ie, gallic acid, ellagic acid, corilagin, and geraniin, which were quantitatively analyzed in the extracts, were evaluated for their effects on immune cells. Among the compounds tested, the lignans, especially phyltetralin and phyllanthin, showed strong inhibition on lymphocyte proliferation with half maximal inhibitory concentration (IC_{50}) values of 1.07 μM and 1.82 μM, respectively. Ethyl 8-hydroxy-8-methyl-tridecanoate and 1,7,8-trihydroxy-2-naphtaldehyde exhibited strong inhibition on nitric oxide production with IC_{50} values of 0.91 μM and 1.07 μM, respectively. Of all the compounds, corilagin was the strongest inhibitor of tumor necrosis factor-α release with an IC_{50} value of 7.39 μM, whereas geraniin depicted the strongest inhibitory activity on interleukin-1β release with an IC_{50} value of 16.41 μM. The compounds constituting the extract of *P. amarus* were able to inhibit the innate immune response of phagocytes at different steps.

Keywords: *Phyllanthus amarus* (Euphorbiaceae), immunosuppressive effects, 1,7,8-trihydroxy-2-naphtaldehyde, 7β, 19α-dihydroxy-urs-12-ene, phyllanthin, hypophyllanthin

Introduction

The immune system is made up of immune cells (neutrophils, monocytes, macrophages, and T-lymphocytes) and specialized immune molecules that are organized to protect the host against foreign invaders such as germs, parasites, and toxins. The immune cells recognize the invading pathogens, leading to the release of several mediators, free radicals, nitric oxide (NO), soluble cytokines, and chemokines, along with the activation of the complement system.[1] Cellular and humoral immune responses have different roles to play in defending the host. Cellular immunity is mediated by T-cell populations and $CD8^+$ and $CD4^+$ T-cells, while humoral immunity is mediated by antibodies produced by B-cells. Upon activation, $CD4^+$ T-cells differentiate into T-helper (Th)1 cells and produce cytokines, including IFN-γ, interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor (TNF)-α, which stimulate delayed-type hypersensitivity and autoimmunity. Th2 cells are characterized by the production of IL-4, IL-5, and IL-10, thereby stimulating humoral immunity.[2] Besides the defensive roles during infections, the phagocyte–microbe interactions

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when excessively or inappropriately deployed can damage host tissues and contribute to the pathogenesis of various immune and nonimmune chronic inflammatory diseases. Immunomodulators are used to enhance or suppress host defense responses in the treatment of those diseases in which defective immune responses play an important role in determining disease outcomes. These include primary and secondary immunodeficiencies that accompany long-standing infections, as well as debilitating diseases such as cancer, rheumatoid arthritis, and systemic lupus erythematosus, leading to acquired immune deficiencies.[3]

Some therapeutic activities of plant extracts or compounds have been proposed owing to their effects on the immune system.[4] Previous studies have indicated that many herbs such as *Phyllanthus debilis, Trigonella foenum graecum, Pouteria cambodiana, Tinospora cordifolia, Centella asiatica, Panax ginseng*, and *Picrorhiza scrophulariiflora* were able to alter the immune function and possess a wide array of immunomodulatory effects.[5–7] Plant extracts that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the immune system, including both adaptive and innate arms of the immune response, can be potential sources of immunomodulators. The immunological effects of compounds can be assessed based on their selective activities on the different components of the immune system. Search for natural immunomodulators from medicinal plants to substitute conventional therapy has gained momentum in recent years.[7,8]

Phyllanthus amarus Schum. & Thonn. (Family: Euphorbiaceae) is a herbaceous plant, widely distributed in most tropical and subtropical countries from Africa to Asia, South America, and the West Indies.[6] It is locally known as "dukung anak" and is highly valued in traditional medicine for its healing properties, to treat inflammatory disorders, viral hepatitis, diarrhea, jaundice, kidney disorders, influenza, diabetes, bronchial infections, sores, swelling, itchiness, and gastric and cardiovascular problems. It is a rich source of phytochemicals, including alkaloids, flavonoids, hydrolyzable tannins, lignans, and polyphenols.[9–13] The ethnopharmacological uses and studies that reported the usefulness of the extracts of *P. amarus* as anti-inflammatory, antioxidant, antiplasmodial, antimalarial, and antidiabetic have prompted researchers to investigate the possible immunomodulatory effects of its isolated compounds on the cellular immune response.[14–19] In addition, several lignans isolated from *P. amarus* such as phyltetralin, nirtetralin, and niranthin revealed important in vivo and in vitro anti-inflammatory activities.[16]

Our previous studies on the standardized extracts of *P. amarus* and *P. urinaria* have revealed that these extracts exhibited strong immunomodulatory activity on human neutrophils.[20] High performance liquid chromatography (HPLC) analysis on *P. amarus* and *P. urinaria* demonstrated the presence of phyllanthin and hypophyllanthin as well as polar compounds such as gallic acid (GA), ellagic acid (EA), corilagin (Cor), and geraniin (Ger), which significantly inhibited the phagocytic activity of human phagocytes.[21] In the current study, the effects of compounds constituting the extract of *P. amarus* on various cellular activities, which include NO production of RAW 264.7 cells, T-cell proliferation, and cytokine release by peripheral blood mononuclear cells (PBMCs), were investigated.

Materials and methods

Chemicals and reagents

Dextran from *Leuconostoc mesenteroides*, phosphate-buffered saline (PBS) tablet, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, gelatin from bovine skin, type B, ficoll, dimethylsulfoxide (DMSO), Trypan blue were obtained from Fluka Chemicals Ltd. (Gillingham, UK). Lymphoprep was obtained from Fresenius Kabi Norge AS (Halden, Norway). Fetal calf serum (FCS) and penicillin/streptomycin (100×) were obtained from PAA Laboratories (North Dartmouth, MA, USA). GA, EA, Cor, and Ger (all standards >98% purity) were purchased from ChromaDex Inc (Irvine, CA, USA). *N*-1(1-Naphthylethylenediamine dihydrochloride was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). [^{3}H]Thymidine and liquid scintillation cocktail solutions, Ultima Gold, were purchased from PerkinElmer Inc. (Waltham, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Sulfanilamide was obtained from MP Biomedicals (Illkirch Cedex, France). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cayman (Ann Arbor, MI, USA). Dexamethasone (99.0% purity) and prednisolone (99% purity) were obtained from Duopharma (Klang, Malaysia). A CO_2 incubator (Shel Lab, Cornelius, OR, USA), light microscope, microplate reader (Thermo Fisher Scientific), and liquid scintillation analysis (PerkinElmer Inc.) were also used in this assay. Molecular weights of the compounds were recorded by ESIMS (electrospray ionization mass spectrometer) using electrospray ionization time-of-flight mass spectrometry (MS; Bruker MicroToF-Q 86; Bruker BioSpin AG, Fällanden, Switzerland) and gas chromatography-MS

(Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA). The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were carried out on a Varian VNMRS 500 MHz with tetramethylsilane as internal standard.

