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RESEARCH ARTICLE

Evaluation of Anti-inflammatory Activity of *Cayratia pedata (Lam.)* Juss. Leaf Extract in Some *In vivo and In vitro* Experimental Models

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ABSTRACT

Cayratia pedata (Lam.) Juss. Family: *Vitaceae*, finds many uses in folklore medicine including in the treatment of scabies, wounds, boils, skin ailments, diarrhoea, fever and inflammatory conditions. It is also used as an emetic, anthelmintic, astringent and rubefacient. The anti-inflammatory activity of the ethanol extract of leaves of *C. pedata* was evaluated using cotton pellet granuloma and granuloma pouch models in rats. An attempt to evaluate the possible mechanisms of action was also done on various in vitro models. It was shown that treatment with the *C. pedata* extract (250 and 500 mg/kg body weight, p.o.) significantly reduced the formation of granuloma and reduced the severity of inflammatory process in both the animal models. It is found that *C. pedata* leaf extract inhibits total cyclooxygenase in lipo-polysaccharide activated macrophages. The extract also exhibited significant proteinase inhibition activity *in vitro*.

KEYWORDS

Cayratia pedata, Granuloma, Anti-inflammatory Activity, Cyclooxygenase, Lipoxygenase and Proteinase

INTRODUCTION

Cayratia pedata (Lam.) Juss. Family: Vitaceae is a weak woody climber naturalized in tropical evergreen and semi-evergreen forests of India, Andaman-Nicobar Islands, Sree Lanka and Malaysia[1]. In folklore medicine, *C. pedata* is used for treating many conditions like scabies, wounds, boils, skin ailments, diarrhoea, and fever and in many inflammatory conditions. It is used also as an emetic, anthelmintic, astringent and rubefacient[2]. The anti-inflammatory activity of *C. pedata* in carrageenan induced rat hind paw edema has been reported[3]. The objectives of this study were to evaluate the Anti-inflammatory activity of ethanolic extract of *C. pedata* in subacute and sub chronic using croton oil induced granuloma pouch and cotton pellet models in rats and to evaluate the possible mechanisms of the anti-inflammatory activity of the extract. Phytochemical studies showed that the leaf extract (ethanol) contains carbohydrates, alkaloids, steroids, flavonoids and terpenes.[4]

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MATERIAL AND METHODS

All chemicals and solvents and standard drugs used for this study were of analytical grade or of HPLC grade. Prior consent from the institutional animal ethics committee was obtained for conducting studies on experimental animals. All the animals were housed in standard conditions of macro and micro environment and were fed with standard animal diet. Animals were accessible to food and water *ad libitum*, unless when the experimental protocols warranted doing so. No animals were subjected to undue pain or sufferings other than the experimental procedures demanded.

Cotton Pellet Granuloma Method[5]

Albino rats of both sexes were divided in to four groups of six animals each. Group I served as control and received 1% Carboxy Methyl Cellulose (CMC) in distilled water in a dose of 1 ml/100 g p.o. body weight. Groups II and III were dosed p.o. with *C. pedata* leaf extract at doses of 250 and 500 mg/kg respectively. The fourth group (standard) was given hydrocortisone acetate, 10mg /kg body weight. The dorsal side of all the animals was shaved before the study. Under ketamine anesthesia, cotton balls weighing 50 mg (which were previously dipped in penicillin solution, squeezed and dried) were implanted subcutaneously in the pectoral region of each rat. Dosing was continued for seven days and on the eighth day the animals were sacrificed, and the pellets together with the granulomatous tissue were removed carefully. The wet weight of all the pellets was taken and then they were dried overnight at 60 ^{0}C to record the dry weight. The percentage inhibition was calculated by the equation

% inhibition of granuloma formation= (C-T) x 100 /C

Where, C and T are the mean increase in weight of cotton pellet implanted in control and test animals respectively

Granuloma Pouch Method[6]

Male albino rats weighing 150- 200 g were weighed, marked for identification and divided in to four groups of six animals each as follows:

Group-I: control, Group-II: standard, Group-III: test group-I and Group-IV : test group-II

On the day of experiment granuloma pouches were induced under ketamine anesthesia on the back of all the animals by injecting 20 ml of sterile air in to the subcutaneous tissue, followed by injection of 1 ml of 1 % v/v solution of croton oil (Fluka AG, Switzerland, CH9470) in olive oil in to the air pouch formed. On the next day the air was removed by the application of vacuum. On the third day the pouches were compressed gently to prevent formation of adhesions.

