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EVALUATION OF PHARMACOLOGICAL ACTIONS OF DERIVATIVES OF 4-THIAZOLIDIONES AND 1,3,4- THIADIAZOLES

Shail Modi* and Gayatri Aggarwal

Vivekanand Education Society's College of Pharmacy, Mumabi – 400074.

***Corresponding Author: Shail Modi**

Vivekanand Education Society's College of Pharmacy, Mumabi, 400074.

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ABSTRACT

The project involves evaluation of pharmacological actions of derivatives of 4-thiazolidinone and 1, 3, 4-thiadiazoles. The derivatives were investigated for various activities such as inflammation, analgesia and some CNS activities such as CNS depressant, anti-psychotic and skeletal muscle relaxant activity. In-vitro test like membrane stabilization and protein denaturation and in-vivo activities such as mice paw edema, cotton pellet granuloma, forced swim test, locomotor activity were carried out to know the pharmacological activities of the test drugs. It was concluded that the derivatives had anti-inflammatory and analgesic property without development of ulcers. They also showed muscle relaxant property and depressant characteristics which could be helpful for sedation. Further investigations were still required for confirmation of CNS activities.

KEYWORDS: Inflammation, 4-thiazolidione, 1,3,4-thiadiazole, forced swim test, catalepsy.

INTRODUCTION

In recent years, synthesis of novel 4-Thiazolidinones and 1, 3, 4-thiadiazoles derivatives and its investigation have increased significantly. 4-thiazolidinone and 1, 3, 4-thiadiazoles are important class of compounds and have demonstrated various biological activities. 4-thiazolidinone derivatives are considered to be biologically active scaffold and possesses activities such as anti-inflammatory, analgesic, antimicrobial, anti-depressant, anti-tubercular, anti-viral (Jain et al., 2012). 1, 3, 4-thiadiazole is an another versatile biologically active scaffold which possesses activities like antimicrobial, anti-cancer, analgesic, anti-inflammatory, anti-tubercular, anti-viral activity (Jain et al., 2013).

Based on literature review and reported studies, the derivatives of 4-thiazolidinone and 1, 3, 4-thiadiazoles were investigated for its anti-inflammatory, analgesic and for few CNS activities in the current research. Currently NSAIDs are the most commonly used drugs for the treatment of acute and chronic inflammation, pain and fever. However, long-term usage causes side effects like gastrointestinal irritation, peptic ulcers, nephrotoxicity and bleeding(Becker et al., 2004). Inflammation is a fundamental, yet complex process designed to protect the human body by eliminating both the causes and consequences of injury, such as microbes, toxins etc. Inflammation is therefore the body's way of attempt for removal of primary cause of inflammation and any damage that may have occurred as a result of it (Granger and Senchenkova, 2010).

The derivatives were also screened for CNS depressant/anti-depressant activity, anti-psychotic and skeletal muscle relaxant activity. According to WHO, globally an estimated 340 million people are affected with depression (Bebbington, 2001). It is a mood disorder which is caused due to deficiency in the amount or function of nor-epinephrine, serotonin and dopamine. The current antidepressant therapy possesses various side effects like sedation, anticholinergic effects, orthostatis, weight gain, and sexual dysfunction (Craig et al, 1997).
The term psychosis refers to a variety of mental disorders like delusions, various types of hallucinations, disorganized thinking or violence. Schizophrenia is a particular kind of psychosis characterized by impaired behavioral function, and disturbance in thinking process. Around 1% of the world population is affected by schizophrenia the current therapy involves typical and atypical antipsychotics but there are side effects related to the medication such as sedation, anticholinergic effects, metabolic and endocrine effects, tardive dyskinesia and extrapyramidal reactions (Craig et al, 1997). Considering the adverse effects and patient response, there is a need for more efficacious and safer alternatives to the currently available drugs.

MATERIALS AND METHODS

1) Membrane stabilization activity

During inflammation, lysosomal enzymes are released which causes damage to the surrounding tissue and organelles, thereby triggering inflammation. The erythrocyte membrane stabilization method involves the

process in which the integrity of the erythrocyte membrane and lysosomal membrane is maintained by anti-inflammatory drugs by stabilizing the membrane. A variety of disorders are initiated by lysosomal enzymes released during inflammation and hence, stabilization of lysosomal membrane is important in limiting the inflammatory response. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane(Rani et al., 2014).

Preparation of standard solution: 100mg diclofenac sodium dissolved in distilled water and volume was made up to 100ml. It was diluted suitably to get a concentration of 10, 20, 40, 60, 80 and 100µg/ml.

Preparation of sample solution: Stock solutions were prepared by using 1% CMC solution in the form of suspension. From this stock solution different concentrations of 10, 20, 40, 60, 80 and 100µg/ml were prepared by using CMC solution as solvent.

Procedure: The reaction mixture (2ml) consisted of 1ml of test sample or standard (diclofenac) of different concentrations (10-100 µg/ml) and 1ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56^{o}C for 30mins. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of supernatant was taken at 560nm. The experiment was performed in triplicates for all the test samples (Leelaprakash and Mohan Dass, 2011).

Calculation

The percent inhibition of hemolysis was calculated using the following formula:

Percent inhibition = [(Acontrol – Asample)/Acontrol] X 100

Where, Acontrol is absorbance of control group. Asample is absorbance of the standard or test drugs.

2) Inhibition of Protein denaturation:

Protein denaturation is a process in which the proteins lose their tertiary and secondary structure by application of external stress or chemicals, such as strong acid or base, a concentrated inorganic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of protein is a well-documented cause of inflammation, and as a part of investigation the ability of the drug to inhibit protein denaturation was studied(Rani et al., 2014).

Preparation of standard solution: 100mg diclofenac sodium dissolved in distilled water and volume was made up to 100ml. It was diluted suitably to get a concentration of 10, 20, 40, 60, 80 and 100µg/ml.

