Evaluation of anti-inflammatory and antioxidant activity of *Ichnocarpus frutescens* root

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ABSTRACT

Background and the purpose of the study: *Ichnocarpus frutescens* has been widely used in the traditional medicine for the treatment of a variety of diseases. In the present study anti-inflammatory and antioxidant property of roots of this plant was investigated.

Methods: The anti-inflammatory activity of methanolic extract of Ichnocarpus *frutescens* (MEIF) was evaluated by carrageenan, and cotton pellet induced granuloma tests to determine its effects on acute and chronic phase of inflammation models in rats.

Results and major conclusion: Preliminary Phytochemical analysis of methanolic extracts showed presence of terpenoids, flavonoids, and sterols. Maximum inhibition (54.63 %) was obtained at the dose of 100 mg/kg after 3 hrs of drug treatment in carrageenan induced paw oedema, whereas indomethacin produced 57.65 % of inhibition. In the chronic model, 300 mg/kg of MEIF like indomethacin and dexamethasone standard drug decreased formation of granuloma tissue by 22.64, 29.63 % and 34.84 % respectively. The successive methanol re-extract of *Ichnocarpus frutescens* root (MEIF) exhibited strong scavenging effects on 2, 2-diphenyl-2-picryl hydroxyl (DPPH) free radical, nitric oxide, super oxide anion, hydroxyl radical and inhibition of lipid peroxidation. These results clearly indicate strong anti-inflammatory and antioxidant properties of *Ichnocarpus frutescens* root.

Keywords: Ichnocarpus *frutescens;* Anti-inflammatory; Carrageenan; Cotton pellet induced granuloma

INTRODUCTION

Ichnocarpus frutescens R. Br (Apocyanaceae), commonly known as siamlata, is an evergreen, laticiferous, woody creeper with rusty red appearance, found almost throughout India. The roots are reported to possess demulcent, alterative, tonic, diaphoretic and diuretic properties and are used in fevers, dyspepsia and skin troubles, usually in combination with bitters and aromatics. The root powder is administered with milk for diabetes, excretion of the stone in the bladder and as blood purifier. Leaves are boiled in oil and applied for headaches and fevers and wounds between fingers. (1,2). A decoction of the roots of Colocynth, Anantamul, Sariva (Sanskrit) and Hedyotis biflora prepared in the usual way is administered with the addition of powdered long pepper bdellium in chronic skin diseases, syphilis, loss of sensation and hemiplegia (3). Pharmacological investigations have demonstrated that *Ichnocarpus frutescens* possess antidiabetes (4), anti tumour (5), anti-inflammatory and analgesic activity (6). Studies on chemical constituents of the plant have revealed the presence of phenylpropanoids, phenolic acids, coumarines, flavonoids, sterols and pentacyclic triterpenoids. (7,8). The aim of this study was to evaluate Anti-inflammatory and antioxidant activity of methanolic extract of *Ichnocarpus frutescens* root

MATERIALS AND METHODS

Plant material

The roots of *Ichnocarpus frutescens* R. Br were collected during the month of June 2005 from Chennai, Tamilnadu, India. The plant material was taxonomically Identified and authenticated by Prof. P. Jayaraman, Taxonomist, Plant Anatomy Research Centre, Chennai, India. A voucher specimen (PARC/24/06) has been deposited in the Herbarium of the Department of Pharmaceutical Technology, Meerut Institute of Engineering and Technology, India, for future reference. The roots of the plants were dried under controlled temperature, powdered and passed through a 40 mesh sieve and stored in an air tight container.

