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Ixora coccinea down Regulate the Inflammation-Associated Prostaglandins (PGE-2), Nitric Oxide (NO) and Cytokines Production in LPS-Stimulated RAW 264.7 Macrophage

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Abstract

Ixora coccinea L. (Rubiaceae) is a traditional herbal medicine for various ailments like cutaneous wounds, infection, hypertension, menstrual irregularities, sprain, chronic ulcer and other skin diseases. The present study investigated the anti-inflammatory effects of *I. coccinea* leaves methanol extract (IxME) on RAW 246.7 macrophage and its effect on Prostagladins E2 (PGE2), Nitric Oxide (NO) and pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) production. The experimental findings revealed that IxME treatment inhibited the Cyclooxygenase 2 (COX-2) mediated PGE2 and Nitric Oxide Synthsae (NOS) catalyzed NO production in LPS-treated RAW 246.7 macrophages in a dose dependent manner. On the other hand, production of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β was significantly decreased as compared to negative control LPS-treated RAW 246.7 macrophages. The findings data coincides and correlated the *in vitro* anti-inflammatory activity of *I. coccinea* with previous reports of *in vivo* anti-inflammatory property.

Keywords: *Ixora coccinea* L., RAW 246.7 Macrophages, Cyclooxygenase, Cytokines

1. Introduction

Inflammation is the protective and ubiquitous defensive reactions to the chemical or mechanical tissue injury, and to invading pathogens. In tissue repair process of wound healing, inflammation phase is the second and crucial phase associated with extravasations of inflammatory polymorphonuclear cells and active participation of a large range of signalling mediators. During inflammatory phase, injured cells/tissue secretes various mediators, such as bradykinin, prostaglandins (such as PGE2), and leukotriene that attracts and activate the various infiltrating inflammatory blood cells including macrophages [1, 2]. Macrophages are considered to impart an important role to re-establishing tissue homeostasis. Activated macrophages secrete excessive amount of Reactive Oxygen Species (ROS), NO PGE2, and pro-inflammatory cytokines, such as Tumour Necrosis Factor-*α* (TNF-*α*) and interleukins (ILs), which aggravate and propagate local inflammation by inducing the activation and nuclear translocation of NF-κB [2]. Polymorphonuclear cells-induced inflammatory response sterile the clot matrix at

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wounded site and initiates the regeneration of granulation tissue. Whereas, the prolonged deregulated inflammatory phase leads to excessive ROS/RNS production that alter the cell structure and functionality by interacting with biological molecules, leading into the cell death and derail the acute tissue repair process [1, 3].

Ixora coccinea L. (Rubiaceae) is a small-medium evergreen shrub, widely cultivated ornamental plant throughout South Asian regions and used in the treatment of various ailments like infection, hypertension, menstrual irregularities, sprain, chronic ulcer and skin diseases including cutaneous wounds [4–6]. Decoction of the flowers is given for hemoptysis, catarrhal bronchitis, and dysmenorrhea. Pharmacological investigations had revealed the antioxidant, antiinflammatory, cardio-protective, chemoprotective, cytotoxic, antitumor, and antimicrobial activities of *I. coccinea* [4–7]. The plant is reported to contain triterpenoids (lupeol, 3-acetyl betulic acid, betunolic acid, α-amyrin, β-amyrin, ursolic acid, 3-acetyl ursolic acid, and oleanonic acid), flavonoids (kaempferol, kaempferol-7-O-α-rhamnoside, kaempferitrin, luteolin, epicatechin, and catechin), coumarins (scopoletin, coumarin, and erythro-10, 20-albiflorin), peptides (Ixorapeptide I and II), trimeric proanthocyanidin named Ixoratannin A-2 and many other polyphenolic compounds procyanidin A-2, cinnamtannin B-1 etc. [8, 9]. Our previous *in vitro – in vivo* investigations for wound healing activity revealed that *I. coccinea* accelerated wound contraction may be a combine effect of antioxidant-antimicrobial and fibroblast proliferation properties [10]. In the present study, *I. coccinea* is investigated for anti-inflammatory activity in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophage cells its effect on PGE-2, NO, and pro-inflammatory cytokine (TNF-α, IL-6, IL-1β) production.

2. Material and Methods

2.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco, Invitrogen Corporation (USA). *Escherichia coli* Lipopolysaccharide (LPS), resazurin and diclofenac were obtained from Sigma Chemicals (USA). All other chemicals not mentioned were from Himedia (India).