Preparation of sample solution: Stock solutions were prepared by using 1% CMC solution in the form of suspension. From this stock solution different concentrations of 10, 20, 40, 60, 80 and 100µg/ml were prepared by using CMC solution as solvent.

Procedure: The reaction mixture (2ml) consisted of 1ml of test sample or standard (diclofenac) of different concentrations (10-100 µg/ml) and 1% aqueous solution of bovine albumin fraction, instead of test sample only saline was added to the control test tube. The test tubes were incubated at 37^{o}C for 20min and then heated to 51^{o}C for 20min. After cooling the test tubes, the absorbance of these solutions was determined by using spectrophotometer at a wavelength of 660nm (Leelaprakash and Mohan Dass, 2011).

Calculation

The percent inhibition of hemolysis was calculated using the following formula:Percent inhibition

= [(Acontrol – Asample)/Acontrol] X 100

Where, Acontrol is absorbance of control group. Asample is absorbance of the standard or test drugs.

In-vivo pharmacological investigations

All pharmacological investigations were carried on following groups consisting 6 animals per group of either sex at doses mentioned below.

Control: 1% Sodium CMC solution as vehicle.

Standards: Diclofenac (10mg/kg), Diazepam (2mg/kg), Haloperidol (2mg/kg), Clozapine (3mg/kg), levodopa (30mg/kg).

Test Drugs: Derivatives of 4-thaizolidinone and 1, 3, 4-thiadiazole were administered in 1/10th dose of LD 50 and half of 1/10th dose of LD 50.

Preparation of doses: All the test drugs were relatively insoluble in water. They were soluble in organic solvents but such solvents were avoided because they may produce the effects of their own. Thus solvent used for test substance was 1% sodium carboxy methyl cellulose (CMC) suspension in water. The doses were prepared by suspending the test drugs in 1% aqueous sodium CMC using a mortar and pestle.

3) Acute Toxicity Studies: OECD (Organisation for Economic Co-operation and Development) guideline 425 was followed for acute toxicity studies. The test procedure described in OECD 425 guideline helps in minimizing the number of animals required to estimate the acute oral toxicity of a drug.

Rationale for initial dose level selection

As no information was available to make a preliminary estimate of the LD50 and the slope of the dose-response curve, starting dose of 175 mg/kg was selected as per the OECD guideline 425. Dose progression factor: The default dose progression i.e. 1.75, 5.5, 17.5, 55, 175, 550, 1750 mg/kg was followed.

Procedure: Toxicity studies were conducted according to the OECD Guideline 425 using Up and Down procedure. Female albino mice weighing between 25-30 g were used in the study. They were housed in groups of three or four mice per cage under standard laboratory conditions for one week before the experiments .The housing conditions were maintained at controlled temperature (230C) and humidity (50%). They received standard diet and water. The animals were transferred to the experiment room 1 hour before the start of experiment. The test drugs were suspended using 1 % w/v sodium carboxymethyl cellulose and were administered orally with a starting dose of 175 mg/kg body weight. The animals were observed continuously for 2 hours and once in 30 min for every 4 hours till another 24 hours for any changes in their behavior, movements, gait, writhing reflex, etc. At the end of 48 hours following drug administration the mortality was calculated. The subsequent doses were then increased by a factor 3.2 if the dose was tolerated, and decreased by a factor 3.2 if it was lethal. One tenth of this dose (LD50) can be considered as the therapeutic dose for in-vivo evaluation (OECD guidelines 425).

4) Mice Paw Edema: Mice paw edema is a widely used screening technique for anti-inflammatory drugs. In this technique, the drugs are evaluated on their ability to inhibit edema which is induced by a phlogistic agent (carrageenan). The percentage reduction in edema volume by a particular drug indicates its anti-inflammatory activity.

Preparation of standard solution: Diclofenac sodium was suspended in 1% CMC solution.

Preparation of sample solution: The quantities of test drugs(A and B)* were suspended in 1% carboxyl methyl to get 1/10th dose of LD 50 and half of 1/10th dose of LD 50.

Procedure: Albino mice weighing between 25-30g were selected. Mice were fasted for 16 hours prior to dosing. Mice were divided into 6 groups of 6 animals each, namely test drugs (A and B of lower and 1/10th dose), Standard (diclofenac) and control. The paw edema was induced with 0.05 ml of 1% suspension of carrageenan in saline, 1 hour after administration of drugs or vehicle dosed orally, into sub-plantar portion of left hind paw. The paw was marked in order to immerge it always till the same extent. The paw volumes were measured plethysmographically immediately after injection, again at 0.5, 1, 3 and 6 h, and eventually 24 h after challenge(Posadas et al., 2004).

Calculation: Percent inhibition of inflammation were calculated using a formula, Percent inhibition= [Vc – Vt / Vc] X 100 Where, Vc is edema volume in control group. Vt is edema volume in groups treated with standard drug/ treated compound.

*Test drug A- Derivative of 4-thiazolidione
*Test drug B- Derivative of 1,3,4-thiadiazoles

5) Cotton Pellet induced granuloma:
In the cotton pellet induced granuloma model, a foreign body like cotton when implanted in sub-cutaneous layer of animal, causes proliferation of undifferentiated connective tissue around it, indicating state of inflammation. This model is an indication for the proliferative phase of inflammation. Inflammation involves proliferation of macrophages, neutrophils and fibroblasts, which are basic sources of granuloma formation. Hence, the decrease in the weight of granuloma indicates that the proliferative phase was effectively suppressed by the anti-inflammatory drug.

Preparation of standard solution: Diclofenac sodium was suspended in 1% CMC solution.

Preparation of sample solution: The quantities of different test drug (A and B) were suspended in 1% carboxyl methyl cellulose to get 1/10th dose of LD50 and half of 1/10th dose of LD50.

