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RESEARCH ARTICLE

Effect of *Indigofera aspalathoides* on complete Freund's adjuvant-induced arthritis in rats

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Abstract

The effect of ethanol extract of stems of *Indigofera aspalathoides* Vahl (Papilionaceae) (EIA) was evaluated for anti-arthritic activity on complete Freund's adjuvant-induced (CFA-induced) arthritis in rats. The EIA was administered orally at doses of 250 and 500 mg/kg daily for 30 days. The paw volume was measured on days 7, 14, 21 and 30. At the end of day 30, the rats were sacrificed and various biochemical parameters such as serum aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol and triglycerides were estimated. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and lipid peroxide (LPO) in the liver and kidney of normal, arthritic control and EIA treated rats were studied. Oral administration of EIA effectively inhibits rat paw edema in a dose-dependent manner. EIA significantly ($P < 0.01$) altered the biochemical parameters which were affected in arthritic rats. There was significant alteration in LPO, SOD, catalase, and GPx levels when compared to arthritic control rats. Our findings showed a significant anti-arthritic effect of EIA against CFA-induced arthritis in rats.

Keywords: *Antioxidants; biochemical parameters; complete Freund's adjuvant-induced arthritis; Indigofera aspalathoides*

Introduction

Rheumatoid arthritis is a chronic progressive inflammatory arthritis. Arthritis has been found to be the most prominent manifestation of rheumatoid disease. It is a generalized connective tissue disorder that involves para-articular structures such as tendons, bursae and the tendon sheath as well as extra-articular tissues such as subcuits, lymph nodes, skeletal muscle, central and peripheral nervous system and eyes.

Complete Freund's adjuvant-induced arthritis has been used as a model of sub-chronic or chronic inflammation in rats and is of considerable relevance for the study of pathophysiological and pharmacological control of inflammatory processes, as well as the evaluation of analgesic potential or anti-inflammatory effects of drugs (Butler et al., 1992; Besson & Guilbaud, 1988). One of the reasons for the wide utilization of this model is due to the strong correlation between the efficiency of therapeutic agents in this model and in rheumatoid arthritis in humans (Besson & Guilbaud, 1988). Anti-inflammatory drugs, currently available for the treatment of joint inflammation of various kinds, have many undesirable side effects such as the induction of peptic ulcers. Therefore, plant remedies have become increasingly popular and are often preferred to synthetically derived pharmaceuticals because they have lesser side effects.

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Indigofera aspalathoides Vahl (Papilionaceae) is a low under-shrub with wide distribution, found mostly in south India and Ceylon. It is popularly known as "sivanar vembu" in Tamil. The stem of the plant is traditionally used for various skin disorders and tumors (CSIR, 2001). In our previous studies, the stem has been found to have anti-inflammatory and anticancer activity against Ehrlich ascites carcinoma tumors and hepatocellular carcinoma (Rajkapoor et al., 2003, 2004, 2005). The phytochemical studies of the plant have revealed the presence of 5,4′-dihydroxy 6,8-dimethoxy 7-*O*-rhamnosyl flavones and pterocarpan (Rajkapoor et al., 2007; Selvam et al., 2004). The present study evaluated the anti-arthritic activity of *Indigofera aspalathoides* on complete Freund's adjuvant-induced arthritis in rats.

Materials and methods

Plant material and extraction

Stems of *Indigofera aspalathoides* were collected in and around Salem district in the month of December 2005 and authenticated by G. Murthy, Botanical Survey of India, Coimbatore, Tamil Nadu, India. The stems were shade-dried for one month, then pulverized. The powder was treated with petroleum ether for de-waxing and removal of chlorophyll. Later, it was packed (250 g) in a Soxhlet apparatus and subjected to hot continuous percolation (78°C) for 8 h using 450 mL of ethanol (95% v/v) as solvent. The extract was concentrated under vacuum, dried in a desiccator (yield, 4.5% w/w) and suspended in 5% gum acacia for the study.

Rats

Male Wistar rats (100-125 g) aged 6 weeks were procured from Tamil Nadu Veterinary College, Chennai, India. They were housed in standard microlon boxes with standard laboratory diet (Hindustan Lever, Mumbai, India) and water *ad libitum*. The experiments were performed in accordance with the guidelines established by the European community for the care and use of laboratory rats and were approved by the Institutional Animal Ethics Committee (IAEC).

Experimental induction of arthritis

The rats were divided into four groups with each group containing six rats. The treatment schedules of rats belonging to the different groups are shown below.

