

## **Design, synthesis and evaluation of specific TNF- $\alpha$ inhibitory novel dithioic acid derivative from natural acids as anti-inflammatory agents**

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### **Abstract**

Novel dithioic acid derivatives have been synthesized from 3 different natural acids having marked anti-inflammatory activities including ferulic acid, cinnamic acid and salicylic acid. The structures of the new compounds were established on the basis of IR, <sup>1</sup>H NMR and mass spectral data. *In silico* molecular analysis of different dithioic acid analogues of natural acids were done, and the compounds which obeyed "Lipinski's rule of five" were taken for computing molecular descriptors. Docking studies were carried out against target, human TNF- $\alpha$  converting enzyme (TACE). Out of the seventeen analogues docked, compound 1b, 2b and 3b exhibited minimum glide score and were taken out for wet laboratory synthesis and validations. Compound 1b (*{5-[(E)-2-(4-hydroxy-3-methoxyphenyl) ethenyl]-1, 3, 4-thiadiazol-2-yl} carbamodithioic acid*) was screened for *in vitro* anti-TNF- $\alpha$  study, and compounds 1b, 2b and 3b for *in vivo* anti-inflammatory activity. *In vitro* specific TNF- $\alpha$  inhibitory activity of the ferulic acid analogue (1b) was evaluated using Reverse transcriptase- polymerized chain reaction. *In vitro* and *in vivo* anti-inflammatory activity was also evaluated using the HRBC membrane stabilization method and carrageenan induced rat paw edema method and compared with standard drug (diclofenac sodium). Among all derivatives 1b (ferulic acid derivative) showed maximum percentage inhibition (60.09%) while the remaining compounds showed moderate inhibition.

**Keywords:** Ferulic acid, natural acids, TNF- $\alpha$ , dithioic acid, anti-inflammatory activity.

## **1. Introduction**

### **1.1 Tumour Necrosis Factor Alpha (TNF- $\alpha$ ) in inflammation:**

Tumor necrosis factor-alpha (TNF- $\alpha$ ), one of the best characterized cytokines, was originally discovered in the mouse serum during endotoxemia and recognized for its anti-tumor activity in 1975. Human tumour necrosis factor was cloned in 1985. TNF has since been implicated in a diverse range of inflammatory, infectious and malignant conditions, and the importance of TNF in inflammation has been highlighted by the efficacy of anti-TNF antibodies or administration of soluble TNF receptors (TNFRs) in controlling disease activity in rheumatoid arthritis and other inflammatory conditions.

TNF-alpha is produced by activated macrophages and T lymphocytes. Other pro-inflammatory cytokines including interleukin (IL)-1, IL-6 and are stimulated by TNF-alpha. It also increases leukocyte migration by inducing expression of adhesion molecules by endothelial cells and leucocytes. Because TNF- $\alpha$  plays a key role in the inflammatory cascade, by activating membrane bound TNFR<sub>1</sub> and TNFR<sub>2</sub> receptors, exogenously administered soluble TNF receptor protein or antibody can neutralize it and interrupt the reaction. TNF inhibitors mainly suppress macrophage and T-cell function; inflammatory changes are slowed. Quicker responses than DMARD's has been obtained. Side effects are few, but susceptibility to opportunistic infections, including tuberculosis and pneumocystis pneumonia is increased. The drugs available in the market include etanercept, adalimumab, infliximab which are very expensive.

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TNF is produced predominantly by activated macrophages and T lymphocytes as a 26 kDa protein, pro-TNF, which is expressed on the plasma membrane, where it can be cleaved in the extracellular domain by the matrix metalloproteinases, which result in the release of a soluble 17 kDa form. Both membrane-associated and soluble TNF's are active in their trimeric forms, and the two forms of TNF may have distinct biological activities. TNF $\alpha$  converting enzyme (TACE, also known as ADAM-17) mediates release of TNF from the cell surface, but is involved in processing several cell membranes associated proteins, including TNF receptors, which are released by its action to produce soluble forms that can neutralize the actions of TNF. Inhibition of this TACE can pave the way to establish a new series of anti-inflammatory agents through specific TNF- $\alpha$  inhibition.

### 1.2 Ferulic acid, Cinnamic acid and Salicylic acid as leads:

Ferulic acid, cinnamic acid and salicylic acid are three different natural acids found to possess the anti-inflammatory activity for which one of the major mechanisms of action is inhibition of tumour necrosis factor.

