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Induction of Carrageenan Paw Oedema with Anti-Inflammatory Serum Enzymes of *Diospyros Ferrea* Leaf Extract

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ABSTRACT

The present study was employed to evaluate the probable anti-inflammatory activity of methanolic leaf extract of *Diospyros ferrea* (willd.) Bakh, using Carrageenan (0.1 ml of 0.1%) induced inflammation and with serum acid phosphatase and alkaline phosphatase enzymes. The paw volume in experimental rats increased steadily for 2 hours after carrageenan administration and Indomethacin (0.5mg/kg bw), the standard drug significantly reduced paw volume. The methanolic leaf extracts at 200mg & 400mg/kg bw (1.631 ± 0.248 & 1.390 ± 0.226) also exhibited similar effect on paw oedema comparable with Indomethacin (1.805 ± 0.139 & 1.690 ± 0.168). The 400mg/kg extract exhibited significantly greater activity than it was noticed with 200mg/kg leaf extract at all tested intervals when compared with control. The two enzymes, acid phosphatase and alkaline phosphatase (2.189 ± 0.149 & 1.843 ± 0.139) present in elevated levels in inflammation-induced rats were significantly brought down by Indomethacin drug and leaf extracts respectively. Hence, the methanolic leaf extracts of *D.ferrea* plant is responsible for reduced inflammation and show similarity in action with drug Indomethacin.

KEY WORDS: Anti-inflammatory, Carrageenan, Indomethacin, Paw oedema, serum enzymes.

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INTRODUCTION

Inflammation is a protective biological response of living tissues to injury caused by the physical trauma, noxious chemicals and microbial agents. It is mediated by an array of enzymes and processes involving in cell migration, tissue break down and repair. Despite of significant studies made in medical research, the inflammatory diseases remain worlds major problem. The NSAIDs (non-steroidal anti- inflammatory drugs) that are being prescribed to treat inflammatory diseases that are too costly and show adverse side effects. So inflammation is a crucial biological response of vascular tissues to harmful stimuli and initiate the healing process. Vasoactive amines, Eicosanoids, Cytokines, Growth factors Reactive oxygen species (ROS) and hydrolytic enzymes are involved.

Therefore, screening of plants for their anti-inflammatory activity and development of plant based drugs are the two main thrust areas for research investigators in recent times. In the present study the effect of *D.ferrea* leaf extract against Carrageenan induced paw oedema is evaluated and it was compared with the activity of anti- inflammatory drug Indomethacin. Neutrophils, monocytes, macrophages, lymphocytes, plasma cells and fibroblasts cells are involved in protecting defence systems, an uncontrolled and persistent inflammation can be controlled by anti-inflammatory compounds. So phytochemical compounds like phytoI (Phenols) was actively involved in the process of immunity Jensen⁷ *et al.*

MATERIAL AND METHODS:

Induction of Carrageenan paw oedema and evaluation of Anti-Inflammatory effect of *D.ferrea* methanolic leaf extract

a) *Animals and drugs*

Male albino rats of wistar strain weighing about 150-250g were used in the study. The animals were housed in group of 6 rats per cage and maintained under standard laboratory conditions at $24 \pm 2^\circ\text{C}$ in light controlled room (12 hrs dark and 12 hrs night) and provided commercial pellet diet. All the experimental protocols used for this study were reviewed by the Institutional Animal Ethics Committee (CPCSEA/2015/ARTI18) and were in accordance with the guidelines of the CPCSEA. The experiments were carried out at Albino Research Lab, Hyderabad.

Carrageenan was procured from SD-Fine Chem. Ltd., India and standard drug Indomethacin was procured from Cipla Ltd. Diagnostic kits used in this study were procured from Span Diagnostics Ltd., India. All the other chemicals used were of analytical grade. Paw oedema was induced by injecting 0.1ml of 0.1% carrageenan in physiological saline into the sub plantar tissues of the right hind paw of each rat in all groups. Control group received Indomethacin (0.5 mg/kg) by oral route as standard drug.