Drugs were administered to all animals in the morning for seven consecutive days as follows:

Group-I: 1% CMC in distilled water, 10 ml/kg body weight, p.o.
Group-II: Prednisolone, 10 mg/kg body weight, p.o.
Group-III: *C. pedata* leaf extracts 250 mg /kg body weight, in 1% CMC, p.o.
Group-IV: *C. pedata* leaf extracts 500 mg /kg body weight, in 1% CMC, p.o.

On the seventh day, 60 min after the last dose, the animals were sacrificed and blood samples were collected for protein estimation. The pouches were carefully cut open, the exudates fluid in the pouch was collected using a syringe and the volume and protein content of the pouch fluid were measured (Lowry's method)[7]. Percentage reduction in exudate volume in standard and test groups was calculated with respect to the exudate volume of control group animals.

Inhibition of Morphine Induced Degranulation of Isolated Rat Peritoneal Mast Cells [8]

Tyrode solution (10 ml) containing 5U/ml heparin was injected by intra peritoneal injection to four rats. After gentle massage around the area of injection for 45 seconds, the mast cells rich fluid was collected over ice and centrifuged at 2000 RPM for 5 minutes. The cells were washed twice with chilled Tyrode solution and re-suspended in 4ml Tyrode solution and the contents of all 4 tubes were pooled together.

To 5 sets of test tubes marked as positive control, negative control, standard, test-I and test-II, pipetted out 1 ml each of the above cell suspension. Then added 1ml 1% CMC in Tyrode solution to negative control, 0.5 ml of 200 µg/ml solution of sodium cromoglycate to standard, 1 ml of 100 µg/ml solution of morphine sulphate to positive control, and 0.5 ml of 250 and 500 µg/ml suspension of *C. pedata* leaf extract in 1% CMC to test I and II respectively.

All the tubes were incubated for 15 minutes at 37º C. Then added 0.5 ml morphine sulphate solution (200µg/ml) to standard and test solutions and was further incubated for 10 minutes. The

cells were then stained with neutral red and % protection of degranulation was calculated.

Effect of *C. Pedata* Leaf Extract on Membrane Stabilization: The SRBC Membrane Stabilization Method

The ability of *C. pedata* leaf extract to prevent haemolysis induced by hyposaline (0.25 g/dl sodium chloride solution in distilled water) at concentrations of 50, 100 and 200 µg /ml were tested (all in triplicates) by sheep RBC membrane stabilization method[9,10] with some modifications. The standard employed was diclofenac sodium at same concentrations. Three fractions were tested: The ethanol extract of *C. pedata* leaves (CPEE), the Flavonoid-rich fraction of CPEE (which was prepared by fractionation with methanol-water mixture) and terpine rich fraction of CPEE (prepared by fractionation with n-Hexane: Ethyl acetate mixture).

Fresh g*oat* blood (from local slaughter house) was collected in Alsever solution (anticoagulant) and erythrocytes were separated by centrifuging at 3000 RPM for 5 minutes. The packed cells so obtained were washed with isosaline (pH 7.2) three times and diluted with more isosaline to get a 10% v/v suspension of erythrocytes.

The assay mixture consisted of 1 ml erythrocyte suspension, 1 ml phosphate buffer (0.15 M, pH 7.4), and various *C. pedata* leaf extract fractions(tests), diclofenac sodium (standard) at varying concentrations, 2 ml isosaline (for negative control) and 1 ml hyposaline (for positive control).

Each sample was tested in triplicate. Hyposaline (1 ml) was added to every tube (except the negative control) and incubated at 37°C for 30 minutes. The hemolysate was then centrifuged at 3000 RPM for 5 minutes and the optical density of the supernatant was measured at 560 nm. The percentage inhibition of hemolysis was calculated by the following formula:

$$\% \text{ inhibition} = 100 - [C/T \times 100]$$

Where C is the optical density of control and T is the optical density of the compound tested.

