Neuroprotective effect of 1, 2, 4- triazine Analogue of Diclofenac on Chlorpromazine induced Parkinson's in Rat

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ABSTRACT:
Parkinson's disease (PD) is a common neurodegenerative disorder, which is characterized by slowly progressive loss of dopaminergic neurons associated with substantial. Twenty-four Albino Wistar rats were randomly divided into four groups of six per each. Group I was set as a vehicle control received 1% Gum acacia solution *p. o.*, Group II-IV treated with Chlorpromazine (3mg/kg/day, *i. p*). for 14 days to induced Parkinson's. Group-II set as negative control. Group III set as standard drug treated group received Diclofenac (10mg/kg/day, *p. o)*. Group IV received with 1, 2, 4-Triazin analogue of diclofenac at (30mg/kg, *p. o)*, for 14 days. All animals were tested on the behavioral activity of rat tests in a familiar environment and were monitored on the 0^{th}, 7^{th}, and 14^{th} day after 30 minutes of drug treatment. On day 14, the selected animals were sacrificed by decapitation and the stomach was isolated for morphological analysis and brain isolated for further antioxidant, neurochemical analysis, and histopathological studies. The treatment groups showed significant increase in locomotion action, muscle coordination, cognitive performance, and dopamine level; also decrease muscle rigidity, oxidative stress, and cholinergic over activity when compared to the disease control rats. Results of our study concluded that 1, 2, 4-Triazin analogue of diclofenac as potential target for Parkinson's disease with minimal GI side effects.

Keywords: Chlorpromazine, GI-toxicity, Parkinson's disease, 1, 2, 4 –Triazin analogue of Diclofenac

INTRODUCTION

Neurodegenerative disease (ND) refers to the selective loss of neurons and the distinct involvement of functional systems within the central nervous system (CNS) [1]. The term "neurodegeneration" etymologically combines the prefix "neuro," referring to nerve cells, and "degeneration," which signifies the loss of structure or function in tissues or organs [2]. ND is considered multifactorial, with genetic, environmental, and endogenous factors, including aging, playing a role [3]. These diseases result in the progressive loss of neurons in the CNS, affecting critical brain functions such as movement disorders, speech, cognitive impairment, dysautonomia, and memory [4]. ND encompasses conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS), and Multiple System Atrophy (MSA) [5].

The etiology and pathogenesis of these diseases are primarily related to neurochemistry and synaptic transmission. The selective vulnerability of neurons in specific areas is a key factor in understanding the pathogenesis, alongside the genetic regulation of neuronal development and the impact of endogenous and exogenous neurotoxins [6]. These pathological conditions stem from the slow, progressive, and irreversible dysfunction and loss of neurons and synapses in targeted regions of the nervous system [7]. Cellular and sub-cellular pathology involves whether

neurons or glial cells (such as astrocytes and oligodendrocytes) show pathological protein deposits and whether these deposits are intracellular or extracellular [8].

The mechanisms behind neurodegeneration include genetic mutations, protein misfolding, proteinopathies, neuronal cell membrane damage, mitochondrial dysfunction, defects in axonal transport, and apoptosis (programmed cell death). Clinical observations and epidemiological studies have implicated age, environmental toxins, and genetics as significant contributing factors [9].

Parkinson's disease (PD) is a common, slowly progressive neurodegenerative disorder, which currently has no cure. It affects a wide range of motor and non-motor functions, leading to significant disability in later stages [10]. The disease is mainly characterized by a diminished facial expression, stooped posture, slowness of voluntary movement, festinating gait (shortened, accelerated steps), rigidity, and a pill-rolling tremor [11]. Clinically, PD is associated with resting tremor, postural instability, rigidity, bradykinesia (slowness of movement), and impaired postural balance, resulting in gait disturbances and frequent falls [12]. The understanding of the etiology of PD has evolved, with the realization that both environmental and genetic factors contribute to its onset. Increasingly, genetic predisposition is seen as a major contributor to the disease's underlying cause [13].

This prospective study explores the potential of a potent, selective, and orally efficacious 1,2,4-triazin analogue of diclofenac as a ligand to improve dopamine levels in PD. Based on previous studies, this 1,2,4-triazin analogue of diclofenac has desirable physicochemical and drug-like properties, including high oral bioavailability and potent in vivo efficacy. Preclinical results show its potential to improve motor deficits in chlorpromazine-induced animal models of Parkinson's disease.

MATERIALS AND METHODS

Chlorpromazine (Suri Pharma Laboratories Ltd, Gujarat) and diclofenac (Novartis Pvt. Ltd, Hyderabad) were both sourced from a local pharmacy. These drugs were freshly prepared daily by dissolving them in a 1% gum acacia solution in distilled water, creating a suspension that was administered orally (p.o.). The analytical grade chemicals used in this experiment are equivalent to American Chemical Society (ACS) grade and are acceptable for drug and chemical use, making them suitable for the purposes of this study.