Cell culture

RAW 264.7 cells, a murine macrophage cell line, were grown in DMEM supplemented with 10% of FCS, 1% of penicillin (100 units/mL), and streptomycin (100 µg/mL). They were maintained at 37°C in a humidified atmosphere of 5% CO_2.[22]

Plant collection

The whole plants of *P. amarus* (UKMB 30075) were collected from Marang, Kuala Terengganu, Malaysia, in June 2012. The plant was identified by Doctor Abdul Latif Mohamad of Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), and deposited at the Herbarium of UKM, Bangi, Malaysia.

Extraction and isolation of compounds

The dried material (667 g) was ground and macerated, and then subjected to sequential extraction method with *n*-hexane, ethyl acetate (EtOAc), and methanol (MeOH) (3×1,500 mL). The solvents were removed under reduced pressure to obtain extracts of *P. amarus* (*n*-hexane extract, 17.2 g, 2.58% w/w; EtOAc extract, 29.8 g, 4.46% w/w; MeOH extract, 54.2 g, 8.12% w/w). Ten grams of *n*-hexane extract was subjected to column chromatography (CC; 5 cm of diameter) on silica gel (40–63 µm) and eluted (100 mL for each elution) with a gradient system of *n*-hexane–EtOAc (10:0 to 0:10, v/v) and EtOAc–MeOH (10:0 to 0:10, v/v) to yield 20 mL of each fraction. Each fraction was checked by thin-layer chromatography, and fractions with same spots were combined to afford 19 fractions (F1–F19). Fraction F1 (1.781 g) was subjected to silica gel CC and eluted with a *n*-hexane–EtOAc gradient to afford fractions F1A–E. Fractions 1A and 1B were further purified by silica CC and eluted with *n*-hexane–EtOAc (9:1 to 8:2) to yield **1** (8.2 mg) and **6** (2.5 mg). Fraction F2 (703.5 mg) was chromatographed on silica CC repeatedly with *n*-hexane–EtOAc (8:2) and purified by recrystallization from *n*-hexane to afford **3** (235.8 mg). Fractions F10 (664 mg), F14 (949 mg), and F15 (579 mg) were treated in a similar way to obtain a mixture of **4** and **5** (47.4 mg), **2** (4.1 mg), and **7** (269 mg), respectively. Further purification of fractions F16 and F18 by silica CC eluted with *n*-hexane–EtOAc (6:4) afforded **8** (10.1 mg) and **9** (115.0 mg), respectively. Ten grams of EtOAc extract was fractionated by vacuum liquid chromatography on silica gel type 60H F_{254} (10–40 µm) and eluted with a gradient system of *n*-hexane:EtOAc (10:0 to 0:10, v/v) and EtOAc:MeOH (10:0 to 0:10, v/v) to afford 12 fractions. Repeated silica gel column of fractions FE4 (1.1 g) and FE6 (0.222 g) with a gradient system of *n*-hexane:EtOAc (10:0 to 0:10, v/v) and EtOAc:MeOH (10:0 to 0:10, v/v) afforded **10** (5.9 mg). Fifteen grams of MeOH extract was treated similarly as the EtOAc extract to yield **10** (2 mg). The structures of the known compounds were determined by a combination of ESIMS, ^{1}H, and ^{13}C NMR techniques, and by comparison of their spectral data with literature values. The purities of all isolated compounds were >98% based on their physicochemical properties, HPLC, NMR, and MS-time-of-flight data.

Ethyl 8-hydroxy-8-methyl-tridecanoate (**1**): White solid, R_f 0.6 in *n*-hexane–EtOAc (8:2); mp 69°C–71°C, ultraviolet (UV) ($CHCl_3$) λ_{max}: weak absorption in 242 nm; infrared (IR) ν_{max} (KBr): 3,436 (OH), 1,736 (C=O) cm^{-1}; gas chromatography-MS: $C_{16}H_{32}O_3$, $[M]^+$ 272. ^{1}H NMR ($CDCl_3$) δ 0.87 (*m*, H-1, H-42, H-43), 1.26 (*bs*), 1.62 (*dd*, H-2, H-4, *J*=14, 7.0 Hz), 2.29 (*t*, H-37, *J*=8.0 Hz), 4.06 (*t*, H-39, *J*=7.0 Hz) ppm and ^{13}C NMR ($CDCl_3$). δ 14.1, 22.7, 25.1, 25.9, 28.7, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 34.4, 64.4, 174.0. ^{1}H and ^{13}C spectra of compound **1** are shown in Figures S1 and S2, respectively.

7β,19α Dihydroxy-urs-12-ene (**6**): White needle-like crystals, R_f 0.6 in *n*-hexane–EtOAc (8:2); mp 59°C–60°C, UV (MeOH) λ_{max}: 251 nm; IR ν_{max} (KBr): 3,504 (OH), 1,650 (C=C) cm^{-1}; HRESIMS (high resolution electrospray ionization mass spectrometry; positive ion mode): *m/z* 442.2888 $[M]^+$, 465.3560 $[M+Na]^+$ (Calcd for $C_{30}H_{52}O_2$, $C_{30}H_{52}O_2+Na$, 442.38, 465.3698, respectively); for ^{1}H NMR and ^{13}C NMR spectroscopic data (Table 1). ^{1}H and ^{13}C spectra of compound **6** are shown in Figures S3 and S4, respectively.