Assay of Total COX and 5-LOX in LPS Activated Macrophages

RAW 264.7 macrophages were cultured in DMEM media containing FBS for 72 hours. The culture was activated by adding 1µl LPS. Incubation was done for 24 hours. After 24 hours *C. pedata* leaf extract (in DMSO) was added in concentrations of 50, 100 and 200 µg/ml concentrations and incubated for 48 hours. The cells were then isolated by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 50µl of cell lysis buffer was added and again centrifugation was done at 6000 rpm for 10 minutes. Supernatant was discarded and anti-inflammatory assay was done in pellet suspended in a small amount of supernatant.

Assay of Total Cyclooxygenase (COX)

COX activity was assayed by the method of Shimizu et al.[11]. Lysed cells isolated from control, standard and test groups were incubated with Tris-HCl buffer (pH 8), 5mM glutathione and 5mM hemoglobin for 1 min at 25°C. The reaction was initiated by the addition of 200µM arachidonic acid and terminated after 20 min incubation at 37°C by addition of 10% trichloro acetic acid in 1N HCl. Following centrifugal separation and addition of 1%thiobarbituric acid, COX activity was determined by reading absorbance at 530 nm.

Assay of 5-LOX on Cultured LPS Activated Macrophages

Determination of LOX activity was done by the method of Axelrod et al.[12]. Briefly, the reaction medium contained Tris-HCl buffer (pH 8.1), cell lysate and a solution of sodium linoleate (10mM). The 5-LOX activity was monitored as an increase of the absorbance at 234nm which reflects the formation of hydroperoxy linoleic acid.

Effect of *C. Pedata* Leaf Extract on Denaturation of Protein[13, 14]

This test is based on the ability of compounds to prevent the heat induced denaturation of albumin.

The reaction mixture consisted of 1 ml 1%w/v solution of Bovine Serum Albumin (BSA) and

the test solutions. *C. pedata* leaf extract was tested in concentrations of 100, 250 and 500 µg/ml. The standard used was acetyl salicylic acid in the same concentrations. For control, 1 ml of distilled water was used. All the concentrations were tested in triplicates.

The mixture of bovine serum albumin (BSA) and test compounds was incubated initially for 37 °C for 20 minutes. Then the temperature was increased to 51°C and maintained at that temperature for another 30 minutes. After cooling to room temperature the optical density of the solutions were measured at 660 nm.

The inhibition of protein denaturation (%) was calculated by the following formula:

% inhibition of denaturation = [1-T/C] x 100,

where C and T are the optical densities of Control and Test respectively.

In Vitro Inhibition of Proteinase[15]

The reaction mixture consisted of various concentrations of *C. pedata* leaf extract, (100, 250 and 500µg/ml, each in triplicate) and1.0 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 0.06 mg trypsin. The final pH was adjusted to 6.3 using 1N HCl incubated at 37^0C for 5 min followed by addition of 1 ml of 0.8 % (w/v) casein and further incubated for 20 minutes.

After the incubation period, the reaction was stopped by the addition of 2 ml of 70% perchloric acid and then centrifuged at 1000 RPM for 3 minutes.

The absorbance of the supernatant was read at 280 nm against the buffer as blank. Diclofenac sodium was used as standard, tested at the same concentrations in a similar manner. The control consisted of all the reagents except the test or standard.

The percentage inhibition of proteinase was calculated using the formula:

Percentage inhibition = [1 – (C-T)/C] x 100,

Where C and T are the optical densities of control and test respectively.

RESULTS

Cotton Pellet Granuloma Method

Subcutaneous implantation of sterile cotton pellet in control, standard and test group animals produced granuloma tissue surrounding the pellet. The dried weight of tissue was determined by subtracting the weight of cotton implanted (50 mg) from the total dry weight of tissue and cotton pellet. Table 1.lists the observations of evaluation of anti-inflammatory activity of *C. pedata* leaf extract in cotton pellet granuloma model in rats.

The observed dry weight of granulomatous tissue formed in the untreated (Control) group animals was 160± 11 mg (Mean ±SEM). Treatment with hydrocortisone acetate in a dose of 10mg/kg body weight in the standard group resulted in significant ($p<0.01$) reduction in formation of granuloma in comparison to the weight of granulomatous tissue formed in the control group. The mean weight of tissue in mg amounted to 34± 2.2, which corresponds to a reduction of 79.59%.