Experimental animals

Healthy adult male Albino Wister Rats (270-300gm) and Female Swiss Albino Mice (25-30 gm) will be used for the present study. They are housed in polypropylene cages and maintained in an air-conditioned well-ventilated animal house with standard lab conditions at temperature (25±2ºC), humidity (50±15%) under a 12-hour light/dark cycle and were obtained from the central animal house of Swamy Vivekanandha College of Pharmacy. They are fed with a standard pellet diet and water *ad libitium*. All animals used in this study were handled with humane care in compliance with the guidelines prescribed by the Committee for control and supervision on experiments on animals (CPCSEA). IAEC Reference No: SVCP/IAEC/PG/1/02/2017.

Acute oral toxicity studies (OECD 423 guidelines)

As per the OECD Test Guideline 423, the substance is tested using a stepwise procedure, with each step involving three animals of a single sex (usually females). Animals should be fasted before dosing, with food withheld for 3-4 hours, but water should remain available. After the fasting period, the animals are weighed, and the test substance is administered. Following the administration, food may be withheld for an additional 1-2 hours in mice. Three animals are used for each step. The starting dose level is chosen from one of four fixed levels: 5, 50, 300, or 2000 mg/kg body weight. In cases where there is no prior information on the substance being tested, a starting dose of 50 mg/kg body weight is recommended for animal welfare reasons. After dosing, animals are observed individually at least once during the first 30 minutes, periodically during the first 24 hours (with special attention during the first 4 hours), and daily thereafter for a total of 14 days. Additional observations are needed if the animals continue to show signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, as well as the cardiovascular system, central nervous system, autonomic nervous system, and gastrointestinal tract [14-16]. By the 14th day of observation, if the animals show no side effects, the 1,2,4-triazin analogue of diclofenac was selected for study at a dose of 1/10 of the dose used in the treatment group. The dose for the in vivo study was set at 30 mg/kg.

Experimental protocol

Twenty four Albino wistar rats were randomly divided into four groups of six per each. Group I was set as vehiclegroup includes six rats received1% Gum acacia solution and distilled water *p.o.* Group II received negative control includes six rats received Chlorpromazine (3mg/kg, *i. p*). Group III received standard group includes six rats received Chlorpromazine (3mg/kg/day, *i. p)* and was treated with Diclofenac (10mg/kg/day, *p.o)*. Group IV received 1, 2, 4-Triazin analogue of diclofenac group includes six rats received Chlorpromazine (3mg/kg/day, *i. p)* and was treated with 1, 2, 4-Triazin analogue of Diclofenac at (30mg/kg, *p. o)*, dose respectively. CPZ was dissolved with 1% gum acacia in distilled water suspension daily. All animals were tested on behavioral activity of rat tests in familiar environment was monitored on 0^{th} , 7^{th} and 14^{th} day after 30 minutes of drugtreatment. At day 14, the brain of selected animals were collected by decapitation for further histopathological and neurochemical studies.

Behavioral Assessment

Locomotor activity by using Actophotometer

To study the locomotor activity, animal behavior was monitored and calculated using an Actophotometer (activity cage), as described by Dews P.B. (1953). In this system, photoelectric cells are connected to a counter. When a beam of light falls on the photocell and is interrupted by the rat's movement, a count is recorded. On the 0th, 7th, and 14th days of the study, rats from each group were placed individually in the activity cage for 10 minutes, and the scores for each rat were recorded [17].

Muscle coordination behavior by using Rota Rod

Dunham and Miya (1957) suggested that neurological depression in rats could be evaluated by testing their ability to remain on a Rota-rod. The Rota-rod apparatus consists of a four-panel device with a timer. Animals (4 at a time) were placed on a rod rotating at a speed of 20-25 rpm. Only rats that were able to remain on the revolving rod (20-25 rpm) for 5 minutes after training

sessions during pretest screening were selected for the study. A decrease in the time before the rat falls off is indicative of CNS depression. On the 0th, 7th, and 14th days of the study, the fall-off time for rats from each group was recorded 30 minutes after drug administration [18][19].

Cognitive performance by using Morris Water Maze Apparatus

Cognitive abilities were assessed using the Morris Water Maze. The chamber consisted of a black circular swimming pool made from nontoxic materials (160 cm diameter, 80 cm high, and 40 cm deep) filled with water. Visual cues were placed around the chamber, which was divided into four equal quadrants. A square, hidden black platform (10 cm in diameter) was submerged 1.5 cm beneath the water surface in the middle of the target quadrant. The water was made opaque using a titanium dioxide suspension and maintained at a temperature of approximately 23°C ± 2°C during the experiment. Each trial began from one of four assigned polar positions, with a different sequence each day. The latency to find the platform was measured as the time from placing the rat in the water until it located the platform. On the 0th, 7th, and 14th days of the study, the rats from each group were tested, and the fall-off time was recorded 30 minutes after drug administration [20][21].