Group 1: Normal (normal saline 2 mL/kg)

Group 2: Complete Freund's adjuvant (0.1 mL)

Group 3: Ethanol extract of *Indigofera aspalathoides* (250 mg/kg, per oral)

Group 4: Ethanol extract of *Indigofera aspalathoides* (500 mg/kg, per oral)

On day zero, arthritis was induced in rats belonging to groups 2, 3 and 4 by injecting 0.1 mL of complete Freund's adjuvant (Sigma, St. Louis, MO, USA) below the plantar aponeurosis of the right foot paw of the rats (Newbould, 1963). After this day, the extract was administered to the rats at the doses of 250 and 500 mg/kg, p.o. for up to 30 days. The paw volume was measured by using plethysmograph apparatus at days 7, 14, 21, and 30. At the end of the experimental period the rats were fasted overnight and then killed by cervical decapitation. Blood was collected and serum separated out. The liver and kidney were immediately removed and suspended in ice-cold saline for antioxidant studies.

Biochemical estimation

Serum was analyzed for the following biochemical parameters: serum aspartate transaminase (AST) (Reitman&Frankel,1957),alanineaminotransferase(ALT) (Reitman & Frankel, 1957), alkaline phosphatas (Kind & King, 1954), total protein (Lowry et al., 1951), cholesterol (Wybenga & Pileggi, 1970), and triglyceride (Hawk et al., 1954). A 10% homogenate of the tissue was used for the assay of lipid peroxidation (LPO) (Devasagayam & Tarachand, 1987), superoxide dismutase (Marklund & Marklund, 1997), catalase (Sinha, 1972), and glutathione peroxidase (GPx) (Rotruck et al., 1973).

Statistical analysis

The results are expressed as mean ± SD and the statistical significance was analyzed by one way ANOVA followed by Tukey's multiple comparison test. P values < 0.05 were considered as significant.

Results and discussion

Table 1 shows the time course of edema and inhibition rate after the administration of CFA and EIA. The hind paw developed edema in the footpad. Edema value of the injected footpad significantly ($P < 0.001$) increased and reached a peak at 30 days. Administration of EIA at a doses of 250 and 500 mg/kg of body weight significantly ($P < 0.001$) inhibited the development of the swelling induced by CFA. The doses of EIA exhibited anti-inflammatory activity in a dose-dependent manner, which was maintained until the experiment was terminated on day 30 (groups 3 and 4).

As a result of arthritis induced by CFA in group 2, the level of AST, ALT and ALP were increased (Table 2). It also showed the lipid profiles following the administration of EIA at 250 and 500 mg/kg in group 3and 4 rats, in which a significant difference in the concentration of total cholesterol and triglyceride was found.

With adjuvant treatment, an increase in lipid peroxide was seen in liver and kidney. An arthritic rat treated with EIA was found to have altered values which were closer to that of controls. A decrease in SOD, catalase and GPx levels in liver and kidney was observed in adjuvant-induced arthritis (Tables 3 and 4). Treatment with EIA in adjuvant-induced rats altered the antioxidant levels near to normal in a dose-dependent manner.

The adjuvant produces arthritis predominantly in the joints of hind limbs, promoting significant reduction of motor activity and an increase in itching and scratching behaviors (Calvino et al., 1987). In addition, increased

Table 1. Effect of EIA on CFA-induced paw edema in rats.

		Paw volume (mL)			
Treatment	Dose (mg/kg)	Day 7	Day 14	Day 21	Day 30
Normal (normal saline)	2 mL/kg	0.21 ± 0.01	0.20 ± 0.01	0.26 ± 0.01	0.24 ± 0.01
CFA	0.1 mL	0.56 ± 0.02[a]	0.64 ± 0.01[a]	0.72 ± 0.02[a]	0.74 ± 0.03 [a]
EIA	250	0.27 ± 0.01[b, d]	0.32 ± 0.01[a,d]	0.36 ± 0.01[a,d]	0.39 ± 0.01[a,d]
	500	0.24 ± 0.01[d]	0.28 ± 0.02[c,d]	0.31 ± 0.01[d]	0.34 ± 0.01[c,d]

N = 6 rats in each group.
[a]P < 0.001; [b]P < 0.05; [c]P < 0.01 versus normal; [d]P < 0.001 versus CFA.
Data were analyzed by one way ANOVA followed by Tukey multiple comparison test.

Table 2. Effect of EIA on biochemical parameters against CFA-induced arthritis in rats.