### 1.3 Importance of sulphur containing compounds in anti-inflammatory activity:

Four different anti-inflammatory sulfur-containing compounds were obtained from garlic, and their chemical structures were identified as Z- and E-ajoene and oxidized sulfonyl derivatives of ajoene. The sulfur compounds inhibited the production of nitric oxide (NO) and prostaglandin<sub>E2</sub> (PGE<sub>2</sub>) and the expression of the pro-inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-1 $\beta$ , and Interleukin-6 in lipopolysaccharide (LPS)-activated macrophages.

### 1.4 1, 3, 4-Thiadiazole moiety:

Several five membered aromatic systems having three hetero atoms at symmetrical positions such as thiadiazoles have been studied extensively owing to their interesting pharmacological activities. Thiadiazole consists of a five membered ring with two Nitrogen atoms and a Sulphur atom as hetero atoms. Thiadiazole derivatives have shown considerable biological actions such as antimicrobial, anti-inflammatory, anticancer, anticonvulsant, antidepressant, antioxidant, radioprotective and anti-leishmanial. 1, 3, 4-Thiadiazole and its derivatives continue to be of great interest to a large number of researchers owing to their great pharmaceutical and industrial importance and it is surprising that the synthetic publication far outweighs in numbers those relating to all other fields.

## 2. Materials and Methods

### 2.1 Materials:

*In silico* molecular modelling studies were carried out on various software like Schrodinger, ACDLABS ChemSketch and Molinspiration.

The chemicals and reagents used in the present work were of AR and LR grade, procured from Merck, Hi-Media, Nice and Sigma-Aldrich. All the chemicals were dried and purified wherever necessary. The melting points of the synthesized compounds were determined by Thiels melting point apparatus (open capillary tube method) and all the compounds gave sharp melting points and were uncorrected. Purity of the compounds was ascertained by thin layer chromatography. The IR spectra of the synthesized compounds were recorded on IR affinity-1 FTIR spectrophotometer Shimadzu in the range of 400-4000. The NMR Spectra of the characteristic compound was recorded by NMR 400 MHZ Spectrophotometer Bruker. The mass spectrum of the characteristic compound was recorded by JOEL GC mass spectrometer using electron ionisation method.

### 2.2 Methods:

#### 2.2.1 *In silico* design procedure:

The 3-D structure of the protein was obtained from PDB using their specific PDB ID (2OI0). The protein structure was prepared using the protein preparation wizard in the Schrodinger software graphical user interface Maestro v9.3. A set of dithioic acid derivatives of various natural acids were selected as ligands and their structures were drawn using the workspace of Maestro and were converted to 3D form for the docking studies. The collected ligands were prepared for docking. Then the prepared ligands were docked into the generated grid in the prepared protein. The best docked pose with lowest Glide score value was recorded for each ligand. Extra precision (XP) was performed using the module Induced Fit Docking of Schrödinger-Maestro v9.3 (2012). Best derivatives with good docking score were selected and their ADME properties were checked using QIKPROP which is a tool available in Schrodinger under Maestro. The Lipinski's rule of five and drug likeness analysis of selected derivatives are also calculated.

### 2.2.2 Procedure for synthesis:

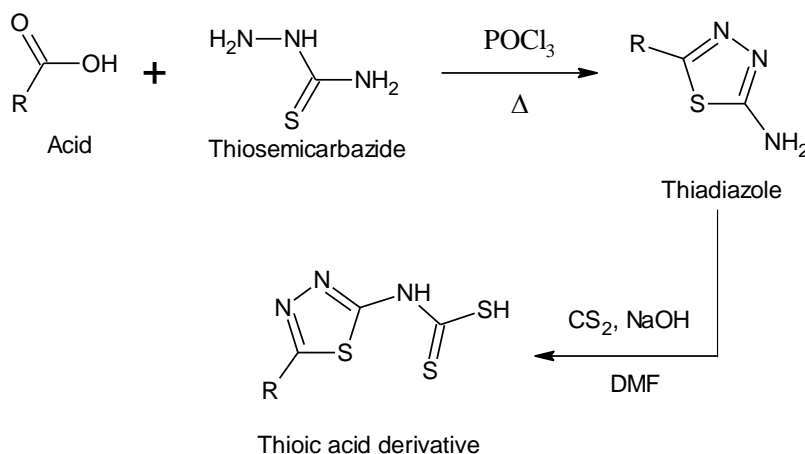
#### Synthesis of 1a, 2a, 3a:

A mixture of substituted acid [0.01mol] and thiosemicarbazide [0.01mol] in phosphorous oxychloride was refluxed for 5 hours. After cooling the solution, added 15ml of water. The reaction mixture was again refluxed for 2 hours. It was then cooled. The mixture was basified to pH 8-9 with aqueous potassium hydroxide. Stirred well and cooled in ice bath. The product [thiadiazole derivative] obtained was filtered, washed with water and re-crystallized from ethanol. Physicochemical properties of different thiadiazole derivatives are given in table 1.