Catalepsy by using a round wooden bar

On the 7th and 14th days of the experiment, the scores were recorded in three stages, and scores for each stage were assigned. Catalepsy behavior, defined as the inability to correct an abnormal posture, was assessed using the standard bar test. In this test, the animal's front limbs were extended and placed on a 9-cm high round wooden bar. The catalepsy endpoint was considered reached when both front paws were removed from the bar or if the animal moved its head in an exploratory manner. The cut-off time for the test was 720 seconds [22].

Biochemical Estimation

On the 14th day of the study, the animals were anesthetized with diethyl ether. Blood was collected through the retro-orbital plexus, and serum was obtained by centrifuging the whole blood (without anticoagulants) at 3000 rpm for 10 minutes. The serum was then used for the analysis of serum glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase, total bilirubin, urea, and creatinine using standard laboratory techniques [23], [24], [25], [26].

Estimation of Antioxidant levels in Rat Brain

On the 14th day after behavioral quantification, all animals were sacrificed by cervical dislocation. The brains were removed, forebrain was dissected. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) and it was used for further estimation of superoxide dismutase (SOD), reduced glutathione (GSH), Nitrite, Protein, Lipid Peroxidation, catalase, and brain glutamate level.

Superoxide dismutase (SOD)

To 1 ml of the sample, 0.25 ml of absolute ethanol and 0.15 ml of chloroform were added. After shaking the mixture for 15 minutes in a mechanical shaker, the suspension was centrifuged, and the supernatant obtained constituted the enzyme extract. The reaction mixture for auto-oxidation consisted of 2 ml of buffer, 0.5 ml of 2 mM pyrogallol, and 1.5 ml of water. The rate of

pyrogallol auto-oxidation was initially measured at 1-minute intervals for 3 minutes. The assay mixture for the enzyme contained 2 ml of 0.1 M Tris-HCl buffer, 0.5 ml of pyrogallol, aliquots of the enzyme preparation, and water, which was then made up to a total volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation, following the addition of the enzyme, was recorded. Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of pyrogallol auto-oxidation at 420 nm for 10 minutes. One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of pyrogallol auto-oxidation. The enzyme activity was expressed as units/min/mg protein [27].

Reduced glutathione (GSH)

1 ml of tissue homogenate was precipitated with 1 ml of 10% trichloroacetic acid (TCA). The precipitate was removed by centrifugation. To an aliquot of the supernatant, 4 ml of phosphate solution and 0.5 ml of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) reagent were added. The absorbance was measured at 412 nm. The concentration of glutathione (GSH) in the supernatant was determined from a standard curve using standard reduced glutathione and expressed in nM/mg of protein [28].

Nitrite

The production of nitric oxide (NO) in the brain may result from oxidative stress and can be determined by estimating the nitrite level. The nitrite level was measured spectrophotometrically using the Griess reagent, which consists of 0.1% N-1-naphthyl ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid. Equal volumes of brain homogenate and Griess reagent were mixed, incubated for 10 minutes, and the absorbance was measured at 546 nm. A standard curve of sodium nitrite was prepared, and the concentration of nitrite in the supernatant was determined from this standard curve [29].

Protein

The protein content of brain tissue was estimated using the method described by Lowry et al. A standard curve was generated using bovine serum albumin, and the protein values are expressed in mg/ml [30].

Lipid peroxidation

Lipid peroxidation in brain tissue was estimated spectrophotometrically by quantifying TBARS. Briefly, the supernatant of the tissue homogenate was treated with Thio barbituric acid-trichloroacetic acid (TBA-TCA) reagent and mixed thoroughly. The mixture was then placed in a boiling water bath for 15 minutes. After cooling, the tubes were centrifuged for 10 minutes, and the supernatant was collected for measurement. The developed color was read at 532 nm using a UV spectrophotometer, with a reagent blank as the reference. The concentration of TBARS in the supernatant was determined from a standard curve using 1, 1, 3, 3-tetramethoxypropane (TMP) and expressed in nM/mg of protein [31].

Catalase

The tissue was homogenized with M/15 phosphate buffer at 1 to 4°C and then centrifuged. The sediment was stirred with cold phosphate buffer and allowed to stand in cold conditions with

occasional shaking. This extraction process was repeated once or twice, and the supernatants were combined and used for the assay. In the assay, 3 ml of H2O2 phosphate buffer was placed in one cuvette. Then, 0.01 to 0.04 ml of the sample was added, and the absorbance was measured at 240 nm against a control cuvette containing enzyme solution without H2O2 phosphate buffer. A decrease in optical density from 0.450 to 0.400 was noted, and this value was used for the calculations [32].

Glutamate

A weighed portion of the brain was homogenized with 2 parts by weight of perchloric acid and centrifuged for 10 minutes at 3000 rpm. To the supernatant (3.0 ml), 1.0 ml of phosphate solution was added to adjust the pH to 9. The mixture was allowed to stand for 10 minutes in an ice bath, then filtered through a small, fluted filter paper. After warming to room temperature, the solution was diluted 1:10, and 1.0 ml was taken for the assay. Absorbance was measured at 340 nm. A blank reading at 340 nm was also recorded. The level of glutamate was expressed as µmol/g tissue [33].