Treatment	Dose (mg/kg)	AST U/L	ALT U/L	ALP U/L	Total protein mg%	Cholesterol mg%	Triglyceride mg%
Normal (normal saline)	2 mL/kg	54.5 ± 1.65	51.25 ± 1.78	177.5 ± 3.52	6.27 ± 0.34	61.75 ± 1.8	67 ± 1.36
CFA	0.1 mL	156.2 ± 3.49[a]	67.8 ± 1.90 [a]	301 ± 5.48 [a]	7.46 ± 0.49	68.8 ± 1.15 [b]	99.2 ± 1.59[a]
EIA	250	122.0 ± 2.64 [a,d]	62.0 ± 1.22 [a]	254.0 ± 7.4[a,d]	6.36 ± 0.19	58.6 ± 1.27[a,d]	78.4 ± 1.60 [a]
	500	98.5 ± 1.54[a,d]	57.6 ± 1.08[b,e]	206.0 ± 4.93[c,d]	6.24 ± 0.35	55.8 ± 1.93[d]	65.4 ± 1.23[d]

N = 6 rats in each group.
[a]P < 0.001; [b]P < 0.05; [c]P < 0.01 versus normal; [d]P < 0.001; [e]P < 0.01 versus CFA.
Data were analyzed by one way ANOVA followed by Tukey multiple comparison test.

Table 3. Effect of EIA on antioxidant status in liver of control and experimental animals.

Treatment	Dose (mg/kg)	LPO	SOD	CAT	GPx
Normal (normal saline)	2 mL/kg	141.8 ± 4.48	4.74 ± 0.03	46.56 ± 1.4	9.65 ± 1.50
CFA	0.1mL	394.2 ± 11.94[a]	1.91 ± 0.02[a]	33.92 ± 2.59[b]	5.15 ± 0.65
EIA	250	179.55 ± 3.18[b,c]	2.51 ± 0.02[a,c]	40.12 ± 1.12[d]	7.12 ± 0.80[c]
	500	158.6 ± 2.10[c]	3.28 ± 0.06[a,c]	44.16 ± 1.16[d]	9.10 ± 0.35[e]

N = 6 rats in each group.
[a]P < 0.001; [b]P < 0.01 versus normal; [c]P < 0.001; [d]P < 0.01; [e]P < 0.05 versus CFA.
Data were analyzed by one way ANOVA followed by Tukey multiple comparison test.
LPO = µmoles of MDA/min/mg protein; GPx = µmoles of GSH oxidized/min/mg protein; SOD = Units/min/mg protein; CAT = µmole of H_2O_2 consumed/min/mg protein.

Table 4. Effect of EIA on antioxidant status in kidney of control and experimental animals.

Treatment	Dose (mg/kg)	LPO	SOD	CAT	GPx
Normal (normal saline)	2 mL/kg	83.62 ± 2.90	3.49 ± 0.46	36.12 ± 1.04	4.65 ± 0.84
CFA	0.1mL	127.04 ± 3.94[a]	1.88 ± 0.11[c]	21.53 ± 1.47 [a]	2.31 ± 0.36[b]
EIA	250	98.77 ± 1.56[b,d]	2.13 ± 0.02[c]	28.42 ± 1.08[b]	3.42 ± 0.28
	500	87.5 ± 1.42[d]	2.96 ± 0.14[f]	31.64 ± 1.35[d]	4.01 ± 0.17

N = 6 rats in each group.
[a]P < 0.001; [b]P < 0.05; [c]P < 0.01 versus normal; [d]P < 0.001; [e]P < 0.01; [f]P < 0.05 versus CFA.
Data were analyzed by one way ANOVA followed by Tukey multiple comparison test.
LPO = µ moles of MDA/ min/mg protein; GPx = µ moles of GSH oxidised/min /mg protein; SOD = Units/min/mg protein; CAT = µ mole of H_2O_2 consumed/ min/mg protein.

sensitivity of the affected paw to pressure or flexion and extension of the inflamed joints and weight loss were observed. The arthritis observed in rats is associated with a hyperalgesia phenomenon and spontaneous behaviors, such as protection of the affected paw, evidenced by curving and/or elevation of the paw, as well as avoidance of supporting the body on the paw (Clatworthy et al., 1995). The hyperalgesia is more evident during the acute inflammatory phase, when spontaneous behaviors, indicative of painful response are more pronounced. Increased paw diameter (posterior and anterior) due to inflammation and edema was also observed (Cain et al., 1997). The initial inflammatory response was developed within hours, but more critical clinical signals emerged from day 10 and thereafter, and the alterations remain detectable for several weeks (Clopaert et al., 1982). The results of the present study indicate that the EIA exhibits anti-arthritic effects in rats with Freund's adjuvant-induced arthritis, on its acute as well as its chronic phases.