2.2 Preparation of the Plant Extract

I. coccinea leaves were collected during September–October (2011) from campus garden of Defence Research Laboratory, Tezpur, Assam, (India), and authenticated at Botanical Survey of India, Shillong (India), and the specimen sample deposited (Acc. no. 081168). About 100 g of shade dried leaves powder was successively extracted with petroleum ether, chloroform, methanol and water at 1500 lb at room temperature in Accelerated Solvent Extractor (ASE 1.5, Dionex, USA). The extraction was considered complete when the initial color of the percolate gradually changed to colorless. After removal of solvent with rotatory evaporator (Rotavac, Heidolph2, Schwabach, Germany) at 45 ± 2°C for the organic extracts and freeze drying for the aqueous extract, yield of extracts were calculated and extracts stored in the fridge till needed for analysis. Among all extracts methanol extract of *I. coccinea* (IxME) showed potent antioxidant-antimicrobial and fibroblast proliferation activity [10], therefore was selected for *in vitro* anti-inflammatory evaluations. The extract was dissolved in distilled water and centrifuged at 10000×g at 4°C for 20 min, and passed through a 0.22 µm filter. The filtrate was used as the starting material for subsequent studies.

2.3 In vitro Anti-inflammatory Evaluation

2.3.1 Cell culture

Murine RAW 264.7 macrophage cell line was obtained from National Centre For Cell Science (NCCS) and cultured in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin at 37°C in a 5% CO_2 incubator. The cells were sub-cultured every 2–3 days at 1:5 split ratios.

2.3.2 Cytotoxicity assay

The macrophage proliferation assay was performed as described elsewhere [11]. Confluent macrophages were harvested, centrifuged and re-suspended in DMEM supplemented with 10% FBS. Cells (1×10^4 cells/well) were seeded per well in 96-well plate excluding first row and maintained at 37°C in humidified 5% CO_2 atmosphere. After 1 h of incubation different concentrations for test samples were added (final concentration ranged from 25 to 200 µg/ml). The cells were incubated for 24 h and thereafter population was estimated by using resazurine and absorbance was estimated at 570 nm in SpectraMax

Plus 384 micro-plate reader (Molecular Devices, USA). Each sample was assayed in triplicate and three independent tests were performed.

2.3.3 Prostaglandin E2 assay

PGE2 is an inflammatory mediators released from arachidoinc acid metabolism catalyzed by COX-2, was estimated as a marker. RAW 246.7 macrophages were plated on 24-well plates at 1×10^5 cells/well. The cells were pre-treated for 1 h with EhME concentrations (25–200 μg/ml) prior to LPS (500 ng/ml) stimulation for 24 h. The culture supernatant was immediately used for PGE2 determinations. The PGE2 quantity in the supernatant was determined using a commercially available PGE2 ELISA kit (Cayman Chemical Co., USA), using the manufacturer's protocols. Briefly, 50μl of diluted standard/sample was pipetted into a 96-well plate precoated with goat polyclonal anti-mouse IgG. Aliquots of a PGE2 monoclonal antibody and PGE2 acetylcholine esterase (AChE) conjugate were added to each well and allowed to incubate at room temperature for 16 h. Next, wells were washed five times with buffer containing 0.05% Tween 20, followed by the addition of 200 μl of Ellman's reagent containing acetylthiocholine and 5,5`-dithio-bis-(2-nitrobenzoic acid). Wells were read after 30 min at 405 nm with an ELISA reader.

2.3.4 Nitric oxide determination

Nitrite accumulation was used as an indicator of NO production in the medium using a commercially available Nitro Oxide estimation kit (Cayman Chemical Co., USA). Cells were plated as 1×10^5 cells/well in 24-well culture plates and pre-incubated with and without indicated concentrations of test sample as described above. About 80 μl of each medium supernatant was mixed with 10 μl of nitrate reductase enzyme and 10 μl of nitrate reductase cofactor. After 1 h incubated at RT, 50 μl of 1% sulphanilamide (in 5% phosphoric acid) and 50 μl of 0.1% naphthylenediamine dihydrochloride was added and allowed to developed colour for 10 min. The absorbance at 540 nm was measured with $NaNO_2$ standard curve and nitrite production was determined.