Estimation of Brain Tissue Extract Neurotransmitters

Antioxidants were examined using the forebrain, while the remaining brain tissue was used for neurotransmitter analysis. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was mixed with 3 ml of HCl-Butanol in a cool environment and then centrifuged for 10 minutes at 2000 rpm. After centrifugation, 0.8 ml of the supernatant was removed and added to an Eppendorf tube containing 2 ml of heptane and 0.25 ml of 0.1 M HCl. The mixture was shaken for 10 minutes and then centrifuged under the same conditions to separate the two phases. The upper organic phase was discarded, and the aqueous phase was used for the dopamine assay.

Acetylcholine

To a 0.4 ml aliquot of the homogenate, 2.6 ml of phosphate buffer (0.1 M, pH 8) and 100 µl of DTNB were added in a cuvette. The contents of the cuvette were mixed thoroughly by bubbling air, and the absorbance was measured at 412 nm using a photoelectric colorimeter (H2 grade). Once the absorbance reached a stable value, it was recorded as the basal reading. Then, 5.0 ml of substrate, acetylthiocholine, was added, and the change in absorbance was recorded for a period of 10 minutes, with measurements taken at 2-minute intervals. The change in absorbance per minute was then determined [34].

Dopamine

To 0.02 ml of the HCl phase, 0.005 ml of 0.4 M HCl and 0.01 ml of EDTA/Sodium Acetate buffer (pH 6.9) were added, followed by 0.01 ml of iodine solution for oxidation. The reaction was stopped after 2 minutes by adding 0.1 ml of sodium thiosulfate in 5 M sodium hydroxide. After 1.5 minutes, 10 M acetic acid was added. The solution was then heated at 100°C for 6 minutes. Once the samples returned to room temperature, the excitation and emission spectra were measured (330 to 375 nm) using a spectrofluorimeter. To compare the tissue values, the fluorescence of the tissue extract (minus the fluorescence of the tissue blank) was compared with an internal reagent standard (fluorescence of the internal reagent standard minus the fluorescence of the internal reagent blank). Tissue blanks for the assay were prepared by adding the reagents

of the oxidation step in reverse order (sodium thiosulfate before iodine). Internal reagent standards were prepared by adding 0.005 ml of bidistilled water and 0.1 ml of HCl-Butanol to 20 ng of dopamine standard [35].

Determination of Ulcerogenecity

At the end of the study stomach of the entire animals was cut open and the ulcerogenecity was assessed by ulcer score as follows 0-Normal Mucosa, 0.5-Red coloration, 1.0-Spot ulcers, 1.5-Hemorrhagic streaks, 2.0-Ulcers >3 but <5, 2.5- Ulcer >5.

Histopathology

At the end of the experiments, all animals were sacrificed. The midbrain region of the brain was carefully removed from each animal and postfixed in formal saline for 24 hours. After washing in tap water, the tissue was subjected to a series of ethanol dilutions (methyl, ethyl, and absolute) for dehydration. The specimens were then cleared in xylene and embedded in paraffin at 56°C in a hot air oven for 24 hours. Blocks were prepared using paraffin wax and sectioned at a thickness of 4 µm using a microtome. The obtained tissue sections were deparaffinized and stained with hematoxylin and eosin (H&E) for histopathological examination under a light microscope [36].

Statistical Analysis

Statistical analyses were performed using SPSS version 20.0 for Windows. Behavioral comparisons of pre- and post-treatment data across the four groups were analyzed using a three-way analysis of variance (ANOVA), followed by the Scheffé test for comparisons between the treated groups. The results are presented as means ± SEM, with n=6. A p-value of <0.05 was considered statistically significant [37].

RESULT

Acute Oral Toxicity Studies

The 1, 2, 4-Triazin analogue of diclofenac did not induce any toxic symptoms or mortality up to an oral dose of 300 mg/kg in mice. Therefore, the compound was considered safe for further pharmacological evaluation. According to OECD-423 guidelines, a 1/10th dose (30 mg/kg) of the 1, 2, 4-Triazin derivative was selected for subsequent pharmacological testing. No lethal toxic reactions were observed throughout the 14-day observation period.

Effect on Locomotor activity

The locomotor activity in the 7^{th} day negative group showed significantly decreased (73.17 ± 6.45) and after the 14^{th} day (78.17 ± 1.88, $P<0.05$) when compared to the vehicle group. The standard group shows a significantly decreased on the 7^{th} day showed (95.50 ± 11.90, $P<0.05$), and after the 14^{th} day showed (82.00 ± 4.68, $P<0.05$). In this no significant difference in the treatment group on the 7^{th} day. On the 14^{th} day 1, 2, 4- Triazin analogue of diclofenac treated group showed (149.2±11.71, $P<0.001$) significantly increases when compared to a negative group and standard group (Table-1).

