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Research Article

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Evaluation of a Novel Thiadiazole Derivative for Anti Inflammatory Activity

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ABSTRACT

Thiadiazoles are heterocyclic compounds of importance that exhibit diverse biological properties as anticancer, antibacterial, fungicidal, antiviral, antiparasitic, anticonvulsant, anticoagulant, antidiabetic, anti-Helicobacter pylori, leishmanicidal agents. The biological activity of thiadiazole is mainly due to the unsaturation of the ring system, which leads greater in vivo stability and lack of toxicity. The present study aimed to synthesize 2, 5 substituted 1,3,4 thiadiazole nucleus and evaluates its anti-inflammatory property using cell lines and membrane stabilization methodalong with in vivo models. The compound, 5-(5-(phenyl amino)-1,3,4 thiadiazole-2yl) benzene 1, 2,3 triol was synthesized via microwave irradiation method and the characterization done by TLC,IR,NMR and Mass spectral analysis. It showed anti-inflammatory activity when tested for cycloxygense, lipoxygenase and myeloperoxidase inhibition using RAW 264.7 macrophage cells cultured in Dulbecco's media. In vivo anti-inflammatory activity of the test drug, thiadiazole derivative was investigated using acute and chronic models of inflammation, carrageenan induced paw edema method and cotton pellet granuloma method respectively.

Keywords: Thiadiazole; Inflammation; COX; LOX; Paw oedema; Granuloma

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INTRODUCTION

Inflammation is a complex process produced by vascularised living tissues to stop the tissue damage caused by a wound or invading pathogen .It serves to remove the injurious agent and try to heal the damaged tissue. The process of inflammation is associated with repair and it begins during the early phases of inflammation but complete healing occurs after the injurious influence has been removed [1]. The drugs which are used nowadays for the treatment of chronic as well as acute inflammation have enormous side effects. The long term use of NSAIDs may leads to the development of indigestion, stomach ulcers and increased blood pressure. Other anti-inflammatory drugs rather than NSAIDs also have some major side effects such as immune suppression, increased skin fragility, osteoporosis, delaying puberty, impaired memory and attention deficits [2]. Due to this, development of new drugs with low toxicity, specificity and good pharmacokinetic profiles are essential. The ring system of thiadiazole shows broad-spectrum biological activity. Thiadiazoles are also known as inhibitors of oxidation, metal complexing agents and anti-corrosion agents apart from its use as anti-inflammatory, antidiabetic ,antiparasitic, anticancer, anti-Helicobacter pylori, antibacterial, fungicidal, antiviral, anticonvulsant, anticoagulant, leishmanicidal agents [3]. 1,3,4-thiadiazoles [4] are mesoionic five-membered heterocyclic compound containing poly-heteroatomic system. The thiadiazole ring associated with conjugated p and p electrons shows distinct positive and negative charges.

Thus, the present study aimed to synthesize and evaluate the thiazole derivative,5-(5- (phenyl amino)-1,3,4 thiadiazole-2yl)benzene 1,2,3 triol for *in vitro* and *in vivo* anti-inflammatory activity.

MATERIALS AND METHODS

Animal selection, maintenance and care

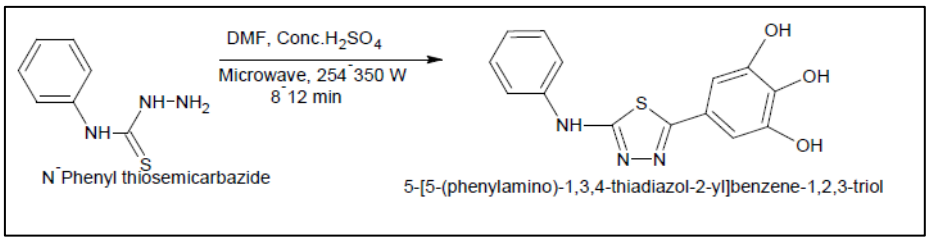
Wistar rats of both sex weighing between 150-250 g were procured from animal house (Reg No: 752/02/a/CPCSEA) of Govt. Medical College, Trivandrum and used for the study. They were housed in 12:12 light dark cycle under standard condition of relative humidity (30-70%) and temperature (25±20c). Animals

were fed with standard rodent pellet diet and water ad libitum except during experimentation. Study protocols were approved by animal ethical committee and the experiments were conducted based on CPCSEA guidelines.