Procedure: Albino mice weighing between 25-30 g were selected. Mice were fasted for 16 hours prior to dosing. Mice were divided into 6 groups of 6 animals each, namely test drugs (A and B of lower and 1/10th dose), Standards (diclofenac) and control. Mice were anesthetized using ketamine and fur of their back was removed using sterile scissors. Subsequently, an incision was made in the lumbar region and with the help of a blunt forceps; subcutaneous tunnel was made on right side in the scapular region and autoclaved cotton pellets weighing 10±1 mg were implanted subcutaneously into the groin region of each mouse. The drugs or vehicle dosed orally for 7 consecutive days. On 8th day the animals were sacrificed by large doses of CO2 and the pellets together with the granuloma tissues were carefully removed, dried in an oven at 60^0 C weighed and compared with control. Increment in the weight of the pellets was taken as a measure of granuloma formation(Goyal et al., 2013).

Calculation: Percent inhibition of inflammation were calculated using a formula,
Percent inhibition= [Wc – Wt / Wc] X 100
Where, Wc is dry granuloma weight in control group. Weightt is dry granuloma weight in groups treated with standard/test drug.

6) Ulcerogenic Studies: Ulcerogenic potential of drug was studied for safety profile. The main side effect of non-steroidal anti-inflammatory drugs is their potential to produce gastric lesions. The ulcerogenic activity of NSAIDs is due to inhibition of COX enzyme responsible for the production of prostaglandins involved in the general housekeeping activities, e.g. maintenance of gastric mucosal integrity. The ulcerogenic potential of orally administered drugs is evaluated in fasted mice.

Procedure: Ulcerogenic activity was studied on the same animal used for cotton pellet granuloma method. The stomach was removed, gently washed with normal saline. The stomach was opened along the great curvature and was examined for severity of ulcer (Sabina et al., 2013).

Evaluation: The ulcerative index was calculated for each animal. For each stomach the mucosal damage was assessed according to the following scoring systems.

Table 1.1: Ulcerogenic potential

Observation	Score
No lesions	0
Hyperaemia	0.5
One or two lesions	1
Severe lesions	2
Mucosa full of lesions	4

Calculation: Ulcer index = Mean score of test – Mean score of control

In-vivo analgesic activity

7) Hot plate method: The hot plate method is used to evaluate analgesic activity. The paws of mice are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The hot plate consists of an electrically heated surface whose temperature is controlled at 55° to 56 °C. This can be a copper plate or a heated glass surface. The animals are placed on the hot plate and the time until either licking or jumping occurs is recorded by a stop-watch.

Preparation of standard solution: Diclofenac sodium was suspended in 1% CMC solution.

Preparation of sample solution: The quantities of different test drug (A and B) were suspended in 1% carboxyl methyl cellulose to get 1/10th dose of LD 50 and half of 1/10th dose of LD50.

Procedure: Albino mice weighing between 25-30g were selected. Mice were fasted overnight, prior to dosing. Mice were divided into 6 groups of 6 animals each, namely test drugs (A and B of lower and 1/10th dose), Standard (diclofenac) and control. Mice were placed on the hot plate and the temperature was maintained at 55±10C. A cut off time of 20 sec was set to prevent any injury to the tissues of the paws. The reaction time was recorded at 0min, 15mins, 30mins, 60mins and 120mins after the administration of drugs. The values were compared with standard drug, diclofenac sodium(Suresha et al., 2014).

Calculation: Maximum possible analgesia = [(Reaction time for treatment – reaction time for control)/ (20 sec – reaction time for control)] * 100

In-vivo CNS activities

8) Locomotor activity

Locomotor activity can be measured using actophotometer. It is use to evaluate the CNS depressant activity of the drug. The animals are placed individually for a period of 10 mins and their basal activity score is measured. The difference in the activity scores, before treatment and after treatment is recorded and percentage decrease in locomotor activity is calculated.

Preparation of standard solution: Diazepam was suspended in 1% CMC solution.

Preparation of sample solution: The quantities of different test drug (A and B) were suspended in 1% carboxyl methyl cellulose to get 1/10th dose of LD50 and half of 1/10th dose of LD50.

Procedure: Albino mice weighing between 25-30g were selected. Mice were fasted for 16 hours prior to dosing. Mice were divided into 6 groups of 6 animals each, namely test drugs (A and B of lower and 1/10th dose), Standard (diazepam) and control. Individual animals were placed in the instrument and basal activity score was recorded over a period of 10 mins. Each group was treated with the test drug or the standard respectively and the score was recorded after a period of 30 mins. Decreased activity score was taken as index of CNS depression(Sugumaran and Quine, 2008).

Calculation: Percent decrease = [(Activity time before – activity time after)/ activity time before] * 100

9) Forced swim test

The forced swim test is a behavioral test used for evaluation of efficacy of antidepressant drugs. The mice are forced to swim in an inescapable tank filled with water and their escape related mobility behavior is measured. This behavior reflects a state of despair which can be reduced by several agents which are therapeutically effective in human depression.

Preparation of standard solution: Haloperidol and Clozapine were suspended in 1% CMC solution.

Preparation of sample solution: The quantities of different test drug (A and B) were suspended in 1% carboxyl methyl cellulose to get 1/10th dose of LD 50 and half of 1/10th dose of LD50.

Procedure: Albino mice weighing between 25-30g were selected. Mice were fasted for 16 hours prior to dosing. Mice were divided into 7 groups of 6 animals each, namely test drugs (A and B of lower and 1/10th dose), Standards (haloperidol and clozapine) and control. Mice were placed in an inescapable transparent tank that was filled with water and their escape related mobility behavior was measured. A cylindrical tank constructed of transparent Plexiglas was used for conducting forced swim test in mice. The water level was maintained 15cm

from bottom. The animals were placed gently in the water for a period of 6 mins and movements were recorded. After the activity, the animals were removed and placed on a drying paper and were dried. The time that each mouse spends mobile was measured. The total amount of mobility time is subtracted from the total of 240 secs, said to be immobility time. The results were than compared with the control group(Can et al., 2011).
Calculation: Immobility time = 240 (secs) – Mobility time (secs)

10) Rota rod: Rota rod apparatus is used to assay the motor abilities of mice/rats. The ability of the mice to remain on a revolving rod helps to evaluate the skeletal muscle relaxation that is induced by the test drug. The mice are placed on horizontal bars which revolve at a set rpm (20-25rpms) and their fall off time is recorded.