b) Acute toxicity studies

Acute oral toxicity study was performed as per Organization for Economic Cooperation and Development (OECD) guidelines Stepwise dose of methanolic leaf extract of *Diospyros ferrea* (50 mg/kg-2000 mg/kg b.w) were administered. Animals were observed individually during the first 30 minutes and periodically during the first 24 hrs with special attention given during the first 4 hours and daily thereafter, for total of 14 days .The dose 2000 mg/kg was found to be safe since no toxicity was observed. There were no toxic effects of mortality observed up to 14 days.The LD50 cut off value found to be 2000 mg/kg. for evaluation of anti-inflammatory activity two dose levels were selected i.e., first dose is one-tenth of LD50 cut off value and second dose was twice that off one-tenth dose (200 mg/kg and 400 mg/kg. b.w)

c) Carrageenan induced paw oedema

The anti-inflammatory activity of methanolic leaf extract was evaluated in wistar rats by employing the standard method Winter¹ et al. All animals were fasted over night and were divided into control, standard and different test groups consisting of six animals each. The different extracts were administered to the animals in the test groups at a dose of 200 and 400 mg/kg. by oral route. Control group animals were received 1% Dimethyl Sulphoxide at the dose of 10 ml/kg body weight. Standard group received Indomethacin (0.5 mg/kg) by oral route. Thirty minutes after administration of the respective drugs a mark was made on the right hind paw just below the tibiotarsal junction. Paw oedema was induced by injecting 0.1 ml of 0.1% carrageenan in physiological saline into the sub planter tissue of the right hind paw of each rat in all groups. The paw volume was measured at intervals of 60, 120, 240 and 360 minuts by the mercury displacement method using a Plethysmograph Ghosh⁵ and Vogel⁴. Reduction in the paw volume compared with negative control, untreated animals considered as the test dose showing effective anti-inflammatory activity.

d) Experimental design

Group	Treatment
Group-II	Carrageenan (0.1ml/0.1%) + DMSO (1%)
Group-III	Carrageenan + Indomethacin (0.5 mg/kg) b.w.
Group-IV	Carrageenan + methanolic leaf extract (200 mg/kg) b.w
Group-V	Carrageenan + methanolic leaf extract (400 mg/kg) b.w

Treatment groups [n=6]

Percentage inhibition of oedema was calculated by the following formula

$$\text{Percentage inhibition of oedema} = \frac{V_c - V_t}{V_c} \times 100$$

V_c = Increase in paw volume in control group of animals

V_t =Increase in paw volume in drug/plant extract-treated animals

e) Biochemical estimation of acid phosphatase and alkaline phosphatase enzyme

Biochemical estimation is carried out after 4th hour in Carrageenan induced paw oedema acute study model. The animals were anaesthetized under light anaesthesia and blood samples were collected by retro orbital plexus puncture. Serum was separated after coagulating the blood at 37°C and centrifuged at 1200-1500 rpm for 15-20 minutes. Serum was analysed for the enzymes acid phosphatase and alkaline phosphatase.

1. Estimation of acid phosphatase

Principle

Acid Phosphatase catalyzes the hydrolysis of alpha-naphthylphosphate, liberating the alpha-naphthol and phosphate. The alpha-naphthol is then coupled with diazotized 2-amino-5-chlorotoluene (Fast Red TR) to form diazo dye which has a strong absorbance at 405 nm. The increase in absorbance is directly proportional to the level of ACP in the sample. The diazo dye is measured bichromatically at 412 nm.

Reagents

Citrate buffer	: 0.1 M pH 4.8
Substrate	: 0.01 M phenyl phosphate disodium salt
Sodium carbonate salt	: 15% Magnesium chloride; 0.1M
Folin-Ciocalteu reagent	: Diluted with distilled water [1:2]
Standard phenyl solution	: Phenol was dissolved in water to give a concentration of 100 mg/ml

Procedure

The incubation mixture consisted of 1.5ml of buffer, 0.1ml of substrate solution, 0.1ml of magnesium chloride solution, 0.1ml of water and 0.1ml of homogenate. The incubation was carried out at 37°C for 20 minutes. The reaction was arrested by the addition of 1.0 ml of folin-ciocalteu reagent. The suspension was centrifuged and 2.0 ml of 15% sodium carbonate solution was added to an aliquot of the supernatant. The solution was incubated at 37°C for 10 minutes. The standard phenol solution was also treated similarly with folin-ciocalteu reagent and alkali. The intensity of the blue colour developed was read at 640 nm in a UV-spectrophotometer.

The enzyme activity was expressed as IU/L of serum.

2. Estimation of alkaline phosphatase

Plasma alkaline phosphatase was estimated by using the diagnostic kit based on Kind P R N and King E J's³ method. ALP catalyses disodium phenyl phosphate into phenol and disodium hydrogen phosphate at a pH 10. Phenol so formed reacts with 4-aminoantipyrine in alkaline medium in the presence of oxidizing agent potassium ferricyanide to form a red coloured complex whose absorbance is proportional to the enzyme activity.