Chemicals

The chemicals and solvents were procured from Sigma Central drug house, India. Melting points were determined on digital melting point apparatus in open capillary tubes and are uncorrected. The characterization of the compounds were carried out by both IR Spectrometer(Thermonicolete Avatar 375) and NMR spectrometer (Bruker Avance III, 400MHz)

Synthesis of 5-(5-(phenyl amino)-1,3,4 thiadiazole-2yl)benzene triol by microwave assisted synthesis [5,6]**:**



N-phenyl thiosemicarbazide (0.01mol, 1.67g) and gallic acid (0.01 mol) in DMF were mixed together and irradiated in a microwave irradiator at 350 W for 10 minutes. The reaction mixture was poured into crushed ice and kept overnight. Solid product was recrystallized from ethanol-water mixture (4:1) to yield off white shining crystals of compound Yield: 51%W/W.

The newly synthesized compound was characterized by Melting point, Vibrational spectra (IR), ^{1}H NMR spectra and Mass spectral analysis.

Biological screening

***In vitro* anti-inflammatory activity**

SRBC membrane stabilisation method [7,8]: The lysosomal enzymes such as proteases and bactericidal enzymes that are released during inflammatory process is very dangerous as it can produce serious disorders. Thus stabilization of lysosomal membrane is significant to limit the inflammatory responses. Erythrocyte membrane or SRBC is analogous to the lysosomal membrane and therefore this method checks the stability of the SRBC membrane which can be easily co related to lysosomal membrane too.

Procedure: Various concentrations of drug (100,250,500,1000 µg/ml) were prepared in isosaline. 2 ml hypo saline ,0.5 ml of SRBC suspension and 1 ml of phosphate buffer were added to each of the concentration above. Incubated at 370^{0}C for a period of 30 minutes. The mixture was centrifuged for 20 min at 3000 rpm. Diclofenac sodium was used as reference standard. The absorbance of supernatant was estimated spectrophotometrically at 560 nm.

Percentage Inhibition of haemolysis
= 100 - (OD of the control - OD of test) / OD of control *100
Where, OD is the optical density

***In vitro* cell line studies for inflammation:** RAW 264.7 macrophage cells were cultured in Dulbecco's modified eagles media supplemented with 10% heat inactivated FBS, antibiotics (Penicillin and streptomycin) and 1.5% sodium bicarbonate. The anti-inflammatory effects of the samples were found by measuring the inhibition of COX, LOX, and myeloperoxidase enzymes spectrophotometrically.

COX-Inhibition assay [7]: The assay mixture contained Tris- HCl buffer, glutathione, hemoglobin & enzyme. Addition of arachidonic acid marked the start of the process and 20 min incubation was done at 37ºc by addition of volume of 0.2ml of 10% TCA in 1N HCl. Mixed well and added 0.2ml of TBA. The contents were allowed to boil in a water bath for 20 min. Later, it was cooled and centrifuged at 1000rpm for a period of 3 min. The COX activity was measured at 632nm. Diclofenac sodium were used as standard with same concentration as that of test.

Lipoxygenase inhibition assay [7]: The reaction was carried out in a quartz cuvette at 25°C with 1cm light path. The assay mixture contain 2.75ml tris buffer of pH 7.4, 0.2ml of sodium linoleate and 50µl the enzyme. The increase in OD was measured in 234nm.

Percentage inhibition was calculated using the formula given bellow
% inhibition = (C-T/C) *100
C = Optical density of control
T = Optical density of Test

Myeloperoxidase assay [7]: Cultured sample was mixed in a solution containing 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 6) and 0.57 hexadecyl trimethyl ammonium bromide(HTAB). After freeze thawing, the samples were centrifuged at 2000 rpm for 30 min at 4°C and resulting supernatant was assayed spectro-photometrically for MPO. Sample was mixed with 50mM phosphate buffer (pH 6). The change in absorbance at 490 nm was measured.

***In vivo* Pharmacologic Studies**
Acute toxicity study [7]: The acute toxicity study was taken as per OECD (Organization of Economic Cooperation and Development) 425 test guideline under a computer-guided Statistical Programme-AOT425 stat. Six female Wistar rat was selected for the study and the test consists of a single ordered dose progression in which single animal dosed, once at a time.