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Chemicals

Rutin was obtained from Acros organics, New Jersey, USA. 1, 1-diphenyl, 2-picryl hydrazyl (DPPH), Nitro blue tetrazolium (NBT), Reduced nicotinamide adenine dinucleotide phosphate (NADH), Phenazine methosulphate (PMS), Trichloroacetic acid (TCA), Butyl hydroxy toluene (BHT) ,Quercetin and Carrageenan were obtained from Sigma chemical Co, USA. 2-Deoxy-d-ribose was from Hi-Media Laboratories Pvt. Ltd., Mumbai, India .Ascorbic acid and Vitamin-E were obtained from SD Fine Chemicals Ltd, Biosar, India. TBA (Thiobarbituric acid) and pyridine were obtained from Loba Chemie, Mumbai, India. Acetic acid, EDTA (Ethylene diamine tetra acetic acid disodium salt) and hydrogen peroxide (H_2O_2) were obtained from Qualigens Fine Chemicals, Mumbai, India. Naphthyl ethylene diamine dihydrochloride was obtained from Roch-light ltd, Suffolk, England. All chemicals used were of analytical grade.

Extraction procedure

The powdered plant material was extracted using 95 % methanol and the solvent was completely removed by vacuum distillation to yield a reddish-brown residue (yield 5.4 %, w/w). This methanolic extract (MEIF) was examined chemically and it was found to contain flavonoids, terpenoids, and sterols which were confirmed by thin-layer chromatography (TLC).The extract was stored in a refrigerator and a weighed amount of MEIF was suspended in 2 % aqueous Tween 80 solution and used for the present study.

Carrageenan induced paw edema

Anti-inflammatory activity was evaluated using the carrageenan induced rat paw oedema according to the technique of Winter et al. (9). After 16hrs fast rats were divided into five groups of six each. Group I served as control group received Tween 80 (5 ml/kg) of 2% w/v, orally. Group II, III and IV animals received MEIF at a dose of 100,200 and 300 mg/kg as a fine suspension in 2 % v/v aqueous Tween 80 solution orally. Group V was orally administered indomethacin at a dose of 10 mg/kg as a standard drug. After 1 h, 0.1 ml of 1 % w/v carrageenan suspension was injected subcutaneously to the planter surface of the right hind paw. The paw volume was measured using a plethysmometer immediately and 3 hrs after carrageenan injection.

Cotton pellet induced granuloma

The rats were divided into five groups, of six animals. Animals were anaesthetized after shaving off the fur. Sterile pre-weighed cotton pellets (50 ± 1 mg) were implanted in the axilla region of each rat through a single needle incision (10). MEIF at doses of 100, 200 and 300 mg/kg), positive control (indomethacin 10 mg/kg) and vehicle control (2 % v/v aqueous Tween 80 solution, 5 ml/kg) were administered to the respective group of animals for seven consecutive days from the day of cotton pellet implantation. On the eighth day, the animals were anaesthetized again; the cotton pellets were removed surgically and made free from extraneous tissues. The pellets were incubated at 37°C for 24 hrs and dried at 60°C to constant weight. The increment in the dry weight of the pellets was regarded as measure of granuloma formation.

Antioxidant Activity

Free radical scavenging activity

The free radical scavenging capacity of the extracts was determined using DPPH. Briefly, 0.15% solution of DPPH in ethanol was prepared and 1 ml of this solution was added to 3 ml of MEIF in methanol at different concentration (1µg/ml to 40 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 515 nm using a spectrophotometer (Systronics-2203, UV-visible double beam spectrophotometer). The inhibition curve was plotted and IC_{50} values obtained (11).

Nitric oxide radical inhibition assay

Nitric oxide radical inhibition was estimated by the use of Griess Illosvoy reaction (12). In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and MEIF (10 µg to 320 µg) or standard solution (rutin, 0.5 ml) was incubated at 25°C for 150 min. Equal volume of methanol without the test compound was used as control.. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completion of diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. The concentration of nitrite was assayed at 540 nm and calculated with reference to the absorbance of the standard nitrite solutions.