Reagents

Buffered substrate: 0.01M Disodium phenyl phosphate dissolved in carbonate- bicarbonate buffer (0.1 M, pH 10)

Colour reagent: 4-aminoantipyrine, sodium hydroxide and potassium ferricyanide Phenol standard: 10 mg/ml

Procedure

The incubation mixture contained 1.0ml of buffered substrate, 3.1ml of deionised water and 0.1ml of serum was incubated at 37°C, after 15 min, 2.0ml of colour reagent was added to all the tubes. Enzyme is added to the control tubes after the addition of colour reagent. 0.1ml of standard and 0.1ml of distilled water (blank) were also treated simultaneously and the colour developed was read at 510 nm. The enzyme activity was expressed as IU/L of serum.

Statistical analysis

All the values are expressed as Mean \pm S.E.M. The data was analyzed for ANOVA and post hoc Dennett's t- test. The results were considered statistically significant when <0.05 . The statistical analysis was carried out using Graph pad instat 3.0 software.

RESULTS:

The NSAIDs (Non-steroidal Anti-inflammatory Drugs) that are being prescribed to treat inflammatory diseases, are too costly and show adverse side effects. Therefore, screening of plants for their anti-inflammatory activity and development of plant based drugs are the two main thrust areas for research investigators in recent times. In the present study, the effect of *D.ferrea* leaf extracts against carrageenan induced paw oedema is evaluated and it was compared with the activity of anti-inflammatory drug Indomethacin.

The paw volume (ml) in experimental rats immediately after the administration of carrageenan was recorded and was used to compare with paw oedema recorded after 1 hour, 2 hours, 4 hours and 6 hours time. The paw oedema increased steadily and significantly upto 2 hrs

duration and there after decreased (Table-1 and Figure-1). Indomethacin treatment reduced the carrageenan induced paw volume and the decrease is statistically significant (Table-1). The methanolic leaf extracts of *D.ferrea* also exhibited reduced effect on paw volume similar to Indomethacin (Table-1). The activity of 400mg/kg leaf extract was relatively greater than the 200mg/kg treatment. The methanolic leaf extract of *D.ferrea* showed significant anti-inflammatory effect.

The amount of two enzymes i.e., acid phosphatase and alkaline phosphatase was recorded in inflammation induced rats, treated with Indomethacin and *D.ferrea* leaf extract (Table-2 & Fig-2). In inflammation induced rats the two enzyme levels are elevated significantly. The treatments with Indomethacin and *D.ferrea* leaf extracts brought down the enzyme level in experimental rats. The effect of 400mg/kg extract was equal when compared with the activity of Indomethacin. Moreover, the methanolic leaf extracts exhibited dose dependent effect on anti-inflammatory enzymes.

DISCUSSION:

The currently used non-steroidal anti-inflammatory drugs (NSAIDs) cause several adverse side effects and toxicity. The greatest disadvantage of (NSAIDs) lies in reappearance of inflammatory disorders after the discontinuation of drugs. Hence, as an alternate measure indigenous medicinal plants are being screened by many investigators to develop anti-inflammatory drugs. In this context, the statement of Durmowicz and Stenmak⁶ gains prominence that a plant contains a multitude of different molecules that affect complex cellular pathways by acting synergistically. Therefore plants have become the best source of wide variety of active compounds extensively used as crude extracts for treating various diseases.

In the present study, the anti-inflammatory activity of methanolic leaf extracts (200mg & 400mg/kg b.w) of *D.ferrea* has been evaluated and compared with the activity of Indomethacin a standard anti-inflammatory drug. Anti-inflammatory activity can be studied through varied experimental models. Of the several models used, carrageenan induced paw oedema appears to be the most commonly employed method to evaluate inflammation Biren and Nayak². The present study results project *D.ferrea* as an important medicinal species in the *Ebenaceae* family with great pharmacological potential.