Carrageenan induced paw oedema in rats: an acute anti-inflammatory study [8-10]: Paw edema method is a convenient method for assessing inflammatory responses to antigenic challenges and irritants. In this model, the test compounds were assessed for their acute inflammatory activity by examining their ability to reduce or prevent the development of carrageenan induced paw swelling by inhibiting the prostaglandin production.
Grouping of animals: Wistar rats weighing between 150-200 g were randomly divided into 4 groups of 6 animals each.

Group I – control (0.5% CMC suspension)
Group II – standard drug indomethacin (10 mg/kg)
Group III – test drug (400 mg/kg)
Group IV – test drug (200 mg/kg).

Procedure: The rats received 5 ml of 0.5% CMC suspension orally for all four groups. Thirty minutes later, they were challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw was marked with ink at the tier of the lateral malleolus and immersed in digital plethysmometer up to this target. Immediately after injection, measured the paw volume plethysmographically &procedure was repeated at 1, 2, 3 and 4 h after challenge.

Percentage of edema inhibition = (Vc -Vt/Vc) *100
Vc- Volume of paw edema in control group
Vt- volume of paw edema in treated group

Cotton Pellet-Induced Granuloma in Rats- chronic model for anti-inflammatory study [11]
The method has been described first by Meier et al(1950) in which foreign body granulomas were provoked in rats by subcutaneous implantation of pellet of compressed cotton. After several days, histologically giant and undifferentiated connective tissue can be observed besides the fluid infiltrations, which were measured by weighing the dried pellets after removal.
Wistar rats weighing between 150-200 g were randomly divided into 4 groups of 6 animals each.
Group I – control (0.5% CMC suspension)
Group II – standard drug Hydrocortisone (10 mg/kg)
Group III – test drug (400 mg/kg)
Group IV – test drug (200 mg/kg).

Procedure: An incision was made in the lumbar region after shaving the back skin and by using a blunted forceps subcutaneous tunnels were formed. Sterilized cotton pellet were placed on both sides in the scapular region. After the implantation of cotton pellet, the animals were treated for 7 days subcutaneously or orally, with standard, control and test drug. The animals were sacrificed on 8^{th} day, the pellets prepared and dried until the weight remains constant. The net dry weight, of the cotton pellet determined and the percentage change of granuloma weight relative to vehicle control group is determined.

Statistical analysis
Values are depicted as mean ± standard error mean for groups of six animals. The results were analysed by one way analysis of variance (ANOVA) followed by Dunnett's t test.

Docking studies

The synthesized compound is subjected to docking by using discovery studio software which is described in another paper.

RESULTS

The compound was synthesized by both microwave irradiation and conventional means. The yield was found to be better in the microwave irradiation method and calculated to be 51% w/w. The synthesised compound was characterised by various methods such as melting point, TLC, IR, NMR and Mass spectrometry.

Compound	MF	MW	Yield	Solubility
(5-(5-(PHENYL AMINO)-1, 3, 4 THIADIAZOLE-2YL) BENZENE 1, 2, 3 TRIOL	C14H11N3O3S	301.328	51%	Ethanol, Methanol, DMSO

Spectral data

IR(KBr):3393.43 (secondary aromatic N-H str) 3030.51 (aromatic C-H str), 3242.74(O-H str), 1601.11 (N-H bend), 1496.41, 1455.68, 1422.31 (aromatic C=C ring str), 1299.00 (secondary aromatic C-N str), 1200.42(phenolic C-O str), 1082.15(N-N=C), 1058.84 (N-N str), 893.34(C_6H_3), 745.58 (C_6H_5), 687.88 (C-S-C), 606.94 (O-H out of plane bend).H^1NMR (δ, ppm): The multiplet at 6.999-7.826 was due to the presence of aromatic protonos and which shows about 7 protons. The peak at 5.089 was due to the presence of aromatic C-OH group and shows about 3 protons. The peak at 3.999 is due to the presence of C-NH group and which shows about 1 proton. Mass spectral analysis the molecular ion peak was observed at the expected m/z 301 corresponding to molecular formula C14H11N3O3S and the base peak was at m/z 135.7426.

***In vitro* screening for anti-inflammatory activity**

SRBC membrane stabilisation method

In vitro anti-inflammatory activity of the 1,3,4 thiadiazole derivative was evaluated by human SRBC membrane stabilization method. Sample showed maximum protection of 77.14% at a concentration of 1000µg /ml. Standard drug diclofenac showed 95.51% at same concentration.

