Therapeutic potential of methyl gallate and cilnidipine in diabetic neuropathy: An integrated in silico, in-vivo approach via regulation of AGE, GAT-1, CaV2.2

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Running Title: Protective effect of methyl gallate and cilnidipine in diabetic neuropathy

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ABSTRACT

Diabetic neuropathy is one of the most common microvascular complications of diabetes mellitus which is associated with neuronal dysfunction and pain. Methyl gallate (MG) is a polyphenolic compound reported for its anti-tumor, anti-inflammatory, anti-oxidation, anti-α-glucosidase properties. Cilnidipine (CLN) is N- and L-type calcium channel blocker used in treatment of hypertension. However, its role in diabetic neuropathy is still unclear. The present study investigated the potential protective effects of MG alone and its combination with CLN against streptozotocin (STZ)-induced diabetic neuropathy using in silico method and to confirm them by in-vivo activities. STZ (55 mg/kg) was injected in adult Wistar rats to induce diabetes. Diabetic animals were treated daily for four weeks with MG (40 mg/kg, p.o), CLN (3 mg/kg, p.o) and the combination of MG (40 mg/kg, p.o.) with CLN (3 mg/kg, p.o) starting from the end of the 4th week of diabetes development. Body weight, blood glucose, thermal hyperalgesia, mechanical allodynia, cold allodynia, locomotor behaviour, motor coordination, and nerve conduction velocity were recorded. Oxidative stress parameters, AGE, Ca^{2+} levels and GAT-1 expression were studied in sciatic nerve. Histopathology of sciatic nerve were also studied at the end study. MG and CLN attenuated STZ induced alteration in metabolic parameters, nociceptive threshold, motor nerve conduction velocity, AGE, Ca^{2+} levels and GAT-1 expression. In addition both drugs suppressed oxidative stress. Furthermore, diabetic rats treated with MG (40 mg/kg, p.o.) and CLN (3 mg/kg, p.o) combination demonstrated more pronounced beneficial effects as compared to either agent alone. Collectively, our results suggest that MG either alone or in combination with CLN therapy could serve as an efficacious agent for treating diabetic neuropathy.

Keywords

Advanced Glycation End Product (AGE), Allodynia, Diabetic neuropathy, GABA Transporter-1(GAT-1), Hyperalgesia, Methyl gallate

INTRODUCTION

International Dibetes Federation (IDF) estimates that currently about 352 million people are at risk of developing diabetes.[1,3] According to WHO, the prevalence of diabetes is predicted to increase by 366 million people worldwide by the year 2025.[2] IDF also projected that 625 million people will suffer from diabetes by the year 2045.[3] Long-standing diabetes mellitus leads to microvascular and neurologic complications such as cardiomyopathy, neuropathy, nephropathy, and retinopathy.[4] Diabetic neuropathy is one of the most common microvascular complications affects 50–70% of the diabetic population.[5,6] After 5 years of diabetes, around 26% of individuals may develop peripheral neuropathy, and this number increases to 41% after 10 years.[7] Clinically, diabetic neuropathic pain can be recognized by persistent burning or tingling sensation in legs and feet.[8]

Diabetic neuropathy is precipitated due to an array of factors including elevated hexosamine shunt, aldose reductase activation, decrease in the nerve myoinositol content, an impaired neurotrophic support, activation of protein kinase C (PKC), activation of poly (ADP-ribose) polymerase (PARP), impaired insulin/C peptide action, and formation of advanced glycation end products (AEG) which modulate various intertwining biochemical and functional aberration of peripheral neurons, spinal glial cells and nerve fibers.[9,10] In diabetic neuropathy, AGEs induce oxidative stress result in upregulation of NF-kappa B-mediated proinflammatory genes, and exaggerate altered pain sensation.[11] Therapeutic strategy to prevent and ameliorate diabetic neuropathy using anti-AGE agents needs to be established. In the spinal cord, the presynaptic distribution of N-type calcium channels supports a role in controlling the transmission of nociceptive signal and upregulated in neurons following nerve inflammation.[12] It is need to reform the current treatment guidelines and one step in this direction seems to block the ongoing activity of high-threshold N-type calcium channels. γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the central nervous system.[13] Neuropathic pain has been attributed to decreased availability of spinal GABA, an inhibitor of spinal pain transmission. Spinal GABA depletion resulting in pain behaviour may possibly be an effect of enhanced presynaptic reuptake through upregulated GAT-1.[14] Hence neuropathy may be resolved by blocking GAT-1.

First-line therapies include tricyclic antidepressants, serotonin–noradrenaline reuptake inhibitors, and anticonvulsants that act on calcium channels. Other therapies include opioids

and topical agents such as capsaicin and lidocaine.[15] Although a wide range of drugs are available, there is still lack of specific drugs and treatment options for DN due to its complex pathogenesis, diverse clinical manifestations. It is estimated that approximately 40% of neuropathic patients are resistant to the currently available analgesics.[13] This necessitates the exploration of novel drug targets to treat neuropathic pain of various region.

Polyphenols, a diverse and extensive collection of phytochemicals with the highest antioxidant and anti-inflammatory effects, with the potential to treat numerous illnesses such as diabetes and its consequences.[16] Methyl gallate is one such kind having reported activities such as antioxidant, anti-inflammatary, anticancer, antidepressant, antiarthritic, anti glycation, antiapoptotic.[17] Drug repurposing is one of the approaches to discover and develop the drugs. Generally, the repurposed drug presents known safety profile and this strategy is associated with reduced development cost and timelines also reduced costs and faster approval procedures.[18] Cilnidipine is N- and L-type calcium channel channel blocker used to treat hypertension. Reported pharmacological activities of cilnidipine are antihypertensice, lipid lowering , antiangina, renoprotective.[19] According to literature, it was found that N-type calcium channel play role in nerve pain.[20] Hence by blocking this receptor, neuropathy may be resolved. The network pharmacology and in silico techniques were used to identify the potential targets of methyl gallate and cilnidipine, which were confirmed through in vivo studies. By linking these predictions with preclinical findings, the aim was to uncover new therapeutic options to improve the quality of life for people with diabetic neuropathy

MATERIALS AND METHODS

Drugs and Chemicals

Methyl gallate (Yucca enterprises, Wadala, Mumbai); Cilnidipine (Windlas Biotech Pvt Ltd, Dehradun); Streptozotocin (Otto chemie Pvt Ltd, Mumbai); Nicotinamide (Kanha Biogenetic, Solan); Pregabalin (Sun pharmaceuticals, Gujrat); Nitrobluetetrazolium Chloride (NBT) (Himedia Laboratories Pvt. Ltd. Mumbai, India); thiobarbituric acid (TBA) (Research-Lab Fine Chem Industries, Mumbai, India); 5, 5'- dithiobis-2-nitro benzoic acid (DTNB) (Alfa Aesar, A Johnson Mathey Company); bovine serum albumin (Spectrochem Pvt. Ltd., Mumbai, India). All the chemicals used were of analytical grade and purchased from standard manufacturers.

Animals

Wistar strain rats (230-250 g of 9 weeks old) of either sex were used for the study. Animals were procured and housed in polypropylene cages and maintained under the standard laboratory environmental conditions; temperature 25 ± 2ºC, 12: 12 h L: D cycle and 50 ± 5% RH with free access to food and water *ad libitum.* Animals were acclimatized to laboratory conditions before the test. All the experimental work was carried out during the light period (08:00-16:00 h). The study was carried out in harmony with the guidelines given by the Committee for the Control and Supervision of Experiments on Animals (CCSEA), New Delhi (India). The Institutional Animal Ethical Committee of MVPS, College of Pharmacy, Nashik, India approved the protocol of the study (IAEC/Oct.2025/06).

