ANTI-INFLAMMATORY ACTIVITY OF *ICHNOCARPUS FRUTESCENS*

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Summary

Leaves and roots of *Ichnocarpus frutescens* is considered to be an important drug in the indigenous system of medicine, used as a substitute of Indian Sarsaparilla. In the present study the anti-inflammatory activity of total hydroalcoholic extract (HAE) of leaves of *Ichnocarpus frutescens* were investigated in various *in vivo* (carrageenan, dextran induced paw edema, cotton pellet granuloma assay) and *in vitro* (inhibition of protein denaturation and protease activity) anti-inflammatory models. The amounts of polyphenolic compounds were also determined. HAE was analyzed and compared with reference antioxidants (α-tocopherol and BHT) for its *in vitro* antioxidative properties such as scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and hydroxyl radicals, as well as inhibition of lipid peroxidation. HAE showed dose dependent anti-inflammatory activity with maximum of 33.10 %, 30.13 % and 39.85 % in carrageenan, dextran induced paw edema and cotton pellet granuloma in rats, at a dose of 300 mg / kg body weight and the effect was comparable to the reference standard drug. *In vitro* anti-inflammatory activity of HAE was examined on the basis of inhibition of albumin denaturation and protease activity. Different concentrations of HAE (50 – 250 μg/ml) also showed ability to inhibit protease activity and denaturation of proteins. Hydroethanolic extract was found to be significantly effective in scavenging 1,1-diphenyl-2-picryl-hydrazyl (IC_{50} 194.06 μg/ml) and hydroxyl radical (163.13 μg/ml). The antioxidant activities of HAE increase with increase amount of extracts (50-250μg/ml). HAE showed different levels of inhibitory activities in various *in vitro* anti-inflammatory and antioxidant models, thereby supporting its anti-inflammatory activity. It is concluded that hydroalcoholic extract of *Ichnocarpus frutescens* may prove to be useful anti-inflammatory product in future.

Keywords: anti-inflammatory activity; albumin denaturation, protease inhibitory activity, radical scavenging activity, *Ichnocarpus frutescens*; hydroalcoholic extract. Lipid peroxidation

Introduction

Plants have yielded many widely used drugs and the current treatment of inflammatory conditions as well as infectious diseases relies heavily on natural products (1). Over 100 chemical substances that are considered to be important drugs that are either currently in use or have been widely used in the world have been derived from different plants(2). Cost of drug discovery and drug development continues to increase at astronomical rates, yet despite these expenditures; there is a decrease in the number of new medicines introduced into the world market. Despite the successes that have been achieved over the years with natural products as a platform for drug discovery has waxed and waned in popularity with various pharmaceutical companies. Natural products today are most likely going to continue to exist and grow to become even more valuable sources of new drug leads. It is believed that current NSAIDs (Non-steroidal Anti-inflammatory Drugs) are not useful in all cases, because of these side effects. As a result, a search for other alternatives seems necessary and beneficial. The study of plants that have been traditionally used for inflammation is still fruitful and logical research strategy in the source of new anti-inflammatory drugs. It is well known that reactive oxygen species (ROS) are involved in many pathological diseases. Most living species have protective systems against stress and toxic effects of ROS. Several studies have demonstrated that the antioxidant properties of plant compounds could be correlated with oxidative stress (3).

Apocynaceae, primarily a tropical family comprising about 300 genera species is considered a very natural taxon. Distribution of various flavonoids and phenolic acids in the leaves of 22 plants belonging to Apocynaceae, have been systematically studied (4). *Ichnocarpus frutescens* (Apocynaceae), commonly known as Siamlata, a medicinally important large evergreen, climbing, much branched shrub and lactiferous woody crooper with rusty red appearance and ascending up to an altitude of 4000 ft, is found almost through out India. Three species occur in India. The roots are reported to be medicinally useful and they are used in medicine as a substitute for Indian sarsaparilla and often are mixed with the later, though neither their therapeutic properties for their suitability for use as a sarsaparilla substitute have been established. Leaves are boiled in oil and applied in headaches and fevers; they are also applied to wounds. It is considered to be an important drug in the indigenous system of treatment (5).

A survey of literature revealed that different pentacyclic triterpenoids (6) and flavonoids (7). Many naturally occurring triterpinoids exhibited a good anti-inflammatory activity have been isolated from various plants (8,9). Pentacyclic triterpinoids have a wide spectrum of biological activities and some of them may be useful in medicine. There is growing interest in natural triterpinoids caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of biological activities, they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, anti-inflammatory, anti-cancer and anti-allergic (10). A review of literature did not reveal any information on the anti-inflammatory study of the plant.