Preparation of standard solution: Diazepam was suspended in 1% CMC solution.

Preparation of sample solution: The quantities of different test drug (A and B) were suspended in 1% carboxy methyl cellulose to get 1/10th dose of LD50 and half of 1/10th dose of LD50.

Procedure: Albino mice weighing between 25-30g were selected. Mice were fasted for 16 hours prior to dosing. Mice were divided into 6 groups of 6 animals each, namely test drugs (A and B of lower and 1/10th dose), Standard (dizepam) and control. Two animals at a time were placed on rod rotating at 20–25 rpm speed. The fall off time was recorded in all the groups before and 30 min after drug administration(Bhosale et al., 2011).

Calculation: Percent decrease = [(fall off time control – fall off time test)/ fall off time control] * 100

11) Haloperidol induced catalepsy
Catalepsy is defined as failure to correct an externally imposed, unusual posture over a prolonged period of time. Cataleptic symptoms are compared to Parkinson-like extrapyramidal side effects seen clinically with administration of neuroleptics. The present study aims at lowering a behavioral state called catalepsy induced by haloperidol by the test drugs(Salam et al., 2011).

Preparation of standard solution: Levodopa was suspended in 1% CMC solution.

Preparation of sample solution: The quantities of different test drug (A and B) were suspended in 1% carboxyl methyl cellulose to get 1/10th dose of LD50 and half of 1/10th dose of LD50.

Procedure: In this experiment, the mice were divided into six groups of six animals each. Group I (control) received haloperidol (0.5mg/kg, i.p). Group II were treated with standard, Levodopa (30mg/kg). Group III, IV, V and VI were treated with Drug A (100mg/kg and 200mg/kg b.w), and Drug B (100mg/kg and 200mg/kg b.w) respectively. The test drugs were given and after 30 mins haloperidol was administered and following procedure were carried out.

Table. 1.2: Procedure for haloperidol induced catalepsy.

Steps	Procedure	Score
Step 1	If the mice fails to move when kept on a platform.	A score of 0.5 is assigned if touched or pushed.
Step 2	Front paws of the mice were placed on 3cm high block.	If unable to correct posture in 15secs, 0.5 score assigned to each paw. Score added to above step.
Step 3	Front paws of the mice were placed on 9cm high block.	If unable to correct posture in 15secs, 1 score assigned to each paw. Score added to above steps.

Percent decrease in catalepsy score was determined(Remya et al., 2013).
Calculation: Cataleptic score = 0.5 + (0.5 * time in sec for 1st paw) + (0.5 * time in sec for 2nd paw) + (1 * time in sec for 1st paw) + (1 * time in sec for 1st paw).

RESULTS AND DISCUSSION

1) Membrane stabilization method

Table. 1.3: Effect of test drugs A, B and standard on membrane stabilization activity.

Sr.No	Concentrations (µg/ml)	% Inhibition of membrane lysis (Mean ± SEM)		
		Diclofenac Sodium	Drug A	Drug B
1	10	22.70 ± 0.020 [a]	25.68 ± 0.010 [a ns]	22.19 ± 0.021 [a ns]
2	20	35.19 ± 0.005 [a]	31.36 ± 0.016 [a ns]	29.89 ±0.025 [a ns]
3	40	37.82 ± 0.005 [a]	37.48 ± 0.028 [a ns]	35.97 ±0.020 [a ns]
4	60	44.59 ± 0.009 [a]	43.19 ± 0.009 [a ns]	39.02 ±0.028 [a ns]
5	80	46.68 ± 0.005 [a]	44.49 ± 0.004 [a ns]	43.80 ±0.024 [a ns]
6	100	50.03 ± 0.001 [a]	49.04 ± 0.005 [a ns]	48.49 ±0.016 [a ns]
All values are Mean ± SEM (n=6). Two way ANOVA followed by Dunnett's test, where ns≥0.05, when compared with standard and [a]P≤0.05, when compared with control (percent inhibition of control =0%)				

The membrane stabilization activity of test drugs A, B and diclofenac were performed at 10, 20, 40, 60, 80, 100 µg/ml. The percent inhibition of membrane lysis exhibited by drug A at 100ppm was found to be 49.04% and by drug B was found to be 48.49% at 100ppm. The percent inhibition of standard drug diclofenac was found to be 50.03% at 100ppm. The erythrocyte membrane is analogous to the lysosomal membrane and stabilization of it implies that the drug has the potential to stabilize the lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bacterial enzymes and proteases which causes further tissue inflammation and damage upon extracellular release(Leelaprakash and Mohan Dass, 2011). The lysosomal enzymes released during inflammation produce various disorders. Therefore, the drugs with membrane stabilizing property should offer protection of cell membrane against injurious agents and thereby exhibiting anti-inflammatory activity. Results in table 1.3 demonstrate membrane stabilization activity of 4-thiazaolidinone and 1, 3, 4-thiadiazole derivatives. The test drugs exhibited significant membrane stabilization activity when compared with control and standard drug diclofenac. Drug A exhibited 49.04% activity at 100ppm which was greater as compared to drug B which was found to be 48.49% at 100ppm. Diclofenac exhibited 50.03% activity at 100ppm. Thus the test drugs have the potential to be used as anti-inflammatory agents and were further subjected to *in-vivo* testing.

2) Inhibition of protein denaturation

Table. 1.4: Effect of test drugs A, B and standard on inhibition of protein denaturation activity.