1,7,8-Trihydroxy-2-naphtaldehyde (**10**): White brown semi-solid, UV (MeOH) λ_{max}: 283.50 nm and 221 nm; HRESIMS (positive ion mode): *m/z* 205.0854 $[M+H]^+$ (Calcd for $C_{11}H_8O_4+H$, 204.0423); for ^{1}H NMR and ^{13}C NMR spectroscopic data (Table 2). ^{1}H and ^{13}C spectra of compound **10** are shown in Figures S5 and S6, respectively.

Quantitative determination of the major components of 80% ethanol extract of *P. amarus* by HPLC

The diluted solutions of 80% ethanol extract and the reference standards (GA, EA, Cor, Ger) were analyzed separately by using a validated reversed-phase HPLC method as described earlier by Jantan et al.[21]

Table 1 NMR spectroscopic data (500 MHz, $CDCl_3$) for compound **6** (δ in ppm)

Position	δ_C	δ_H, multiplicity (J in Hz)	HSQC	HMBC	NOESY
1	34.1	1.42, m	C-1, H-1	C-11	–
		1.45, m		C-11	
2	18.1	1.39, m	C-2, H-2	C-5	–
		1.52, m		C-5	
3	28.9	1.14, m	C-3, H-3	–	–
		1.35, m		–	
4	27.8	1.60, m	C-4, H-4	–	–
		1.67, m		–	
5	51.7	1.58, m	C-5, H-5	–	–
6	28.3	1.77, m	C-6, H-6	–	–
		1.83, m		–	
7	76.4	3.45, m	C-7, H-7	–	H-24, 6
8	42.8	–		–	–
9	40.8	–		–	–
10	38.6	–		–	–
11	24.0	1.81, m	C-11, H-11	–	H-21
		1.91, m		–	
12	122	5.58, d (5.5)	C-12, H-12	C-18, 17, 11, 9	H-26, 24, 6
13	141.9	–		–	–
14	39.3	–		–	–
15	19.9	0.83, m	C-15, H-15	–	–
		1.18, m		–	
16	35.4	1.56, m	C-16, H-16	C-22, 19, 15, 8	–
17	44.3	1.46, m	C-17, H-17	–	H-25, 21
18	50.2	2.05, d (15)	C-18, H-18	–	–
19	60.0	0.97 (s)	C-19, H-19	–	–
20	30.8	1.32	C-20, H-20	–	–
21	29.1	1.06, m	C-21, H-21	–	H-30, 17
		1.18, m		–	
22	34.8	–		–	–
23	14.9	0.89, s	C-23, H-23	C-10, 5	H-25
24	25.5	1.11, s	C-24, H-24	C-26, 13, 9, 7	–
25	16.1	0.75, s	C-25, H-25	C-16, 10, 8	–
26	29.4	1.01, s	C-26, H-26	C-24, 13, 9, 7	–
27	15.7	0.97, s	C-27, H-27	C-22, 21, 17	H-28, 16
28	22.9	0.79, d (6.5)	C-28, H-28	C-29, 20, 19	–
29	21.9	0.85, s	C-29, H-29	C-28, 20, 19	–
30	17.9	0.86, s	C-30, H-30	C-18, 17	C-21, 16

Note: – indicates data not applicable.
Abbreviations: HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single-quantum correlation; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; s, singlet; d, doublet; m, multiplet.

Table 2 NMR spectroscopic data (500 MHz, MeOD) for compound **10** (δ in ppm)

Position	δ_C	δ_H (J in Hz)	HMBC	NOESY
1	164.1	–	–	–
2	128.7	–	–	–
3	132.1	7.78, d (8.5)	C-1, 4, 11	H-4, 5
4	115.6	6.92, d (9.0)	C-2	H-3, 6
5	115.6	6.92, d (9.0)	C-7	–
6	132.1	7.78, d (8.5)	C-8	–
7	127.6	–	–	–
8	164.1	–	–	–
9	114.4	–	–	–
10	103.4	–	–	–
11	191.4	9.76, s	C-2, 3	H-3

Note: – indicates data not applicable.
Abbreviations: HMBC, heteronuclear multiple bond correlation; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy.

Isolation of PBMCs

PBMCs were isolated by using a modified method of Gmelig-Meyling and Waldmann.[23] Briefly, venous blood (10 mL) was obtained in heparin-containing tubes containing equal amount of physiological saline (1:1) by aseptic vein puncture from healthy human volunteers who were nonsmokers, had fasted overnight, and did not take any medicine or supplements. Written informed consent was provided for protection of the rights and welfare of human subjects involved in this research. The diluted blood was then centrifuged at 400× g for 20 minutes after carefully layered on lymphoprep. The monocytes, formed as a distinct band at the medium interface, were collected carefully using a Pasteur pipette. The collected

cells were diluted with PBS and centrifuged at 250× *g* for 10 minutes for the purification of cells. The cells were then suspended in PBS and counted using a hemocytometer. The number of cells was adjusted to 5×10^5 cells/mL. The use of human blood was approved by the Human Ethical Committee of UKM (approval number: FF/2012/Ibrahim/23-May/432-May 2012–August 2013).

Cell viability

Cytotoxicity of samples and positive controls (dexamethasone and prednisolone) on PBMC and RAW 264.7 cells was determined using MTT test as previously described by Jantan et al.[21] One hundred microliters of RAW 264.7 cells (1×10^6/mL) or PBMC (5×10^5/mL) suspensions in RPMI 1640 supplemented with 10% of FCS were incubated with 100 µL samples of five different concentrations (3.125–100 µg/mL) in a 96-well round bottom microplate at 37°C, 5% CO_2, for 2 days and 4 days, respectively. Plates were incubated again for 4 hours at 37°C, 5% CO_2, with 25 µL of MTT reagent (1 mg/mL). Supernatant was discarded, the formazan produced in each well was dissolved in DMSO, and the optical densities were measured by ELISA readers at 570 nm.