Induction and assessment of diabetes

Prior of induction of diabetes, animals were fasted for 16 h, the rats were not allowed food although they had access to drinking water. Diabetes was developed in rats by first administering nicotinamide (NA) (100 mg/kg i.p.), following 15-minute duration. streptozotocin (STZ) (55 mg/kg, i.p.) mixed in citrate buffer (0.1M) at pH 4.5 was injected to ensure diabetes. Blood glucose levels were checked after 72 h. Rats were restrained and their tails were washed with a warm soap solution and cloth. Under light isoflurane anaesthesia, blood was obtained from the tail vein. All experimental animals with glucose level ≥ 250 mg/dL were chosen for the study. A glucometer (Dr. Morepean Gluco one) was used to monitor blood glucose levels to confirm hyperglycemia.[16]

Experimental design

After a basal recording of nociceptive reaction at week 4, after streptozotocin injection, the control and diabetic rats were randomly selected and divided into seven groups (n = 6) based on body weight and blood glucose level as follows. Group I: Normal control animals receiving vehicle, 0.5% (W/V) sodium carboxymethyl cellulose in 0.5 ml/kg distilled water, Group II: STZ (55 mg/kg, i.p), Group III: STZ + Methyl gallate (40 mg/kg, p.o.) Group IV: STZ + Cilnidipine (3 mg/kg, p.o.), Group V: STZ + Methyl gallate (40 mg/kg, p.o.) + Cilnidipine (3 mg/kg, p.o.), Group VI: STZ + Metformin (200 mg/kg, p.o.), Group VII: STZ + Pregabalin (30 mg/kg, p.o.). The suspension of methyl gallate and cilnidipine was prepared in 0.5% (w/v) sodium carboxymethyl cellulose and doses of metformin, pregabalin were prepared in distilled water. The doses of methyl gallate, cilnidipne were selected in accordance to previous studies.[21,22] All the treatments were administered for 4 weeks, starting from week 5 of streptozotocin injection. The behavioural parameters were measured at the

end of 0^{th} week, 4^{th} week, 6^{th} and 8^{th} weeks of treatment. After 8 weeks, rats were sacrificed by CO_2 euthanasia chamber on the last day, 1 h after all behavioral tests, and sciatic nerves were immediately isolated and tissue homogenate was prepared in 0.1 M Tris–HCl buffer (pH 7.4) for the biochemical and molecular estimations.

Computational in-silico studies

In order to find out, the exact mechanism of neuroprotection of methyl gallate and cilnidipine, theoretical binding studies of target prediction were performed using Swiss target prediction, Super-PRED, Software ProTox 3.0 for toxicity prediction, SwissADME for Log p value. The molecular docking is considered as significant methods to validate the predictive neuroprotection mechanism of phytoconstituents as these softwares highlight the interactions between ligand and protein molecules. For molecular docking PyRx software and for visualization of active sites Biovia discovery studio software were used. Binding affinity of test compounds (Methyl gallate, Cilnidipine,) were examined against various targets such as adavanced glycation end product (AGE), N type calcium channel (CaV2.2) and GABA transporter-1(GAT-1), having PDB ID- 2e5e, 7VFV, and 7Y7V respectively and their binding affinities were compared with standard drugs (Metformin and pregabalin)

Evaluation parameters

Body weight and blood glucose level

Body weight of experimental animals were recorded at the start (Week 0) and end (Week 8) of the study. The blood glucose levels of experimental animals were recorded at the 0^{th} week, 2^{nd} week, 4^{th} week, 6^{th} week and 8^{th} week of the treatment. The blood was collected from tail vein for the estimation of blood glucose levels. A glucometer (Dr. Morepean Gluco one) was used to monitor blood glucose levels.

Behavioural assessments

Rota Rod Test (Motor Coordination)

This test was done to assess the motor coordination, balance, grip strength and motor learning of animals at rotarod device. Animals with impaired coordination will fall off when the rotation speed exceeds their capacity. Fall of latency is automatically recorded.[23] The device comprises of a horizontal wooden rod or metal rod with a 3 cm diameter rubber coating coupled to a motor with a speed set to 2 rpm. Six mice or rats could be tested at once because

of the 75 cm long rod that was separated into six portions by plastic discs.The rats were placed on the revolving rod 30 or 60 minutes after intraperitoneal or oral injection, respectively. The rod was rotated at 4 rpm at first, then at 20 rpm. During this time, it was recorded how much amount of time took the rat to fall off from rotating rod.[24]

Actophotometer Test (Locomotor Activity)

The locomotor activity measured using an actophotometer, which operates on photoelectric cells which are connected in circuit with a counter when the beam of light falling on the photocell is cut off by the animal, a count is recorded. Decreased locomotor count was taken as an index of intense peripheral neuropathy. An actophotometer could have either circular or square arena in which the animal moves. The actophotometer contains a square arena (30×30 cm) with walls that are fitted with photocells just above the floor level. The photocells were checked before the beginning of the experiment. The number of times each animal crossed the light beam was recorded automatically for a period of 10 minutes.[25]

Hot Plate Test (Thermal Hyperalgesia)

The Eddy's hot plate test was used to evaluate a condition of altered perception of temperature (thermal hyperalgesia), Operates on the principle of measuring an animal's withdrawal response to a controlled heat stimulus applied to their paws. where a shorter withdrawal time indicates increased pain sensitivity.[26] In this test, animals were individually placed on Eddy's hot plate with the temperature adjusted to 55 ± 1°C. The first sign of jump response or paw licking latency to avoid heat was taken as an index of the pain threshold (the cut-off time was 10s in order to avoid damage to the paw).[27]

Acetone Drop Test (Cold Allodynia)

When acetone is applied to the skin, it rapidly evaporates, causing a sudden drop in temperature. In animals experiencing cold allodynia, even a small drop in temperature due to the acetone can trigger a noticeable withdrawal response, indicating increased sensitivity to cold stimuli.[28] The cold allodynia was assessed by spraying a 100 µL of acetone on to the surface of the paw, without touching the skin. The response of the rat to acetone was noted for 20 s and was graded to a 4-point scale as defined by Flatters and Bennett, 2004; *viz* 0, no response; 1, quick with-drawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking; 3, repeated flicking of the paw with licking of the paw. Acetone was applied thrice to the hind paw, with a gap of 5 min between the acetone applications and the

individual scores noted in 20 s interval were added to obtain a single score over a cumulative period of 60 s. The minimum score was 0, while the maximum possible score was 9.[29]

Von Frey Test (Mechanical Allodynia)

The mechanical allodynia was assessed using von Frey filaments (Aesthesio, Samitek Instruments, New Delhi). In brief, the individual rat was placed in a transparent box made from plexiglass having mesh at the bottom. The rat was allowed to acclimatize for 30 min before applying the von Frey filaments. In the context of pain, the sudden withdrawal of a paw or flinching was regarded as a positive response, and failure to withdraw a paw was considered a negative response. First, the filament with 2.0 g force was applied perpendicularly to the ipsilateral (left) hind paw for 3 s until the filament bend. As per the method described by Dixon's up-down, if the rat showed a positive response, a weaker force was applied and if the rat showed a negative response, the filament with increased force (a stronger stimulus) was applied. Each filament was applied thricely keeping a time interval of 5 min between each application and an average of three applications was used for further analysis.[30]

Motor Nerve Conduction Velocity (MNCV)

A nerve conduction velocity (NCV) is an electrical test used to determine nerve impulse conduction down to the nerve that detects nerve injury. These abnormalities in motor nerve conduction may be due to blood flow reduction induced by hyperglycemia.[31] MNCV assessment was done using an 8-channel powerab (AD instruments, Australia) with animal nerve stimulating electrodes and needle electrodes of AD instruments, Australia. Animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg i.p) and Xylazine (10 mg/kg i.p.). Action potential was generated by applying a stimulating electrode at the proximal end and recording was done from the distal end. The distance between the stimulating electrode at the proximal end and the recording electrodes divided by the latent period is calculated as conduction velocity.[32]

Biochemical estimation

Tissue homogenate preparation

The animals were sacrificed using a CO_2 euthanasia chamber on the last day, 1 h after all behavioral tests, the portions of the sciatic nerve (from the left legs) were isolated immediately and rinsed in an isotonic saline solution. Then homogenized (10% w/v) with 0.1

M Tris HCl buffer (pH 7.4). The homogenates were kept in ice water for 30 min, followed by centrifugation at 2000×g for 10 min at 4 °C was done and post nuclear fraction was collected for catalase assay (Remi - C30, Remi Industries Ltd. Mumbai, India); for other enzyme assays, centrifugation was at 12000×g for 60 min at 4ºC, was used for subsequent assays. Following biochemical parameters were performed using Microplate reader (BMG LABTECH Spectrostar ^{Nano})[30]

Estimation of catalase (CAT)

Catalase activity was assessed by the method of Luck (1971), where the breakdown of H_2O_2 was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer (0.0125 M H_2O_2) and 0.05 ml of supernatant of sciatic nerve homogenate and the change in the absorbance was measured at 240 nm. The enzyme activity was calculated using the millimolar extension coefficient of H_2O_2 (0.07). The results were expressed as micro moles of H_2O_2 decomposed per minute per milligram of protein.[33]

Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) in the sciatic nerve was assayed according to the method of Ellman (1959). A 0.75 ml sample of homogenate was precipitated with 0.75 ml of 4% sulphosalicylic acid. The samples were centrifuged at 1200×g for 15 min at 4 ºC. The assay mixture contained 0.5 ml supernatant and 4.5 ml of 0.01 M DTNB [5-5'- dithiobis (2-nitrobenzoic acid)] in 0.1 M phosphate buffer, pH 8.0. The yellow colour developed was read immediately at 412 nm. The results were expressed as micro moles of GSH per milligram of proteins.[34]

Estimation of superoxide dismutase activity (SOD)

Superoxide dismutase activity was assayed according to the method of Kono (1978), where in the reduction of nitrobluetetrazolium chloride (NBT) was inhibited by the superoxide dismutase and measured at 560 nm spectrophotometrically. The reaction was started by adding 0.1ml of 1 mM hydroxylamine hydrochloride to a reaction mixture containing 0.1ml of 0.1 mM ethylene diamine tetra acetic acid (EDTA), 0.1 ml of 24 µM NBT, 0.1 ml of 0.03% v/v Triton X 100 reagent, and 1 ml of post nuclear fraction of brain homogenate. After 20 min of incubation at 37°C, the absorbance was measured at 560 nm. The results were expressed at percentage inhibition of reduction of NBT.[35]

Estimation of malondialdehyde (Lipid peroxidation assay)

The quantitative measurement of lipid peroxidation in sciatic nerve was done by the method of Wills (1966). By reacting with thiobarbituric acid at 532 nm, this method evaluated the amount of malondialdehyde (MDA) produced. 1.5 ml of 20% acetic acid, 0.1 ml of tissue homogenate, 0.2 ml of 8% sodium lauryl sulphate (SLS), and 1.5 ml of a 0.8% thiobarbituric acid (TBA) solution make up the reaction mixture. Then, the mixture was heated for one hour in a water bath at 95℃. Further, 5 ml of 15:1 n- butanol and pyridine mixture were added. After a vigorous shaking, the mixture was centrifuged for 5 min. The organic layer's upper layer's absorbance was measured at 532 nm. The molar extension coefficient of the chromophore was used to convert the values into nM of MDA per milligrams of protein (1.56 × 105 M-1 cm-1).[36]

Estimation of nitric oxide (NO)

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO) was determined by the method of Titheradge (1998). Sodium nitroprusside (5mM, 0.1 ml) in phosphate buffer (pH 7) (50mM) was incubated with sciatic nerve homogenate at room temperature for 30 min. After 30 min, 0.5 ml of incubated solution was added with 0.5 ml of griess reagent and Abs was measured at 543 nm. Nitrite concentration was calculated from std. curve using sodium nitrite as std. & expressed as micromoles nitrite per milliliter of homogenate.[37]

Estimation advanced glycation end product (AGEs)

AGEs levels in the sciatic nerve were determined by a method as previously described by Sensi et al. (1996). Briefly, sciatic nerve was homogenized in 2 ml of 0.25 M sucrose followed by centrifugation at 900 gm at 5℃ and the supernatant was separated.The pellet was resuspended in 2 ml sucrose solution and centrifuged and the supernatant obtained was mixed with the previous one. The proteins present were precipitated by adding equal volume of trichloroacetic acid (TCA).Following centrifugation at 4℃ with 900 gm, the protein pellet obtained was mixed with 1 ml methanol twice to remove the lipid fraction. The insoluble protein, after washing with 10% cooled TCA was centrifuged and the residue was solubilized in 1 ml of 1 M NaOH and the protein concentration was estimated by measuring the absorbance at 280 nm against BSA standard curve.[38]

Estimation of total calcium level (Ca^{2+})

Total calcium levels were estimated in sciatic nerve as described by Severinghaus and Ferrebee (1950). Briefly, sciatic nerve homogenate was mixed with 1 ml of trichloroacetic acid (4%) in ice-cold conditions and centrifuged at 2000 rpm for 10 min. The clear supernatant was used for the estimation of total calcium by flame photometry method. Total calcium level in sciatic nerve homogenate was calculated from standard curve using calcium carbonate and expressed as ppm per milligram of protein.[39]

Estimation of neurotransmiter (Brain GABA content)

The animals were sacrificed using a CO_2 euthanasia chamber on the last day, 1 h after all behavioral tests, and brains were removed. The brains were separated and weighed after being rinsed in an isotonic saline solution. The tissue homogenate (10% w/v), with 0.1M phosphate buffer (pH 7.4) was made. Centrifugation of the homogenate at 2000 g for 20 minutes at 4°C the supernatant was used for the estimation of GABA levels. About 0.1 ml of sample was added with 0.2 ml of 0.14 M ninhydrin (Prepared with 0.5M carbonate-bicarbonate buffer, pH 9.95). This mixture was heated for 30 min at 60 °C. After heating, the mixture was allowed to cool and 5 ml of copper tartarate reagent was added. Then the fluorescence was measured at 377/455 nm in a spectofluorimeter after 10 min.[40]

Histopathological analysis

The rat sciatic nerve were examined for histopathological evaluation. The sciatic nerve were removed and immediately fixed in 10% buffered formalin. The sciatic nerve, which had been alcohol- dehydrated and embedded in paraffin. Using a microtome, five-micrometer thick serial histological slices were cut from paraffin blocks and stained with hematoxylin and eosin (H&E). Digital microscope (Olympus, Japan), was used to analyses the sections under a light microscope while taking photomicrographs.[41]

Statistical analysis

Data was expressed as mean ± standard error mean (SEM). Data analysis was performed using Graph Pad Prism 10.4.1 software (Graph Pad, San Diego, USA). Data of behavioural tests were statistically analyzed using two-way repeated analysis of variance (ANOVA) and Tukey's multiple comparison test was applied for post-hoc analysis, while data of biochemical parameters were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test was applied for post-hoc analysis. A value of $P < 0.001$ were considered to be statistically significant

RESULTS

Computational In-silico studies

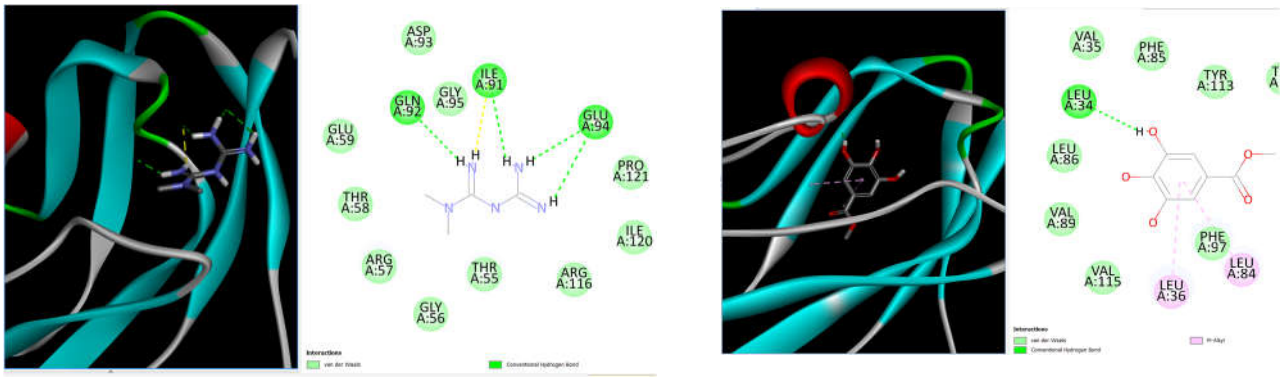
LD_{50} for methyl gallate and cilnidipine was found to be1700 mg/kg, and 2968 mg/kg respectively as per predicted by Software ProTox 3.0. which indicates that both the drugs was found to be safe. Also acute toxicity of both the drugs was already performed. Log p values of methyl gallate (0.93) and cilnidipine (3.17) were estimated using SwissADME software. These log p values indicate that both drug have good permeability. Targets for methyl gallate was found to be Cox-2, VEGF, aldose reductase, NT-3 growth factor, AMPK, caspase-6, glucose transportor, Nf-kb, Insulin like growth factor1 and for cilnidipine includes voltage gated calcium channel, glucose transporter, GABA receptor, G-protein couple receptor 55, VEGF, NT-3 growth factor, Nrf2, Nf-kb, TRPV1. According to literature, these targets were involved in pathogenesis of diabetic neuropathy. Hence methyl gallate and cilnidipine could be potential to treat neuropathy by targeting these pathways.