The mean (±SEM) weight of granulomatous tissue formed in the two test groups treated with 250 and 500 mg/kg of *C. pedata* extract were 120±2.2mg and 81±3.1 mg respectively. The reduction in granuloma was to the extent of 27.21% and 50.66% respectively. (Significant when compared to control $p<0.01$)

Table 1: Evaluation of anti-inflammatory activity of *C. pedata* leaf extract in cotton pellet granuloma method

Groups & Dose	Dry weight of granuloma tissue in mg (Mean ± SEM)	% inhibition in granuloma
Control (A)	160±11	-
Standard (B) Hydrocortisone 10mg/kg	34±2.2*	79.59
Test-I (C) *C. pedata* leaf extract 250 mg/kg	120±2.2*	27.21

Test-II (D) *C. pedata* leaf extract 500 mg/kg	81±3.1*	50.66
* Significant decrease when compared with control. (p<0.01), N=6, 10df.		

Granuloma Pouch Method

Injection of sterile air and the subsequent injection of croton oil into the air pouch in rats lead to the formation of granuloma pouch in all of the animals of the four groups studied in the experiment. The volume of the pouch exudate as well as the content of protein in the exudate fluid was determined, the results of which are listed in the table 2.

There was considerable reduction of exudate fluid volume in the standard (Prednisolone treated) group animals- the mean value was 1.2 ± 0.13 ml/100g body weight. When expressed in percentage, this reduction corresponds to a decrease of inflammation by 66.31%, which is Statistically significant (P<0.01) in comparison to the control group.

Treatment with *C. pedata* leaf extract also showed to decrease the volume of exudate fluid- the mean volume in test-I (*C. pedata* leaf extract 250 mg/kg) and test-II (*C. pedata* leaf extract 500 mg/kg) were 2.3±0.11 and 1.8±0.17 ml/100g respectively. When compared to the volume of pouch exudate in the control group, the reduction in volume was found to be significant (p<0.01).

The observed value of protein content in the serum and pouch exudates were also determined using Lowry's method. The total protein content in the serum of control and standard group animals were 7.4 ±0.19 and 7.1±0.19 g/dl respectively. The total protein content in the serum of test group animals I and II were 7±0.09 and 6.9±0.09 g/dl respectively. No significant deviation (at 99% confidence level) in the serum protein content of standard and test group animals were observed when compared with that of the control group animals. (Table3).

Table 2: Evaluation of anti-inflammatory activity of *C. Pedata* leaf extract by granuloma pouch method

Groups & Dose	**Pouch exudate volume in ml (Mean ± SEM)**	**Exudate volume in ml/ 100g body weight (Mean ± SEM)**	**% inhibition**
Control (A)	5.8±0.14	3.6±0.15	
Standard (B) Prednisolone 250 µg/kg	1.9 ±0.16	1.2±0.13	66.31*
Test-I (C) *C. pedata* leaf extract 250 mg/kg	3.63±0.13	2.3±0.11	36.9*$
Test-II (D) *C. pedata* leaf extract 500 mg/kg	2.9±0.12	1.8±0.17	49.69*$
* Significant decrease with respect to control (p<0.01). $ Significant difference in comparison with standard p<0.01. N=6,10df			

Table 3: Estimation of total protein in pouch exudate in granuloma pouch method

Groups	Protein content in serum in g/dl (Mean ± SEM)	Value of t and R^2 for N=6, 10df At 99%CL & Columns compared	Protein content in exudate in g/dl (Mean ± SEM)
Control (A)	7.4±0.19		4±0.12
Standard (B) (Prednisolone 500 µg/kg)	7.1±0.19	t=1.3, R^2=0.15 (A & B)	2.7±0.10*
Test-I (C) *C. pedata leaf extract* 250 mg/kg	7 ±0.09	t=2, R^2=0.28 (A & C) t=0.3, R^2=0.009 (C& B)	3.5±0.09*
Test-II (D) *C. pedata leaf extract* 500 mg/kg	6.9 ±0.09	t=2.3, R^2=0.34 (A & D) t=0.58, R^2=0.03 (D & B)	3.2±0.12*
* Significant reduction in protein content ($p<0.01$), N=6, 10 df, .when compared with control.			