NO production

The NO assay was performed as described earlier with slight modification.[24] Briefly, RAW 264.7 cells (1×10^6 cells/mL) were incubated with different concentrations of compounds ranging from 3.125 µg/mL to 50 µg/mL or dexamethasone as positive control (0.0004–4 µg/mL) followed by stimulation with LPS (1 µg/mL). The compounds or the positive control were dissolved in DMSO and diluted with DMEM into the appropriate concentrations. The final concentration of DMSO was adjusted to 0.1% (v/v) to eliminate the effect of the solvent on cytotoxicity and NO production. The control wells contained the same amount of DMSO but no compounds. The plates were incubated for 24 hours at 37°C, 5% CO_2. Amounts of nitrite were measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Then, 100 µL of culture supernatant was added to 100 µL of Griess reagent, followed by incubation for 10 minutes in the dark. Absorbance was measured at 550 nm in a microplate reader. Nitrite concentrations were calculated using standard solutions of $NaNO_3$.

Lymphocyte proliferation

The antiproliferation effect of compounds was determined by measuring the inhibition of phytohemagglutinin (PHA)-induced T-cell proliferation by incorporation of radioactive thymidine following a methodology reported by Amirghofran et al.[25] Briefly, 50 µL of mononuclear cells suspension was added to each well of 96-well plates containing 50 µL RPMI 1640 medium supplemented with 10% of FCS, 1% of penicillin (100 units/mL), and streptomycin (100 µg/mL). Then, 50 µL of different concentrations of the compounds ranging from 3.125 µg/mL to 50 µg/mL or prednisolone (0.0005–5 µg/mL) and 50 µL of media containing PHA (20 µg/mL) were added to the mixture. The compounds and prednisolone were dissolved in DMSO and adjusted to the appropriate concentrations by the cell culture medium. The final concentration of DMSO at the cells was 0.1%. At this concentration, DMSO did not show any effects on the lymphocyte proliferation. The plates were incubated in a CO_2 incubator for 3 days. The amount of 25 µL of [^{3}H]thymidine (0.5 µCi/well) was then added to the wells and incubated again for 18 hours. The cells were harvested using a cell harvester. Harvested cells were dissolved in 2.5 mL of liquid scintillation cocktail. Then, [^{3}H]thymidine incorporation was measured as count per minute using liquid scintillation analysis.

Cytokine release assay

The effect of the bioactive isolates on the synthesis of inflammatory cytokines, TNF-α, and IL-1β in mononuclear cells was measured by ELISA. LPS (1 µg/mL) and isolated compounds (3.125–100 µg/mL) or dexamethasone (0.0005–5 µg/mL) were incubated with PBMC suspension for 12 hours at 37°C, 5% CO_2. The compounds and standards were dissolved in DMSO and diluted in cell culture medium to the required concentrations. Then, they were centrifuged at 300× *g* for 10 minutes at 4°C, and the supernatant was transferred to 96-well plates. The assessment of cytokine levels in the supernatant of human blood cell cultures was accomplished using the appropriate ELISA kit. The cytokine concentration in the sample was determined from appropriate calibration curves.[26]

Statistical analysis

All the data were analyzed using Statistical Package for Social Sciences Version 15.0. Each sample was measured in triplicate, and the data were presented as mean ± standard error of the mean. The half maximal inhibitory concentration (IC_{50}) values were calculated using Graph Pad Prism 6 software. The values were obtained from at least three determinations. Data were analyzed using a one-way ANOVA for multiple comparisons. $P\leq0.05$ was considered to be different significantly.

Results and discussion

The whole plants of *P. amarus* were sequentially extracted with *n*-hexane, EtOAc, and MeOH. The *n*-hexane extract

was subjected to repeated silica CC to yield two new compounds, ethyl 8-hydroxy-8-methyl-tridecanoate (**1**) and 7β,19α dihydroxy-urs-12-ene (**6**), in addition to the known compounds, isolintetralin (**2**), 1-triacontanol (**3**), stigmasterol (**4**), β-sitosterol (**5**), hypophyllanthin (**7**), phyltetraline (**8**), and phyllanthin (**9**). Fractionation of the EtOAc extract by vacuum liquid chromatography on silica gel type 60H, followed by successive separations on silica CC, afforded a new compound, 1,7,8-trihydroxy-2-naphtaldehyde (**10**). The MeOH extract was treated similarly as the EtOAc extract and afforded compound **10**. The structures of the new compounds are shown in Figure 1. The structures of the known compounds were elucidated by a combination of ESIMS, ^{1}H, and ^{13}C NMR techniques, and by comparison of their spectral data with literature values.[27–29]

Analysis of the NMR spectrum of compound **1** showed the presence of a long-chain hydrocarbon, and one carbon was attributed to a carbonyl group. The ^{1}H NMR of **1** showed a singlet at δ_H 0.87 for three methyl groups, a multiplet at δ_H 1.61 for four $-CH_2$ protons next to a CHOH group, and a triplet at δ_H 2.29 for two protons of CH_2 adjacent to a C=O group, while two protons at δ_H 4.05 were assigned to CH_2 attached to CH_2OCO. The remaining methylene appeared as a broad singlet at δ_H 1.26. The ^{13}C NMR spectrum of **1** exhibited signals for carbonyl carbon at δ_C 174.03 and hydroxymethylene carbon at δ_C 64.41. The mass spectrum of **1** exhibited a molecular ion peak with undetectable two ion radicals at *m/z* 270 corresponding to a molecular formula of an ethyl ester, $C_{16}H_{32}O_3$, $[M]^+$ 272. The ion fragments arising at *m/z* 74 (73+1 $[CH_3CH_2COO]^+$), 87 $[CH_2]^+$, 143 $[(CH_2)_4]$, and 155 $[CH_2]$ were due to two undetectable radicals. The loss of 44 ions demonstrated the fragment ion of methyl-hydroxy branched $[COHCH_3]^+$, whereas the loss of 71 ions demonstrated the fragment ion of $[(CH_2)_4CH_3]^+$. Based on the data, we concluded that **1** was a new ethyl ester, namely ethyl 8-hydroxy-8-methyl-tridecanoate. The long-chain ethyl esters have been previously identified in several essential oils. For example, ethyl tridecanoate was identified in the essential oils from two species of *Houttuynia*.[30]

