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Immunomodulatory effects of *Tinospora crispa* extract and its major compounds on the immune functions of RAW 264.7 macrophages

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ABSTRACT

The in vivo immunomodulatory activities of *Tinospora crispa* have been reported but its molecular mechanisms underlying its immunomodulatory properties remains obscure and the active constituents contributing to the activities have not been identified. The present study was aimed to investigate the immunomodulatory effects of *T. crispa* extract (TCE) and its chemical constituents on RAW 264.7 macrophages. Six known compounds including magnoflorine and syringin were isolated by various chromatographic techniques from TCE and their structures were determined spectroscopically. A validated HPLC method was used to quantify magnoflorine and syringin in the extract. The immunomodulatory effects of TCE and its isolated compounds on chemotaxis, phagocytosis, production of inflammatory mediators including reactive oxygen species (ROS), nitric oxide (NO), prostaglandin E2 (PGE2) and pro-inflammatory cytokines which include tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and monocyte chemoattractant protein-1 (MCP-1) on macrophages were assessed. TCE increased the chemotaxis and phagocytic activity of macrophages and significantly enhanced the production of ROS, NO and pro-inflammatory cytokines. All alkaloids isolated, specifically magnoflorine showed remarkable inducing effects on the chemotaxis, phagocytic activity, ROS and NO productions and the secretions of IL-1β, TNF-α, IL6, PGE2 and MCP-1. In contrast, syringin potently reduced the chemotaxis, phagocytic activity, ROS and NO productions and secretions of IL-1β, TNF-α, IL6, PGE2 and MCP-1. TCE showed strong immunostimulant effects on various components of the immune system and these activities were possibly contributed mainly by the alkaloids specifically magnoflorine. TCE has potential to be developed as an effective natural immunostimulant for improvement of immune-related disorders.

1. Introduction

Immune system is the natural defense mechanism of body against infections and it involves dynamic cellular and molecular actions regulated by several modulatory systems [1]. Diseases such as carcinogenesis, chronic inflammation diseases, sepsis, anaphylactic shock, systemic vasodilatation, and autoimmunity display highly active immune system [2]. Conversely, immunosuppression results in more susceptibility to diseases and is also involved in tumor development [3]. Immune system can be classified into two main categories on the basis of function, i.e. innate (non-specific) and adaptive (specific or acquired) [4]. The first line of defense against pathogens is innate immunity which is facilitated by phagocytes such as neutrophils and macrophages. Macrophages are one of the important components of immune system and have important roles in antigen presentation, processing and phagocytosis, hematopoiesis, clearance of apoptotic cells, wound repair, tissue remodeling during embryogenesis, secreting cytokines and antibody-dependent cell-mediated cytotoxicity. Macrophages may be produced due to malignancy, infections, normal cell turnover or wounding and disturbance of tissue homeostasis. Macrophages are activated after recruitment. Varying phenotypes of the activated macrophages are present depending on the type of stimulus and location. In addition to playing a role in host's defense against infections, activated macrophages produce numerous inflammatory mediators such as nitric oxide (NO), reactive oxygen species (ROS) and cytokines including interferon, interleukin (IL) and tumor necrosis factor (TNF) [4].

IL-1β is an important and multi-functional pro-inflammatory cytokine component in the investigation and improvement of inflammatory response to infection by microbes [5]. It is secreted by various cells

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including monocytes, macrophages and fibroblasts. Activated macrophages primarily produce TNF-α. The roles of TNF-α include initiation of adhesion molecules (intercellular adhesion molecule-1) expression on endothelial cells and neutrophils, triggering and chemotaxis of leukocytes [6]. IL-6 is an interleukin which acts both as a pro- and anti-inflammatory cytokine. IL-6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. IL-6 plays part in acute-phase inflammatory response. IL-6 can be secreted by macrophages in response to certain microbial molecules, known as pathogen-associated molecular patterns (PAMPs) [7].