To predict the therapeutic targets and mechanism of neuroprotection of methyl gallate and cilnidipine, theoretical binding studies (molecular docking) were performed on AGE, CaV2.2, GAT-1 pathways which involved in pathogenesis of diabetic neuropathy. The results showed that values for calculated binding affinities of methyl gallate and cilnidipine (Kcal/mol) was found to be comparable to the standard drugs pregabaline and metformin (Table 1 and Fig.1).

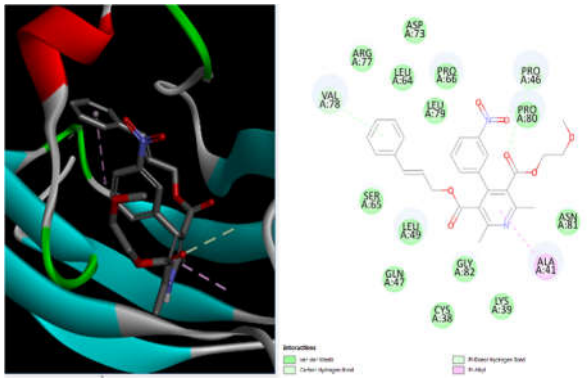
Table 1- Calculated affinity of drugs

Targets	Calculated affinity of drugs (Kcal/mol)		
	I. Methyl Gallate	II. Cilnidipine	III. Standard
1. AGE	- 5.7 (A)	- 4.6 (B)	-6.4 (Metformin) (C)
2. CaV2.2	- 5.3 (D)	-5.9 (E)	- 5.1 (Pregabalin) (F)

(A) (B)



(C)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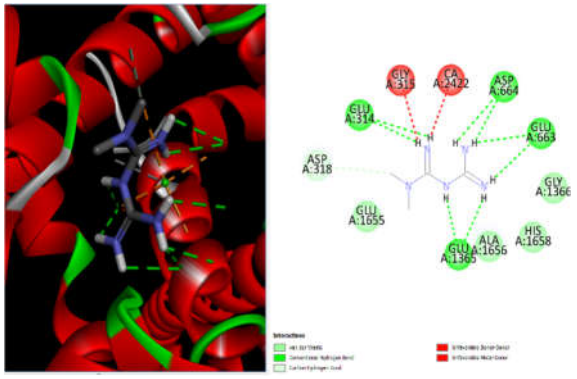
ALA A:41
CYS A:38
LYS A:39

(D)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GLU A:314
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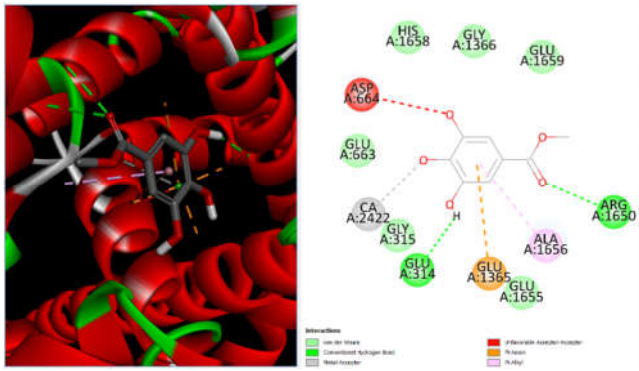
GLU A:1365

(E)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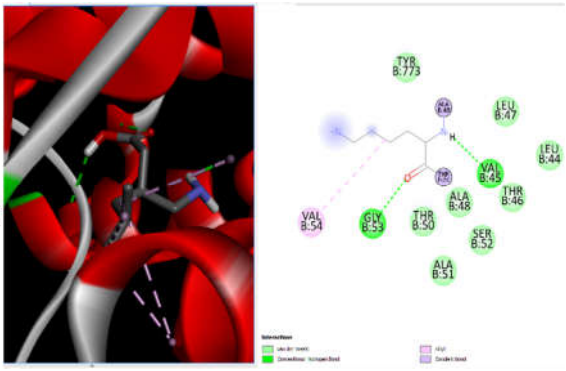
GLU A:1655

(F)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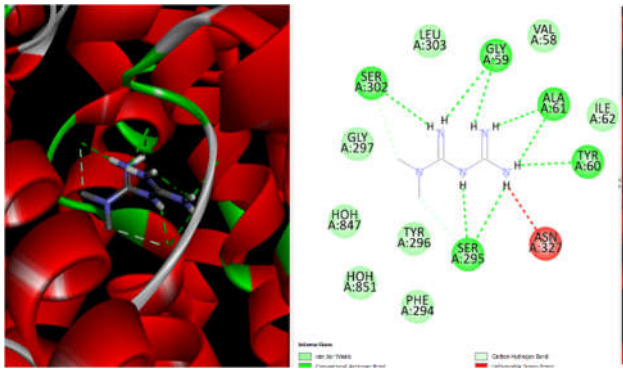
ALA B:51

(G)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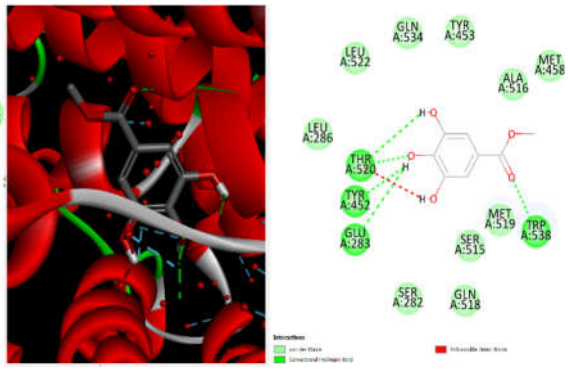
HOH A:851
PHE A:294

(H)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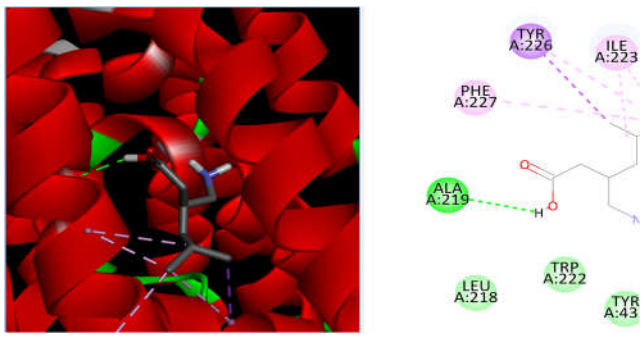
(I)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ILE A:223
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LEU A:218
TYR A:43

Fig.1: 3D and 2D representation of the interaction of (A) methyl gallate, (B) cilnidipine, (C) metformin with human advanced glycation end products receptor (AGE- 2e5e).

3D and 2D representation of the interaction of (D) methyl gallate, (E) cilnidipine, (F) pregabalin with human N-type VGCC (CaV2.2- 7vfv).

3D and 2D representation of the interaction of (G) methyl gallate, (H) cilnidipine, (I) Pregabalin with human GAT-1 (7y7e).

Effect of methyl gallate and cilnidipine on body weight

There was no significant difference in the body weight in the STZ control rats and normal non diabetic rats before induction of diabetic neuropathy. Eight weeks after STZ administration resulted in significant decrease ($P < 0.001$) in body weight in STZ control rats as compared to normal control rats. Chronic treatment with methyl gallate, cilnidipine in diabetic rats from the 5th to 8^{th} week significantly ($P < 0.001$) restored the body weight as compared with STZ treated rats. However, methyl gallate in combination with cilnidipine also produced significant ($P < 0.001$) effect on body weight at the end of 8^{th} week as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. Also pregabalin and metformin treated diabetic rats also significantly ($P < 0.001$) restored body weight (Fig 2A).

Effect of methyl gallate and cilnidipine on blood glucose level

There was no significant difference in blood glucose level in the STZ control rats and normal nondiabetic rats before induction of diabetic neuropathy. However, results indicated that starting from week two after STZ administration there was singnificant ($P < 0.001$) increase in blood glucose level in STZ control rats when compared to the normal control rats. Treatment with methyl gallate, cilnidipine in diabetic rats from the 5^{th} to 8^{th} week significantly ($P < 0.001$) ameliorated increased glucose levels as compared with control rats. However, methyl gallate in combination with cilnidipine showed more significant improvement in blood glucose level at the 8^{th} week as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. Also metformin treated diabetic rats produced significant ($P < 0.001$) effect on blood glucose level. In addition threre was no significant difference in blood glucose level in the pregabalin treated rats as compared to STZ treated rats (Fig. 2B).