Compound **6** was obtained as white needle-like crystals and has a molecular formula of $C_{30}H_{52}O_2$ as determined by HRESIMS (*m/z* 442.2888 $[M]^+$, 465.3560 $[M + Na]^+$). ^{13}C NMR exhibited 30 signals in accordance with the molecular formula $C_{30}H_{50}O_2$. ^{1}H-NMR (Table 1) showed eight methyls at δ_H 0.75, 0.79, 0.85, 0.86, 0.89, 0.97, 1.01, and 1.11. All these peaks appeared as singlets except one methyl at

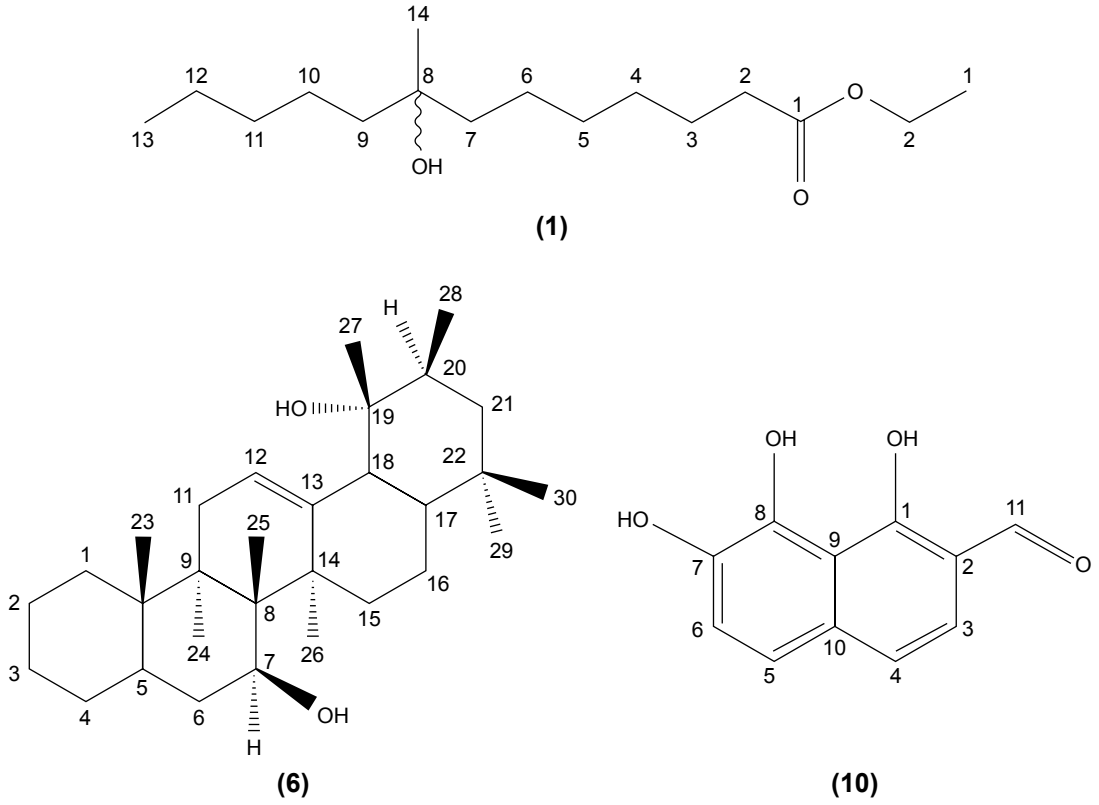

Figure 1 Structures of new compounds isolated from *P. amarus*.
Abbreviation: *P. amarus*, *Phyllanthus amarus*.

δ_H 0.79 as doublet. A signal at δ_H 3.45 (H-7) was attributable to a proton which was carried by a carbon bonded to a hydroxyl group. In the spectrum, there was also present an olefinic proton at δ_H 5.58. In the heteronuclear multiple bond correlation (HMBC) spectrum, the protons resonating at δ_H 0.79 (H-28) showed ^3J correlation with C-19 (δ_C 60.1) and ^2J coupling with C-20 (δ_C 30.8). The methyl at C-25 was observed to correlate with C-8 (δ_C 42.8) (^2J) and C-10 (δ_C 51.7) (^3J), whereas the methyl protons at C-26 (δ_C 1.01) showed ^3J correlation with C-13 (δ_C 141.9) and C-7 (δ_C 76.4). The relative configuration of **6** was determined using nuclear Overhauser effect spectroscopy (NOESY) correlation and was supported by comparison with literature values for similar compounds. The correlations between H-7 and H-24, H-6, as well as between H-12 and H-26, H-24, H-6 implied that these protons accepted α-orientation. The OH position at C-7 has been assigned by HMBC analysis and supported by NOESY correlations. In the HMBC spectrum, the protons resonating at δ_H 1.11 (H-24) showed ^3J correlation with δ_C 76.4 (C-7) and δ_H 1.01 (H-26) showed ^3J correlation with δ_C 76.4 (C-7). Meanwhile, δ_H 1.01 (H-26) also showed ^3J correlation with δ_C 141.9 (C-13) and δ_C 40.8 (C-9). NOESY correlations displayed the correlations between H-7 and H-24, H-6, as well as between H-12 and H-26, H-24, H-6. The 7_β-hydroxyl position was indicated by NOESY from the H-7_α. The relative configuration of the hydroxyl at C-19 was deduced to be α by NOESY correlations at the H-27_β position which correlated with C-19 with H-28, H-16. Heteronuclear single-quantum correlation (HSQC) and HMBC spectra of compound **6** are shown in Figures S7 and S8, respectively. Figure 2 shows HMBC and NOESY correlations, respectively, of compound **6**. The complete data of NOESY, HSQC, and HMBC experiments are shown in Table 1. Based on these data, we concluded that compound **6** was a new pentacyclic triterpene, 7β,19α dihydroxy-urs-12-ene.