Materials and Methods

Plant Materials

The fresh leaves of *Ichnocarpus frutescens (*L.) R.Br. were collected from Thiruchirappalli, India, in February 2004 and was authenticated at Botanical Survey of India (BSI), Central National Herbarium (CNH), Howrah, India (REF NO: CNH/I-I/87/2005-TECH/1326). An authentic voucher specimen was deposited in the Herbarium of Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Preparation of hydroalcoholic extracts (HAE)

The leaves were air dried under room temperature without exposure to sunlight and coarsely powdered. The dried, powdered leaves (200 g) were maceration with 70% aqueous/ethanol (500 ml) by stirring at room temperature for 7 days. The extract was filtered before drying using Whatman filter paper no. 2 and the solvent removed under vacuum, concentrated in a rotary evaporator at $35 \pm 2\,^\circ$ C under reduced pressure (SUPERFIT, India) and then lyophilized, and the resulting powder extract (yielded, 23% w/v) was used in the present study. Hydroalcoholic extract was stored at - 4° C. Extracts and reference drugs were supended in 5 % Tween 80 solution.

Drugs and Chemicals

Carrageenan, Dextran, 1,1-diphenyl-2-picryl-hydrazyl (DPPH)**,** 2-deoxy-2-ribose Thiobarbituric acid and Trypsin were obtained from Sigma Chemical Co (MO, USA). Egg albumin, α-Tocopherol, Tris-HCl,, trichloroacetic acid, ascorbic acid and casein were purchased from Sisco Research Laboratories (SRL) Pvt Ltd. Indomethacin (IMMECIN 50) was purchased from E.M. Pharmaceuticals Pvt Ltd, Mumbai, India. All other chemicals were used analytical grade.

Animals

Healthy, male albino rats (Wistar Strain) weighing from 150 – 200 g were obtained from M/S Ghosh Enterprises, Kolkata, India. The animals were housed individually in a room maintained under environmentally controlled conditions of $24 \pm 1^\circ$ C and 12 hr light – 12 hr dark cycle with free access to food and water *ad libitum* during the course of experiments. Animals were fed with standard laboratory diet (SLD) provided by Hindustan Limited, Mumbai, India. The experiments were authorized by the ethical committee for animal care of Jadavpur University, in accordance with Care Prevention Control for Experimental Animals (CPCEA), New Delhi, India.

Determination of Total Phenolics.

The total concentration of phenolics in the Hydro alcoholic extract was determined according to the method Singleton et al. (11). Briefly, 0.1 mL of extract solution (contains 500 μg of extract) was transferred to a 100 mL Erlenmeyer flask, and then the final volume was

adjusted to 46 mL by the addition of distilled water. Afterward, 1 mL of Folin-Ciocalteu reactive (FCR) (Fluka) was added into this mixture and after 3 min 3 mL of Na_2CO_3 (2%) was added. Subsequently, the mixture was shaken on a shaker for 2 h at room temperature, and then absorbance was measured at 760 nm. Pyrocatechol (Sigma) was used as the standard for the calibration curve. The estimation of phenolics in the fractions was carried out in triplicate, and the results were averaged. The phenolic compound content was determined as pyrocatechol equivalents using the following linear equation based on the calibration curve: $A = 0.0034C - 0.058$, $R2$) 0.9996. A is the absorbance, and C is pyrocatechol equivalents (μg).

Acute Toxicity study

Acute oral toxicity study was performed as per OECD – 423 guidelines (OECD 1996; acute toxic class method), albino rats (n = 6) of either sex selected by random sampling technique were used for acute toxicity study (12). The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose level 5 mg/kg body weight by gastric intubation and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2000 mg/kg body weight.

Anti-inflammatory Activity-*In vivo* Models

Carrageenan and Dextran induced paw edema in rats

Pedal inflammation in male rats (150 - 200 g) was produced according to the method of Winter et al. (13) Male rats (150 - 200 g) were divided into four groups of six animals each. An injection (s.c) was made of 0.1 ml of carrageenan (1% w/v) or 0.1 ml of Dexran (1% w/v) in to the right paw of each rat under the subplantar aponeurosis. Test groups of rats were administered orally with 150 and 300 mg/kg of the extracts 1 h before carrageenan injection. At the time, the control group received 5ml/kg of normal saline and the reference group received 10 mg/kg. The paw volume was measured by dipping the foot in the mercury bath of the plethysmograph up to the anatomical hairline on lateral malleolus and compared with control animals, which received only the vehicle. Measurement was done immediately before, first and third hour following carrageenan injection. The edema inhibitory activity was calculated according to the following formula. Percent inhibition (%) = Vc - Vt/ Vt × 100.Where Vc and Vt were mean edema volumes of control and treated groups respectively.

