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Original article

Phyllanthin from *Phyllanthus amarus* inhibits cellular and humoral immune responses in Balb/C mice

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ABSTRACT

Background: Phyllanthin found in many *Phyllanthus* species has various biochemical and pharmacological properties especially on its hepatoprotective effects. However, its effect on the immune system has not been well documented.
Purpose: In the present study, phyllanthin isolated from *Phyllanthus amarus* was investigated for its immunosuppressive effects on various cellular and humoral immune responses in Balb/C mice.
Methods: Male mice were treated daily at 20, 40 and 100 mg/kg of phyllanthin for 14 days by oral gavage. The effects of phyllanthin on cellular immune responses in treated /non treated mice were determined by measuring CD 11b/CD 18 integrin expression, phagocytosis, nitric oxide (NO) production, myeloperoxidase activity (MPO), T and B cells proliferation, lymphocyte phenotyping, serum cytokines production by activated T-cells and delayed type hypersensitivity (DTH). Its effects on humoral immune responses were evaluated by determining the serum levels of lysozyme and ceruloplasmin, and immunoglobulins (IgG and IgM).
Results: Phyllanthin dose-dependently inhibited CD11b/CD18 adhesion, the engulfment of *E. coli* by peritoneal macrophages molecules, NO and MPO release in treated mice. Phyllanthin caused significant and dose-dependent inhibition of T and B lymphocytes proliferation and down-regulation of the Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines. Phyllanthin at 100 mg/kg caused a significant reduction in the percentage expression of $CD4^+$ and $CD8^+$ in splenocytes and the inhibition was comparable to that of cyclosporin A at 50 mg/kg. At 100 mg/kg, phyllanthin also dose-dependently exhibited strong inhibition on the sheep red blood cell (sRBC)-induced swelling rate of mice paw in DTH. Significant inhibition of serum levels of ceruloplasmin and lysozyme were observed in mice fed with higher doses (40 and 100 mg/kg) of phyllanthin. Anti-sRBC immunoglobulins (IgM and IgG) antibody titer was down-regulated in immunized and phyllanthin-treated mice in a dose-dependent manner with maximum inhibition being observed at 100 mg/kg.
Conclusion: The strong inhibitory effects of phyllanthin on the cellular and humoral immune responses suggest that phyllanthin may be a good candidate for development into an effective immunosuppressive agent.

Introduction

Among the bioactive constituents of *Phyllanthus amarus* Schum and Thonn (Euphorbiaceae), phyllanthin has been well investigated for its biochemical and pharmacological properties especially on its protective effects on the liver. The hepatoprotective effects of phyllanthin have been reported based on many *in vitro* and *in vivo* studies (Sethiya et al., 2015; Kheng et al., 2015; Jia et al., 2016). Phyllanthin has also been much investigated for its antitumor effects on various cancer cell lines (Leite et al., 2006; Islam et al., 2008). The anti-apoptotic, antioxidant and anti-inflammatory effects of the standardized aqueous extract of *P. amarus* in rat colitis induced by acetic acid were due to the protective role of

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Con A, concanavalin A; Cys A, cyclosporin A; DMEM, Dulbecco's Modified Eagle Medium; DTH, delayed type hypersensitivity; FBS, fetal bovine serum; fMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balance salt solution; IgM and IgG, immunoglobulins M and G; IL-6, interleukin-6; LPS, lipopolysaccharide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MPO, myeloperoxidase; NO, nitric oxide; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PMN, polymorphonuclear neutrophil; TNF-α, tumour necrosis factor-alpha; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute-1640; sRBC, sheep red blood cell.

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phyllanthin and hypophyllanthin (Kiemer et al., 2003). Phyllanthin reduced the whole brain acetyl cholinesterase activity in diazepam- and scopolamine-induced amnesic and aged mice when compared to piracetam (Londhe et al., 2012). The inhibitory activity of phyllanthin and hypophyllanthin from *P. amarus* against distinctive enzymes and proteins which play crucial role in the life cycle of HIV including competent interference with the reverse transcription process has been reported (Notka et al., 2004).

Our previous *in vitro* studies indicated that the standardized extract of *P. amarus* and its major constituents inhibited the chemotaxis of neutrophils and monocytes, phagocytic capacity of phagocytes, nitric oxide (NO) and reactive oxygen species (ROS) release from peritoneal macrophages (Yuandani et al., 2013; Jantan et al., 2014). Furthermore, phyllanthin exhibited *in vitro* inhibition of PHA stimulated T-cell proliferation and the release of pro-inflammatory cytokines including TNF-alpha and IL-6. The standardized extract of *P. amarus* also showed strong immunosuppressive effects on cellular immune and humoral immune responses in Wistar–Kyoto rats and Balb/C mice. In our previous studies, phyllanthin was used as one of the active chemical markers in the standardized extract of *P. amarus* (Ilangkovan et al., 2015; Ilangkovan et al., 2016). In order to further investigate the immunosuppressive properties of phyllanthin, we studied the effects of phyllanthin isolated from *P. amarus* on selected parameters of specific and non-specific cellular and humoral immune responses in Balb/C mice, which included the CD 11b/ 18 integrin expression, nitric oxide release, and phagocytosis of leukocytes and, T- and B-cell proliferation and $CD4^+$ and $CD8^+$ T-cell subset expression in splenocytes, and serum cytokine production by activated T-cells, myeloperoxidase activity, swelling of footpad in delayed hypersensitivity (DTH), serum immunoglobulins, ceruloplasmin and lysozyme levels.

