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Anti-inflammatory effects of royal poinciana through inhibition of toll-like receptor 4 signaling pathway

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ABSTRACT

Inflammation is part of the non-specific immune response that occurs in reaction to any type of bodily injury. In some disorders the inflammatory process, which under normal conditions is self-limiting, becomes continuous and chronic inflammatory diseases develop subsequently including cardiovascular diseases, diabetes, cancer etc. Barks of *Delonix regia* is used traditionally in the treatment of inflammatory diseases. Therefore, in this study we evaluated the therapeutic potential of *D. regia* ethanol extract and its active constituent β-Elemene with special interest in inflammation model using standard in vivo anti-inflammatory models: Carrageenan-induced paw edema, Cotton pellet granuloma, and Acetic acid-induced vascular permeability. To explicate the mechanism of action for the possible anti-inflammatory activity, we determined the level of major inflammatory mediators (NO, iNOS, COX-2-dependent prostaglandin E2 or PGE2), and pro-inflammatory cytokines (TNF-a, IL-1b, IL-6, and IL-12). Additionally, we determined the toll-like receptor 4 (TLR4), Myeloid differentiation primary response gene 88 (MyD88), by mRNA expression in drug treated LPS-induced murine macrophage model. To explore the mechanism of anti-inflammatory activity, we evaluated expression of c-Jun N-terminal kinases (JNK), nuclear factor kappa-B cells (NF-kB), and NF-kB inhibitor alpha (IK-Ba). Furthermore, we determined the acute and sub-acute toxicity of *D. regia* extract in BALB/c mice. This study established a significant anti-inflammatory activity of *D. regia* extract and β-Elemene along with the inhibition of TNF-a, IL-1b, IL-6 and IL-12 expressions. Further, the expression of TLR4, NF-kBp65, MyD88, iNOS and COX-2 molecules were reduced in drug-treated groups, but not in the LPS-stimulated untreated or control groups, Thus, our results collectively indicated that the *D. regia* extract and β-Elemene can efficiently inhibit inflammation.

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1. Introduction

Inflammation can be defined as a pathophysiological response of living tissue to injury that leads to local accumulation of plasmatic fluid and blood cells. Although it is a defence mechanism, the complex events and mediators involved in the inflammatory reaction can aggravate many diseases [1]. According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience which is primarily associated with tissue damage or described in terms of such damage or both" [2]. Fever or pyrexia may be a result of infection of tissue damage, inflammation or other disease states. Hypothalamus regulates the body temperature by maintaining a balance between production and loss of heat [3]. Majority of the anti-inflammatory drugs available for clinical use are costly, and have adverse effect including gastrointestinal and respiratory irritation, nephrotoxicity, physical dependence and constipation in long-term use. Therefore, a search for cost-effective natural agents with low toxicity and better tolerance is in demand. Inflammation is a complex biological response of the damaged vascular tissues with protective attempt of healing, and classified as acute or chronic. The acute inflammation is the initial response of the body to the harmful stimuli, when increased movement of plasma and granulocytes takes place from blood to the injured tissues [4]; followed by a cascade of events involving the propagation and maturation of vascular and immune system, along with the cells of the injured tissues [5]. The affected cells are then activated to release several mediators including eicosanoids, cytokines and chemokines to elicit the inflammatory response from acute to the chronic phase. In chronic inflammation, a progressive shift of injured cells occurred at site with simultaneous destruction and healing of the injured tissues [6], along with the release of cyclooxygenase (COX)-mediated prostaglandins (PGs), leading to the pain, edema and fever. Thus, COX inhibitors are used as antiinflammatory drugs. However, many COX inhibitors have serious adverse effects and conventional nonsteroidal anti-inflammatory drugs (NSAD) are unsuitable for the management of chronic and silent inflammations [7].

Toll-like receptors (TLRs) play a critical role in the early innate immune response to invading pathogens by sensing microorganism and

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are involved in sensing endogenous danger signals; whereas lipopolysaccharide (LPS), a component of the bacterial cell wall, LPS mediated danger signal causes dendritic cells to migrate into the regional lymph nodes, where they present the antigens picked up in an infected organ to T lymphocytes.

Toll-like receptors (TLRs) are known to recognize pathogen-associated molecular patterns and induce innate immune responses; while lipopolysaccharides (LPS) of bacteria potently activate the dendritic cells (DCs) and monocyte/macrophages [8]. In professional immune cells, TLR4 induces tightly regulated inflammatory response to avoid tissue damage in response to lipopolysaccharide (LPS) re-stimulation after a prior LPS exposure followed by inducing vigorous productions of various cytokines [8,9]. TLRs are type I transmembrane proteins, and like other TLRs, TLR4 contains a characteristic leucine-rich repeats (LRRs) extracellular domain and a cytoplasmic tail that contains a conserved Toll/IL-1 receptor (TIR) domain region [10,11]. Upon activation, TLR4 binds TIR domain containing adaptor proteins including myeloid differentiation primary response gene 88 (MyD88) [12,13]and TIR domain-containing adaptor inducing IFN-β (TRIF) for its signal transduction [14]. MyD88 is an essential adaptor molecule TLR4, and the canonical MyD88-dependent pathway activates NF-kB and MAPK signaling to AP-1, both of which collectively regulate the expression of numerous inflammatory cytokines [15]. Scientists are in search of new phytochemicals from higher plants that could be a therapeutic potential in inflammatory diseases [16–20]. In the developing countries, people depend on traditional healers and their knowledge of natural medicines for primary health care. Herbal medicine is regaining its importance, since it has no side effects and also improves the basic health needs, however most of the herbs used in folkloric system of medicine have not yet been evaluated scientifically. *Delonix regia* (Fabaceae) is an example of similar promising traditional medicinal source found in Indian subcontinent commonly known as royal poinciana, flamboyant tree, flame tree, peacock flower.