Cotton-pellet granuloma in rats

This study was carried out by cotton pellet implantation method in rats with a light modification of using only male rats (14). Under light ether anesthesia, sterile cotton (Bengal Surgicls Limited, Kolkata) pellets (10 mg) were implanted subcutaneously in the axilla and groin regions of the rats. The animals were treated orally with HAE extract (150, 300 mg/kg) daily for 7 consecutive days. Animals in the control group received normal saline. Indomethacin (10 mg/kg, i.p.) was given to animals in the control group. They were sacrificed on day 8, the cotton-

pellet removed, free from extraneous tissue and dried overnight at 60 º C and weighed. The percent inhibition of dry weight of the granuloma were calculated and compared.

Anti-inflammatory Activity-*In vitro* Models

Inhibition of protein denaturation

Test solution (1ml) containing different concentration (50 - 250 µg/ml) of drug was mixed with 1ml of egg albumin solution (1mM) and incubated at $27 \pm 1°$ C for 15 min. denaturation was induced by keeping the reaction mixture at 70 ° C in a water bath for 10 min. after cooling the turbidity was measured spectrophotometrically at 660 nm (15,16). Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and taken the average.

Protease inhibitory activity

The reaction mixtures (2.0 ml) contained 0.06 mg trypsin, 1.0 ml of 25 mM tris-HCl buffer (pH 7.4) and 1.0 ml of aqueous solution of HAE (50 - 250 µg/ml). The mixtures were incubated at 37 ° C for 5 minutes. Then 1.0 ml of 0.8 % (w/v) casein was added. The mixtures were incubated for an additional 20 minutes. 2.0 ml of 70 % perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged. Absorbance of the supernatant was read at 280 nm against buffer as blank (17). The percentage of inhibition as calculated. Each experiment was done in triplicate and taken the average.

Scavenging activity against DPPH radical

Scavenging activity on DPPH (1,1-diphenyl-2-picryl-hydrazyl radicals) of HAE was measured according to the method reported by Blois et al. (18). Each sample stock solution was diluted to final concentrations of 250, 200, 150, 100, and 50 µg/ml, and 0.2 ml of methanol and 0.3 ml of various concentrations of the samples in methanol were mixed in a 10 ml test tube. To this was added 2.5 ml of 75 µM DPPH (1,1-diphenyl-2-picryl-hydrazyl) in methanol to achieve a final volume of 3 ml. The solution was kept at room temperature for 90 min, and the absorbance at 517 nm was measured. α -Tocopherol was used as a reference compound. The DPPH (1, 1-diphenyl-2-picryl-hydrazyl) scavenging effect and IC_{50} were calculated using linear regression method.

Inhibition of hydroxyl radical

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds (MEGP) for hydroxyl radical generated by Fe^{3+}-Ascorbate–EDTA–H_2O_2 system (Fenton reaction) according to the method of Kunchandy and Rao (19). The reaction mixture contained in a final volume of 1.0 ml, 100 µl of 2-deoxy-2-ribose (28mM in KH_2PO_4-KOH buffer, 20mM, pH 7.4), 500µl of the various concentrations of MEGP (50 - 250 µg) in KH_2PO_4-KOH buffer (20mM, pH 7.4), 200µl of 1.04mM EDTA and 200mcM $FeCl_3$ (1:1 v/v), 100µl of 1.0mM H_2O_2 and 100µl of 1.0mM ascorbic acid was incubated at 37^0C for 1h.

1.0ml of thiobarbituric acid (1%) and 1.0ml of trichloroacetic acid (2.8%) were added to the test tubes and were incubated at 100^0C for 20min. After cooling, absorbance was measured at 532nm against control containing deoxyribose and buffer. α - Tocopherol was used as a positive control. Reactions were carried out in triplicate. The percentage inhibition was determined by comparing the results of the test and control compounds.

Lipid Peroxidation by Thiobarbituric Acid (TBA) Assay.