Table 1: Effect of 1, 2, 4-Triazin analogue of diclofenac on Locomotors Activity by Using Actophotometer

TREATMENT	0^{th}day	7^{th}day	14^{th}day
Group I (vehicle group)	112.7±11.79	120.3±12.21	113.0±11.42

Group II (Negative group)	126.7±10.13	73.17±6.45	78.17±1.88^{c}
Group III (Standard group)	118.5±11.53	95.50±11.90^{c}	82.00±4.68^{c}
Group IV (treatment group)	129.0±10.36	130.0±12.48^{f}	149.2±11.71^{d}

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II, III, IV symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d -***P<0.001, e - ** P<0.01, f- * P<0.05.

Effect on Muscle Coordination behavior

The mean fall-off time of vehicle group animals from the rotarod was the observation of the treatment. The 7th day and 14th day showed significantly decreased (91.0 ± 3.23, P<0.05 and 66.33±5.83, P<0.001) when compared to the vehicle group. On 14th day standard group showed a significantly increased (142.7 ± 11.50, P<0.001) when compared to the negative group. The treatment group showed a significantly increased on the 7th day (125.0±11.00, P<0.001) and after the 14th day (140.7 ± 13.35, P<0.001) when compared to the negative group (Table-2).

Table 2: Effect of 1, 2, 4-triazin analogue of diclofenac on Muscle Coordination Behaviour by Using Rota Rod

TREATMENT	0thday	7thday	14thday
Group I (vehicle group)	116.7±13.80	123.0±10.78	138.7±13.40
Group II (Negative group)	119.0±11.62	91.0±3.23^{c}	66.33±5.83^{a}
Group III (Standard group)	99.33±7.41	124.2±10.53	142.7±11.50^{d}
Group IV (treatment group)	113.5±10.29	125.0±11.00d	140.7±13.35^{d}

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II, III, IV, symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d -***P<0.001, e - ** P<0.01, f- * P<0.05.

Effect on Cognitive Performance

Antipsychotic-related effects on swim speeds on the 7thday chlorpromazine negative group showed significantly increased (35.95 ± 1.27, P<0.05) and after 14th day showed (36.99 ± 1.13, P<0.001) when compared to vehicle group. The standard group shows a significantly increased on the 7th day showed (27.99 ± 1.98, P<0.05) and after the 14th day showed (27.21 ± 1.91, P<0.001) when compared to the negative group. The treatment group showed a significantly decreased on the 7th day (24.45±1.25, P<0.001) and after the 14th day (25.02 ± 1.03, P<0.001) when compared to the negative group (Table-3).

Table 3: Effect of the 1, 2, 4-triazin analogue of diclofenac on cognitive performance by using morris water maze

TREATMENT	0^{th}day	7^{th}day	14^{th}day
Group I (vehicle group)	29.01±1.90	29.67±1.88	26.63±1.43
Group II (Negative group)	23.43±2.71	35.95±1.27^{c}	36.99±1.13^{a}
Group III (Standard group)	25.65±1.51	27.99±1.98^{f}	27.21±1.91^{d}
Group IV (treatment group)	28.82±1.60	24.45±1.25^{d}	25.02±1.03^{d}

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II, III, IV, symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d -***P<0.001, e - ** P<0.01, f- * P<0.05.

Effect on Catalepsy

Cataleptic behavior of all animals was evaluated on the 7th day chlorpromazine negative group showed a significantly increased (132.50 ±25.20, P<0.05) and after the 14th day showed (131.7 ± 17.12, P<0.01) when compared to vehicle group. The standard group shows a significantly decreased on the 7th day showed (30.90 ± 1.63, P<0.05), and after the 14th day showed (27.83 ± 2.27, P<0.001) when compared to the negative group. On the 7th and 14th day these results showed cataleptic latency time was reduced by 1, 2, 4- Triazin analogue of diclofenac treated group showed significantly (13.57 ± 1.58, P<0.001 and 14.02 ± 1.018, P<0.001) decreased when compared to negative group. (Table-4)

Table 4: Effect of the1, 2, 4-triazin analogue of diclofenac on catalepsy in chlorpromazine induced Parkinson's rat

TREATMENT	0^{th}day	7^{th}day	14^{th}day
Group I (vehicle group)	0.87±0.19	1.11±0.18	1.13±0.15
Group II (Negative group)	129.00±22.49	132.50±25.20^{c}	131.7±17.12^{b}
Group III (Standard group)	29.14±3.45	30.90±1.63^{c}	27.83±2.27^{a,e}
Group IV (Treatment group)	12.42±1.58	13.57±1.58^{c,d}	14.02±1.018^{a,e}

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II, III, IV, symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d -***P<0.001, e - ** P<0.01, f- * P<0.05.

Effect on biochemical parameter

The results in the table 5 indicates the serum glutamate pyruvate (SGPT), glutamate oxaloacetate transaminase (SGOT), Alkaline phosphates, and Total bilirubin were of the negative group showed significantly ($P<0.001$) increase in SGPT, SGOT, ALP, Total bilirubin level when compared to vehicle group. The 1, 2, 4-Triazin treatment group shows a significantly low level when compared to the standard group.