Sr.No	Concentrations (µg/ml)	% Inhibition of protein denaturation (Mean ± SEM)		
		Diclofenac Sodium	Drug A	Drug B
1	10	28.32 ± 0.001 [a]	41.13 ± 0.001 [a *]	21.67 ± 0.002 [a ns]
2	20	34.72 ± 0.003 [a]	48.02 ± 0.001 [a *]	25.36 ± 0.002 [a ns]
3	40	37.19 ± 0.001 [a]	55.41 ± 0.000 [a **]	27.58 ± 0.000 [a ns]
4	60	39.40 ± 0.003 [a]	56.65 ± 0.001 [a **]	37.68 ± 0.001 [a ns]
5	80	42.11 ± 0.005 [a]	56.89 ± 0.001 [a *]	39.65 ± 0.004 [a ns]
6	100	50.24 ± 0.002 [a]	62.56 ± 0.001 [a *]	49.01 ± 0.002 [a ns]
All values are Mean ± SEM (n=6). Two way ANOVA followed by Dunnett's test, where ns≥0.05, *P≤0.05, **P≤0.001 when compared with standard [a]P≤0.05, when compared with control (percent inhibition of control =0%)				

The inhibition of protein denaturation activity of test drugs A, B and diclofenac were performed at 10, 20, 40, 60, 80, 100 µg/ml. Drug A at 100ppm exhibited 62.56% inhibition of protein denaturation and drug B exhibited 49.01% inhibition of protein denaturation at 100ppm. The percent inhibition of standard drug diclofenac was found to be 50.24% at 100ppm. Most of the biological proteins lose their biological function when they get denatured. By application of external stress or chemicals, such as strong acid or base or heat, proteins tend to lose their secondary or tertiary structure. Denaturation of protein is a well-documented cause of inflammation. Production of auto-antigens in certain anti-inflammatory diseases like arthiritic disease may be due to denaturation of protein. The mechanism of denaturation involves alteration in electrostatic hydrogen, hydrophobic and disulphide bonding(Rani et al., 2014). Result in table 1.4 demonstrates inhibition of protein denaturation activity of 4-thiazaolidinone and 1, 3, 4-thiadiazole derivatives. The test drugs exhibited significant activity when compared with control and standard drug diclofenac. Drug A exhibited 62.56% activity at 100ppm which was highest as compared to drug B which was found to be 49.01% at 100ppm. Percent inhibition of diclofenac was found to be 50.24% at 100ppm. Drug A was found to be more active as compared to the standard. With increase in concentration there was increase in percent inhibition, the drugs are concentration dependent. It can be stated that drug A is capable of inhibiting denaturation of proteins. Thus the test drugs have the potential to be used as anti-inflammatory agents and were further subjected to *in-vivo* testing.

3) Acute toxicity studies

Table 1.5: Results of acute toxicity studies

Test Drugs	Lower (mg/kg)	Higher (mg/kg)
A	100	200
B	100	200

RESULT

Table 1.5 demonstrates the acute toxicity results of test drugs A and B. The maximum toxic dose was found to be 2000mg/kg for both the drugs. The $1/10^{th}$ (higher) dose was found to be 200mg/kg for both the drugs and half of $1/10^{th}$ (lower) dose was found to be 100mg/kg for both the drugs. Acute toxicity studies were determined for *in-vivo* studies according to OECD guidelines 425. LD_{50} for *in-vivo* studies was considered as 2000 mg/kg. Table 4.3 demonstrates the $1/10^{th}$ and half of $1/10^{th}$ value of LD_{50}.

***In-vivo* anti-inflammatory activity**

6) Mice paw edema activity

Table. 1.6: Effect of test drug A, B and standard on mice paw edema activity.

Time (hrs)	% Decrease in paw edema (Mean ± SEM)				
	Diclofenac Sodium	Drug A(lower)	Drug A(higher)	Drug B(lower)	Drug B(higher)
0	13.04 ± 0.00[a]	9.09 ± 0.002[ns a]	9.09 ± 0.002 [ns a]	9.09 ± 0.002 [ns a]	13.04 ± 0.00[ns a]
0.5	27.27 ± 0.00 [a]	13.04 ± 0.00 [ns a]	13.04 ± 0.00 [ns a]	13.04 ± 0.00 [ns a]	27.27 ± 0.00[ns a]
1	68.57 ± 0.00 [a]	28.57 ± 0.00 [ns a]	28.57 ± 0.00 [ns a]	28.57 ± 0.00 [ns a]	28.57 ± 0.00 [ns a]
3	80.00 ± 0.00 [a]	50.00 ± 0.00 [ns a]	50.00 ± 0.00 [ns a]	50.00 ± 0.00 [ns a]	50.00 ± 0.00 [ns a]
6	66.67 ± 0.00 [a]	16.67 ± 0.00 [ns a]	54.16 ± 0.00 [ns a]	16.67 ± 0.00 [ns a]	50.00 ± 0.00 [ns a]
9	66.67 ± 0.00 [a]	16.67 ± 0.00 [ns a]	54.16 ± 0.00 [ns a]	16.67 ± 0.00 [ns a]	50.00 ± 0.002 [ns a]
24	66.67 ± 0.002 [a]	16.67 ± 0.00 [ns a]	66.67 ± 0.00 [ns a]	16.67 ± 0.00 [ns a]	54.16 ± 0.00 [ns a]
All values are Mean ± SEM (n=6). Two way ANOVA followed by Dunnett's test, where ns≥0.05, *P≤0.05, when compared with standard [a]P≤0.05, when compared with control (percent inhibition of control =0%)					