Table 1. Effect of *D.ferrea* m ethanolic leaf extract on Carrageenan induced Paw oedema (200mg & 400mg/kg) Indomethacin treated rats

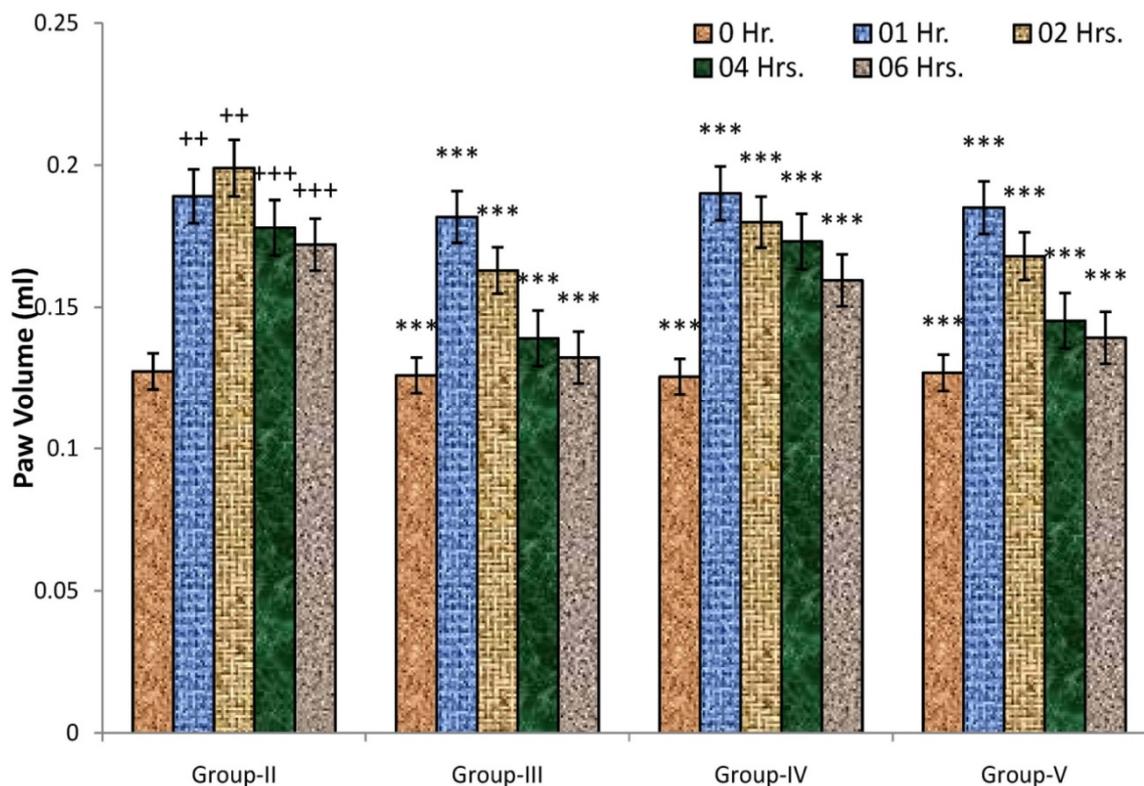
Group-I	Treatment	Paw Volume[ml]				
		0 Hr.	01 Hr.	02 Hrs.	04 Hrs.	06 Hrs.
Group-II	Carrageenan induced paw	0.1275 ± 0.001	0.1891 ± 0.002 ⁺⁺⁺	0.1990 ± 0.001 ⁺⁺⁺	0.1780 ± 0.002 ⁺⁺⁺	0.1721 ± 0.001 ⁺⁺⁺
Group-III	Indomethacin (0.5mg/kg)	0.1261 ± 0.000	0.1818 ± 0.001 ⁺⁺⁺	0.1630 ± 0.001 ⁺⁺⁺	0.1391 ± 0.002 ⁺⁺⁺	0.1324 ± 0.001 ⁺⁺⁺
Group-IV	Methanolic leaf extract (200mg/kg)	0.1256 ± 0.000	0.1901 ± 0.000 ⁺⁺⁺	0.1800 ± 0.001 ⁺⁺⁺	0.1732 ± 0.001 ⁺⁺⁺	0.1595 ± 0.001 ⁺⁺⁺
Group-V	Methanolic leaf extract (400mg/kg)	0.127 ± 0.001	0.1851 ± 0.001 ⁺⁺⁺	0.1680 ± 0.001 ⁺⁺⁺	0.1453 ± 0.001 ⁺⁺⁺	0.1393 ± 0.000 ⁺⁺⁺

All values were expressed as Mean ± SE. (n =6)

⁺⁺⁺ means p<0.001, ⁺⁺ means p<0.01, ⁺ means p<0.05 when compared with Normal Control.