The protein content in the pouch exudate in the control group animals were found to be 4.0±0.12g/dl, the same for standard being 2.7±0.1 g/dl. (Mean ±SEM). The corresponding values in the test group-I and II were 3.5±0.09 and 3.2±0.12 g/dl respectively. It was seen that the treatment with prednisolone and *C. pedata* leaf extract lead to a significant reduction in protein content in pouch exudate, when compared to that of the control group animals.

Inhibition of Morphine Induced Degranulation of Isolated Rat Peritoneal Mast Cells

The effect of *C. pedata* leaf extract on morphine induced degranulation of rat peritoneal mast cells were evaluated and compared with that of sodium cromoglycate against positive and negative controls and the observations of this study are summarized in table 4.

It was noted that in the positive control (morphine sulphate) tubes maximum degranulation of mast cells occurred and the observed mean was 93.56±1.7 %. This value was significantly higher than the percentage degranulation occurred in all other groups ($p<0.05$).

The percentage degranulation in the standard tubes (Mean ± SEM in % =15.27±1.6) which was exposed to sodium cromoglycate 200µg/ml was found to be significantly low ($p<0.05$) when compared with the % degranulation occurred in the positive control and *C. pedata* leaf extract treated tubes. This infers that sodium cromoglycate can prevent the morphine induced degranulation significantly.

The effect of *C. pedata* leaf extract in a concentration of 500µg/ml on morphine induced degranulation of mast cells found to be insignificant when compared with the effect of the standard group. The % degranulation in the *C. pedata* leaf extract treated cells were 90 ± 1.3, which was not a significant difference from the value obtained in the positive control tubes (93.56± 1.7) at 95% CL.

Table 4: Inhibition of morphine induced degranulation of isolated rat peritoneal mast cells

Group	Treatment	Total No. of mast cells	% degranulation Mean ± SE	Value of t and R^2 for N=3, 4 df at 95% CL
Positive control (A)	0.5 ml mast cell suspension +1 ml Morphine sulphate (100µg/ml)	39.33± 4.05	93.56 ±1.7	t=50.6, R^2=0.99 (A & B)
Negative control (B)	0.5 ml mast cell suspension +1ml Tyrode solution	46.33± 3.84	1.3 ±0.67	
Standard (C)	0.5 ml mast cell suspension +0.5 ml Morphine sulphate (200µg/ml)+ 0.5 ml sodium cromoglycate (200 µg/ml)	43.67 ±1.45	15.27 ±1.6	t=30.2, R^2=0.99 (A & C) * t=6.7, R^2=0.92 (B & C)* t=31.7, R^2=0.99 (C & D)*
Test (D)	0.5 ml mast cell suspension +0.5 ml Morphine sulphate (200µg/ml)+ 0.5 ml *C. pedata* leaf extract (200 µg/ml)	41 ±2.08	90. ±1.3	t=1.5, R^2=0.37 (A & D) t=59.92, R^2=0.99 (B & D)*
* significant, p<0.05				

Table 5: Effect of *C. pedata* leaf extracts on membrane stabilization: The SRBC membrane stabilization method

Concentration (µg/ml)	% inhibition of hemolysis			
	Diclofenac Sodium	CPEE	CPFF	CPTF
50	61±0.23*	17±1.4*	16± 0.7*	25±0.20*
100		25±0.58*	25±0.20*	34±1.3*
200		30±0.51*	29±0.82*	41±0.49*

CPEE: Ethanol extract of leaves of *C. pedata*, CPFF: Flavonoid rich fraction of *CPEE*, CPTF: terpine rich fraction of CPEE.

- Significant, P<0.001, N=3 for 4 df, with respect to control (NOVA followed by Dunnet's multiple comparison against optical density of control tubes.