Materials and methods

Chemicals and reagents

3-Aminophthalhydrazide (luminol), concanavalin A (Con A), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent, fluorescein isothiocyanate (FITC)-labeled opsonized *Escherichia coli*, fetal bovine serum (FBS), ficoll, Hank's balance salt solution (HBSS), lipopolysaccharide (LPS), Lymphoprep™ gradient (1077 mg/ml), phosphate-buffered saline (PBS) tablet, phorbol 12-myristate 13-acetate (PMA), Roswell Park Memorial Institute (RPMI)-1640, streptomycin, penicillin and trypan blue reagent were purchased from Sigma–Aldrich Corp (St Louis, MO, USA). Tween 20 and 3% Brewer's thioglycollate medium were procured from Sigma (St Louis, MO, USA). Cyclosporin A (Cys A) was obtained from Sigma–Aldrich (Milano, Italy). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase – alanine aminotransferase (ALP), colorimetric activity assay kit were obtained from Cayman Chemical (Ann Arbor, MI, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Myeloperoxidase (MPO) and fluorometric activity assay kit were purchased from BioVision, Inc. (Milpitas, CA, USA). Mouse ceruloplasmin and lysozyme kits were available from Cusabio Biotech Co., Ltd, (Wuhan, P.R. China). Mouse anti-sRBC IgG and IgM ELISA kit were purchased from Life Diagnostics (West Chester, PA, USA). Sheep red blood cell (sRBC) was purchased from Innovative Research Inc. (Novi, MI). The chemical reagents used for tissue fixation and the preparation of tissue block 10% formaldehyde in phosphate buffered saline, xylene histological grade, absolute ethanol paraffin wax, were all purchased from Sigma–Aldrich. Plethysmometer with standard equipment was purchased from Ugo Basile (Gemonio, Varese Italy). A CO_2 incubator was purchased from Shell Lab; Sheldon Manufacturing, Inc. (Cornelius, OR, USA). A flow cytometry (BDFACS Canto II equipped with 488 nm argon-ion laser, BD Biosciences) and a phagotest kit (Glycotope Technology, Berlin, Germany) were utilized in this study.

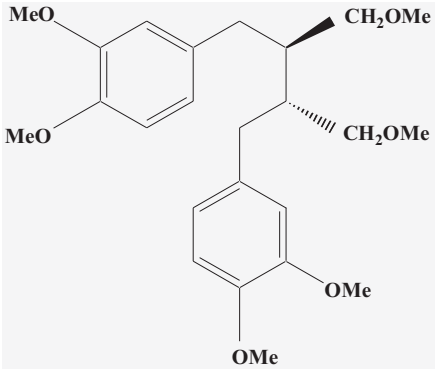

Fig. 1. Chemical structure of phyllanthin.

Plant collection and isolation of phyllanthin

The whole plants of *P. amarus* were collected at midday in the month of February 2012 from Kuala Trengganu, Malaysia. The plant was identified and a voucher specimen (UKMB 30078) was deposited at the Herbarium of Universiti Kebangsaan Malaysia (UKM). The plant sample was cleaned of foreign matters by gentle brushing, followed by washing under cold water tap to remove water soluble contaminants. The sample was air-dried under shade at room temperature. Phyllanthin was isolated from the whole plant according to a method reported previously (Yuandani et al., 2013). Briefly, 500 g of dried plant material was ground and macerated in methanol at room temperature at the ratio of 1:10 (w/v) for three times. The extract obtained was vacuum evaporated to obtain 55.2 g (11.04%) of crude extract. The extract (10 g) was initially chromatographed on silica gel type H (10–40 µm, 7 × 30 cm) by vacuum through gradient elution with *n*-hexane: CH_2Cl_2 (10:0 - 1:9, v/v) and CH_2Cl_2: MeOH (10:0 - 0:10, v/v). This was later followed by repeated silica gel column (40–63 µm, 3 × 60 cm) with a gradient elution of *n*-hexane:ethyl acetate (10:0 - 1:9, v/v), and recrystallization from *n*-hexane-EtOAc to yield 228.5 mg (2.29% on a dry weight basis) of phyllanthin. The chemical structure of phyllanthin is shown in Fig. 1. Purity of phyllanthin (> 98%) was determined based on their physicochemical properties, ESI-MS and NMR data. ^{13}C NMR spectrum of phyllanthin is shown in Fig. 2. ^1H NMR and HRESI-MS spectra of the compound are shown in Fig. 1S and 2S, respectively. Phyllanthin was resuspended in 3% Tween 20 solution before being fed orally to mice.

Experimental animals

The male Balb/C mice of inbred strains (age 6–8 weeks; 18–22 g) were procured from the Faculty of Science and Technology, UKM. The methods used in this study were approved by the UKM Animal Ethics Committee (No. FF/2014/IBRAHIM/22-JAN.-2014-MAY-2015). The animals were placed under controlled environmental conditions in plastic cages (8 mice in each cage) with 23 ± 1 °C temperature, 60% humidity and 12 h dark/light cycle. Animals were subjected to acclimatization for at least 1 week before the experiment. They were provided with commercially available laboratory rodent diet which was obtained from Bell Laboratories, Inc. (Sudbury, UK) and water was provided freely throughout the period of the study *ad libitum*.