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Modulation of inflammatory markers by the ethanolic extract of *Leucas aspera* in adjuvant arthritis

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ABSTRACT

Aim of the study: To investigate the anti-inflammatory and antioxidant potential of ethanolic extract of *Leucas aspera* (EELA) in adjuvant arthritis.
Materials and methods: Complete Freund's adjuvant served to induce arthritis. EELA was administered in two doses along with vehicle control (0.1% carboxymethyl cellulose) and positive control (Diclofenac). Levels of tumour necrosis factor (TNF-)-α, C-reactive protein (CRP), Interleukin-2 (IL-2), Cathepsin D, activities of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were estimated in plasma/hemolysate and tissue. HPLC analysis of EELA was also performed.
Results: EELA exhibited significant anti-inflammatory ($p < 0.001$) and antioxidant activity ($p < 0.001$). It did not show mortality up to 2000 mg/kg body weight. Histopathological studies confirmed complete cartilage regeneration and near normal joint in EELA2 treated arthritic rats.
Conclusion: This study highlighted the antioxidant and anti-inflammatory potential of *Leucas aspera*. Three major families of compounds present in EELA may explain these activities: catechins (epicatechin, beta epicatechin), flavonoids (procyanidin), phytosterols (beta-sitosterol) apart from glycosides, phenolic compounds and tannins.

1. Introduction

Free radicals and the associated oxidative stress have been implicated in the etiology of rheumatoid arthritis (RA). The pro-inflammatory cytokines released by arthritic joints activate neutrophils that play a key role in the pathogenenesis of synovial inflammation. They generate ROS (reactive oxygen species) which cause the release of collagenases and elastases that contribute to cartilage destruction (Mythilipriya et al., 2007). Recently, there has been an increasing interest in free radical scavenging substances derived from herbs and the role of potential herbs in anti-arthritic therapy has been evaluated (Jiang and Qiang, 2003). *Leucas aspera* (thumbai) belonging to the family Labiateae, is an herbaceous annual distributed throughout India. In traditional medicine, the leaves find their use in chronic rheumatism and juice for skin eruptions (Kirtikar and Basu, 1991). Literature reveals that extracts of *Leucas aspera* exhibit analgesic, anti-inflammatory and anti-pyretic efficacies (Saundane and Satyanarayanan, 2000). The anti-inflammatory activity of different fractions of *Leucas aspera* in cotton pellet and carrageenan induced paw edema has been proved (Goudgaon et al., 2003). Extracts prepared from root parts of the plant showed significant antinociceptive, antioxidant and cytotoxic activities (Rahman et al., 2007). Compounds isolated from *Leucas aspera* include a hydroxyl tetra triacontan-4-one, aliphatic ketones (Misra et al., 1993), nicotine (Mangathayaru et al., 2006b), alpha-farnesene, alpha-thujene, menthol from leaf volatiles and amyl propionate, isoamyl propionate from flower volatiles (Mangathayaru et al., 2006a). *In vitro* antioxidant activity guided fractionation of *Leucas aspera* yielded eight lignans and four flavonoids (Sadhu et al., 2003).

The present study focuses on the effect of *Leucas aspera* on adjuvant induced arthritic rats by analysing the markers of inflammation, antioxidant status and histological changes of knee joints. HPLC analysis was performed to detect the phenolic and bioflavonoid constituents of EELA.

2. Materials and methods

2.1. Drugs and chemicals

Complete Freund's adjuvant (CFA) was purchased from Sigma Chemical Company, USA. ELISA kits for TNF-α from Pierce Endogen, USA; CRP from Immunology Consultants Laboratory, USA and IL-2

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from Bender Med Systems Gmbh, Europe. Other chemicals used were of analytical grade.

2.2. Plant material

Aerial parts of the plant *Leucas aspera* were collected from Kanchipuram district, Tamilnadu, India and taxonomically identified by the Plant Anatomy Research Center, Chennai (PARC/2007/362).

2.2.1. Preparation of extract

Shade dried, coarsely powdered plant material (1 kg) was subjected to successive extraction in solvents of increasing polarity (n-hexane, chloroform, ethyl acetate and ethanol). The maceration period in each solvent was 72, 48 and 24 h (Chamundeeswari et al., 2010). Solvents were filtered, distilled and dried in a vacuum desiccator, and extractive yields calculated, yields being 1.7, 3.2, 2 and 4.6% (w/w) respectively.

2.3. In vitro antioxidant studies

The *in vitro* antioxidant activity of the extracts was determined by their ability to scavenge DPPH (1,1-diphenyl-2-picryl-hydrazyl) radicals (Sreejayan and Rao, 1996). Percentage inhibition was expressed in terms of IC_{50} value calculated by linear regression method. Based on this, the ethanolic extract of *Leucas aspera* (EELA) was selected for further studies.