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (20). Normal albino rats of the Wister strain were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared using a Potter Elvehjem homogenizer at 0-4 °C with 0.15 M KCl. The homogenate was centrifuged at 800g for 15 min, and clear cell-free supernatant was used for the study of in vitro lipid peroxidation. Different concentrations (10, 50, 100, 150, 200, and 250 µg/ml) of HAE (dissolved in Double distilled water) were taken in various test tubes. One milliliter of 0.15 M KCl and 0.5 mL of rat liver homogenates were added to the test tubes. Peroxidation was initiated by adding 100 µL of 0.2 mM ferric chloride. After incubation at 37 °C for 30 min, the reaction was stopped by adding 2 mL of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA, and 0.5% BHT. The reaction mixtures were heated at 80 °C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. An identical experiment was performed to determine the amount of lipid peroxidation obtained in the presence of inducing agents without any extract. The percentage inhibition of lipid peroxidation is calculated by the following formula, Inhibition of lipid peroxidation (%) = 1 — (sample OD/blank OD) × 100.

 Statistical analysis

The experimental data were expressed as mean ± SEM. the significance of difference among the various treated groups and control group were analyzed by means of one-way ANNOVA followed by Dunnett's multiple comparison test using Graphat Instat Software (San Diego, CA, USA). The level of significance was set at $p < 0.05$. IC$_{50}$ (inhibitor concentration which produce 50 % inhibition) were estimated using linear regression method of plots of the percent of antiradical activity against the concentration of the tested compounds using Microsoft Excel Software Programme.

Results

The hydroalcoholic extract of *Ichnocarpus frutescens* did not cause any mortality up to 2000 mg/kg and were considered as safe (OECD, 1996). Total phenolic contents compounds of hydroalhocolic extract were expressed as mg of pyrocatechol equivalent pergram of dry weight. 1000 µg of hydroalholic extract were used to determine the amount of total polyphenolic contents. The level of total polyphenolic compounds was 100.51mg of pyrocatechol equivalent per gram of hydroalholic extract.

The *in vivo* anti-inflammatory effects of the HAE were assayed by using carrageenan, dextran induced paw edema and cotton pellet granuloma. The administration of subplantar injection of carrageenan (0.1 ml, 1% w/v), dextran (0.1 ml, 1% w/v) produced significant edema in the rat paws, reaching maximum at 3 h (Table 1 and 2). Oral administration of HAE caused dose related inhibition of carrageenan and dextran induced inflammation. The extract with dose of 150 mg/kg induced significant (p< 0.05) anti-inflammatory activity at 3 h after carrageenan administration. The dose of 300 mg/kg abolished inflammation more significantly (p <0.01) at 3 h and it was comparable to the untreated control group. Where as in the dextran induced paw edema both doses of HAE produced significant anti-inflammatory activity at 3 h after dextran injection. A standard drug Indomethacin (10 mg/kg) showed potent inhibition in both the models. Carrageenan treatment after 3 h, the highest dose of HAE, inhibited edema by 33.10 % and 30.13 %, in carrageenan and dextran induced paw edema, respectively.

Table 1.Effect of hydroalcoholic extract of *I. frutescens* on carrageenan induced paw edema in rats

Treatment	Dose (mg/kg)	Paw volume (ml)			Edema inhibition (%)
		Initial	1 h	3 h	
Normal saline	3 ml/kg	3.13 ± 0.19	3.85 ± 0.18	4.38 ± 0.20	----
HAE	150	3.11 ± 0.18	3.65 ± 0.18	3.38 ± 0.22*	22.38
HAE	300	3.16 ± 0.15	3.38 ± 0.21	2.93 ± 0.23**	33.10
Indomethacin	10	3.21± 0.24	3.40 ± 0.19	2.73 ± 0.24**	37.67

HAE – Hydroalcoholic extract; Data represents mean ± S.E.M. (n=6). * $p < 0.05$, ** $p < 0.01$ significant as compared to control group. One way ANOVA using Dunnett's t- test.

Table 2. Effect of hydroalcoholic extract of *I. frutescens* on Dextran induced paw edema in rats

Treatment (p.o)	Dose (mg/kg)	Paw volume (ml)			Edema inhibition (%)
		Initial	1 h	3 h	
Normal saline	3 ml / kg	2.61 ± 0.19	3.20 ± 0.19	3.56 ± 0.14	----
HAE	150	2.65 ± 0.17	3.01 ± 0.14	2.95 ± 0.13*	17.13
HAE	300	2.73 ± 0.15	2.98 ± 0.15	2.48 ± 0.16**	30.13
Indomethacin	10	2.70 ± 0.16	2.98 ± 0.17	2.33 ± 0.20**	34.55

HAE – Hydroalcoholic extract; Data represents mean ± S.E.M (n=6).* $p < 0.05$, ** $p < 0.01$ significant as compared to control group, One way ANOVA using Dunnett's t- test.