Tissue damage was assessed by measuring the activity of enzymes in the serum and in the respective organ, since liver impairment is also a feature of adjuvant arthritis (Marylatha et al., 1998). The increase in aminotransferase is due to its release from cells of the damaged organ (Rainsford, 1982). Subrata et al. (1994) reported a similar increase in aminotransferases observed in arthritic rats. Alkaline phosphatase has been reported to be present mainly in the blood vessels, pia-arachnoid and choroid plexes. Alkaline phosphatase activity has been reported to increase during the morphological and functional development of the tissues. Aminotransferases and alkaline phosphatase were significantly reduced in arthritic rats after the administration of EIA. This reducing effect may be related to their anti-inflammatory activity. Arthritis is characterized by inflammation and an increased cardiovascular risk. It was recently shown that active early rheumatoid arthritis is associated with dyslipidemia, which may partially explain the enhanced cardiovascular risk (Van Halm et al., 2007). Table 2 shows the lipid profiles following the administration of EIA at 250 and 500 mg/kg in group 3 and 4 rats, in which there was found a significant difference in the concentration of total cholesterol and triglyceride. Therefore, we can speculate that EIA may reduce the risk of inflammation-related diseases.

The increased lipid peroxide level noticed in arthritic rats in our study (group 2) may be due to its release from neutrophils and monocytes during inflammation (Greenwald & Moy, 1980). At the onset of inflammation there is a rapid fall in the total iron content of blood plasma followed by decreased deposition of iron proteins in the synovial fluid. The drop in plasma iron correlates closely with the activity of the inflammatory process. In the synovial fluid of inflamed joints, the iron released during necrosis, might catalyze the formation of OH (hydroxyl) radicals from H_2O_2, thus contributing increased lipid peroxidation in arthritis. From the literature reviewed, it is apparent that RA is exposed to oxidative stress and is prone to lipid peroxidation (Heliovaara et al., 1994). In the present study, the concentration of lipid peroxidation was significantly altered in arthritic rats after the administration of EIA, when compared with arthritic control rats.

Many cellular defense mechanisms are directed against the toxic effects of these radicals in the inflammatory process. SOD which converts super oxide radicals to H_2O_2 is widely distributed in cells having oxidative metabolism and is believed to protect such cells against the toxic effects of super oxide anion. Increased delivery of NADPH from the stimulated hexose monophosphate (HMP) shunt during inflammation is proposed to lead to the activation of SOD in arthritic rats. A decrease in superoxide dismutase in the liver and kidney has been observed in adjuvant-induced arthritis (Tables 3 and 4). Increased production of NADPH from HMP shunt during arthritis may cause a decrease in SOD activity (Marklund et al., 1987). This increase in enzyme activity appears to be protective against the intracellular oxygen free radicals (Kasama et al., 1988). Administration of EIA to arthritic rats caused a significant increase in SOD activity.

Catalase in the erythrocytes functions to protect Hb against oxidation. Liver has been reported to be a major site of lipid peroxide metabolism. The main function of catalase is to detoxify H_2O_2, although catalase is significantly decreased in rheumatoid arthritis. Its concentration is very low to expect considerable protection against H_2O_2 in arthritic rats (Blake et al., 1981). Treatment of arthritic rats with EIA significantly increased the catalase enzyme levels. Glutathione peroxidase is localized in the cytoplasm and mitochondria, which catalyses the degradation of various peroxides by oxidizing glutathione with the formation of its conjugates. The increased activity of glutathione peroxidase in liver and kidney of arthritic rats, as shown by the present results, indicates the free radical defense system against oxidative stress and may help to explain the pathogenesis associated with arthritis. After EIA treatment, GPx levels were significantly decreased.

After EIA treatment, the alterations produced in arthritic rats with respect to lipid peroxidation and antioxidant concentrations were modulated to nearly normal levels. The antiperoxidative action observed in EIA treated arthritic rats might be due to the presence of compound-like flavonoids in EIA. These compounds have been shown to scavenge free radicals, including hydroxyl and superoxide anions and reduce the levels of lipid peroxidation in stress-induced rats (Jovanovic & Simic, 2000). In the present study we thus conclude that

oral administration of EIA has been shown to modulate the above biochemical changes observed in the adjuvant-induced arthritic rats. Further studies are in progress to isolate and characterize active principles.

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