Compound **10** was obtained as a white brown semisolid. The HRESIMS spectrum exhibited a molecular ion peak $[M + H]^+$, at *m/z* 205.0854 suggesting a molecular formula of $C_{11}H_8O_4$. The ^1H and ^{13}C NMR spectra of **10** indicated that it was a naphthalene derivative. The ^1H spectrum showed three proton peaks: two doublets for two protons and one doublet for one proton. There were three protons in one benzene ring and two protons in another ring showed ortho coupling to each other, based on their NOESY correlations and coupling constants; δ_H 6.91 (d, H-4, H-5, *J*=9.0 Hz), 7.77 (d, H-3, H-6, *J*=8.5 Hz), 9.76 (s, CHO) (Table 2). The chemical shifts of the quaternary aromatic carbons indicated that three of the carbons were hydroxylated (δ_C 127.61, 164.09) and one carbon was an aldehyde group (δ_C 191.44). The connectivities between protons and carbons in the HSQC and HMBC spectra indicated that the proton H-3 was ^2J coupled with C-4 (δ_C 115.56) and ^3J coupled with C-1 (δ_C 164.09) and C-11 (δ_C 191.44); the proton H-4 showed ^3J coupling with C-2 (δ_C 128.73); the proton H-5 showed ^3J coupling with C-7 (δ_C 127.61); the proton H-11 showed ^2J coupling with C-2 (δ_C 128.73) and ^3J correlation with C-3 (δ_C 132.05). Conclusively, we identified **10** as a new compound, 1,7,8-trihydroxy-2-naphtaldehyde. Naphthalene derivatives have previously been isolated from other plants such as from the rhizome of *Eleutherine americana*[31] and roots of *Diospyros assimilis*.[32]

The phenolic compounds in the 80% ethanol extract of *P. amarus*, ie, GA, EA, Cor, and Ger, were identified and quantified by a validated reversed-phase HPLC method as previously reported by Jantan et al.[21]

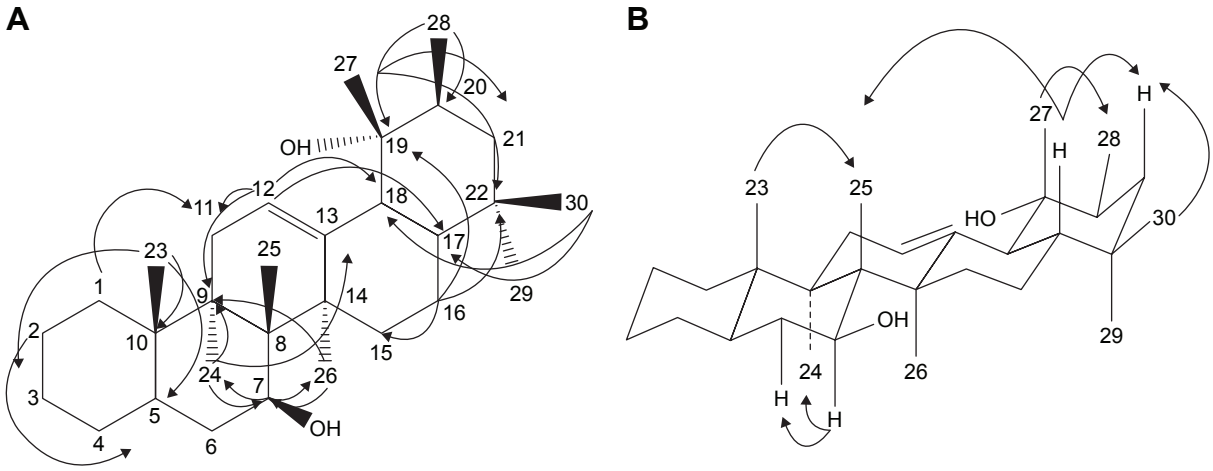

Figure 2 HMBC (**A**) and NOESY (**B**) correlation of 7β,19α dihydroxy-urs-12-ene (**6**).
Abbreviations: HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy.

Table 3 IC_{50} values (μg/mL) of lymphocyte proliferation, cytokine release, and NO inhibitory activities of *P. amarus* extract and its chemical constituents on phagocytes (mean ± SEM, n=3)

Samples	Lymphocyte proliferation	Nitric oxide	TNF-α	IL-1β
P. amarus extract	0.52±1.05	4.84±3.14	12.09±3.06	7.58±3.21
Ethyl 8-hydroxy-8-methyl-tridecanoate (**1**)	3.78±1.43 (13.93±3.77)	0.25±0.01 (0.91±0.03)	–	–
Isolintetralin[a] (**2**)	–	–	–	–
1-triacontanol (**3**)	2.36±0.20 (5.39±0.46)	1.01±0.28 (2.30±0.68)	–	–
Stigmasterol[a] (**4**)	–	–	–	–
β-sitosterol[a] (**5**)	–	–	–	–
7β,19α dihydroxy-urs-12-ene[a] (**6**)	–	–	–	–
Hypophyllanthin (**7**)	0.93±0.38 (2.18±0.89)	1.36±0.01 (3.16±0.02)	12.75±3.45 (29.65±8.03)	–
Phyltetralin (**8**)	0.44±0.03 (1.07±0.07)	1.23±0.38 (2.95±0.93)	–	–
Phyllanthin (**9**)	0.76±0.13 (1.82±0.33)	2.49±0.81 (5.95±1.94)	17.00±5.47 (40.67±0.66)	–
1,7,8-trihydroxy-2-naphtaldehyde (**10**)	5.84±0.89 (28.67±4.37)	0.22±0.10 (1.07±0.31)	–	–
GA	0.96±0.69 (5.66±4.05)	4.12±0.80 (24.17±2.50)	13.92±4.65 (76.32±7.12)	35.28±3.62 (194.99±6.35)
EA	13.23±1.24 (43.77±1.54)	5.54±0.22 (18.36±1.09)	32.47±3.66 (105.95±6.65)	39.20±3.61 (134.10±5.02)
Ger	12.31±1.68 (22.60±1.32)	4.25±0.47 (7.81±0.31)	7.51±5.31 (8.66±2.51)	14.80±2.56 (16.41±0.74)
Cor	11.08±1.17 (17.41±2.12)	5.07±1.75 (7.98±2.05)	4.60±2.99 (7.39±2.53)	27.40±1.17 (43.19±2.53)
Predni	0.038±0.002 (0.09±0.06)	–	–	–
Dexa	–	0.14±0.76 (0.35±0.23)	0.77±0.27 (1.96±0.44)	0.26±1.02 (0.67±1.32)