Table 3 shows the effect of HAE on cotton pellet- induced granuloma formation in rats. The results of the cotton pellet granuloma model indicated that HAE, when administered orally in dose of 150 mg/kg and 300 mg/kg showed 19.97 % and 39.85 % reduction of the granuloma tissue formation, respectively, where as 49.36 % inhibition of granuloma was shown by standard drug Indomethacin (10 mg/kg) for 7 days.

Table 3. Effect of hydroalcoholic extract of *I. frutescens* on cotton pellet granuloma in rats

Treatment (p.o)	Dose	Weight of the granuloma (mg)	Percentage inhibition (%)
Normal saline	3 ml / kg	38.67 ± 1.21	--
HAE	150 mg / kg	27.08 ± 0.91 **	29.97
HAE	300 mg / kg	23.26 ± 0.84 **	39.85
Indomethacin	10 mg / kg	19.91 ± 0.69 **	49.36

HAE – Hydroalcoholic extract; Data represents mean $\pm$ S.E.M. (n=6). ** $p < 0.01$, significant as compared to control group. One way ANOVA using Dunnett's t- test.

Table 4. Effect of hydroalcoholic extract of *I.frutescens* on *in vitro* assays

Drug (HAE) (μg /ml)	*In vitro* assay of HAE	
	Inhibition of Preotein denaturation (%)	Protease inhibition (%)
50	22.02 ± 0.81	17.46 ± 1.59
100	28.59 ± 0.84	28.59 ± 0.91
150	35.29 ± 0.66	35.88 ± 1.92
200	52.80 ± 0.93	48.83 ± 1.77
250	62.86 ± 0.86	53.26 ± 1.12

The effect of hydroalholic extract of *Ichnocarpus frutescens* on ascorbate induced lipid peroxidation of rat liver was investigated. As shown in figure. 3, the hydroalcoholic extract exhibited a dose dependent inhibitory activity on ascorbate induced lipid peroxidation. The effect of hydroalcolic extract is comparable to that of butylated hydroxyl toluene (BHT), which produced an inhibition of 82.40 %.
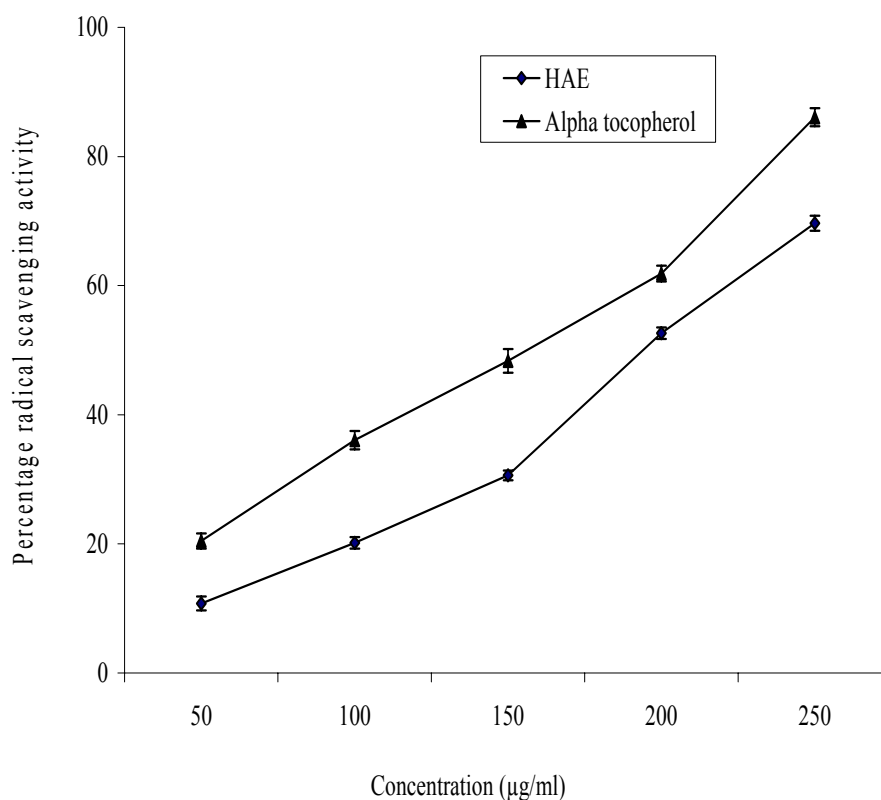
Fig.1. DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity of hydroalcoholic extract of Ichnocarpus frutescens. HAE- Hydroalcoholic extract.Each values represents the mean ± SEM of triplicate experiments .