The results from Table 1.6 demonstrated comparison of lower and higher doses of drug A and B with standard diclofenac. It was observed that the maximum decrease in paw edema was obtained at three hours with respect to diclofenac (80%), drug A at lower dose (50%) and drug B (50%) at lower dose. The activity of drug A (66.67%) at higher concentration and drug B (54.16%) at higher concentration increased upto 24hrs. The mice paw edema method is a suitable test for evaluating anti-inflammatory drugs. Mice paw edema method is a method which is evaluated for acute phase of inflammation. The edema is induced by a phlogistic agent, in this case by carrageenan. The method is based on the inhibition of swelling of paw induced by carrageenan. The percentage reduction in edema volume by a particular drug indicates its anti-inflammatory activity. This model is based on principle release of various inflammatory mediators by carrageenan. Carrageenan induced paw edema is biphasic event. In the first phase there are incidences like release of histamine, serotonin and kinins which lasts for one hour, whereas the second phase of edema is imposed by release of prostaglandins, protease and lysosome. The second phase lasts for 2-4 hours after injection. The test drugs were studied at two dose levels i.e. at a higher and a lower dose level(Posadas et al., 2004). The results as in table 4.4 demonstrated that there was gradual increase in edema paw volume till 3 hours after carrageenan challenge in the control group. While the test drugs showed inhibition of edema formation in first as well as second phase. It was seen that drug A at higher dose level showed better activity as compared to drug B. Drug A at higher dose exhibited 66.67% activity upto 24hours. Thus it can be suggested that the drugs exhibit anti-inflammatory property in acute phase of inflammation and it may be associated with inhibition of some inflammatory mediators such as histamine, serotonins etc.

7) Cotton pellet induced granuloma activity

Table. 1.7: Effect of drug A, B and standard on cotton pellet induced granuloma activity.

% Decrease in weight of cotton pellet (Mean ± SEM)				
Diclofenac Sodium	Drug A(lower)	Drug A(higher)	Drug B(lower)	Drug B(higher)
43.36 ± 0.91[a]	18.58 ± 1.58[** a]	38.05 ± 1.70[ns a]	17.69 ± 1.58 [**a]	30.09 ± 1.70 [ns a]
All values are Mean ± SEM (n=6). One way ANOVA followed by Dunnett's test, where ns≥0.05, **P≤0.01, when compared with standard and [a]p≤0.05, when compared with control. (percent inhibition of control =0%, one way ANOVA followed by Dunnett's test)				

The results in table 1.7 demonstrated the comparison of lower and higher doses of drug A and B with standard diclofenac. Higher doses of both drugs A and B exhibited greater activity as compared to the lower doses of drug A and B respectively. Drug A at higher dose exhibited 38.05% activity while drug B at higher dose exhibited 30.08% activity. Standard drug diclofenac exhibited 43.26% activity. The cotton pellet-induced granuloma method is a well-known model to screen the anti-inflammatory activity in the chronic phase of inflammation. It is widely used to assess the transudative, exudative and proliferative components of chronic inflammation. The moist weight of the pellets correlates with transude, the dry weight of the pellet correlates with the amount of granulomatous tissue formed. The transudative phase is defined as increase in net weight of the pellet which occurs during first three hours. The exudative phase occurs between 3 to 72 hours after implanting the pellet and proliferative phase occurs between three and six days (Anosike et al., 2012). The results as seen from table 4.5 demonstrated that anti-inflammatory activity was indicated by a significant reduction in the granuloma tissue formation. It was found that drug A at higher dose level was found to be more active as compared to drug B. Activity was found to be 38.05% for drug A and for drug B it was found to be 30.09%. The standard drug, diclofenac gave an activity of 43.36%. The decrease in granuloma weight indicates the suppression of proliferative phase. Proliferation of macrophages, neutrophils and fibroblasts are the basic

sources of granuloma formation. Cotton pellet induced granuloma method is based on the principle of implantation of a foreign body like cotton pellet subcutaneously in animals and the ability of the drugs to inhibit the increase in number of fibroblasts and synthesis of collagen during granuloma tissue formation(Goyal et al., 2013).

8) Ulcerogenic Activity

Table. 1.8: Evaluation of ulcerogenicity.

Test Groups	Ulcer score	Ulcer index
Control	-	-
Diclofenac	0.5	0.5 ±0.0
Drug A (lower)	-	-
Drug A (higher)	-	-
Drug B (lower)	-	-
Drug B (higher)	-	-

It was observed that standard drug diclofenac showed ulcer score of 0.5 which indicated hyperaremia and the test drugs did not show any ulceration as compared to diclofenac.Anti-inflammatory drugs exhibit their activity through the inhibition of an enzyme cyclooxygenase (COX) which is responsible for production of prostaglandin synthesis. Prostaglandins play an important role in protection of mucosa from injury induced by irritants. They play a role in regulation of mucus and bicarbonate secretion, mucosal blood flow, proliferation of epithelial cells and modulation of mucosal immunocyte function. Due to the ability of the anti-inflammatory drugs to inhibit prostaglandin synthesis they are responsible for causing gastric damage(Wallace, 2000). The main side effect of NSAID's is to produce gastric lesions; hence the ulcerogenic potential of such drugs is studied for safety profile. The mice are treated with the drugs and are sacrificed after a predetermined time interval. The stomachs are removed and inspected for irritation and ulcers. The ulcerogenic potential is scored along the scale, 0: no lesions, 0.5: hyperaemia, 1: one or two lesions, 2: severe lesions, 4: mucosa full of lesion(Sabina et al., 2013). The results from table 1.8 demonstrate that the test drugs A and B did not show any ulcerogenic potential at the studied dose over a given period of time.

9) *In-vivo* analgesic activity: Hot plate method

Table 1.9: Effect of drug A, B and standard on hot plate method

Time (mins)	% Increase in reaction time (Mean ± SEM)				
	Diclofenac Sodium	Drug A(lower)	Drug A(higher)	Drug B(lower)	Drug B(higher)
30	19.04 ± 0.288 [a]	23.80 ± 0.577 [a ns]	23.80 ± 0.577 [a ns]	23.80 ± 0.577 [ns a]	28.57 ± 0.288 [ns a]
45	34.78 ± 0.288 [a]	39.13 ± 0.577 [a ns]	45.83 ± 0.288 [a ns]	33.33 ± 0.577 [ns a]	39.13 ± 0.577 [ns a]
60	37.50 ± 0.866 [a]	45.83 ± 0.288 [a ns]	47.82 ± 0.577 [a ns]	34.78 ± 0.288 [ns a]	50.00 ± 0.577 [ns a]
120	33.33 ±1.154 [a]	38.09 ± 0.288 [a ns]	42.85 ± 1.154 [a ns]	19.04 ± 0.866 [ns a]	38.09± 0.866 [ns a]
180	22.72 ± 0.866 [a]	27.27 ± 0.577 [a ns]	27.27 ± 0.577 [a ns]	18.18 ± 0.00 [ns a]	31.81± 0.288 [ns a]