Notes: IC_{50} values (μM) are in parentheses. [a]Not determined. – indicates data not applicable.
Abbreviations: Cor, corilagin; Dexa, dexamethasone; EA, ellagic acid; GA, gallic acid; Ger, geraniin; IC_{50}, half maximal inhibitory concentration; IL-1β, interleukin-1β; NO, nitric oxide; *P. amarus*, *Phyllanthus amarus*; Predni, prednisolone; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

P. amarus extract was shown to possess inhibitory effects on phagocytic activity of human neutrophils in our previous study.[20,21] In this study, ten compounds were isolated from *P. amarus,* but only six compounds were investigated for their immunomodulatory activity due to very limited amounts of compounds isolated (**2** and **6**) and also one sample was a mixture of stigmasterol (**4**) and β-sitosterol (**5**). The reference standards, GA, EA, Cor, and Ger were also investigated for their immunomodulatory activity. The cell viability test was performed using MTT assay to determine the nontoxic concentrations for PBMCs and RAW 264.7 cells. Only the viable and metabolically active cells can cleave the MTT to produce formazan, hence the more the color intensity of the medium the more the cells were viable.[33,34] PBMCs were viable (>90%) at 3.125 μg/mL and 100 μg/mL of the compounds after 4-day incubation, while at similar concentrations RAW 264.7 cells were also >90% viable after 2-day incubation.

In agreement with the reactive oxygen species production activity as previously reported by us,[20,21] most of the compounds tested exhibited strong inhibition on NO production, as compared with LPS-stimulated macrophages (Table 3, Figure 3). Both inhibition of NO and superoxide production consequently inhibited peroxynitrite formation, a highly reactive compound with various harmful effects on cells.[35] Cells were pretreated with the different concentrations of compounds (3.125–100 μg/mL) before activation with LPS to activate inducible nitric oxide synthase (iNOS) synthesis

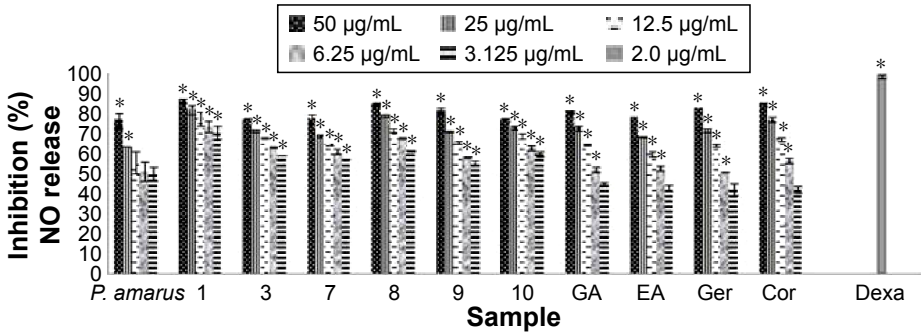

Figure 3 Percentage inhibition of NO release by *P. amarus* and its major compounds on LPS-stimulated RAW 264.7 cells.
Notes: Data are mean ± SEM (n=3). Significance of differences with respective control: *$P<0.05$. For Dexa, only percentage inhibition at 4 μg/mL is shown.
Abbreviations: Cor, corilagin; Dexa, dexamethasone; EA, ellagic acid; GA, gallic acid; Ger, geraniin; LPS, lipopolysaccharide; NO, nitric oxide; *P. amarus*, *Phyllanthus amarus*; SEM, standard error of the mean.

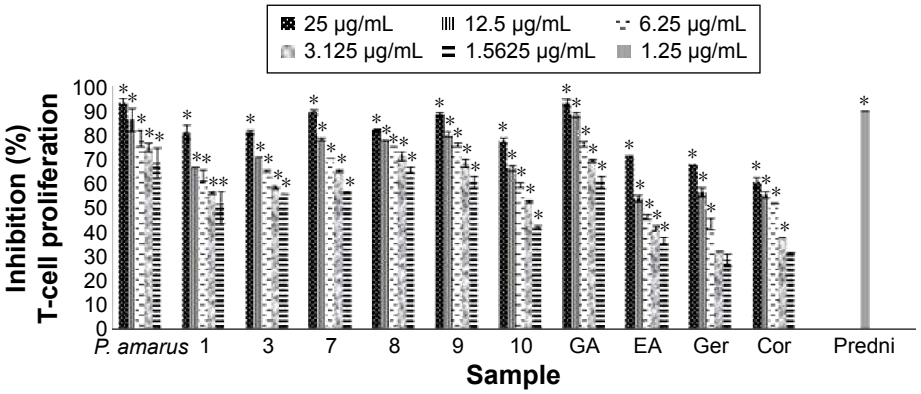

Figure 4 Percentage inhibition of T-cell proliferation by *P. amarus* and its major compounds on PHA-stimulated human PBMCs.
Notes: Data are mean ± SEM (n=3). Significance of differences with respective control: *$P<0.05$. For Predni, only percentage inhibition at 5 µg/mL is shown.
Abbreviations: Cor, corilagin; Dexa, dexamethasone; EA, ellagic acid; GA, gallic acid; Ger, geraniin; *P. amarus*, *Phyllanthus amarus*; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; Predni, prednisolone; SEM, standard error of the mean.

and eventually NO release. Among the compounds, ethyl 8-hydroxy-8-methyl-tridecanoate (**1**) and 1,7,8-trihydroxy-2-naphtaldehyde (**10**) showed strong inhibitory activity on NO production by macrophages with IC_{50} values of 0.91 µM and 1.07 µM, respectively, which were higher than dexamethasone (0.35 µM; Table 3). Dexamethasone was reported to have inhibitory activities on iNOS expression and NO production by destabilizing mRNA in LPS-treated macrophages.[36]