The free radical scavenging activity of the HAE and α-Tocopherol was observed in the presence of 1,1-diphenyl-2-picryl-hydrazyl radical and hydroxyl radicals (Fig.1 and 2). Reduction of both free radicals can be observed by the decrease in absorbance at 517 and 532 nm, respectively. The 1,1-diphenyl-2-picryl-hydrazyl radical hydroxyl free radical scavenging capacity of the HAE was found to be with the IC_{50} being 194.06 μg/ml and 123.93μg/ml respectively, with respect to reference compound α-tocopherol (147.91μg/ml and 31.03 μg/ml), used as positive control. The 1,1-diphenyl-2-picryl-hydrazyl and hydroxyl free radical scavenging activity of hydroalcoholic extract was shown to be strongly concentration dependent.
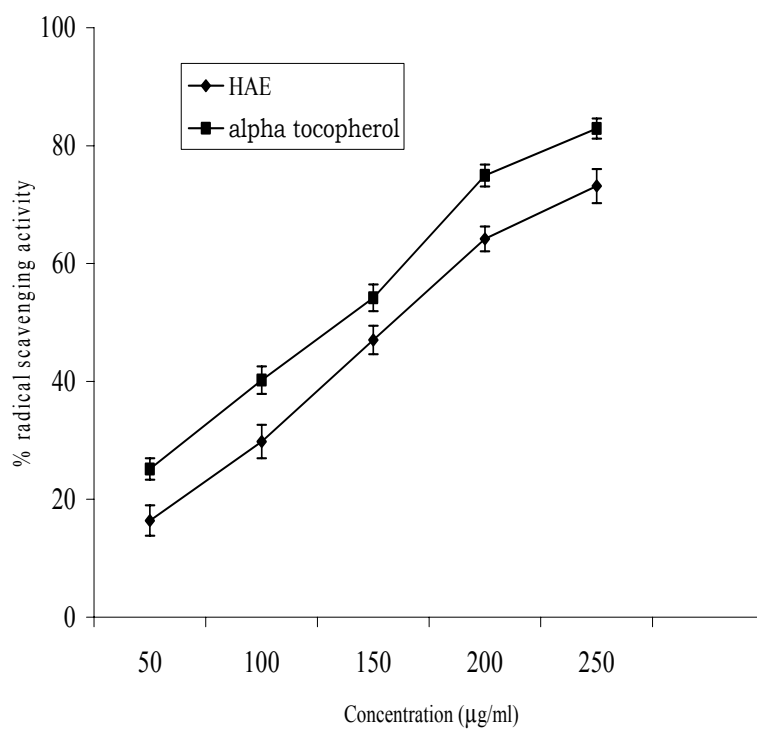
Fig.2. Hydroxyl radical scavenging activity of hydroalcoholic extract of *Ichnocarpus frutescens* and alpha tocopherol . Each values represents the mean ± SEM   of three experiments. HAE - Hydroalcoholic extract.

Table 5. Antioxidant activities of hydroalcolholic extract (HAE) and α –tocopherol as expressed (µg/ml) by inhibitory concentration (IC$_{50}$).

Antioxidant reaction	Antioxidant	IC$_{50}$ (µg/ml)
DPPH radical	HAE	194.06 (µg/ml)
	α –tocopherol	147.91 (µg/ml)
Hydroxyl radical	HAE	163.13 (µg/ml)
	α –tocopherol	132.03 (µg/ml)