Effect of *C. Pedata Leaf Extract* on Membrane Stabilization: The SRBC Membrane Stabilization Method

Many researchers have utilized erythrocytes to study the effects of drugs on biological membranes.[16,17] It is a well-established fact that the pharmacological action of many drugs depend up on the interaction between the drug molecules and biological membranes.[18]

The protection from hyposaline induced hemolysis offered by diclofenac sodium was found to be 61±0.23% (Mean± SEM) in a concentration of 50µg/ml. *C. pedata* leaf extract offered a protection of 17±1.4%, 25±0.58% and 30±0.51% at concentrations of 50 µg/ml, 100 µg/ml and 200 µg/ml respectively. In similar concentrations the terpine rich fraction offered better protection than the ethanol extract and were found to be 25±2.0%, 34±1.3% and 41±0.49% respectively (Table 5).

The flavonoid rich fraction of *C. pedata* leaf extract also showed significant inhibition in hyposaline induced hemolysis of erythrocytes. The percentage inhibition for concentrations of 50 µg/ml, 100µg/ml and 200 µg/ml were 16±0.7, 25±0.2 and 29±0.82 respectively (Fig. 1).

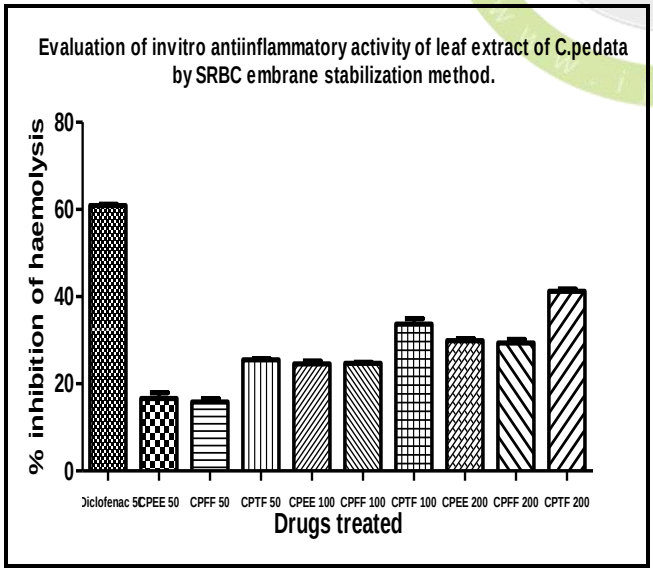

Figure 1: Effect of *C.* pedata leaf extract and diclofenac sodium on haemolysis of sheep erythrocytes by hypotonic saline. CPEE: Ethanol extract of *C. pedata leaves*, CPFF: flavonoid rich fraction of CPEE, CPTF: terpine rich fraction of CPEE

Assay of total COX and 5-LOX in LPS activated macrophages.

a) Assay of COX

The *in vitro* inhibition of total cyclooxygenase by LPS activated macrophages by *C. pedata* leaf extract was studied and compared with the effect of diclofenac sodium as standard. The result of the study is given in table 5. The inhibition of COX is directly proportional to the decrease in optical density of the reaction mixture. The mean OD of the control tubes were found as 0.68±0.004. *C. pedata* leaf extract as well as diclofenac sodium were found to inhibit total COX in vitro. The mean percentage inhibition of COX exerted by diclofenac sodium in a concentration of 250 µg/ml was 85.93, which was significant at $p<0.05$ level in comparison with the control group.

Inhibition of COX produced by the *C. pedata* leaf extract at concentrations of 100, 250 and 500 µg/ml was 31.24%, 48.38% and 57.92 % respectively. All these values are significant at 95% confidence level, when compared to the control group.

Table 5: In vitro inhibition of total cyclooxygenase in lipopolysaccharide activated macrophages by C. *pedata* leaf extract

Group	Optical Density (Mean ± SE)	% inhibition
Control (A)	0.68 ± 0.004	00
Standard (B) Diclofenac Sodium250 µg/ml	0.096 ± 0.003	85.93*
Test-1 (C)) *C. pedata* leaf extract 100 µg/ml	0.47 ± 0.012	31.24*

Test-2 (D) *C. pedata* leaf extract 250 µg/ml	0.35 ± 0.005	48.38*
Test-3 (E) *C. pedata* leaf extract 500 µg/ml	0.29 ± 0.003	57.92*
Significant, p<0.01, N= 3, 4 df.		

b) Assay of 5-LOX on cultured LPS activated macrophages

It was also observed that diclofenac sodium exhibited an inhibition of lipooxygenase enzyme in vitro significantly. The extent of inhibition was up to the level of 78.96% with respect to the activity of LOX in the control. *C. pedata leaf* extract in concentrations of 100, 250 and 500 µg/ml exhibited inhibition of 20.59%, 26.9 % and 34.69 % respectively. Statistical analysis shows that all these inhibitions are significant (p<0.05) when compared to the enzyme activity in the control culture. It can also be noted that the inhibition exerted by diclofenac was higher than that of all the three concentration levels of *C. pedata leaf* extract (significant at 0.05 levels).