The host's immune response can be suppressed or strengthened by the use of herbal medicines. Both specific and non-specific immunities are either suppressed or stimulated by botanical drugs due to their immunomodulatory characteristics [8]. Plants such as *Tinospora cordifolia*, *Withania somnifera*, *Actaea racemosa*, *Phyllanthus amarus* and *P. urinaria* have been known to possess immunomodulating activities [9–12]. *T. crispa* (Wild.) Hook. f. & Thomson (family: Menispermaceae) has been shown to possess several biological and pharmacological activities such as immunomodulatory, cardiovascular, anti-proliferative, anti-bacterial, anti-oxidant, anti-hyperglycemic, anti-atherosclerotic and anti-parasitic [13–15].

Recently, in an in vitro experiment comprising lipopolysaccharide (LPS)-stimulated mouse macrophages (RAW 264.7), *T. crispa* extract (TCE) has shown promising activity to enhance intracellular expression of cytokines (TNF-α, IL-6 and IL-8) [15]. Previously, we discovered that TCE could augment innate immunity in rats. The chemotactic and phagocytic activities of neutrophils in rats were enhanced by the standardized TCE. The Th1 (IFN-γ, TNF-α and IL-2) and Th2 (IL-4) cytokines levels in serum of rats were increased after daily treatment with several oral doses of TCE [16]. We reported that TCE could enhance the innate and adaptive immune response in Balb/c mice. The study revealed that NO production and phagocytic activity of peritoneal macrophages in Balb/c mice were enhanced after TCE treatment. In addition to serum immunoglobin and lysozyme levels, delayed type hypersensitivity reaction was also stimulated after TCE administration [17]. However, the active constituents of TCE contributing to the strong stimulatory effects on both humoral and cellular responses have not been identified. In the present study, the TCE was further investigated to get a better insight into mechanisms of action and correlate the effectiveness of the plant with its active components. The immunomodulatory effects of TCE and its isolated compounds on RAW 264.7 macrophage were assessed based on their effects on chemotaxis, phagocytosis, production of inflammatory mediators such as ROS, NO, prostaglandin E_2 (PGE_2) and pro inflammatory cytokines which include TNF-α, IL-1β, IL-6 and monocyte chemoattractant protein-1 (MCP-1).

2. Materials and methods

2.1. Chemicals and reagents

The mouse macrophage RAW 264.7 cell line was obtained from American Type Culture Collection (ATCC, VA, Manassas). Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline (PBS) were obtained from Gibco (Grand Island, NY, USA). Lipopolysaccharide (LPS), serum opsonized zymosan A (*Saccharomyces cerevisiae*) (SOZ), Lymphoprep™ gradient, luminol (3-aminophthalhydrazide), Hanks Balance Salt Solutions (HBSS), ficoll, N-formyl-methionylleucyl-phenylalanine (fMLP), acetylsalicylic acid (purity 99%), ibuprofen (purity 99%), dexamethasone (99%), phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO) and N-1(1-naphthylethylenediamine dihydrochloride) were purchased from Sigma–Aldrich (St. Louis, MO, USA). AlamarBlue® reagent was obtained from Thermo Fisher Scientific (MA, USA). Cytoselect 24-well cell migration assay kits, Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS) were obtained from Cell Bio labs, Inc. (San Diego, CA, USA). Penicillin/streptomycin (100×) and fetal calf serum (FCS) were procured from PAA Laboratories (USA). FACS lysing solution was purchased from Becton Dickinson (USA). Phagotest assay kit and fluorescein isothiocyanate (FITC)-labeled opsonized *Escherichia coli* were procured from Glycotope Technology (Heidelberg, Germany). Levamisole (purity 99%) and ELISA kits for TNF-α, IL-6 and PGE_2 were purchased from Cayman Chemicals (Ann Arbor, MI USA). IL-1β and MCP-1 ELISA kits were purchased from eBioscience (Vienna, Austria). Methanol, acetonitrile, and trifluoroacetic acid of HPLC grade were purchased from Fisher Scientific (Loughborough, UK). Silica gel type 60 (40–63 μM) and type 60HF254 (10–40 μM) were purchased from Merck (Germany). All the chemicals and reagents including biologicals and synthetics used in this study were endotoxin free.