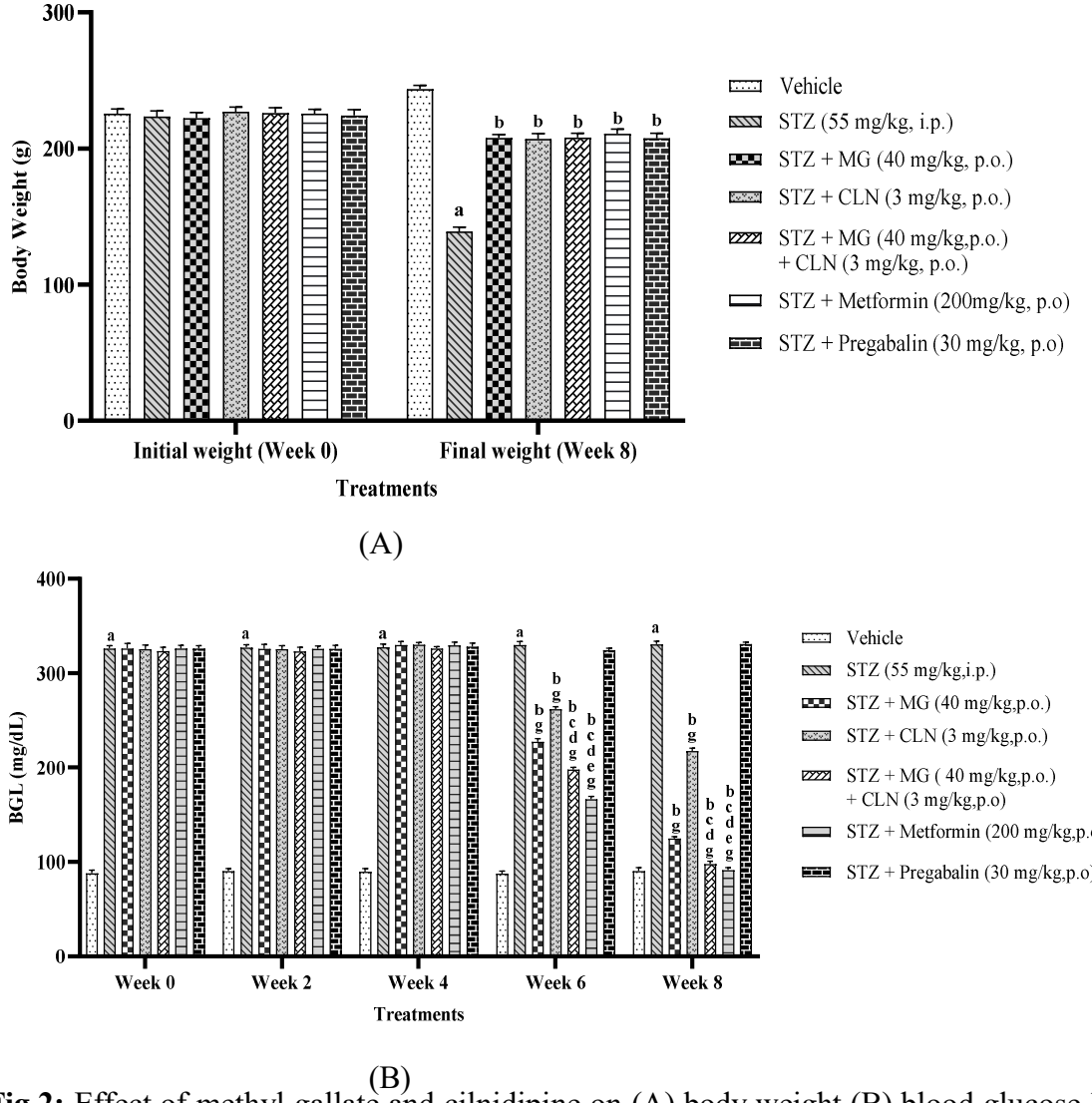


Fig.2: Effect of methyl gallate and cilnidipine on (A) body weight (B) blood glucose level in STZ induced diabetic neuropathy in rats. All values were expressed as mean ± SEM (n=6) and analyzed by two-way ANOVA followed by Tukey's test. *ns*- non significant, $^{a}P < 0.001$ as compared to control group, $^{b}P < 0.001$ as compared to STZ treated group, $^{c}P < 0.001$ as compared to methyl gallate (MG), $^{d}P < 0.001$ as compare to cilnidipine (CLN), $^{e}P < 0.001$ as compared to methyl gallate + cilnidipine (MG + CLN), $^{g}P < 0.001$ as compared to pregabalin.

Effect of methyl gallate and cilnidipine on fall of latency in rota rod test

Diabetic rats showed motor in-coordination as indicated decrease in fall latency (s). There was no significant difference in the fall of latency in the STZ control rats and normal non control rats before induction of diabetic neuropathy. Significant (P< 0.001) decrease in fall latency was observed in STZ control rats from week 2 after STZ administration as compared with normal control rats. Treatment with methyl gallate, cilnidipine in diabetic rats from the 5th to 8th week led to a significant (P< 0.001) improvement in motor coordination,indicated

by increased fall in latency as compared with STZ control rats. Moreover, combined methyl gallate and cilnidipine treatment showed more significant (P< 0.001) improvement in motor coordination in diabetic rats at the end of the 8th week as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. Also pregabalin, metformin treated diabetic rats produced significant (P< 0.001) improvement in motor coordination as compared with STZ control rats (Fig.3A).

Effect of methyl gallate and cilnidipine on locomotor counts in actophotometer test

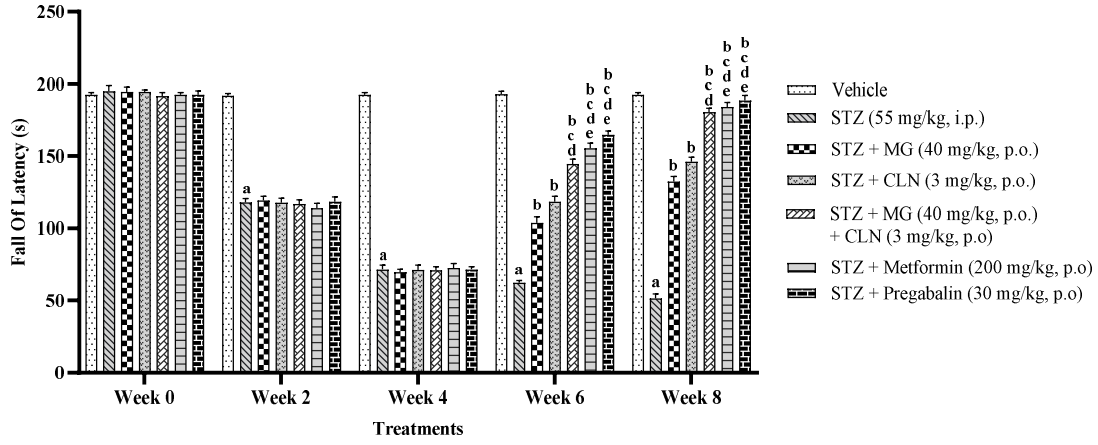
There was no significant difference in total counts in actophotometer test in the STZ control rats and normal nondiabetic rats before induction of diabetic neuropathy. Significant (P < 0.001) decrease in locomotor behavior which is indicated by decrease in total counts in STZ control rats from week 2 after STZ administration as compared with normal control rats. Treatment with methyl gallate, cilnidipine in diabetic rats from the 5th to 8th week led to a significant (P < 0.001) improvement in locomotor behaviour indicated by increased total counts as compared with STZ control rats. Moreover, combined methyl gallate and cilnidipine treatment showed more significant (P < 0.001) improvement in locomotor behaviour in diabetic rats at the end of the 8th week as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. In addition metformin and pregabaline treated diabetic rats also showed significant (P < 0.001) improvement in locomotor counts when compared with STZ control rats (Fig. 3B).