Effect of *C. Pedata* Leaf Extract on Denaturation of Protein

When proteins are exposed to heat, chemicals like strong acids, alkalis or organic solvents or high concentrations of electrolytes, they lose their quaternary structure; a process called denaturation. Most biological proteins lose their function on denaturation. Ability of substances to inhibit denaturation of albumin is taken as a measure of its anti-inflammatory activity.[19]

It was observed that acetyl salicylic acid inhibited the heat induced denaturation of BSA by 33.5%, 50.46% and 66.32 % at concentrations of 100, 250 and 500 µg/ml respectively. The calculated value of IC_{50} of diclofenac sodium was 282.13µg/ml.

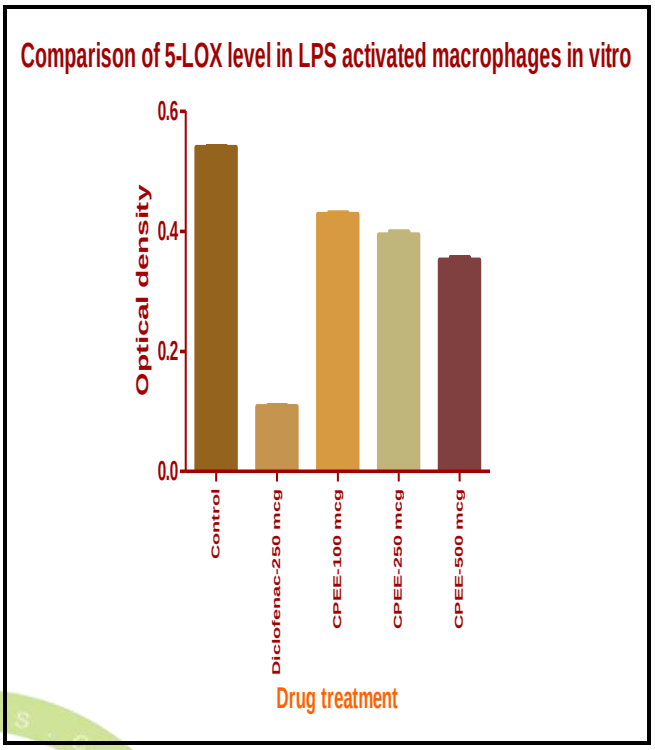

Figure 2: Effect of *C. pedata* extract on total level of 5-LOX

C. pedata leaf extract did not show significant inhibition of thermal denaturation of proteins up to a concentration of 500µg/ml concentrations.

***In Vitro* Inhibition of Proteinase[20]**

Proteinases are digestive enzymes and produce tissue damage during inflammation. Leukocyte proteinases are stored inside the granules of neutrophils and are released during inflammation. Proteinase inhibitors show anti-inflammatory activity.

The role of proteinase in inflammation is well established. It has been reported that protease inhibitors can interfere with liposomal functions and activation of inflammatory cells by microbial lipo-poly saccharides.[21] It is known that leukocyte proteases play an important role in inflammation and protease inhibitors can suppress inflammatory process[22].

Table 7 describes the in vitro inhibition of proteinase by the extract and diclofenac sodium (standard) at various concentrations. It was observed that both the standard and test substances produce significant reduction in proteinase activity *in vitro*.