Table 5: Effect of 1, 2, 4-triazine analogue of diclofenac on biochemical parameter

TREATMENT	SGPT(IU/L)	SGOT (IU/L)	ALP (IU/L)	BILIRUBIN (mg/dl)	UREA (mg/dl)	CREATININE
Group I (vehicle group)	49.89 ±3.27	17.99 ±2.92	80.25±8.04	0.158±0.01	12.68±1.91	0.5117±0.07
Group II (Negative group)	75.17±9.79[c]	38.24±8.47[c]	142.7±4.39[a]	0.681±0.08[a]	20.94±1.30[b]	0.9117±0.02[b]
Group III (Standard group)	64.64 ±4.77	32.91 ±4.05	97.32±2.82	0.1967±0.02	20.24±1.05[b]	0.805±0.07[c]
Group IV (treatment group)	46.80±2.04[f]	17.30±2.96[f]	60.73±6.96[a,d]	0.1317±0.01[a,d]	12.74±1.70[e]	0.4217±0.06[d]

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II, III, IV, symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d - ***P<0.001, e - ** P<0.01, f- * P<0.05.

Effect on Antioxidant Levels in Rat Brain

In this study SOD, GSH, protein, and catalase of the negative group showed a significant (P<0.001) decrease when compared to the vehicle group. Standard group and 1, 2, 4- Triazin analogue of diclofenac treated group showed significantly (P<0.001) increase when compared to negative group. Nitrite, lipid peroxidation, and glutamate of the negative group showed a significant (P<0.001) increase when compared to the vehicle group. Standard group and 1, 2, 4- Triazin analogue of diclofenac treated group showed significantly (P<0.001) decrease when compared to negative group (Table-6).

Table 6: Effect of 1, 2, 4-triazine analogue of diclofenac on antioxidant

TREATMENT	SOD (Units/ml)	GSH (Umol/L)	NITRITE (mmol/l)	PROTEIN (mg/dl)	LIPIDPEROXIDASE (µmol\L)	CATALASE (mg/dl)	GLUTAMATE (mg/dl)
Group I (vehicle group)	205.2 ±10.22	4.417 ±0.23	3.670 ±0.25	0.6583± 0.05	1.665±0.18	272.8 ±15.16	12.09±0.82
Group II (Negative group)	129.5 ±10.00^{a}	2.003 ±0.21^{b}	60.74 ±3.82^{b}	0.2050±0.05^{a}	2.336±0.22^{c}	189.8 ±12.92^{a}	16.93 ±1.08^{b}
Group III (Standard group)	201.5±12.25^{e}	5.877 ±0.69$^{a, d}$	6.280±1.29^{a}	0.6733±0.05^{a}	0.8133±0.27^{b,d}	243.3 ±8.99^{f}	5.588±0.83$^{a, d}$
Group IV (treatment group)	220.0±13.55^{d}	8.028 ±0.43^{b}	3.467±0.74^{a}	0.8300±0.01^{d}	1.152±0.36^{a,d}	327.8±5.07$^{c, d}$	3.577 ±0.77^{a}

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II, III, IV, symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d - ***P<0.001, e - ** P<0.01, f- * P<0.05.

Effects on Acetylcholine and Dopamine

In acetylcholine negative group showed a significant (13.04 ± 2.24, P<0.01) increase when compared to the vehicle group. Standard group and 1, 2, 4- Triazin treated group showed a significant (20.36 ± 1.89, P<0.05 and 30.10 ± 1.19, P<0.05) decrease when compared to the negative group. In dopamine levels, the negative group was reduced significantly (13.04 ± 2.24, P<0.001) when compared to the vehicle group. Standard and treated groups significantly showed an increased (20.36 ± 1.89, P<0.001 and 30.10 ± 1.19, P<0.01) when compared to the negative group (Table-7).

Table 7: Effect of 1, 2, 4-triazine analogue of diclofenac on acetylcholine and dopamine

TREATMENT	ACETYLCHOLINE (µmol/mg)	DOPAMINE(pg/ml)
GroupI (vehicle group)	2.848±0.42	26.34±2.20
GroupII (Negative group)	5.197±0.28^{b}	13.04±2.24^{a}
Group III (Standard group)	3.907±0.58	20.36±1.89^{d}
Group IV (treatment group)	3.052±0.48^{f}	30.10±1.19^{e}

Values are expressed as mean ± SEM, n=6. Comparisons were made between Group I VS II,III, IV, symbols represent statistical: a- ***P<0.001, b- ** P<0.01, c- * P<0.05. Comparisons were made between Group II VS III, and IV, symbols represent statistical: d -***P<0.001, e - ** P<0.01, f- * P<0.05.

Effect on Ulcerogenecity

In ulcerogenecity negative group shows an ulcer score denoted as 0.5. This standard group shows ulcer score was increased were denoted as 2.0. 1, 2, 4- Triazin analogue of diclofenac and vehicle group shows ulcer score was denoted as normal 0 (Fig. 1).