2.4. HPLC analysis and secondary metabolite estimation

EELA was subjected to HPLC analysis (Lachrom L-7000 system, Hitachi) to estimate the total flavonoid content using C18 column. Orthophosphoric acid (0.3 g/l) and methanol served as mobile phase A and B. Procyanidin (Pcy) and epicatechin (EC) were among the reference standards. UV detector was used, set at 350 nm with a flow rate of 1.5 ml/min of 20 μl sample. Total polyphenols and tannins were estimated at 760 nm, compared with pyrogallol as standard. Glycosides were estimated at 540 nm using digitoxin in alcohol (0.1%) as reference standard.

2.5. In vivo studies

2.5.1. Animals

The study was performed with female wistar rats (125–150 g) and swiss albino mice of either sex (25–30 g). Rats were acclimatised, fed commercial pelleted feed and water *ad libitum*. Experimental protocols were approved by the Committee for the purpose of Control and Supervision of experiments on animals (CPCSEA), New Delhi, India (IAEC-XII/SRU/73/2008).

2.5.2. Experimental design

2.5.2.1. Preliminary acute toxicity studies. Mice were divided into two groups of three each. Vehicle control group received 0.2% CMC and second group 2000 mg/kg b.w. of EELA suspended in 0.2% CMC. Immediately after the dose, animals were observed continuously for first 4 h and next 14 days to record mortality.

2.5.2.2. Anti-arthritic studies. Animals were divided into five groups of six each. Group I: control rats orally treated with vehicle. Test groups were injected with 0.1 ml of CFA intradermally. Group II: disease control. Group III: treated with standard drug diclofenac sodium (DF) (0.3 mg/kg/day p.o.) while Group IV and Group V with EELA1 and EELA2 (100 and 200 mg/kg/day p.o. respectively) from day 15th to 35th of induction. Inflammation was assessed by measuring the right hind paw volume every week using mercury displacement plethysmograph (Chattopadhyay et al., 1986).

On the 42nd day, animals were sacrificed, blood collected, liver excised and 10% homogenate prepared (Tris–HCl, 0.01 M, pH 7.4) and used for biochemical estimations. Histopathological studies were done in hind limb joints. Tissues were fixed in formalin, decalcified, embedded in paraffin blocks and sections stained with hematoxylin and eosin (HE).

2.6. Biochemical estimations

Lipid peroxidation (LPO) was estimated by the method of Ohkawa et al. (1979); Cathepsin D by Sapolsky et al. (1973); SOD by Marklund and Marklund (1974); GPx by Rotruck et al. (1973); Catalase by Sinha (1972); GSH by Moron et al. (1979) and tissue protein by Lowry et al. (1951). Plasma CRP, IL-2 and TNF-α levels were assayed by ELISA according to the manufacturer's (Section 2.1) instructions.

2.7. Statistical analysis

Data were expressed as mean ± SD from six rats per group. Statistical analysis of biochemical parameters were performed using one-way ANOVA (SPSS software) and values of $p < 0.05$ were considered to be statistically significant. Results of paw volume inhibition were analysed using Student's *t*-test.

3. Results and discussion

3.1. In vitro antioxidant studies

In the antioxidant investigation, EELA showed maximum efficiency in scavenging DPPH, the stable free radical donor. The antioxidant activity expressed in terms of IC50 value was found to be 929, 1114, 77.29 and 71.4 μg/ml for n-hexane, chloroform, ethyl acetate and ethanolic extracts. Since the antioxidant property of EELA was favourable, it was subjected to further studies.

3.2. HPLC analysis

HPLC analysis of EELA demonstrated the presence of EC (4.66 mg), β, 1-4 EC (1.98 mg), Pcy (2.78 mg) and beta-sitosterol (0.89 mg) per 100 g of extract. Total flavonoid content was 56.78 mg/100 g and total glycosides inclusive of nudiposide and lyoniside was 1.067% (w/w). Appreciable amounts of phenols (0.4021%, w/w) and tannins (0.2567%, w/w) have also been demonstrated.

3.3. Preliminary acute toxicity studies

Preliminary acute toxicity studies showed no mortality and no adverse effects in animals of both sexes indicating that EELA was nontoxic in mice up to an oral dose of 2000 mg/kg b.w.

3.4. Anti-inflammatory studies

Arthritic rats showed soft tissue swelling around ankle joints due to edema of periarticular tissues caused by PMNL accumulation (Ramprasath et al., 2006). The ability of EELA to reduce paw edema ($p < 0.001$) (Fig. 1) can be related to the inhibitory role of its constituents-EC on cyclo-oxygenases and lipoxygenases, the predominant enzymes of prostaglandin biosynthesis.

The study showed increased levels of CRP, TNF-α and IL-2 in disease control while DF and EELA treated rats (Groups III, IV and V) exhibited marked decline ($p < 0.001$ each) which might be due to the suppressed production of inflammatory cytokines, the effect of EELA being dose dependant. Cathepsin D, an important protease in cartilage degradation showed increased