Effect of methyl gallate and cilnidipine on paw withdrawal lateny in hot plate test

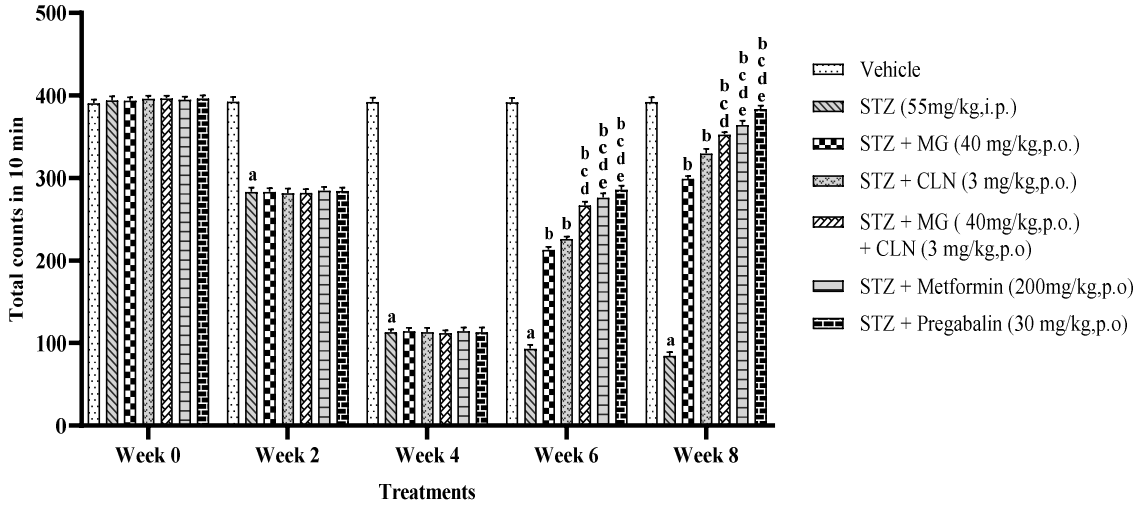
Paw withdrawal latency to thermal stimuli in STZ control rats before the induction of diabetic neuropathy was not significantly different than that in normal control rats. However, results indicate that starting from week two after STZ administration there was singnificant (P < 0.001) decrease in paw withdrawal latencies to thermal stimuli in STZ control rats when compared to the normal control rats. Treatment with methyl gallate, cilnidipine in diabetic rats from the 5th to 8th week led to a significantly (P < 0.001) longer paw withdrawal latency as compared to STZ control rats. Moreover, combined methyl gallate and cilnidipine treatment significantly (P < 0.001) raised paw withdrawal latency at the end of the 8th week and were more effective in potentiating the paw withdrawal latency when compared to diabetic rats treated with either methyl gallate or cilnidipine alone. In addition metformin and pregabaline treated diabetic rats also showed significant (P < 0.001) improvement in paw withdrawal latency when compared with STZ control rats (Fig. 3C).

Effect of methyl gallate and cilnidipine on allodynia score in acetone drop test

Increased allodynia score indicate that there is increased sensation to cold stimuli which is observed in neuropathic pain. Allodynia score in STZ control rats before the induction of diabetic neuropathy was not significantly different than that in normal control rats. However, results indicated that starting from week two after STZ administration, allodynia score due to cold stimuli were significantly ($P < 0.001$) increased in STZ control rats as compared with normal control rats. Treatment with methyl gallate, cilnidipine in diabetic rats from the 5th to 8th week led to a significant ($P < 0.001$) amelioration of increased allodynia score as compared to STZ control rats. Moreover, combined methyl gallate and cilnidipine treatment showed more significant ($P < 0.001$) attenuation of raised allodynia score in diabetic rats at the end of the 8th week as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. Also metformin and pregabaline treated diabetic rats showed significant ($P < 0.001$) decrease in allodynia score when compared with STZ control rats (Fig. 3D).



(A)



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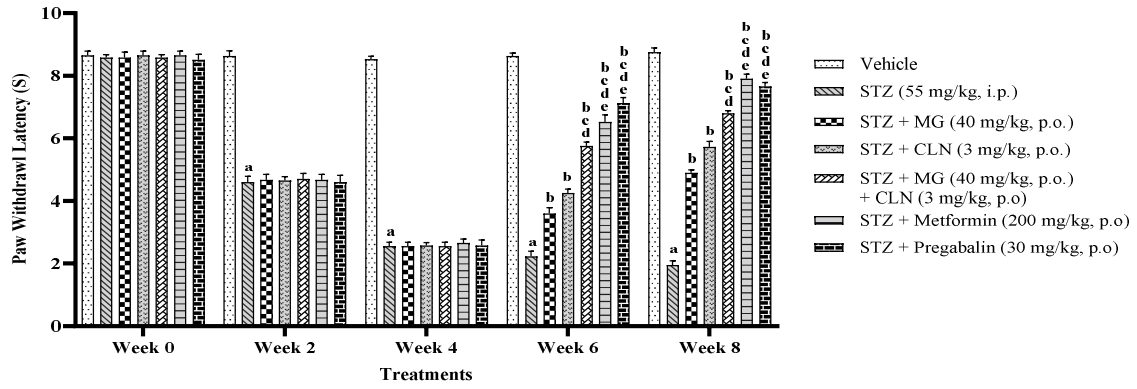

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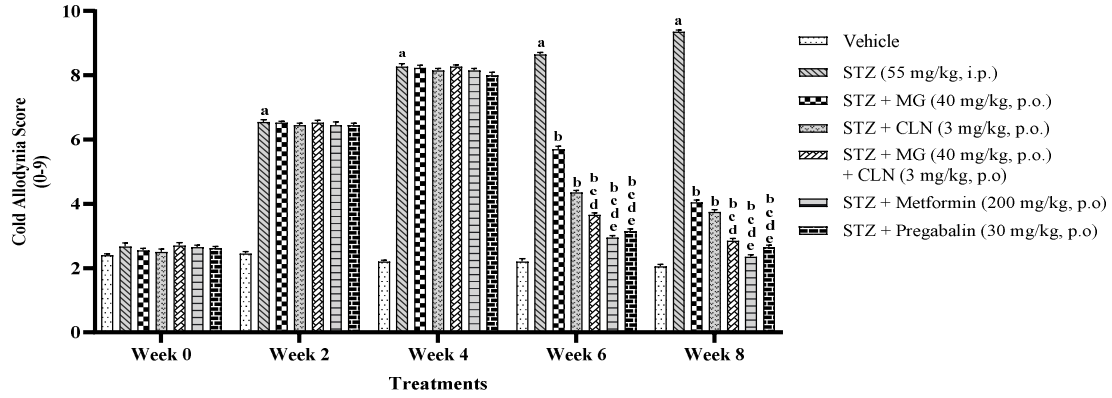


(D)

Fig.3: Effect of methyl gallate and cilnidipine on (A) fall of latency (B) locomotor count (C) paw withdrawl latency (D) allodynia score in STZ induced diabetic neuropathy in rats. All values were expressed as mean ± SEM (n=6) and analyzed by two-way ANOVA followed by Tukey's test. *ns*- non significant, $^{a}P < 0.001$ as compared to control group, $^{b}P < 0.001$ as compared to STZ treated group, $^{c}P < 0.001$ as compared to methyl gallate (MG), $^{d}P < 0.001$ as compared to cilnidipine (CLN), $^{e}P < 0.001$ as compared to methyl gallate + cilnidipine (MG + CLN), $^{f}P < 0.001$ as compared to metformin , $^{g}P < 0.001$ as compared to pregabalin.