The leaf of the plant is used for diabetes, as anti-microbial, as anti-psychotic [21–23] and also the seed gum as a novel tablet binder [24]. Antiinflammatory activity has been suggested for *D. regia*, however, there is no scientific validation of such claims with proper understanding of the mechanism of action. In the present study we have analysed the immunotherapeutic potential of the alcoholic extract of *D. regia* against in vivo carrageenan- (acute) and dextran-induced (sub-acute) paw edema, cotton pellet granuloma (chronic), acetic acid induced vascular permeability, and the in vitro protein denaturation, to correlates its traditional use. β-Elemene is the major component of *D. regia* was tested for its anti-inflammatory activity by acetic acid induced vascular permeability. Further, we have studied its immunomodulatory potential in murine macrophage model through the regulation of COX-2-dependent prostaglandin-E2 (PGE2) and TLR4 signaling pathways, including the p38 MAPK, JNK and NF-kB. Our results showed that *D. regia* and β-Elemene treatment could restrict the inflammation in mice, and its anti-inflammatory effect is due to the down-regulation of TLR4/MyD88 signaling pathway. Furthermore, the acute and sub-acute toxicity studies in mice model showed that the *D. regia* extract is safe at its antiinflammatory dosage.

2. Materials and methods

2.1. Plant material and preparation of extract

Authentication of the specimen was done by Dr. P. Brindha, CARISM, SASTRA University, Thanjavur. The voucher specimens was matched with already determined specimens at Pachaiyappa's College, Chennai, Tamil Nadu, India, with voucher number T- 1586 [25]. For this study, bark samples were shade dried and then powdered. The powder was passed through sieve no. 40 and stored in an airtight container for further use. The dried powder material (150 g) was defatted with petroleum ether (60–80°) to remove waxy substances and chlorophyll. The marc, after defatted with petroleum ether, was dried and extracted with ethanol (99.9% v/v) in a Soxhlet extractor for 72 h. The solvent was then distilled off, and the resulting semisolid mass was dried in a vacuum evaporator to get a yield of 17% w/w.

2.2. Chemicals and drugs

The drugs used as standard for comparing the activity of the extracts were Diclofenac sodium, Pentazocine and Paracetamol for anti-inflammatory, analgesic and antipyretic activities respectively were purchased from Sigma Aldrich, India. Tween 80, carboxy methyl cellulose (CMC), acetic acid and other reagents of analytical grade were purchased from S. D. Fine Chem. Ltd, India.

2.3. Animals

For the in-vivo studies, Wistar rats weighing around 200–230 g and swiss albino mice weighing around 35–50 g of either sex were used. Animals were kept in plastic cages (six per cage) under standardized animal house conditions with temperature 19–25 °C, photoperiod of approximately 12 h light and 12 h dark cycle and a relative humidity of 30–70%. Animals had free access to food (Nutre Plus Feeds Pvt Ltd, Chennai, India) and water; all the animals were acclimatised for a week before experiment start. Animals were fasted (water was provided) 18 h prior to oral administration for each experiment. All the procedures for in-vivo studies were reviewed and approved by the Institutional Animal Ethics Committee at SASTRA University. The IAEC approval number is 111/SASTRA/IAEC/RPP.

2.4. Preliminary phytochemical screening

The ethanolic extract of *D. regia* was screened for various chemical constituents following the standard screening tests including test for alkaloids, steroids, reducing sugars, carbohydrates, saponins, glycosides, phenols, flavones, quinones, amino acids, tannins [26,27].

2.5. Acute toxicity studies

Healthy adult Swiss albino mice of either sex weighing between 20 and 25 g were subjected to acute toxicity studies as per guidelines (AOT no. 425) suggested by the Organization for Economic Cooperation and Development. Animals ascribed as fasted were deprived of food for 16 h, but had free access to water. Forty-two mice including both male and female weighing 20–25 g were selected for the study. Overnight faster mouse were divided into seven groups including six for alcoholic extract and one control group each consists of six mice. Different doses of extract (100, 200, 500, 1000, 2000 and 5000 mg/kg) were administered to seven experimental groups and control group received vehicle. The animals observed continuously for their general behaviour, such as motor activity, tremors, convulsions, Straub reaction, piloerection, loss of lighting reflex, sedation, muscle relaxation, hypnosis, analgesia, ptosis, lacrimation, diarrhoea, skin colour, and mortality intermittently for next 48 h.

2.6. Protein denaturation

A modified method is being used for assessing the anti-denaturation effects of natural products with anti-inflammatory properties [28]. Bovine serum albumin (BSA) of concentration 1 mM was prepared using Phosphate buffer saline - pH 6.3. Reaction mixture (1 ml) consists of 0.5 ml of 1 mM BSA and 0.5 ml of different concentration of *D. regia* extract (50–200 μg/ml) or β-Elemene (5–20 μg/ml) or Diclofenac Sodium (100 μg/ml) and product control (1 ml) consists of 0.5 ml of distilled water and 0.5 ml of test solution was used for evaluation of protein denaturation. Test control solution (1 ml) was prepared using 0.5 ml of bovine serum albumin and 0.5 ml of distilled water and standard solutions

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