#### Synthesis of 1b, 2b, 3b:

Equimolar mixture of thiadiazole derivatives and CS<sub>2</sub> in ethanolic KOH (50ml, 5%) and DMF (50 ml) was warmed for 3 hours and then cooled. The reaction mixture was acidified using dilute acetic acid. The resulting product was filtered and recrystallised from ethanol. Physicochemical properties of different dithioic acid derivatives are given in table 2.

#### Scheme:



Substituent details in synthesized compounds are given in table 3.

## 3. Results and Discussion

The current research revealed the significance of rational designing for the development of novel dithioic acid derivatives of the thiadiazole moieties from three natural acids. The newly synthesized dithioic acid derivatives were anticipated as promising leads having many biological activities. Keeping this view in mind, the new analogues were designed, synthesized and evaluated for anti-inflammatory activity.

#### *In silico* design:

#### Docking result:

Glide scores of designed analogues of dithioic acid derivatives were calculated. Docking scores of dithioic acid derivatives are given in table 4 and docking image of the ferulic acid derivative with the enzyme TACE is given in figure 1.

#### Analysis of Lipinski's rule of five:

**Lipinski's rule of the synthesized compound:** All the synthesized derivatives obey Lipinski's rule of five and the values are given in table 5.

#### Characterization:

**{5-[(*E*)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]1,3,4thiadiazol2yl}carbamodithioic acid (1b) :-** Yield 62%; M.P 221<sup>0</sup>C; IR: 1635.61 (C=N), 1549 (C=C), 2847 (OH), 1300(C-N), 3381 (N-H), 648 (C-S); <sup>1</sup>H NMR: 1.465-1.500 (s, 1H, SH), 8.450 (s, 1H, NH), 7.263-7.561 (m, 3H, Ar-H), 3.705 (s, 3H, OCH<sub>3</sub>), 4.611-4.644 (s, 1H, OH); MS: molecular ion peak is 325.6469 and base peak is 224.3041.

#### Anti-TNF activity:

The synthesised analogue (1b) was selected for anti-TNF activity by using Reverse Transcriptase Polymerised Chain Reaction (RT-PCR) method. RAW 264.7 cell lines (Mouse leukaemic monocyte macrophage cell line) was used for the study. 60% confluent cells were treated with the synthesized compound DT-1 (100mcg/ml) and incubated overnight. RNA was isolated using Trizol (Invitrogen) reagent as per manufacturer's

instruction and then the RT-PCR was carried out. The expression of the amplified cDNA of TNF- $\alpha$  was visualized using agarose gel electrophoresis method. The cells treated with the test compound DT-1 (100 $\mu$ g/ml) was ascertained to reduce the expression of TNF- $\alpha$  when compared to the cells treated with the control. The visualised DNA is shown in figure 2.

### Anti-inflammatory activity

#### *In vitro* anti-inflammatory activity by HRBC membrane stabilization method:

Different concentration of synthesized compound (100-500 $\mu$ g/ml), reference standard (25-500 $\mu$ g/ml) and control were separately mixed with 1ml of phosphate buffer, 2ml of hypo saline and 0.5ml of HRBC suspension. Diclofenac sodium (25-500 $\mu$ g/ml) was used as standard drug. All the assay mixtures were incubated at 37 $^{\circ}$ C for 30 minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm. The percentage haemolysis was estimated by assuming the haemolysis produced in the control as 100%. The membrane stabilization activity of all the 3 derivatives is given in the table 6, 7 and graph is shown in the figure 3 and 4.

$$\text{Percentage inhibition} = \frac{\text{Absorbance of the control} - \text{absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

#### *In vivo* anti-inflammatory activity by Carrageenan induced paw oedema in rats:

##### Experimental procedure:

Wistar albino rats weighed around 150 to 250 were used for this study. The initial right hind paw volume of the rats was measured using a plethysmometer. They were divided into 5 groups consists of 6 animals each.