All values are Mean ± SEM (n=6). Two way ANOVA followed by Dunnett's test, where ns≥0.05, when compared with standard and [a]p≤0.05, when compared with control. (percent inhibition of control =0%, two way ANOVA followed by Dunnett's test)

The result in Table 1.9 demonstrates the comparison of lower and higher doses of drug A and B with standard drug diclofenac. The maximum activity was seen till 60 mins which was exhibited by drug B at higher dose and was found to be 50% as compared to diclofenac which was 37.50%. The activity of drug A at higher dose at 60 mins was found to be 47.82%. The hot plate method is a technique to measure central pain. The hot plate consists of an electrically heated surface whose temperature is controlled at 55° to 56 °C. The plate can be either copper plate or a heated glass surface. The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The animals respond to pain either by jumping or withdrawal of the paws or licking of the paws. The results from table 4.7 demonstrate the analgesic effect via hot plate method of 4-thiazolidinone and 1, 3, 4-thiadiazole derivatives. There was increase in reaction time of mice in this study. There was an increase in activity till 60 mins and drug B at higher concentration exhibited maximum activity of 50%. The activity of the standard drug diclofenac was found to be 37.50 at 60 mins. Hence, the results indicate that the test drugs have the potential to be used as analgesics and act on the CNS component of pain.

In-vivo CNS activities

10) Locomotor activity

Table 1.10: Effect of drug A, B and standard on locomotor activity

% Decrease in locomotor score (Mean ± SEM)			
Groups	**Locomotor Activity**		**% Decrease in activity**
	Pre-treatment	**Post-treatment (after 30 mins)**	
Diazepam	719.25 ± 53.10	306.25 ± 24.19	57.38%
Drug A (Lower)	733.75 ± 81.69	564.75 ± 77.40****	23.14%
Drug A (Higher)	826.50 ± 27.24	620.25 ± 21.12***	24.70%
Drug B (Lower)	789.75 ± 60.39	584.00 ± 35.67***	25.68%
Drug B (Higher)	697.75 ± 72.94	504.5 ± 72.70***	28.41%
All values are Mean ± SEM (n=6). One way ANOVA followed by Dunnett's test, where, ***P≤0.001, ****P≤0.0001 when compared with standard.			

The result in Table 1.10 demonstrates the comparison of lower and higher of drugs A and B with standard drug diazepam. Drug A exhibited 24.70% decrease in locomotor activity while by drug B exhibited 28.41% decrease in locomotor activity. The standard diazepam exhibited 57.38% decrease in locomotor activity. Locomotor activity can be studied using actophotometer. Actophotometer is a rectangular box which contains photocells and photo sensors. Every time the animal passes through the beam of light, score is recorded. Decrease in locomotor activity is used as a measure of CNS depression. Decrease in activity score indicates depressant or sedative effect. The results from table 4.8 demonstrate the locomotor activity of 4-thiazolidinone and 1, 3, 4-thiadiazole derivatives. Drug B at higher dose was found to have greater activity as compared to drug A. Drug B exhibited 28.41% decrease in locomotor activity which was comparable to the standard drug diazepam, which was found to be 57.38%. Locomotor activity is a measure of CNS depression; both the drugs produced a decrease in the motor activity which attributes to the CNS depression activity. Drug B is a derivative of 1, 3, 4-thiadiazole and has better activity as compared to drug A. The possible reason could be the structural similarity with the standard drug diazepam. Also, drug B has more log P value as compared to drug A, i.e. it can penetrate blood brain barrier.

11) Forced Swim Test

Table 1.11: Effect of drug A, B, standard and control on forced swim test.

Groups	**Immobility time (Mean ± SEM)**
Control	13.75 ± 1.75
Standard (Haloperidol)	211.5 ± 4.56^{a}
Standard (Clozapine)	66.00 ± 8.66^{a}
Drug A (Lower)	183.25 ± 6.60$^{*b\,h\,a}$
Drug A (Higher)	206.5 ± 4.78$^{*b\,a}$
Drug B (Lower)	170.5 ± 6.06$^{*b\,h\,a}$
Drug B (Higher)	205.0 ± 4.56$^{*b\,a}$
All values are Mean ± SEM (n=6). One way ANOVA followed by Dunnett's test, where, ^{*b}P≤0.05 when compared to standard clozapine, and ^{h}P≤0.05 when compared to standard haloperidol and ^{a}P≤0.05 when compared to control.	

The result in Table 1.11 demonstrates the comparison of lower and higher of drugs A and B with standard drugs haloperidol and cloazapine. The immobility time for drug A at lower dose was found to be at 183.25secs and immobility time of drug B at lower dose was found to be 170.5ecs. The immobility time for drug A at higher dose was found to be 206.5secs and drug B at lower dose was found to be 205secs. The immobility time for clozapine was found to be 66.0secs and for haloperidol it was found to be 211.5secs. The forced swim test is used as a model to screen anti-depressant drugs. The forced swim test is based on the observation that rats or mice when forced to swim or suspended in a restricted space from which there is no possibility of an escape, eventually cease to struggle, surrendering themselves (despair or helplessness) to the experimental conditions. This suggested that this helplessness or despair behavior reflected a state of lowered mood in laboratory animals and could serve as a valuable test for screening antidepressant drugs. The animals are placed for duration of 6 mins, the first two mins are not considered because of the fact that most mice are very active at the beginning of the FST, and the potential effects of the treatment can be obscured during the first two minutes. Hence the last four mintues are used to evaluate the forced swim test(Patil et al., 2013). Also, forced swim test model has the capacity to differentiate between typical and atypical anti-psychotic drugs. It is seen that the negative symptoms of psychosis overlap with depressive symptoms and atypical antipsychotics display a superior efficacy in the treatment of negative symptoms as compared to the typical antipsychotics. This difference between the two classes of drug helps to differentiate via force swim test(Weiner et al., 2000). The results from table 4.9 demonstrate the immobility time of 4-thiazolidinone and 1, 3, 4-thiadiazole derivatives. Drug B at higher concentration had greater activity as compared to drug A. The immobility time of drug B was found to be 205.0secs and at lower concentration it was found to be 170.5secs. Drug A at higher concentration exhibited immobility time of 183.25 secs while at lower concentration was found to have 204.5 secs. Two standards have been, haloperidol and clozapine, the first one is typical antipsychotic while the latter is atypical. Immobility time is a measure of force swim test, which is a test to screen antidepressant drugs. It was observed that