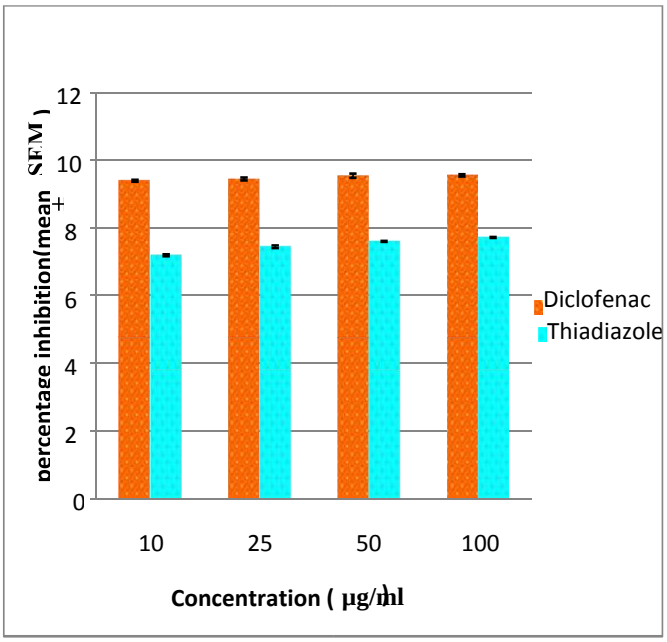


Figure 1: Evaluation of membrane stabilization activity of the compound using SRBC method

COX inhibition assay

Cyclooxygenase (COX) enzymes are responsible for the formation of prostaglandins, prostacyclins and thromboxanes. The inhibition of COX by thiadiazole derivative is responsible for its anti-inflammatory potential. Test drug showed 55.31% percentage protection at 100µg/ml concentration and the standard showed 84.91 % protection at the same concentration.

Table 1: COX inhibition assay

Concentration (µg/ml)	Absorbance (Sample) (nm)	Percentage Inhibition% (Sample)	Absorbance (standard) (nm)	Percentage inhibition (Standard)
Control	0.1638	-	-	
6.25 µg/ml	0.1191	27.29	0.114	64.16
12.5 µg/ml	0.1002	38.9	0.091	71.39
25 µg/ml	0.0885	45.97	0.082	78.02
50 µg/ml	0.0795	51.47	0.063	80.19
100 µg/ml	0.0732	55.31	0.048	84.91

LOX inhibition assay

Inhibition of LOX is an indication of potential anti-inflammatory agents. Test drug showed 41.87% inhibition at 100µg/ml concentration and standard drug showed 98.02% inhibition at 100µg/ml concentration.

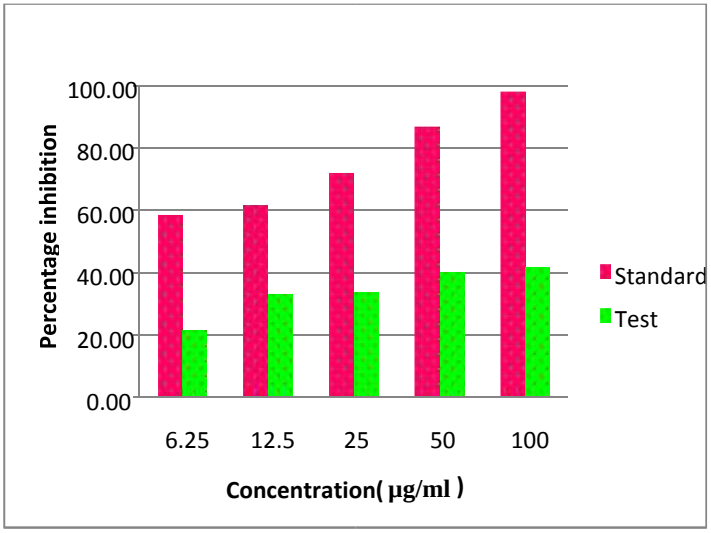


Figure 2: Comparison of LOX inhibition

Myeloperoxidase assay

The amount of myeloperoxidase is an indication of inflammation in the cells. Decreased amount of enzyme level in cells treated with anti-inflammatory agents indicates the drug's potential to reduce the inflammation. The enzyme activity in test drug was 0.0010(U/ml) at 100µg/ml concentration and standard was 0.00040 (U/ml) at the same concentration.