Superoxide anion scavenging activity

The superoxide scavenging activity was determined by the slightly modified PMS-NADH superoxide generating system (13). About 1 ml of

nitro blue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4) 1 ml NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of MEIF (5µg to 80 µg) in water were mixed and treated with 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Lipid peroxidation assay

The effect of crude extract on Fe_2SO_4-H_2O_2 induced lipid peroxidation in rat liver homogenate was determined by malondialdehyde (MDA) - TBA adducts formation (14). The reaction mixture containing 0.5 ml of liver homogenate, 0.05 ml of potassium phosphate buffer (pH 7.4), 0.025 ml of 5nM $FeSO_4$, 0.025 ml of 0.3% H_2O_2 and different concentration of MEIF (50 µg-200 µg) was incubated for 1h at 37° C. After incubation, TBA (0.4% in 0.2 M HCl) and BHT (0.2% in 95% ethanol) at a ratio of 1: 2: 0.3 were added, and the mixture was heated at 90 ° C for 30 min. After cooling, 5 ml of *n*-butanol was added, and the mixture was separated by centrifugation at 1000 × g for 10 min, and MDA production was measured at 532 nm. The ability of the test extract to inhibit MDA was determined by following formula by using tetramethoxy propane as external standard.

$$\% \text{ Inhibition} = \frac{MDA \text{ in homogenate without test - MDA in homogenate with test}}{\text{MDA in homogenate without test}}$$

Hydroxyl radical assay

The assay was performed as described by Halliwell ***(***15) with minor changes. All solutions were prepared freshly and 1.0 ml of the reaction mixture contained 100 µl of 28 mM 2-deoxy-2-ribose (dissolved in KH_2PO_4-K_2HPO_4 buffer of pH 7.4), 500µl solution of various concentrations of the MEIF (10 to 80 µg), 200µl of 200µM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 100 µl H_2O_2 (1.0 mM) and 100µl ascorbic acid (1.0 mM). After an incubation period of 1 hr at 37 °C, the extent of deoxyribose degradation was measured by the TBA reaction by determination of the absorbance at about 532 nm against the blank solution. Vitamin E was used as a positive control.

Statistical Analysis

The results are presented as mean ± SEM. One way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons were used for statistical evaluation. P values less than 0.05 were considered significance

RESULTS AND DISCUSSION

MEIF at doses of 100, 200 and 300 mg/kg significantly ($p < 0.01$) inhibited the edema formation of rat paw at 3 hrs after carrageenan challenge (Table 1). Maximum inhibition (54.63 %) was obtained at the dose of 100 mg/kg after 3 hrs of drug treatment whereas the reference drug, indomethacin at a dose of 10 mg/kg markedly reduced the paw edema and produced 57.65 % of inhibition. Granuloma formation was inhibited in animals treated with MEIF at a dose of 100, 200 and 300 mg/kg significantly ($p < 0.01$) (Table 2). In this chronic model the MEIF at the does of 300 mg/kg, indomethacin and dexamethasone as standard drug decreased formation of granuloma tissue by 22.64, 29.63 % and 34.84 % respectively.

MEIF as well as ascorbic acid produced significant quenching of DPPH radical due to its increase in the ability (Table 3). Free radical scavenging activity was also increased by an increase in concentration. MEIF had strong hydrogen donating ability with an IC_{50} value of 17.4 µg/ml and the value was found to be less than the vitamin C as standard (IC_{50} value of 4.1 µg/ml). The percentage inhibition of 40 µg/ml concentration of MEIF in DPPH radical scavenging model was found as 86.7%.The scavenging of nitric oxide by plant extracts was concentration dependent as illustrated in Table 3. The IC_{50} value of MEIF was found to be 172.8µg/ml. The IC_{50} value of rutin was 161.7 µg/ml. MEIF elicited significant and concentration-dependent superoxide radical scavenging effect in PMS-NADH-NBT system (Table 3). MEIF as well as standard curcumin exhibited IC_{50} values of 37.4 µg/ml and 5.84µg/ml, respectively. MEIF inhibited the OH radical-mediated lipid peroxidation by the $FeSO_4$-H_2O_2 system in a concentration-dependent manner that was determined by the amount of MDA in liver homogenate as given in the Table 3. The percentage inhibition of MDA formation by 200 µg/ml of MEIF was found as 58.13 and 82.7%, respectively. IC_{50} were determined for MEIF 130.7µg/ml and for vitamin-E 109.4 µg/ml. MEIF demonstrated significant scavenging activity of OH^-radical generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Table 3). The IC_{50} values of MEIF and vitamin-E were 49.2 and 32.5µg/ml, respectively.