**Group-1:** Served as negative control which received vehicle (Distilled water p.o).

**Group-2:** Served as positive control which received (carrageenan, s.c) only.

**Group-3:** Served as standard which received only Diclofenac sodium p.o

**Group-4:** Test group which has received the test compound 100 mg/kg p.o

**Group-5:** Test group which has received the test compound 200 mg/kg p.o

The 2 different groups were treated with the test compound solution (100, 200mg/kg) and control vehicle orally. Diclofenac was used as a Standard drug. After 30 min, the rats were challenged with intra plantar injection of 0.1 ml of 1% w/v solution of carrageenan into the sub plantar region of left paw. The paw was marked with ink at the level of lateral malleolus. The paw volume was measured at 0, 1, 2, 3, 4, 5,6 and 24 hr after carrageenan injection using a Plethysmograph. The difference between initial and subsequent reading gave the actual edema volume. The anti-inflammatory activity in animals that received the test compounds (100, 200mg/kg) and Diclofenac (25mg/kg) was compared with that of vehicle control groups. The percentage inhibition of edema was calculated as follows:

$$\text{Percentage inhibition of oedema} = 1 - Vt/Vc \times 100$$

Where Vt is the inflammatory increase in paw volume in drug - treated rats, Vc is the inflammatory increase in paw volume in control group of rats. The percentage inhibition of the rat paw oedema of compound 1b is given in the table 8 and graph is shown in the figure 5.

**Table 1: Physicochemical parameters of different thiadiazole derivatives**

R	Molecular formula	Mol. wt	Appearance	M.P	TLC system	% yield
1a	C <sub>11</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub> S	249	Brown	195-197 $^{\circ}$ C	Toluene: ethyl acetate	65
2a	C <sub>10</sub> H <sub>9</sub> N <sub>3</sub> S	203	Yellow	180-181 $^{\circ}$ C	Toluene: ethyl acetate	85
3a	C <sub>8</sub> H <sub>7</sub> N <sub>3</sub> OS	193	Yellow	186-188 $^{\circ}$ C	Hexane: ethyl acetate	70

**Table 2: Physicochemical parameters of different dithioic acid derivatives**

R	Molecular formula	Mol.wt	Appearance	M.P	TLC system	% yield
1b	C <sub>12</sub> H <sub>11</sub> S <sub>3</sub> O <sub>2</sub> N <sub>3</sub>	325.4	Brown	221-223 $^{\circ}$ C	Toluene: ethyl acetate	62
2b	C <sub>11</sub> H <sub>9</sub> S <sub>3</sub> N <sub>3</sub>	279.4	Yellow	210-213 $^{\circ}$ C	Toluene: ethyl acetate: chloroform	80
3b	C <sub>9</sub> H <sub>7</sub> OS <sub>3</sub> N <sub>3</sub>	269.3	Brownish Yellow	160-165 $^{\circ}$ C	Hexane: ethyl acetate	55

Table 3: Substituent details

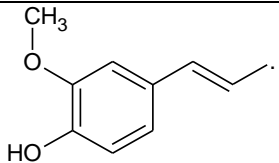
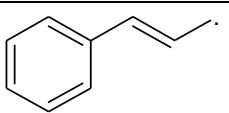
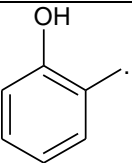
Substituent, R		
1	2	3
		

Table 4: Docking score of dithioic acid derivatives

Sl. No:	Compound	Docking score
1	1b	-8.1977
2	2b	-7.8681
3	3b	-6.5945

Table 5: Parameters and its values in Lipinski's rule of five

Compound	Log P	Mol. wt	nHDon	nHAcc	Nrotb	Lipinski rule alert index
1b	4.463	325.429	1	6	3	0
2b	4.522	279.404	0	7	3	0
3b	4.564	269.366	1	7.25	3	0

Table 6: Effect of the synthesized derivatives (Test) on membrane stabilization

Sl. No	Concentration (µg/ml)	%vinhibition		
		1b	2b	3b
1	Control	0	0	0
2	100	28.97	26.36	20.9
3	200	38.24	35.86	25.17
4	300	44.18	50.1	40.38
5	400	56.76	52.73	41.8
6	500	60.09	57.24	47.7

Table 7: Effect of various concentration of diclofenac sodium (standard) on membrane stabilization