The inhibitory effect of different concentrations of HAE on protein denaturation and protease inhibition was concentration dependent are shown in Table 4. HAE at different dose levels (50 - 250 µg/ml) showed higher ability to inhibit denaturation of egg albumin. It also exhibited significant antiprotease activity.
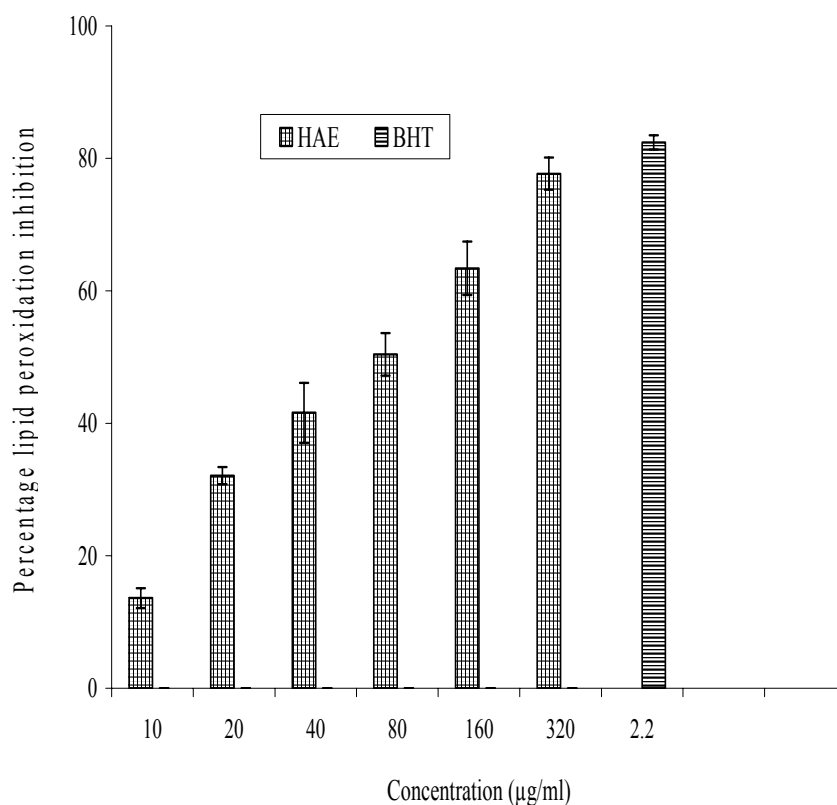

Fig. 3. Inhibitory effect of hydroalcoholic extract of *Ichnocarpus frutescens* on lipid peroxidation in rat liver homogenate. HAE - Hydroalcoholic extract (5-320 µg/ml); BHT- Butylated hydroxy toluene (2.20 µg/ml). Each value represents the mean ± SEM of triplicate experiments.

Discussion

The present study established the anti-inflammatory activity of the hydroalcoholic extract of *Ichnocarpus frutescens*. carrageenan induced acute inflammation in animals is one of the most suitable test procedure to screen anti-inflammatory agents. The irritant effect of carrageenan is a result of activation of the kinin and complement cascades and consequently of the release of anti-inflammatory mediators such as vasoactive amines (Histamine, 5-Hydroxytryptamine and

Bradykinins), eicosanoids (21, 22). The extract showed dose dependent inhibitory activity over a period of 3 hr and extract showed maximum inhibition of inhibition of carrageenan induced paw edema at the end of 3 h (Table 1). This indicates, HAE capable of attenuating both early and delayed phases of carrageenan induced inflammation.

With regard to dextran induced paw edema, it has been reported mainly by histamine and 5-HT released by the mast cells (23). Taken together it suggests that as HAE possess potent acute anti-inflammatory activity, it is mediated by possibly due to the inhibition of release of inflammatory mediators. In case of cotton pellet induced granuloma, there was significant reduction in granular tissue formation. Indomethacin was significantly decreased the dry weight of the cotton-pellets correlates well with the reduction of granuloma developed during a period of 7 days (24). This showed the ability of the HAE in reducing the synthesis of proteins, collagen and infiltration of macrophages.

Many studies have revealed that the leaves of *I. frutescens* have high contents of triterpinoids, flavonoids and phenolic acids (25). Naturally occurring triterpinoids often exhibit a variety of biological activities such as anti-inflammatory (26,27) anti-tumour activity (28), hepatoprotective activity (29), and anti-mycobacterial (30). Most of the anti-inflammtory triterpenes isolated have lupane, oleanane, ursane and taraxastane. Lupanes are widespread in the families of Apocynaceae, Euphorbiaceae and Labiateae (31). Triterpinoids, the rich ingredients of *I. frutescens*, are well known for their physiological anti-inflammation (32), while among lupeol and ursolic acid showed significant anti-inflammatory activity in various models (33,34). Ursolic acid is potent anti-inflammatory agent and it has been recommended for use in burn ointments (35). Lupeol has been reported to possess dose dependent suppression of PGE_2 without any effect of LTC_4 release. Thus, ursolic acid and lupeole (36) were able to prevent the production of some inflammatory mediators which likely contributed to *in vivo* anti-inflammatory effect of *I. frutescens*.