Carrageenan induced rat paw oedema is commonly used as an experimental animal model

Table1. Effect of MEIF on carrageenan-induced rat paw oedema

Treatment	Dose	% Increase in paw volume	% inhibition
Carrageenan control	-	61.89 ± 0.40	-
Indomethacin	10 mg/kg	26.27± 0.24[b]	57.55
MEIF	100 mg/kg	28.07 ± 0.21[a]	54.63
MEIF	200 mg/kg	29.68 ± 0.25[a]	52.04
MEIF	300 mg/kg	30.70 ± 0.12[a]	50.39

Each value represents the mean ± S.E.M., n = 6. [a]*P< 0.01,*[b]*p<0.05* compared with control, Dunnett's *t*-test after analysis of variance.

Table2. Effect of MEIF on cotton pellet-induced granuloma in rats

Treatment	Dose	Weight of granulation (mg)	% Inhibition
Control	-	91.01 ± 0.17	-
Dexamethasone	0.5mg/kg	62.94 ±0.19*	34.84
Indomethacin	10 mg/kg	68.59 ± 0.20*	29.63
MEIF	100 mg/kg	81.96 ± 0.20*	9.94
MEIF	200 mg/kg	74.49 ± 0.27*	18.15
MEIF	300 mg/kg	70.40± 0.24*	22.64

Each value represents the mean ± S.E.M., n = 6. **P* < 0.01 compared with control, Dunnett's *t*-test after analysis of variance.

Table3. *In vitro* antioxidant activity of *Ichnocarpus frutescens* root extracts

Extract/compound	IC_{50} Values ± SEM (µg/ml)				
	DPPH	Nitric oxide	Superoxide	Lipid peroxidation	Hydroxyl radical
Methanol extract	17.4 ± 0. 75	172.8±1.95	37.4 ± 0.34	130.7 ± 2.50	49.2 ± 0.64
Vitamin-C	4.1 ± 0.12	-	-	-	-
Rutin	-	161.7± 1.60	-	-	-
Curcumin	-	-	5.84 ± 0.24	-	-
Vitamin-E	-	-	-	109.4 ± 2.04	32.5 ± 0. 47

Data are presented as the mean ± SEM (n = 3)

for evaluation of the anti-inflammatory potential of natural products (9) and is believed to be biphasic. The initial phase is due to the release of histamine, serotonin and kinin in the first hour after the administration of carrageenan, a more pronounced second phase is attributed to release of bradykinin, prostaglandin and lysosome. The later phase is reported to be sensitive to most of the clinically effective anti-inflammatory agents (16).

The cotton pellet granuloma bioassay is considered a model for studies on chronic inflammation and considered as a typical feature of established chronic inflammatory reaction (17). MEIF exhibited significant reduction in the granuloma formation in the cotton pellet-induced granuloma in rats which means that it may be effective in chronic inflammatory conditions.

CONCLUSION

This study shows that methanol extract of roots of *Ichnocarpus frutescens* possess significant anti-inflammatory effects and antioxidant property. Phytochemical screening of this extract indicated the presence of flavonoids, terpenoids, and sterols. It may be assumed that the anti-inflammatory effects and antioxidant property of the methanolic extract of *Ichnocarpus frutescens* could be due to the presence of various flavonoids, terpenoids. These preliminary results lend to support to the use of this plant in folk medicine for inflammation, mainly because of low toxicity. Further, work is required to clarify the exact active constituents responsible for anti-inflammatory action and their mechanism of action.

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