Sl. No	Concentration (µg/ml)	% inhibition
1	Control	0
2	25	23.51
3	50	47.68
4	100	69.35
5	250	72.68
6	500	80.77

Table 8: The effects of compound 1b and diclofenac sodium on rat hind paw edema induced by carrageenan

Treatment	Dose (mg/kg)	Mean increase in paw volume (ml)				Percentage inhibition (%)			
		1hr	3hr	6hr	24hr	1hr	3hr	6hr	24hr
Group 1 (control)	-	0.1	0.12	0.1	0.11	-	-	-	-
Group 2 (positive control)		0.5533 ± 0.0307	0.733 ± 0.0301	0.58 ± 0.0339	0.5116 ± 0.0510				
Group 3 (standard)	10	0.2733 ± 0.0282***	0.2483 ± 0.0340**	0.18833 ± 0.0212**	0.1483 ± 0.0144**	52.37	68.06	67.25	65.96
Group 4	100	0.415 ± 0.02717**	0.545 ± 0.0286**	0.43166 ± 0.0241**	0.345 ± 0.0271**	25.46	26.39	25.87	31.54
Group 5	200	0.325 ± 0.0295***	0.4116 ± 0.03135**	0.29 ± 0.02556**	0.1933 ± 0.0324**	41.82	43.06	51.72	55.96