2.3.5 TNF-α, IL-6 and IL-1β assays

The generation of TNF-*α*, IL-6 and IL-1β by the RAW 264.7 cells was assayed with ELISA kits (USCN Life Science Inc., USA), according to the manufacturer's instructions. 100 μl of culture supernatant was used for the estimation of TNF-*α*, IL-6 and IL-1β, and absorbance was determined at 450 nm. All estimation was performed in triplicates.

2.4 Statistical analysis

The results were expressed as means ± S.D. Data were statistically analyzed using one way Analysis Of Variance (ANOVA) followed by Dunnett test. A p-value ≤ 0.05 was considered statistically significant as compared to non-treated and vehicle treated group.

3. Results

3.1 Cytotoxicity assay

As shown in Figure 1, treatment of unstimulated RAW 264.7 cells to increasing concentrations (25–200 μg/ml) of IxME does not significantly inhibit the cell viability and showed 91.73% and 89.42% cell viability at 150 and 200 μg/ml concentrations, respectively. Therefore, IxME concentrations upto 200 μg/ml were used in the subsequent experiments.

3.2 Inhibition of LPS-induced PGE2 Production by IxME in RAW 246.7 Macrophages

PGE-2 is a major product of archidonic acid metabolism via COX pathway in stimulated macrophages cells.

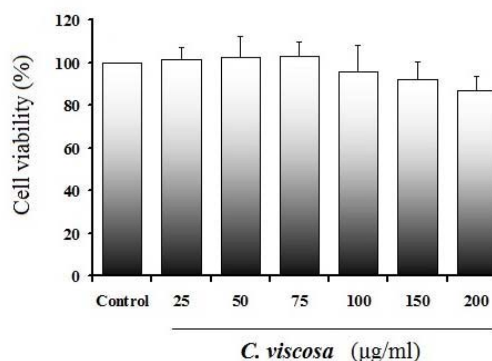

Fig. 1. Cytotoxicity of IxME in RAW 246.7 macrophage cells. Cells were incubated in the presence of IxME and cell viability was assayed by using resazurin. Data are expressed as the mean ± S.D. of three individual experiments.

Unstimulated RAW 246.7 cells in culture medium (24 h) produced basal amount of PGE-2 (87.15 ± 15.52 pg/ml) and LPS (500 ng/ml) treatment increased the PGE-2 up to 550.84 ± 34.44 pg/ml (Figure 2). LPS treatment with simultaneous IxME intervention decreased the PGE-2 production significantly ($p < 0.05$) to 349.61 ± 47.730 pg/ml (reduced ~ 36.53% compared to LPS-treated negative control) at 100 µg/ml concentration.

3.3 Inhibition of LPS-induced NO Production by IxME in RAW 246.7 Macrophages

The extract showed the strong inhibition on NO production in RAW 246.7 macrophages. The amount of nitrite, a stable metabolite of NO, was determined via the Griess reagent. In unstimulated RAW 246.7 cells, NO level (2.36 ± 1.15 mmol/ml) was significantly low and 24 h LPS treatment markedly increased the NO concentration (27.59 ± 3.94 mmol/ml) in medium (Figure 2). Pre-treatment with IxME significantly ($p < 0.05$) decreased LPS-induced NO content to 19.73 ± 2.07 and 18.20 ± 3.79 mmol/ml at 100 and 200 µg/ml concentrations, respectively.

3.4 Inhibition of LPS-induced Pro-inflammatory Cytokine release by IxME in RAW 246.7 Macrophages

TNF-*α*, IL-6 and IL-1β are the major pro-inflammatory cytokines [12]. As shown in Figure 3, LPS treatment significantly stimulated the production of pro-inflammatory cytokines TNF-*α* (2387.51 ± 253.17 pg/ml), IL-6 (160.08 ± 11.94 pg/ml) and IL-1β (379.37 ± 20.01 pg/ml), whereas IxME intervention to LPS-induced RAW 246.7 macrophage significantly ($p < 0.05$) decreased the secretion of TNF-*α* (1280.85 ± 353.33 pg/ml, 100 µg/ml), IL-6 (114.25 ± 11.30 pg/ml, 200 µg/ml) and IL-1β (192.50 ± 25.52 pg/ml, 200 µg/ml). The level of pro-inflammatory cytokines was decreased with increasing doses of IxME.

