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Effect of an extract of *Andrographis paniculata* leaves on inflammatory and allergic mediators *in vitro*

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ABSTRACT

Aim of study: Andrographis paniculata has been known to possess widespread traditional application in the treatment of allergy and inflammatory diseases. In the current study, we sought to examine the effects of an extract of *Andrographis paniculata* leaves on inhibition of lipopolysaccharide (LPS) induced [nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-1beta (IL-1 beta), and interleukin-6 (IL-6)] and calcimycin (A23187) induced [leukotriene B_4 (LTB_4), thromboxane B_2 (TXB_2) and histamine] mediators in diverse cell based models.

Materials and methods: Effect of an extract of *Andrographis paniculata* leaves (AP) was studied on inhibition of LPS induced NO, PGE_2, IL-1 beta and IL-6 in J774A.1 murine macrophages; A23187 induced LTB_4 and TXB_2 in HL-60 promyelocytic leukemic cells and histamine in RBL-2H3 rat basophilic leukemia cells.

Results and conclusion: AP illustrated significant alleviation of pro-inflammatory, inflammatory, and allergic mediators. However, no inhibition was observed against histamine release. This outcome has been summed up to deduce that AP is fairly potent in attenuating the inflammation by inhibiting pro-inflammatory (NO, IL-1 beta and IL-6), inflammatory (PGE_2 and TXB_2) and allergic (LTB_4) mediators.

1. Introduction

Andrographis paniculata (Acanthaceae) is a well-known medicinal plant and has long been used in Chinese official herbal medicine against wide spectrum of ailments (Coon and Ernst, 2004). Our recent study has reported the safety of *Andrographis paniculata* (KalmCold™) in a battery of genotoxic tests and also the LD_{50} value was determined to be more than 5 g/kg rat body weight in an acute oral toxicity study (Chandrasekaran et al., 2009). In a randomized double blind placebo controlled clinical trial, AP (KalmCold™) demonstrated significant relief of common cold symptoms in human populations (Saxena et al., 2010). *Andrographis paniculata* has been reported to have anti-inflammatory (Sheeja and Kuttan, 2008), anti-allergic (Madav et al., 1998), immunostimulatory (Iruretagoyena et al., 2005) activity. The anti-inflammatory action of the plant is attributed to andrographolide, the major active principle of the plant (Madav et al., 1996; Abu-Ghefreh et al., 2009). However, little is known about the pharmacological mechanisms underlying these actions, although some of its anti-inflammatory effects have been investigated (Shen et al., 2002).

In order to extend the understanding of its mechanism on anti-inflammatory activity, we investigated inhibitory activity of AP on LPS induced NO, PGE_2, IL-1 beta and IL-6 levels; A23187 activated LTB_4, TXB_2, and histamine levels using mammalian cell lines *in vitro*. We used murine macrophages (J774A.1) as cellular model, since they represent population of macrophages that can be stimulated *in vitro* by LPS. In the current study, we have utilized human promyelocytic leukemic (HL-60) cells and rat basophilic leukemic cells RBL-2H3 cells in order to study the anti-inflammatory activity of AP. These cell lines have been proved as an appropriate cell assay system to study the anti-inflammatory/anti-allergic nature of the compounds (Ikawati et al., 2001; Zaitsu et al., 2002).

2. Materials and methods

2.1. Source of materials

Lipopolysaccharide (LPS), calcimycin (A23187), acetyl salicylic acid, MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan], ketotifen fumarate, dexamethasone (D-2915), 1400W dihydrochloride, sulphanilamide, naphthyl ethylene diamine dihydrochloride (NEDD) and captopril were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Iscove's modified Dul-

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becco's media (IMDM), Earle's minimum essential media (EMEM) and Dulbecco's modified Eagle's medium (DMEM) were supplied by Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA).

2.2. Plant material

Andrographis paniculata (Burm.f.) Wall. ex. Nees (part used – leaves) belonging to the family Acanthaceae was collected from regions of West Bengal, India. Botanical identification was carried out at NISCAIR (National Institute of Science Communication And Information Resources). A voucher specimen (No. 110) was deposited in our herbarium.

2.3. Preparation of extract of Andrographis paniculata (AP)

Coarse ground leaves of *Andrographis paniculata* were extracted with methanol (4 times of the raw material quantity) for 3 h, in a stainless steel jacketed extractor fitted with reflux condenser. The liquid extract was removed and the remaining raw material was re-extracted two more times with methanol in a similar manner. The resulting extracts were combined, concentrated and dried under vacuum (at <55 °C). The yield of the dried extract was 6% (w/w). After extraction with methanol, the left over raw material was extracted with water (4 times of the raw material quantity) for 3 h under reflux conditions. This water extract was separated and concentrated under vacuum at around 75 °C until the total solid content in the liquid reached about 15% to 20% (w/v), followed by spray drying. Two parts of methanolic and one part of successive water extract were blended to get AP, which was analyzed for phytoconstituents by HPLC as described earlier by us (Chandrasekaran et al., 2009).