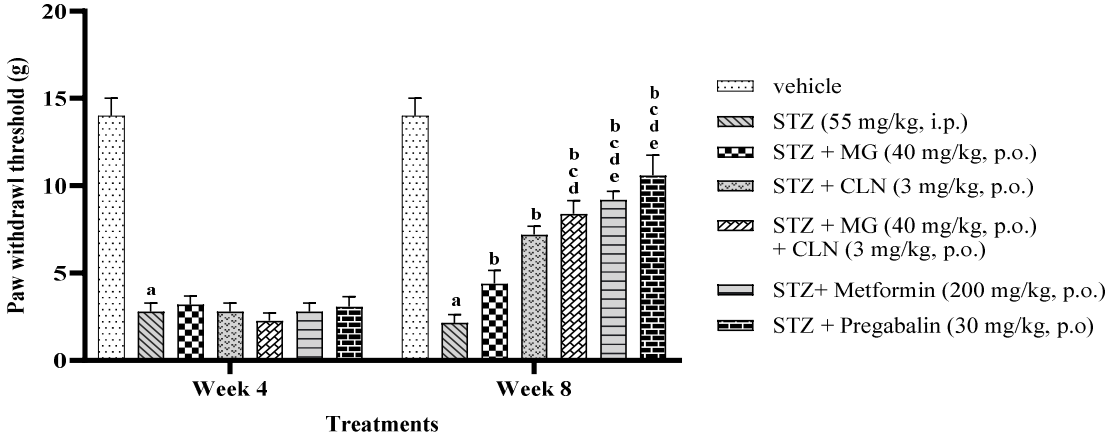
Effect of methyl gallate and cilnidipine on paw withdrawal threshold in von frey hair test

A significant ($P < 0.001$) reduction in mean paw withdrawal threshold (i.e., mechanical allodynia) was observed in STZ control rats after 4 weeks of STZ administration, in response to von frey hair stimulation, as compared to normal control rats. The 4 week treatment of

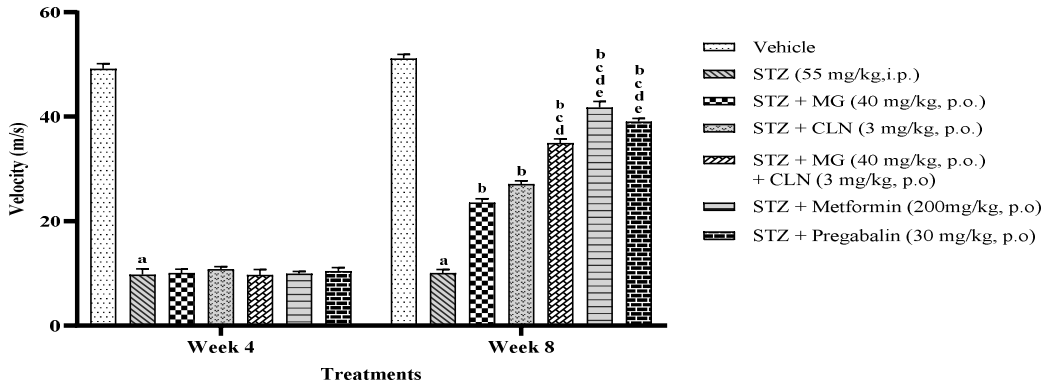
methyl gallate, cilnidipine showed significant ($P < 0.001$) amelioration of the reduced mean paw withdrawal threshold as compared with STZ control rats. However, methyl gallate in combination with cilnidipine showed more significant ($P < 0.001$) improvement in paw withdrawal threshold at the end of week 8 as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. Also metformin and pregabaline treated diabetic rats showed significant ($P < 0.001$) improvement in paw withdrawal threshold when compared with STZ control rats (Fig. 4A).

Effect of methyl gallate and cilnidipine on motor nerve conduction velocity (MNCV)

There was no significant difference in MNCV in the STZ control rats and normal control rats before induction of diabetic neuropathy. However, results indicated that four week after STZ administration there was singnificant ($P < 0.001$) reduction in MNCV in STZ control rats when compared to the normal control rats. Treatment with methyl gallate, cilnidipine in diabetic rats from the 5^{th} to 8^{th} week significantly ($P < 0.001$) ameliorated reduced MNCV as compared with STZ control rats. However, methyl gallate in combination with cilnidipine showed more significant ($P < 0.001$) improvement in MNCV at the end 8^{th} week as compared to diabetic rats treated with either methyl gallate or cilnidipine alone. Also metformin and pregabalin treated diabetic rats showed significant ($P < 0.001$) improvement in MNCV when compared with STZ control rats (Fig. 4B).



(A)



(B)

Fig.4: Effect of methyl gallate and cilnidipine on (A) paw withdrawl threshold (B) motor nerve conduction velocity in STZ induced diabetic neuropathy in rats. All values were expressed as mean ± SEM (n=6) and analyzed by two-way ANOVA followed by Tukey's test. *ns*- non significant, $^{a}P < 0.001$ as compared to control group, $^{b}P < 0.001$ as compared to STZ treated group, $^{c}P < 0.001$ as compared to methyl gallate (MG), $^{d}P < 0.001$ as compared to cilnidipine (CLN), $^{e}P < 0.001$ as compared to methyl gallate + cilnidipine (MG + CLN), $^{f}P < 0.001$ as compared to metformin, $^{g}P < 0.001$ as compared to pregabalin.

Biochemical parameters

All biochemical parameters were examined 1 h after all behavioral tests on last day of week 8 by sacrificing the rats

Effect of methyl gallate and cilnidipine on catalase (CAT)

Catalase in sciatic nerve of STZ control rats was significantly decreased at the 8th weeks after STZ administration as compared to normal control group. The neural catalase levels in pregabalin, metformin, methyl gallate, and cilnidipine treated diabetic rats were significantly increased as compared to STZ control rats. Moreover, diabetic rats treated with methyl gallate and cilnidipine combination more significantly attenuated these reduced catalase levels as compared to either methyl gallate or cilnidipine alone treated groups (Table 2).

Effect of methyl gallate and cilnidipine on reduced glutathione (GSH)

Reduced glutathione in sciatic nerve of STZ control rats was significantly decreased at the 8th weeks after STZ administration as compared to normal control rats. GSH levels in pregabalin, metformin, methyl gallate, cilnidipine treated diabetic rats were significantly increased as compared to STZ control rats. However, diabetic rats treated with methyl gallate

and cilnidipine ccombination more significantly inhibited this reduced levels of GSH as compared to methyl gallate or cilnidipine alone treated groups (Table 2).

Effect of methyl gallate and cilnidipine on superoxide dismutase activity (SOD)

SOD in sciatic nerve of STZ control rats was significantly decreased at the 8^{th} weeks after STZ administration as compared to normal control rats. SOD in pregabalin, metformin, methyl gallate, cilnidipine treated rats were significantly increased as compared to STZ control rats. Moreover, diabetic rats treated with methyl gallate and cilnidipine combination more significantly attenuated these reduced SOD amount as compared to either methyl gallate or cilnidipine alone treated groups (Table 2).

Effect of methyl gallate and cilnidipine on lipid peroxidation (LPO)

After 8 weeks of STZ administration, lipid peroxidation content in STZ control rats was significantly increased as compared to normal control rats. The lipid peroxidation content in pregabalin, metformin, methyl gallate, cilnidipine treated rats was decreased significantly as compared to STZ control rats. However, diabetic rats treated with methyl gallate and cilnidipine combination more significantly attenuated elevated lipid peroxidation content as compared to methyl gallate or cilnidipine alone treated groups (Table 2).

Effect of methyl gallate and cilnidipine on nitric oxide (NO)

Rats treated with STZ resulted in significant increase in NO levels in sciatic nerve of the STZ control rats as compared to normal control rats. Diabetic rats treated with pregabalin, metformin, methyl gallate, cilnidipine significantly attenuated this increase in the levels of NO compared to STZ control rats. However, diabetic rats treated with methyl gallate and cilnidipine combination significantly attenuated these elevated NO levels as compared to methyl gallate or cilnidipine alone treated groups (Table 2).

Table:2 Effect on biochemical parameters such as CAT, GSH, SOD, LPO, NO in sciatic nerve

Treatments	**CAT (µ moles of H_2O_2 decomposed/ mg protein/min)**	**GSH (µ moles of GSH/mg proteins)**	**SOD (% inhibition of reduction of NBT)**	**LPO (n moles of MDA/mg proteins)**	**NO (µ moles of nitrite/mg proteins)**
Vehicle	8.69 ± 0.104	7.82 ± 0.42	92.56 ± 0.87	1.31 ± 0.08	23.7 ± 0.81
Streptozotocin (55 mg/kg, i.p.)	2.68 ± 0.09^{a}	1.83 ± 0.04^{a}	28.97 ± 1.28^{a}	6.66 ± 0.09^{a}	52.8 ± 0.83^{a}
STZ + MG (40 mg/kg, p.o.)	4.32 ± 0.12^{b}	4.28 ± 0.03^{b}	45.11 ± 1.48^{b}	4.37 ± 0.07^{b}	45.7 ± 1.18^{b}
STZ + CLN (3 mg/kg, p.o.)	5.61 ± 0.10^{b}	4.74 ± 0.05^{b}	56.57 ± 1.46^{b}	3.72 ± 0.09^{b}	39.54 ± 0.68^{b}
STZ + MG (40 mg/kg, p.o.) + CLN (3 mg/kg, p.o.)	7.34 ± 0.11^{bcd}	7.21 ± 0.07^{bcd}	80.38 ± 0.98^{bcd}	2.107 ± 0.08^{bcd}	36.86 ± 0.7^{bcd}
STZ + Metformin (200 mg/kg, p.o.)	8.07 ± 0.11^{bcde}	7.53 ± 0.06^{bcde}	89.07 ± 1.34^{bcde}	1.71 ± 0.08^{bcde}	30.63 ± 1.05^{bcde}
STZ + Pregabalin (30 mg/kg, p.o.)	8.31 ± 0.11^{bcde}	7.54 ± 0.04^{bcde}	87.36 ± 0.95^{bcde}	1.86 ± 0.07^{bcde}	33.66 ± 0.66^{bcde}