4. Discussion

Wound healing is a complex cascade of regeneration and restoration that involves a series of co-ordinated events of bleeding & coagulation, chemotaxis of inflammatory cells, migration and proliferation of fibroblasts, neovascularization, synthesis, deposition and maturation of new extracellular matrix, and remodelling of scar [1]. These tissue repair process can be broadly categorized into four phases of wound healing named: haemostasis, inflammation, proliferation, and remodelling phase, which are regulated by several mediators including cytokines, and various secreted growth factors. Inflammation is the body's reaction to injury that happens a few minutes after injury and lasts up to more than 24 hours [13]. Inflammation is the second and crucial phase of wound healing aimed to eliminate dead cells and micro-organisms from fibrin clot matrix, and to initiate the regeneration of wound tissue (proliferation phase). Inflammation is a predictable complex sequence of cellular, biochemical and molecular reactions that induce classic signs of heat and redness, pain and swelling

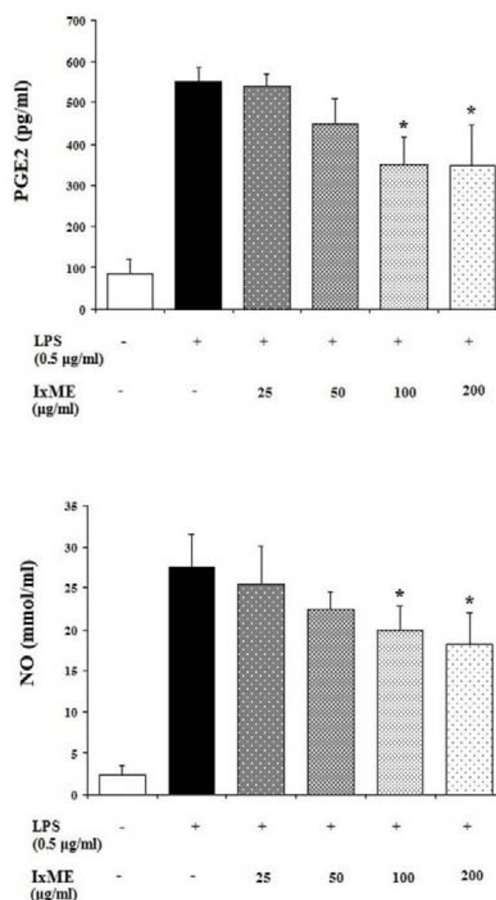

Fig. 2. Effect of IxME on PGE2 and NO production in LPS-stimulated RAW 246.7 cell. Data are expressed as mean ± S.D. Asterisk(*) indicates significantly difference ($p < 0.05$) as compared to control unstimulated RAW 246.7 cells.