AP was found to contain andrographolide (31.3%, w/w), isoandrographolide (0.4%, w/w), neoandrographolide (3.2%, w/w), andrograpanin (0.6%, w/w), 14-deoxy-11,12-didehydroandrographolide (2.8%, w/w), skullcapflavone-I (0.05%, w/w) and 7-O-methylwogonin (0.05%, w/w). This herbal extract is indexed as KalmCold™, manufactured by Natural Remedies Pvt. Ltd., Bangalore.

AP was solubilized in DMSO and filter sterilized through 0.2 μM positively charged nylon DMSO compatible filter to remove the endotoxins. The filtered solution was aliquoted and stored at −80 °C for further use in all the assays. DMSO was used as a solvent control in all the assays up to a maximum concentration of 0.2%. DMSO up to 0.2% did not influence the stimulant induced release of any of the inflammatory mediators. All the assays were well controlled by using respective reference standards.

2.4. Cell lines and culture conditions

J774A.1 murine macrophage cell line (TIB-67™), HL-60 human promyelocytic leukemia cell line (CCL-240™) and RBL-2H3 rat basophilic leukemia cell line (CRL-2256™) were procured from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in appropriate ATCC recommended medium and maintained at 37 °C under 5% CO_2 humidified air.

2.5. Preparation of Ringer's buffer

The components of Ringer's buffer were 118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, 1.10 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 5.0 mM HEPES, 0.1% BSA and 11.1 mM D-glucose. The pH was adjusted to 7.4.

2.6. Cytotoxicity assay

Cytotoxic effect of AP was checked in the respective cell lines by using MTT. Based on the cell viability results, different non-cytotoxic concentrations were selected for each study. All the experiments were conducted in quadruplicates per treatment using 96-well tissue culture plates.

2.7. NO scavenging and PGE_2 inhibition assay

J774A.1 murine macrophages (passage number between 10 and 25) were seeded at a density of 1×10^5 cells/well and incubated overnight. The macrophages were then pretreated with AP at different non-cytotoxic concentrations (1.25–30 μg/mL for NO and 1.6–50 μg/mL for PGE_2) and incubated for 1 h, thereafter, LPS (5 μg/mL) was added followed by further incubation for 24 h. The reference standards used for NO scavenging and PGE_2 inhibition assay were 1400W dihydrochloride and dexamethasone respectively. The cell supernatant was collected for nitrite and PGE_2 estimation.

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of cultured macrophages by Griess reaction. Briefly, equal volumes of treated culture supernatant and Griess reagent (1% sulphanilamide, 0.1% NEDD and 5% orthophosphoric acid) were mixed and incubated at room temperature for 5 min, and then the absorbance was measured at 540 nm in a microplate reader. The amount of nitrite in the sample was determined using sodium nitrite standard curve.

PGE_2 levels in macrophage supernatants were quantified as per the method described by the kit manufacturer [Homogenous Time Resolved Fluorescence (HTRF) kit, CisBio, France].

2.8. IL-1 beta inhibition assay

J774A.1 murine macrophages were seeded at a density of 1×10^5 cells/well and incubated overnight. The macrophages were then pretreated with AP at different non-cytotoxic concentrations (5–40 μg/mL) and incubated for 1 h, thereafter, LPS (5 μg/mL) was added followed by further incubation for 6 h. The treated cells were lysed using cell lysis buffer [0.1% Triton X-100 and protease cocktail inhibitor (1×)] in combination with repeated freeze thaw cycles. The plates were centrifuged and the supernatant was collected for estimating the levels of IL-1 beta (ELISA kit, R&D Systems, Minneapolis, USA). Dexamethasone was used as a reference standard.

2.9. IL-6 inhibition assay

J774A.1 murine macrophages were seeded at a density of 1×10^5 cells/well and incubated overnight. The macrophages were then pretreated with AP at different non-cytotoxic concentrations (2.5–40 μg/mL) and incubated for 1 h, thereafter, LPS (0.1 μg/mL) was added followed by overnight incubation. The supernatant was collected and used to estimate the levels of IL-6 as per the method described by the kit manufacturer (ELISA kit, OptEIA™, BD Biosciences, USA). Dexamethasone was used as a reference standard.

2.10. LTB_4 and TXB_2 inhibition assay

HL-60 promyelocytic leukemia cells (passage number between 2 and 10) were first allowed to undergo differentiation into metamyelocytes and neutrophils, as this enabled the cells to produce higher levels of LTB_4 and TXB_2. Cell differentiation was attained by seeding the cells at a density of 5×10^5 cells/mL in IMDM enriched with 20% FBS and 1.3% DMSO in a T-25 flask followed by incubation for 5 days (Collins, 1987). The Giemsa stained cells were examined under microscope to visualize the differentiated granulocytes. These differentiated cells were seeded at a density of 1×10^5 cells/well in Ringer's buffer. The cells were pretreated with AP at different non-cytotoxic concentrations (1.25–40 μg/mL for LTB_4 and 2.5–40 μg/mL for TXB_2) and incubated