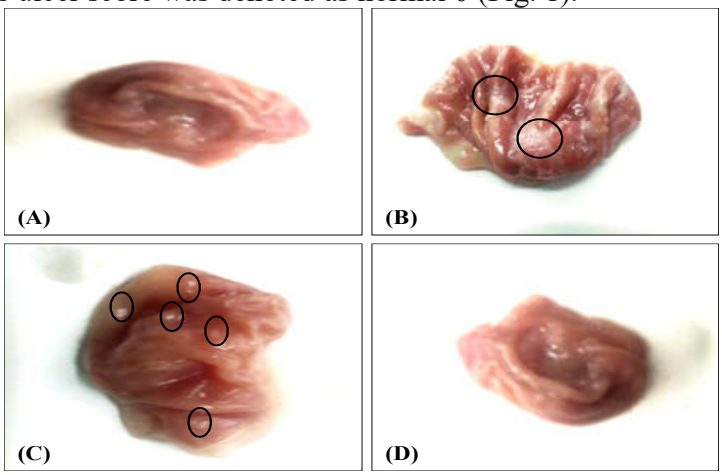

Figure 1: Effect of 1, 2, 4-triazin analogue of diclofenac on the determination of ulcerogenecity

A= Vehicle group shows no ulcerogenecitywas assessed by ulcer score-0 (pointed by a circle). B= Negative group shows mild smooth muscle damage ulcerogenecitywas assessed by ulcer score-0.5 (pointed by a circle). C= Standard group shows severe smooth muscle damage and ulcerogenecity was assessed by ulcer score-2 (pointed by a circle). D=1, 2, 4 –Triazin analogue of diclofenac group shows without injury similar to vehicle group was assessed by ulcer score-0 (pointed by a circle).

Effect of 1, 2, 4-Triazin analogue of diclofenac on histological studies

Histopathological examination of our samples revealed that chlorpromazine induced degeneration in the midbrain region of rats, where neurons were subjected to oxidative stress, as evidenced by the presence of hyperchromatic nuclei and eosinophilic vacuolated cytoplasm, indicating edema. In contrast, sections from the 1, 2, 4-Triazin analogue of diclofenac-treated group exhibited well-preserved tissue architecture, with only occasional hyperchromatic nuclei and mild vacuolization, suggesting minimal degenerative changes in the treated animals (Fig. 2).

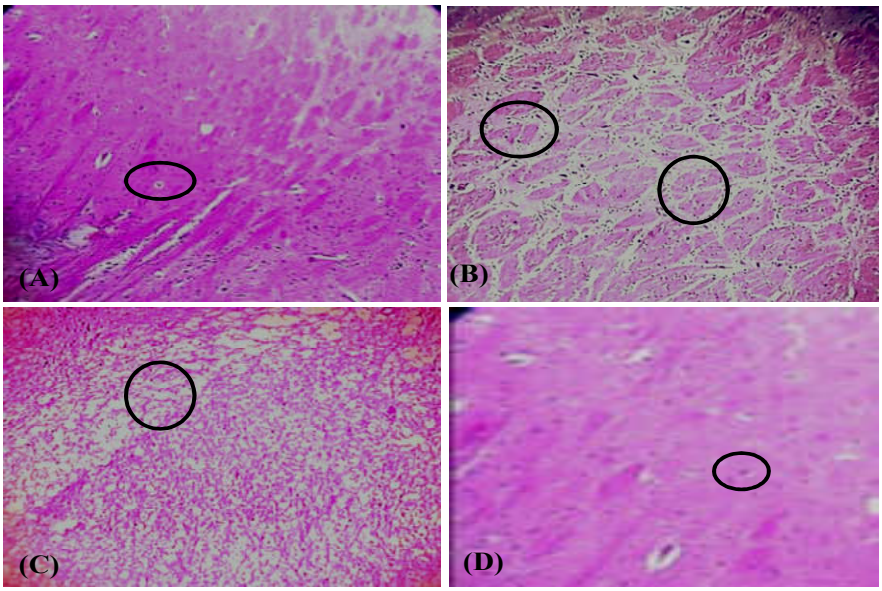

Figure 2: Effect of 1, 2, 4-triazin analogue of diclofenac on histological studies

A= Histopathology of vehicle group shows normal neurons with obvious nuclei (pointed by a circle). B=Histopathology of the negative group shows stained small cells have irregular shapes (pointed by a circle). C= Histopathology of the standard group shows proliferative blood vessels and neurons (pointed by a circle). D=Histopathology of 1, 2, 4- Triazin analogue of diclofenac shows similar to the vehicle group (pointed by a circle).

DISCUSSION

NSAIDs are commonly used in the treatment of neuroinflammatory disorders, with diclofenac being one of the prominent drugs used for Parkinson's disease. However, diclofenac is known to

cause significant gastrointestinal irritation, bleeding, and ulceration. Previous studies have suggested that the 1, 2, 4-Triazin analogue of diclofenac might possess appropriate anti-Parkinson's action [38]. This analogue is synthesized by replacing the carboxyl group of diclofenac with 2-chloroacetamide, forming the 1, 2, 4-Triazin analogue. Chlorpromazine, a centrally acting drug, has a high affinity for dopamine D2 receptors and acts as an antagonist [39][40].