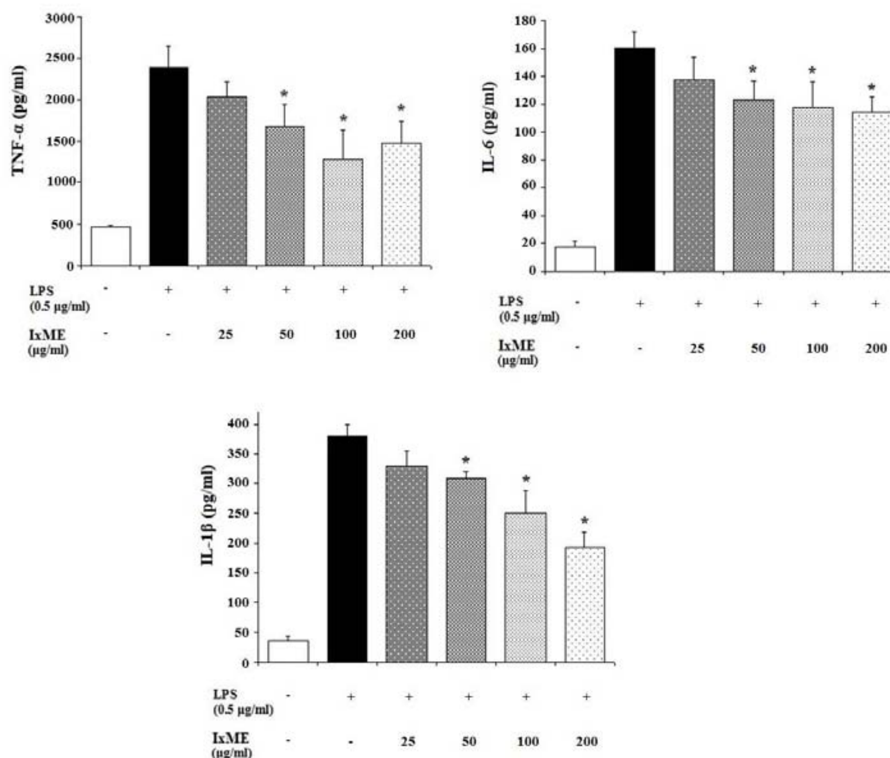

Fig. 3. Effect of IxME on TNF-α and IL-β production in LPS-stimulated RAW 246.7 cells. Data are expressed as mean ± S. D. Asterisk(*) indicates significantly different ($p < 0.05$) as compared to control unstimulated RAW 246.7 cells.

in wound [2]. Activated platelets, entrapped in fibrin clot matrix release various chemoattractant-chemokine molecules that induce the extravasations of inflammatory Polymorphonuclear (PMN) cells at wounded site.

Neutrophils are the first appeared at wounded site, after platelets and followed by macrophages. PMNs are avidly phagocytic and show extremely high oxygen-dependent metabolic rate. PMNs remove invading foreign particles including bacteria and other material from the wound by releasing a large array of anti-microbial molecules such as oxidants, granule proteins, and iron-withholding enzymes. During inflammatory phase, PMN cells through oxygen-dependent NADPH-linked oxygenase mechanism produce ROS like Peroxide Anion (HO_2^-), Hydroxyl ion (HO^-) and Superoxide Anion (O^{2-}). During the inflammatory phase macrophages and neutrophils cells release cytokines including Interleukins (IL-1, IL-6, IL-8) and TNF-*α*. Excessive extravasations and activation of inflammatory cells delay the proliferation phase of wound healing process [13, 14].

Previous study on *I. coccinea* revealed the antioxidant, antimicrobial, fibroblast proliferation properties and present study showed that *I. coccinea* methanol extract also alter the inflammatory phase of wound healing. The IxME treatment decreased the production decreased the production of PGE2, NO and pro-inflammatory cytokine such as TNF-*α*, IL-1β and IL-6 in LPS-induced RAW 246.7 mouse macrophage cell line. Activated macrophages perform crucial functions in inflammatory phase including phagocytosis of foreign pathogen and injured cells at wounded site. Various secreted mediators regulate and amplify the response of other inflammatory cells. RAW 246.7 macrophages provide a useful model for evaluating anti-inflammatory property of new compounds [2].

Activated macrophages produced large amount of pro-inflammatory mediators NO and PGE2 generated by the activated NOS and COX2 enzymes, respectively

[15]. NO, is an endogenous free radical and important signalling molecule generated via oxidation of L-arginine to L-citrulline catalyzed by NOS. NO involved in multifunctional pathophysiological process such as inflammation, apoptosis, regulation of enzyme activity and gene expression [16]. High level of NO in inflammatory conditions mediates pro-inflammatory effect. Experimental data showed that LPS treatment significantly elevate the NO level in comparison to unstimulated control sample, and IxME down regulated the NO production in dose dependent manner in LPS-treated RAW 246.7 macrophages (Figure 2).

Cyclooxygenase-2 (COX-2) catalyzed the production of Prostaglandins (PGs) from arachidoinc acid released form injured cellular debris. PGs cause classical symptoms pain, fever, and edema of inflammation [17]. Inflammatory signals enhance COX-2 expression, especially in PMN cells including monocytes, macrophages etc. IxME dose-dependently inhibits the PGE2 production induces by LPS, indicating the COX-2 down regulating potential of IxME (Figure 2). Inflammatory signals on injury site including increased level of PGs, NO and released cytokines, activates the NF-κB like transcription factor, which translocate from cytosol to nucleus and activates the expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and mediators [18]. Unstimaulted RAW246.7 macrophages produced a basal quantity of TNF-α, IL-1β and IL-6 cytokines. Treatment of LPS significantly stimulated the cytokines level in culture media and pre-treatment with IxME showed significant ($p < 0.05$) dose-dependent down regulation in cytokines production.

In conclusion, *in vitro* anti-inflammatory activity of *I. coccinea* on LPS-stimulated RAW 246.7 macrophage indicated that IxME down regulated the pro-inflammatory cytokine production. These findings suggest that the anti-inflammatory activity may also be important attribute in wound healing activity of *Ixora coccinea*.

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