Denaturation of proteins is well documented cause of inflammation and rheumatoid arthritis (37). Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation (38). Ability of HAE to bring down thermal denaturation of protein is possibly a contributing factor with the mechanism of action.

Enzymes and proteins play an essential role in inflammation and other functions of the immune system. Proteolytic enzymes, such as bromelain, papain, trypsin and chymotrypsin are essential regulators and modulators of the inflammatory response (39). Compelling evidence that has accumulate in recent years also indicates that certain proteases such as thrombin, tryptase and, tryspsin, can signal to cells through the activation of protease activated receptors (PAR family). Trypsin has been shown to induce *in vivo* epidermal proliferation, vasodilatation and inflammatory infiltration in the upper epidermis by the activation of PAR_2 family. PAR_2 activation promotes the first signals leukocyte rolling, adhesion and translocation across the wall of blood vessel (40, 41). The expression of PAR_2 on endothelial cells and inflammatory cells including neutrophils and macrophages, determines the involvement of PAR_2 in both pro-inflammatory and anti-inflammatory responses of different experimental models of inflammation (42). An earlier report indicates lupeol to be a competitive inhibitor of trypsin and chymotrypsin (43,44) .

Constituents from the major pentacyclic triterpenoid classes have been shown to inhibit trypsin activity (45). HAE exhibited significant antiprotease activity; it may be due to the triterpinoids constituents. This finding justifies the usefulness of the HAE in the treatment of inflammation associated diseases like arthritis.

To investigate if the anti-inflammatory effect could be also related to antioxidant activity, the HAE was evaluated by the DPPH° test. This particular plant is said to contain high phenolic acids and flavonoids as the other major constituents. It seems that the phenolic acids that are the active principles of some orally administered medicinal plants might merely act synergistically, without other active substances, for eg., sinapic acid, syringic acid, vanillic acid and protocatechuic acid, isolated from *I. frutescens* might be other active compound involved in this action. The anti-inflammatory activity of these compounds might also because of their behavior as free radical scavengers (46, 47).

The presence of wide variety of flavonoids in the leaves (flavones, and flavonol) of *Ichnocarpus frutescens* has been reported earlier. Flavonoids comprise a large group of secondary metabolites have been shown to possess various biological properties related to antioxidant mechanism (48-51). The most widely acknowledged behavior of antioxidants is the interaction with oxidative free radicals. There is increasing interest in antioxidants such as flavonoids, particularly in those intended to prevent the presumed deleterious effect of free radicals in the human body and prevent the inhibition of lipid peroxidation. The antioxidant effect of flavonoids, can be reside both in their radical scavenging activity or in their metal chelating properties, of which the former may dominate. Some flavonoids included in this plant showed free radical scavenging activity (52). Dose dependent interaction of HAE with 1,1-diphenyl-2-picryl-hydrazyl radical and hydroxyl radicals establishes the capability of the constituents to scavenge the free radicals. Thus, it indicates that some of the therapeutic constituents of leaves of *Ichnocarpus frutescens* may be due t its free radical scavenging property. Free radical scavenging property and total phenolic contents of hydroalcoholic extract can also be accepted an indication of antioxidant potential (53).

Lipid peroxidation is complex and natural deleterious process. The peroxidation of membrane lipids initiated by oxygen radicals may be lead to cell injury. HAE was found to significantly exhibith the lipid peroxidation induced non enzymatically iin the rat liver homogenate. The presnt results clearly exhibit the dose depent protective reponse afforded by the HAE against Fe^{2+}-ascorbate induced lipid peroxiation.

Therefore, the anti-inflammatory activity of hydroalcoholic extract of *I. frutescens* seems to be due to the high flavonoids and triterpinoids constituents, whose effectiveness as free radical scavengers and anti-inflammatory agents, respectively is well known. Both these active groups can be considered of therapeutic relevance of *I. frutescens* and the combination of these properties could help to support the usefulness of the plant in the treatment of inflammation related diseases. Further studies are needed to evaluate the *in vivo* potential of *Ichnocarpus frutescens* in various animal models and to analyze the synergism among the constituents.

Acknowledgement

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