haloperidol increased immobility time while clozapine decreased immobility time. The immobility time for haloperidol was found to be 211.5secs while for clozapine it was 66secs, indicating that clozapine is better in controlling negative symptoms of psychosis as compared to haloperidol. Drug B has structural similarity with haloperidol and also it can penetrate blood brain barrier. Drug B also increased immobility time like haloperidol. This indicates that both drug A and B lack anti-depressant activity. The result is also supported by the findings that the drugs decreased locomotor score and thus have CNS depressant activity.

12) Rota-rod activity

Table1.12: Effects of drug A, B and standard on Rota- rod activity.

Groups	% Decrease in fall off time (Mean ± SEM)
Standard (Diazepam)	$56.49 \pm 5.77^{*}$
Drug A (Lower)	$38.13 \pm 6.92^{*}$
Drug A (Higher)	$39.04 \pm 2.88^{*}$
Drug B (Lower)	$39.96 \pm 6.06^{*}$
Drug B (Higher)	45.17 ± 4.61^{ns}
All values are Mean ± SEM (n=6). One way ANOVA followed by Dunnett's test, where ns≥0.05, *P≤0.05, when compared with standard.	

The result in Table 1.12 demonstrates the comparison of lower and higher doses of drugs A and B with standard drug diazepam. Test drug A exhibited activity of 39.04% while test drug B exhibited activity of 45.17%. The activity exhibited by standard drug, diazepam was found to be 56.49%.

13) Haloperidol induced catalepsy

Table 1.13: Effects of drug A, B, standard and control on haloperidol induced catalepsy.

Time (mins)	Cataleptic Score (Mean ± SEM)					
	Control	Standard	Drug A (lower)	Drug A (higher)	Drug B (lower)	Drug B (higher)
30	235 ± 4.13	105.2 ± 2.34^{a}	$145 \pm 3.23^{ns\ a}$	$132 \pm 2.13^{ns\ a}$	$165 \pm 3.23^{ns\ a}$	$108 \pm 1.63^{ns\ a}$
60	215 ± 2.13	98.5 ± 1.20^{a}	$110 \pm 2.13^{ns\ a}$	$116 \pm 3.03^{ns\ a}$	$142 \pm 3.45^{ns\ a}$	$92 \pm 1.20^{ns\ a}$
120	198 ± 4.18	59.8 ± 3.45^{a}	$87 \pm 2.12^{ns\ a}$	$96 \pm 1.24^{ns\ a}$	$98 \pm 1.20^{ns\ a}$	$86 \pm 2.14^{ns\ a}$
All values are Mean ± SEM (n=6). Two way ANOVA followed by Dunnett's test, where ns≥0.05, *P≤0.05, when compared with standard and ^{a}P≤0.05 when compared to control.						

The result in Table 1.13 demonstrates the comparison of lower and higher doses of drugs A and B with standard drug levodopa. The cataleptic score was observed to decline with increase in time. At 120mins, the cataleptic score of drug A at higher dose was found to be 96 while the cataleptic score of drug B at higher dose was found to be 86. Standard drug levodopa had cataleptic score of 59.8 at 120 mins. Haloperidol is an antipsychotic drug, which is used in the treatment of schizophrenia and other psychotic disorders. However, haloperidol induces a behavioral state known as catalepsy. Catalepsy is defined as failure to correct an externally imposed, unusual posture over a prolonged period of time. Cataleptic symptoms are compared to Parkinson-like extrapyramidal side effects seen clinically with administration of neuroleptics. Decrease in catalepsy is correlated to antipsychotic activity. The results from table 1.13 demonstrated the cataleptic score of 4-thiazolidinone and 1, 3, 4-thiadiazole derivatives. Drug B at higher concentration exhibited greater decrease in cataleptic score as compared to drug A. There was a decrease in the cataleptic score with increase in time. It was observed that the test drugs were able to decrease the catalepsy induced by haloperidol, thus indicating potential anti-psychotic activity. The forced swim test indicates lack of antidepressant activity, thus the drugs may tend to behave like typical antipsychotics.

CONCLUSION

The test drugs A and B belong to the family of 4-thiazolinone and 1, 3, 4-thaidiazloes respectively. The *in-vitro* anti-inflammatory activity was found to be dose dependent and the drugs exhibited activity comparable to standard drug diclofenac. The test drugs have potential to be anti-inflammatory agents. Based on acute toxicity studies, LD_{50} was calculated and used for further *in-vivo* testing. Test drug A exhibited higher *in-vivo* anti-inflammatory activity as compared to drug B. The test drugs were found to have no ulcerogenic potential at the given dose. The test drugs were able to manage pain which was tested using hot plate method and could be potential analgesics. Test drug B was found more active in *in-vivo* CNS activities as compared to drug A. Drug B is capable of crossing lipophilic barrier such as blood brain barrier due to its high log P value. Also, it was found to have structural similarities with the standard drugs like diazepam and haloperidol. The test drugs had CNS depressant, anti-psychotic and skeletal muscle relaxant activity as per the results.

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