Fig 1: Docking image of TACE with compound 1b

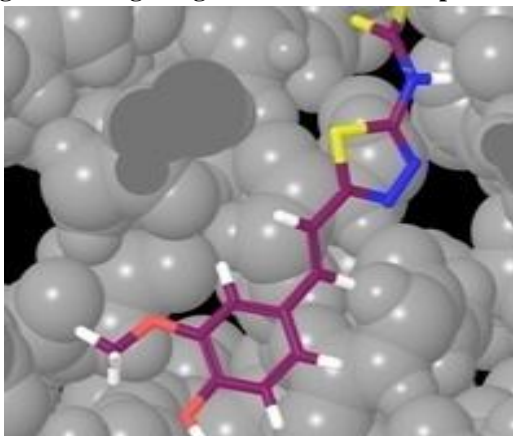


Fig 2: visualization of TNF-  $\alpha$  inhibition of compound 1b

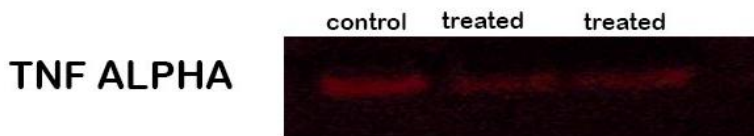


Fig 3: Effect of test compounds on membrane stabilization

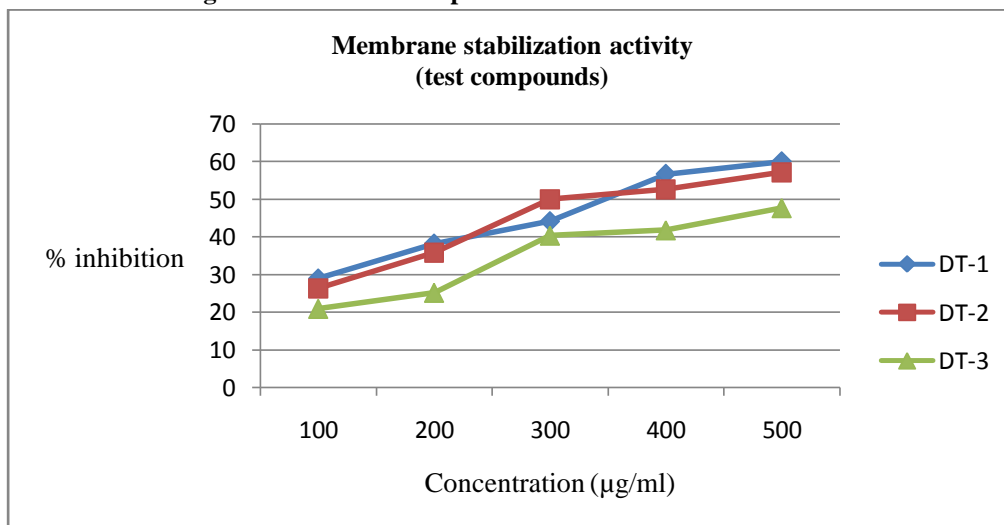
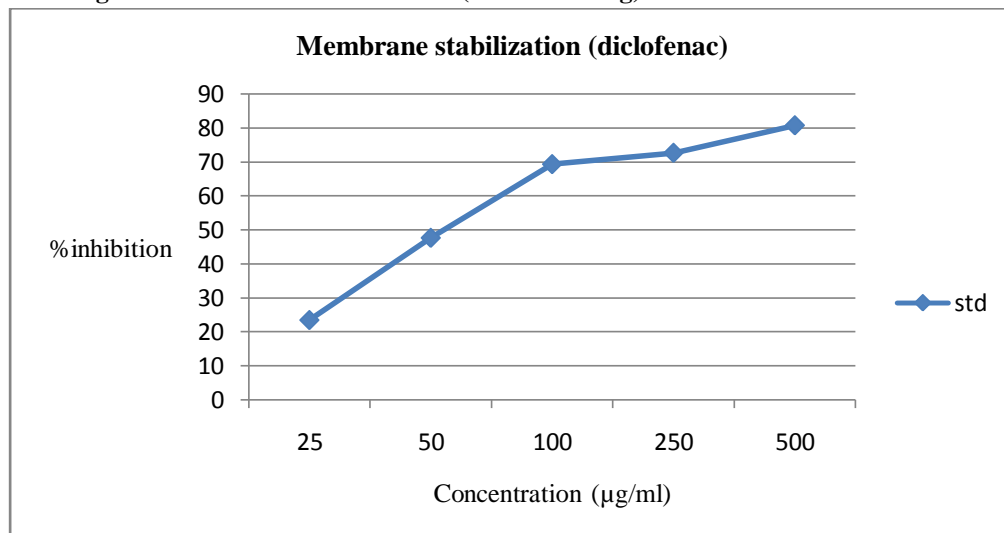
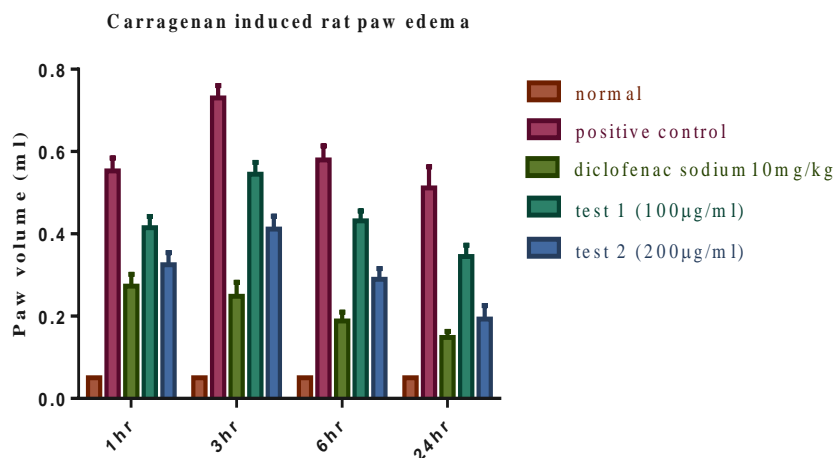


Fig 4: Effect of diclofenac sodium (standard drug) on membrane stabilization



**Fig 5: Effect of the test compound 1b on rat paws volume.**

#### 4. Conclusion

The research was focused on the systematic approach in design and development of dithioic acid derivatives for novel anti-inflammatory drugs having specific TNF- $\alpha$  activity. It involved the preliminary *in silico* designing of the various novel dithioic acid analogues for quantifying their drug likeness using Molinspiration software. The candidates which obeyed Lipinski's rule of 5 were taken for wet lab synthesis. Molecular docking experiments were carried out to identify potential drug candidates among the derivatives. From the results obtained, it may be concluded that the ferulic acid, cinnamic acid and salicylic acid analogues of dithioic acid showed good receptor binding with the selected targets. Three different compounds were synthesised and were characterised using different analytical techniques. Analogue which showed promising glide score i.e. ferulic acid derivative was screened for anti-TNF activity in comparison with the control and was found to be inhibiting the TNF- $\alpha$  expression. All the three synthesised compounds were analysed for *in vitro* and *in vivo* anti-inflammatory activity and the ferulic acid derivative was shown to possess markable activity when compared with the standard drug (diclofenac sodium). Compound 1b showed a maximum inhibition of 60.09% *in vitro* and 55.96% *in vivo*.

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