^{***} means p<0.001, ^{**} means p<0.01, ^{*} means p<0.05 when compared with Inflammatory contro

Figure 1. Effect of methanolic leaf extracts (200 mg & 400 mg/kg) on Carrageenan induced Paw oedema



Group - I: Normal Control

Group – II: STZ (60mg /kg b.w) induced diabetic rats

Group – III: Glebenclamide (0.5mg/kg b.w) treated rats

Group – IV: Methanolic leaf extract (200mg/kg)

Group - V: Methanolic leaf extract (400mg/kg)

Table 2. Effect of *D.ferrea* methanolic leaf extracts on Acid Phosphatase and Alkaline Phosphatase levels in inflammation (200mg & 400mg/kg) Indomethacin induces rats.

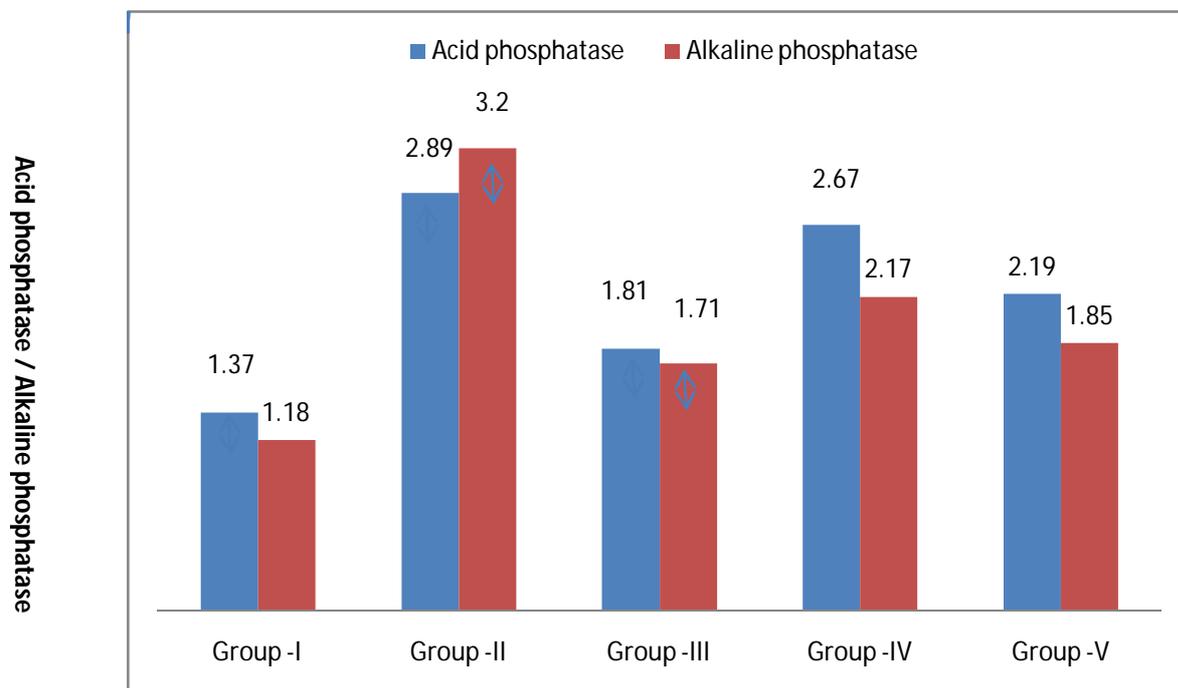
Group	Treatment	Acid Phosphatase	Alkaline Phosphatase
Group I	Normal	1.373±0.222	1.181±0.175
Group II	Carrageenan Induced	2.985±0.066**	3.260±0.470**
Group III	Indomethacin (0.5mg/kg) bw	1.855±0.107	1.720±0.174*
Group IV	Methanolic leaf extract (200mg/kg) bw	2.725±0.148**	2.170±0.331**
Group V	Methanolic leaf extract (400mg/kg) bw	2.195±0.155*	1.863±0.143*

The values expressed as mean + SE (n=6)

Indomethacin treated group, extract treated were compared with the carrageenan treated control (Inflammatory Control)

*** P<0.001 Statistically highly significant, ** P<0.01 very significant * P<0.05 Significant

Figure 2. Effect of methanolic leaf extract on Acid Phosphatase and Alkaline Phosphatase levels in inflammation induced rats.



Group - I: Normal Control

Group – II : STZ (60mg /kg b.w) induced diabetic rats

Group – III: Glebenclamide (0.5mg/kg b.w) treated rats

Group – IV: Methanolic leaf extract (200mg/kg)

Group - V: Methanolic leaf extract (400mg/kg)

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