2.2. Collection of plant material

The whole plant of *T. crispa* was collected from Marang, Kuala Terengganu, Malaysia in June 2014. A specimen (UKMB 40178) of the plant was certified by Dr. Abdul Latif Mohamad of Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM) and was deposited at the UKM herbarium.

2.3. Extraction of plant material and isolation of compounds

The dried stems of *T. crispa* (0.4 kg) were ground and soaked in 80% ethanol (1.5 L) at 25 °C for 72 h. The combined ethanol extracts were concentrated in vacuo at 55–60 °C to yield 38.3 g of dry extract. The extract was fractionated into alkaloid and non-alkaloid fractions by acid-base extraction method. Briefly, TCE (38.3 g) was acidified with 5% H_2SO_4 to pH 2 and the mixture was left to soak for 2 h and was later partitioned with $CHCl_3$. The $CHCl_3$ layer was concentrated in vacuo to obtain a brown gummy residue of non-alkaloidal fraction (26.1 g). The aqueous layer was then basified with 10% Na_2CO_3, adjusted to pH 9 and extracted with $CHCl_3$. The $CHCl_3$ phase-containing alkaloids were collected and concentrated to a dark brown gummy residue (4.3 g). The crude alkaloid residue was subjected to column chromatography over silica gel type 60 (40–63 μM) with a gradient system of *n*-hexane-$CHCl_3$ (10:0 to 0:10, v/v) and $CHCl_3$-MeOH (10:0 to 0:10, v/v) to obtain 20 mL of each fraction. Thin-layer chromatography (TLC) was used to check the fractions and fractions with similar TLC pattern were combined to yield 19 subfractions (F1–F19). The subfractions F7, F11, F13 and F19 were further subjected to silica gel column chromatography eluting with a gradient system of $CHCl_3$-MeOH (10:0 to 0:10, v/v) to yield compounds **1** (32.6 mg), **2** (90 mg), **3** (5 mg) and **4** (13 mg), respectively. The non-alkaloid fraction (26.1 g) was sequentially extracted with *n*-hexane, CH_2Cl_2, EtOAc and MeOH. The CH_2Cl_2 fraction was fractionated using vacuum liquid chromatography (VLC) over silica gel type $60H_{F254}$ (10–40 μM), by using a gradient system of *n*-hexane-MeOH (10:0 to 0:10, v/v) to yield 13 subfractions (FH1–FH13). Fraction FH7 was subjected to column chromatography and eluted with a gradient system of *n*-hexane-$CHCl_3$ (10:0 to 0:10, v/v) to yield compound **6** (82.9 mg). Fraction FH11 was chromatographed on silica gel type 60 column with a gradient system of CHCl3-MeOH (10:0 to 0:10, v/v) to yield compound **5** (8 mg). The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a NMR 500 MHz (JOEL Ltd., Japan), with tetramethylsilane (TMS) as internal standard. Molecular weights of the compounds were determined by using electrospray ionization time-of-flight mass spectrometer (ESI-TOF-MS) (Bruker MicroToF-Q 86, Switzerland). The purity of the isolated compounds was > 98% as determined by NMR and MS-TOF data and based on their physicochemical properties. TCE and the isolated compounds were evaluated for possible endotoxin contamination by Limulus Amebocyte Lysate (LAL) assay (Cambrex BioScience, Walkersville, MD) following the manufacturer's recommended instructions. The samples were not found to contain significant amounts of endotoxin which could potentially interfere with the study at the concentrations used.

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