Table 2: Myloperoxidase assay

Concentration (µg/ml)	Absorbance (Sample) (nm)	Enzyme activity (U/ml) (Sample)	Absorbance (standard) (nm)	Enzyme activity (U/ml) (Standard)
Control	0.0527	0.0701		
6.25 µg/ml	0.0165	0.0039	0.0074	0.00244
12.5 µg/ml	0.0016	0.0021	0.006	0.00198
25 µg/ml	0.0014	0.0019	0.0043	0.00142
50 µg/ml	0.0008	0.0011	0.003	0.00059
100 µg/ml	0.0003	0.001	0.0023	0.0004

Screening for *in vivo* anti-inflammatory activity

Acute toxicity

Acute oral toxicity studies were carried out according to the main test of OECD 425 guidelines. LD50 value was determined by oral administration of thiadiazole to rats. No mortality was observed even at higher concentration 5000mg/kg.

Carrageenan induced paw oedema in rats

Acute anti-inflammatory activity was measured by using carrageenan induced paw oedema method. The sub-plantar injection of carrageenan induce an increase in paw volume in control rats (0.2383±.0195).Pre-treatment

with thiadiazole derivative (200and400 mg/kg p.o) 1 h before carrageenan inhibited paw oedema in a dose dependent fashion. Indomethacin (10 mg/kg) also inhibited carrageenan induced increase in paw volume.
The comparison of progression of inflammation up to 4th hour shows that the drug can inhibit the both phases in inflammation as compared to the standard. The test doses 200 and 400 mg/kg showed reduction in paw volume in first phase this indicate that the drug can inhibit the release of histamine and serotonin release as compared to the standard. They also showed significant (P<0.0001) reduction in paw volume at final phase of inflammation.

Table 3: Paw volume in carrageenan induced paw edema method

	30 Minutes	1 Hour	2 Hour	3 Hour	4 Hour
Control	0.048	0.132	0.18	0.258	0.238
Indomethacin	0.03	0.073	0.107	0.135	0.070****
Test 200mg/kg	0.037	0.075	0.088	0.143	0.132****
Test 400mg/kg	0.022	0.06	0.11	0.157	0.128****

****P<0.0001.n=6, values are expressed in mean as compared to vehicle control
(One way ANOVA followed by Dunnet test)

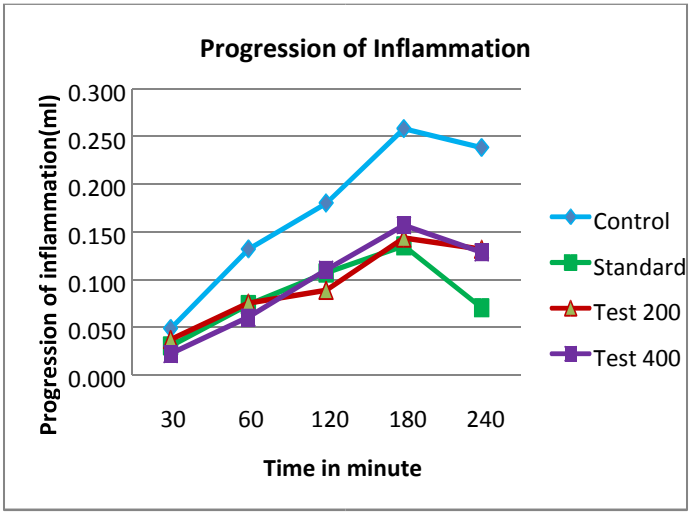


Figure 3: Progress of inflammation up to 4th hour after induction of paw oedema

Cotton pellet granuloma in rats

The chronic proliferative phase of inflammation was studied by cotton pellet granuloma. Different percentage of each type of leukocyte gives an index of inflammation. Decreased total leukocyte count in both test and hydrocortisone treated group compared to control is a good indication of reduced inflammation.