Lymphocytes can be activated to proliferate in vitro by mitogens such as PHA. The addition of compounds from *P. amarus* at the initiation of lymphocyte cultures inhibited proliferative responses (Table 3, Figure 4). Inhibition by the compounds could have resulted from a failure to initiate lymphocyte transformation or interference with the exponential increase in the number of dividing cells.[37] All compounds significantly decreased the [^{3}H] thymidine incorporation of proliferating (PHA-stimulated) lymphocytes in a dose-dependent manner, when compared to prednisolone, the positive control (Figure 4). Of all compounds, the lignans especially phyltetralin (**8**) exhibited the strongest inhibition on lymphocyte proliferation with an IC_{50} value of 1.07 µM, which was about ten times higher than prednisolone (0.09 µM). Prednisolone was reported to have inhibitory activity on whole blood lymphocyte proliferation.[38]

The MAPK cascade regulates both NF-κB- and AP-1-associated gene transcription. The activation of this system by extracellular stimuli such as the bacterial product LPS resulted in increasing levels of the inflammatory enzymes cyclooxygenase-2 (COX-2) and of cytokines.[38] Cor and Ger exhibited moderate inhibitory activity on TNF-α from PBMCs with IC_{50} values of 7.39 µM and 8.66 µM, respectively, whereas Ger depicted the strongest inhibitory activity on IL-1β release from PBMCs with an IC_{50} value of 16.41 µM (Table 3, Figures 5 and 6). These compounds selectively inhibited TNF-α release. The results suggest that these compounds reduced the translocation of NF-κB induced by LPS and subsequently decreased the level of proinflammatory cytokines, especially TNF-α production. Blockage of TNF-α receptor is another possible mechanism. The inhibitory effects of Cor

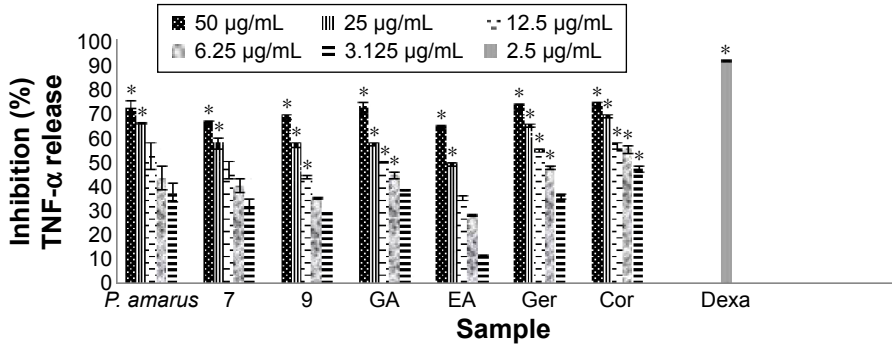

Figure 5 Percentage inhibition of TNF-α by *P. amarus* and its major compounds on LPS-stimulated human PBMCs.
Notes: Data are mean ± SEM (n=3). Significance of differences with respective control: *$P<0.05$. For Dexa, only percentage inhibition at 5 µg/mL is shown.
Abbreviations: Cor, corilagin; Dexa, dexamethasone; EA, ellagic acid; GA, gallic acid; Ger, geraniin; LPS, lipopolysaccharide; *P. amarus*, *Phyllanthus amarus*; PBMCs, peripheral blood mononuclear cells; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

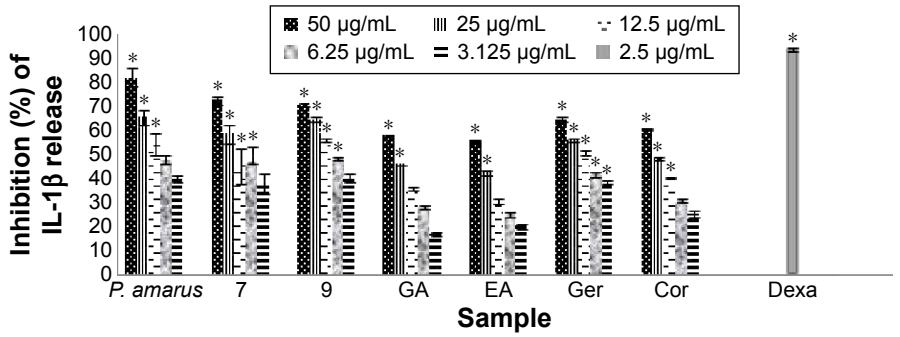

Figure 6 Percentage inhibition of IL-1β by *P. amarus* and its major compounds on LPS-stimulated human PBMCs.
Notes: Data are mean ± SEM (n=3). Significance of differences with respective control: *P<0.05. For Dexa, only percentage inhibition at 5 µg/mL is shown.
Abbreviations: Cor, corilagin; Dexa, dexamethasone; EA, ellagic acid; GA, gallic acid; Ger, geraniin; IL-1β, interleukin-1β; LPS, lipopolysaccharide; *P. amarus*, *Phyllanthus amarus*; PBMCs, peripheral blood mononuclear cells; SEM, standard error of the mean.

and Ger on the innate immune response of phagocytes are comparable with previous studies. Cor has been reported to significantly reduce production of proinflammatory cytokines and mediators TNF-α, IL-1β, IL-6, NO (iNOS), and COX-2 on both protein and gene level by blocking NF-κB nuclear translocation in RAW 264.7 cell line.[39] Cor has also been shown to be able to inhibit the double-strand break-triggered NF-κB pathway in irradiated microglial cells.[40] Ger and other polyphenols have been shown to suppress LPS-induced NF-κB activity through downregulation of IκB kinase activity in macrophages.[41] Our previous study has demonstrated that Ger, Cor, and EA had lower IC_{50} values than that of ibuprofen in blocking the migration of polymorphonuclear leukocytes.[21]

Conclusion

Among the compounds tested, the lignans, especially phyltetralin (**8**) and phyllanthin (**9**), showed strong inhibition on lymphocyte proliferation, while ethyl 8-hydroxy-8-methyltridecanoate (**1**) and 1,7,8-trihydroxy-2-naphtaldehyde (**10**) exhibited strong inhibition on NO production. Cor was the strongest inhibitor of TNF-α release, whereas Ger depicted the strongest inhibitory activity on IL-1β release. The compounds constituting the extract of *P. amarus* were able to inhibit the innate immune response of phagocytes at different steps.

The results suggest that the strong immunosuppressive effect of the plant extract was mainly contributed by these compounds, which could be further developed into leads for the development of immune-related disorders including inflammation.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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