Table 6: Effect of ethanol extract of *C. pedata* and diclofenac sodium on thermal denaturation of bovine serum albumin fraction

Drugs and concentration	**Optical density at 560 nm (Mean ±SEM)**	**% inhibition of Denaturation of albumin**	**Groups compared and Value of t and R^2**
Control (C)	0.304 ±0.001	0	-
Test-1 (T_1) *C. pedata* leaf extract- 100 µg/ml	0.304±0.004	1.09	C and T_1 t=0.099, R^2= 0.002
Test-2 (T_2) *C. pedata* leaf extract- 250 µg/ml	0.299. ± 00	1.2	C and T_2 t=3.08, R^2= 0.66
Test-3 (T_3) *C. pedata* leaf extract- 500 µg/ml	0.298 ±.005	1.86	C and T_3 t=1.03, R^2= 0.215
Standard-1 (S_1) Acetyl salicylic acid 100 µg/ml	0.202 ±0.01	33.55*	C and S_1 t=10.7, R^2= 0.966
Standard-2 (S_2) Acetyl salicylic acid 250 µg/ml	0.151±0.001	50.46*	C and S_2 t=111, R^2= 0.99
Standard-3 (S_3) Acetyl salicylic acid 500 µg/ml	0.102±0.001	66.32*	t=136, R^2= 0.99
* Significant, p<0.05, for N=3, 4 df.			

Table 7: The effect of *C. pedata* leaf extract on proteinase activity *in vitro*

Group	**Concentration (µg/ml)**	**Mean OD ± SE**	**% Inhibition**
Control	Nil	0.664 ± 0.0003	0
Standard (Diclofenac sodium)	100	0.51± 0.0003	76.30*
	250	0.564 ± 0.0003	84.98*
	500	0.611 ± 0.0005	92.05*
C. pedata leaf extract	100	0.147 ± 0.0003	22.21*
	250	0.173 ± 0.003	26.09*
	500	0.219± 0.0004	32.93*
*significant, p<0.001.N=3, 4df.			

DISCUSSION

Inflammation is a response of the body to a derangement in the milieu interior that may be caused by injuries, trauma, and infections by pathogens, toxic substances, ischemia etc, which may lead to both local as well as systemic effects. The course of underlying pathological process, cells involved and mediators that trigger inflammation may vary in acute and chronic inflammation. There are many in vitro and in vivo experimental models to study inflammation and anti-inflammatory activity. The ability of drugs to reduce granuloma formation is taken as its anti-inflammatory potential. Granuloma is a form of localized nodular inflammatory response characterized by accumulation of immune cells called histocytes. Granuloma formation occurs due to a variety of factors including chemical, physical and biological irritants. The primary signs of granuloma formation include infiltration of large number of neutrophils and accumulation of protein-rich fluid at the site of irritation. This will progress to formation of granulomatous tissues by about one week, characterized by formation of a well vascularized fibrous capsule. The capsule is rich in fibroblasts and mono nuclear cells. The content of N-acetyl glucosamine and nucleic acid in the granulomatous cells are higher than the normal cells.[23]

Experimental granuloma may be produced by implanting materials like cotton pellet or asbestose pellets which cannot be metabolized by the animals subcutaneously. Drugs that reduce granuloma formation at the early stages will reduce the weight of granuloma. Treatment after 4th day will not cause much effect on granuloma formation.[24]

Mast cell is a store house of histamine, which is an important mediator triggering the vascular events in inflammation. Agents that can stabilize the mast cell membrane can prevent the degranulation and release of histamine thereby contributing to the anti-inflammatory activity. The present study suggests that the *C. pedata* leaf extract does not prevent the degranulation of mast cells significantly and this mechanism does not contribute to the anti-inflammatory activity of *C. pedata.*

Proteinases and proteinase activated receptors-1 are important in many inflammatory disorders. PRP-1 is a G-protein coupled receptor, which is activated by proteinases like thrombin. Inhibition of proteinases thus may reduce activation of immune system in inflammatory responses.[25,26]

It is known that the leaf extract (ethanolic) of *C. pedata* contains carbohydrates, alkaloids, steroids, flavonoids and terpines. It is established that terpines can modify inflammation[27,28]

Many flavonoids containing plants possess anti-inflammatory activities.[29] It is already established that flavonoids can modify the expression of COX and LOX and can contribute to the anti-inflammatory activity of many plant drugs.[30]

The present study reveals that *C. pedata leaf* extract reduces the weight of granulomatous tissue and protein content in the treated group in comparison to the control group. It was also found that the extract can inhibit the activity of proteinase and can also lower the total COX and LOX activity. These activities might be contributing to the anti-inflammatory activity of *C. pedata* leaf extract.

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