The negative control group exhibited CNS depressant effects, likely due to impaired cholinergic transmission, oxidative/nitrergic stress, neuroinflammation, and dyslipidemia [41]. Muscle coordination in the negative group was significantly impaired, as sensory fibers that detect muscle lengthening failed to initiate contraction of the corresponding motor neurons [42]. The Morris Water Maze (MWM) test showed significantly decreased performance in the negative group, likely due to immersion stress, fatigue, and sensory-motor deficits [43]. Furthermore, catalepsy, an inability to correct an abnormal posture while maintaining the righting reflex, was observed in the negative group, indicating non-selective action and blockade of postsynaptic D2 receptors in the nigrostriatal pathway, which leads to extrapyramidal side effects [44].

In this study, liver function markers such as serum SGPT, SGOT, ALP, and total bilirubin were elevated in Parkinson's disease rats, indicating liver cell damage [45]. In contrast, the standard group and the 1, 2, 4-Triazin analogue of diclofenac-treated group exhibited significantly lower levels of liver enzymes, suggesting a non-toxic effect on the liver by both diclofenac and its analogue. In Parkinson's disease, elevated levels of creatinine and urea indicate impaired renal function [46]. However, the standard and 1, 2, 4-Triazin groups showed no significant kidney damage when compared to the negative group.

Superoxide dismutase (SOD) is an important enzyme in the brain that neutralizes the toxic effects of free radicals by dismutating superoxide into nonreactive oxygen species and hydrogen peroxide [47]. In Parkinson's disease, a depletion of glutathione (GSH) in the substantia nigra may contribute to neuronal loss. A positive correlation exists between neuronal loss and GSH depletion, as reduced glutathione impairs the detoxification of hydrogen peroxide and increases the risk of free radical formation and lipid peroxidation [48]. Nitric oxide (NO) is involved in cytotoxicity through macrophage activation and excessive neuronal stimulation by glutamate in Parkinson's disease [49]. Lipid peroxidation, a sensitive marker of oxidative stress, was measured by TBA levels. This process leads to oxidative degradation of polyunsaturated fatty acids in biological membranes, resulting in impaired membrane function, decreased fluidity, and inactivation of membrane-bound enzymes [50].

The catalase enzyme helps to neutralize free radical precursors [51], and in Parkinson's disease, the accumulation of this enzyme is seen. A significant increase in brain glutamate levels, due to increased density of dopamine receptors and decreased dopamine levels, was observed in the negative control group [52]. Moreover, the negative group exhibited significantly elevated brain acetylcholine levels compared to the vehicle group [53].

Synaptic neuroplasticity, which leads to modifications in dysfunctional brain circuitry, improved dopaminergic function, and increased D2 dopamine receptor expression, is crucial in Parkinson's

disease [54]. The 1, 2, 4-Triazin analogue of diclofenac treatment led to a significant recovery in dopamine levels, indicating improved neurodegeneration recovery.

Neutrophils, lymphocytes, and monocytes/macrophages at the inflammatory site in the stomach are activated by local cytokines such as IL-6, leading to oxidative bursts that cause tissue damage in peptic ulcer disease [55]. Diclofenac induces more ulceration compared to the control, while the 1, 2, 4-Triazin analogue of diclofenac treated group exhibited fewer incidences of ulceration compared to both the standard and negative groups [56].

Histopathological examination of midbrain sections from Parkinson's rats showed hyperchromatic nuclei and eosinophilic vacuolated cytoplasm, indicative of edema and neurodegeneration. However, in the treatment group, hyperchromatic nuclei and mild vacuolization were observed, suggesting only mild degenerative changes, which were less pronounced compared to the negative group [57]. These findings indicate that the 1, 2, 4-Triazin analogue of diclofenac has a greater protective effect on neuronal inflammation and degeneration.

CONCLUSION

In conclusion, this study indicates that the 1, 2, 4-Triazin analogue of diclofenac has the potential to reverse the motor deficits and neuronal damage induced by chlorpromazine. This effect seems to be related to the modification of diclofenac's carboxyl group, creating a 1, 2, 4-Triazin analogue, which functions as a potent ligand in the treatment of Parkinson's disease. Importantly, this analogue demonstrates minimal gastrointestinal toxicity, unlike conventional diclofenac. However, further clinical research, both in vitro and in vivo, is necessary to validate the 1, 2, 4-Triazin analogue of diclofenac as a potential therapeutic option for Parkinson's disease.

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Ethical Issue: These studies were reviewed and approved by the institutional animal ethical committee of Swamy Vivekanandha College of Pharmacy, (IAEC Reference No: SVCP/IAEC/PG/1/02/2017) and performed according to the guidelines of the care and use of Laboratory Animals in Ethics Committee

Conflict of Interest: None

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