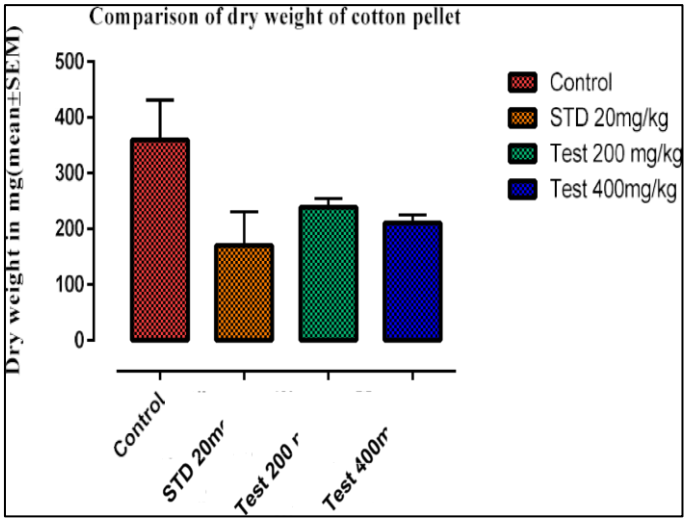


Figure 4: Comparison of dry weight of cotton pellet

DISCUSSION AND CONCLUSION

Thiadiazoles consists of five membered hetero cyclic ring with sulphur, nitrogen, oxygen and hydrogen atoms. The literature reveals that thiadiazole compounds show less toxicity due to higher unsaturation in the ring system and the attachments in the 2 and 5th positions of the ring system are important for various biological activities. The present study aimed to synthesise 2, 5 substituted 1, 3, 4 thiadiazole with less toxicity and evaluated its anti-inflammatory property both *in vivo* and *in vitro.*

The synthesis of 5-(5-(phenyl amino)-1, 3, 4 thiadiazole-2yl) benzene 1,2,3 triol was carried out based on the evidence that they may bind effectively to the target and have less toxicity. The intermediate was synthesised by conventional as well as microwave assisted synthesis and the percentage yield of the product was 51%W/W.

SRBC membrane stabilization assay is an *in vitro* bioassay method to screen the anti-inflammatory potential of synthesised 1,3,4 thiadiazole. The thiadiazole derivative showed significant membrane potential ($p<0.0001$). The maxium protection showed by the thiadiazole derivative was at 1000µg/ml and the percentage protection at that concentration was 77.14%. diclofenac used as the standard and showed 95.51% protection at 1000µg/ml concentration.

Cyclooxygenase are important enzyme involved in the synthesis of prostaglandins, prostacyclins and thromboxanes. These prostaglandins are major components involved in the inflammatory pathway. The inhibiton COX by the drugs indicate its potential anti-inflammatory activity. The inhibition of leukotrines by the drug also indicates its potential anti-inflammatory role. Myeloperoxidase are another class of enzyme released during the process of inflammation. The level of enzyme indicate the inflammation in the cell. Decreased amount of enzyme level in cells treated with anti-inflammatory agents indicates the drug's potential to reduce the inflammation.

The anti-inflammatory data provided by the *in vitro* methods are considerable, so further *in vivo* studies on inflammation were done.

In vivo anti-inflammatory activities are selected based on the phases of inflammation. For acute phase of inflammation carrageenan induced paw oedema was selected and for chronic proliferative phase cotton pellet granuloma.

Carrageenan-induced rat paw oedema is biphasic. The first phase is early mediated by mast cell degranulation, histamine release, serotonin release (1-2 h), bradykinin release and pain followed by eicosanoid production in the late phase. The standard anti-inflammatory drug indomethacin showed significant reduction in paw volume ($P<0.0001$). The drug showed significant reduction in paw volume but less than that of standard drug. .

The comparison of progression of inflammation up to 4^{th} hour shows that the drug can inhibit the both phases in inflammation as compared to the standard. The test doses 200 and 400 mg/kg showed reduction in paw volume in first phase this indicate that the drug can inhibit the release of histamine and serotonin release as compared to the standard. They also showed significant ($P<0.0001$) reduction in paw volume at last phase of inflammation also.

The chronic proliferative phase of inflammation was studied by cotton pellet granuloma. In cotton pellet granuloma method the implantation of cotton pellet provoke the formation of inflammation by activating leukocytes. Different types of leukocytes present in the blood have different functions. Percentage of each type of leukocyte gives an index of inflammation. Decreased total leukocyte count in both test and Hydrocortisone treated group were compared to control. Both the test and standard group showed reduced leukocyte count compared to the control which indicates the anti-inflammatory effect of drug.

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