Effect of methyl gallate and cilnidipine on AGE content

After 8 weeks of STZ administration, AGE content in sciatic nerve of diabetic control rats was significantly increased as compared to normal control rats. The AGE content in pregabalin metformin, methyl gallate, and cilnidipine treated diabetic rats was significantly decreased as compared to STZ control rats. However, diabetic rats treated with methyl gallate and cilnidipine combination more significantly attenuate elevated content of AGE in sciatic

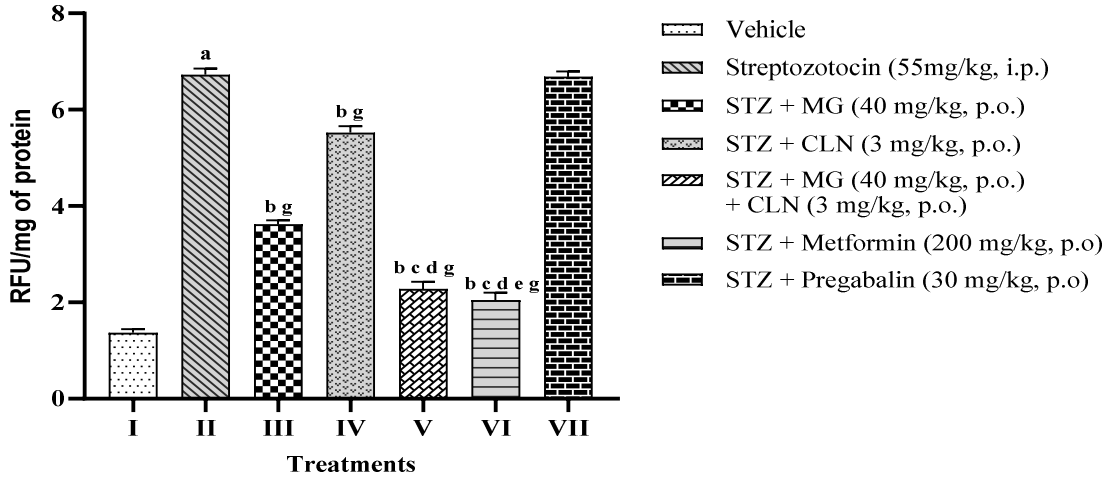
nerve of diabetic rat as compared to methyl gallate or cilnidipine alone treated groups (Fig. 5A).

Effect of methyl gallate and cilnidipine on total calcium (Ca^{2+})

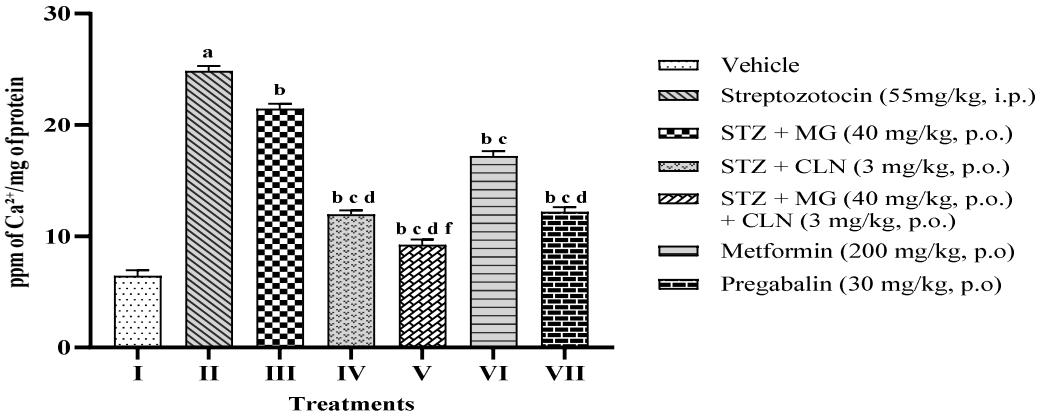
After 8 weeks of STZ administration, Ca^{2+} levels in sciatic nerve of STZ control rats was significantly increased as compared to control group. The Ca^{2+} levels in metformin, methyl gallate, cilnidipine treated diabetic rats was significantly decreased as compared to STZ control rats. However, diabetic rats treated with methyl gallate and cilnidipine combination more significantly attenuate increased level of Ca^{2+} in sciatic nerve of diabetic rat as compared to methyl gallate or cilnidipine alone treated groups (Fig. 5B).

Effect of methyl gallate and cilnidipine on neurotransmiter (brain GABA Content)

After 8 weeks of STZ administration, GABA concentration in sciatic nerve of diabetic control rats was significantly decreased as compared to normal control rats. The GABA concentration in pregabalin, metformin, methyl gallate, cilnidipine treated diabetic rats was significantly increased as compared to STZ control rats. However, diabetic rats treated with methyl gallate and cilnidipine combination more significantly attenuate this decreased GABA concentration in sciatic nerve of diabetic rat as compared to methyl gallate or cilnidipine alone treated groups (Fig. 5C).



(A)



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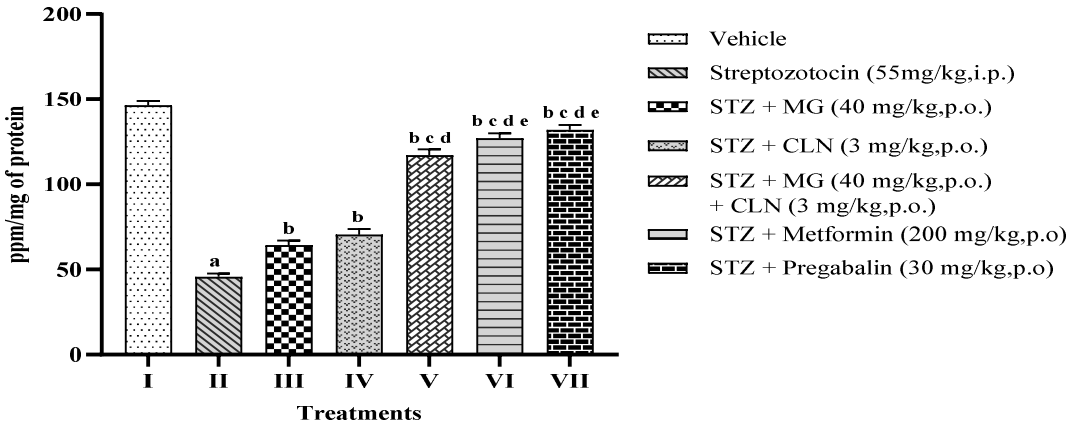

(C)

Fig.5: Effect of methyl gallate and cilnidipine on (A) AGE level (B) Ca^{2+} (C) brain GABA content in STZ induced diabetic neuropathy in rats. All values were expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA followed by Tukey's test. *ns*- non significant, $^{a}P < 0.001$ as compared to control group, $^{b}P < 0.001$ as compared to STZ treated group, $^{c}P < 0.001$ as compared to methyl gallate (MG), $^{d}P < 0.001$ as compared to cilnidipine (CLN), $^{e}P < 0.001$ as compare to methyl gallate + cilnidipine (MG + CLN), $^{f}P < 0.001$ as compared to metformin, $^{g}P < 0.001$ as compare to pregabalin.

Histopathological analysis

In the control group, the sciatic nerve had a well-organized myelin sheath and schwann cells, with no degeneration or vacuolization. In diabetic rats, nerve fibers were widely separated, showing axonal degeneration, cellular infiltration, and reduced schwann cells. Methyl gallate treated group showed reduce degeneration but showed mild axonal damage. Treatment with cilnidipine, there was vacuolization and nerve fiber swelling, with slightly improved schwann cell count. Combined treatment with methyl gallate and cilnidipine showed better myelin organization, recovered Schwann cells, and reduced swelling and infiltration. Metformin and

pregabalin treatment restored nerve fibers with a uniform, dense myelin sheath similar to the control group (Fig.6).

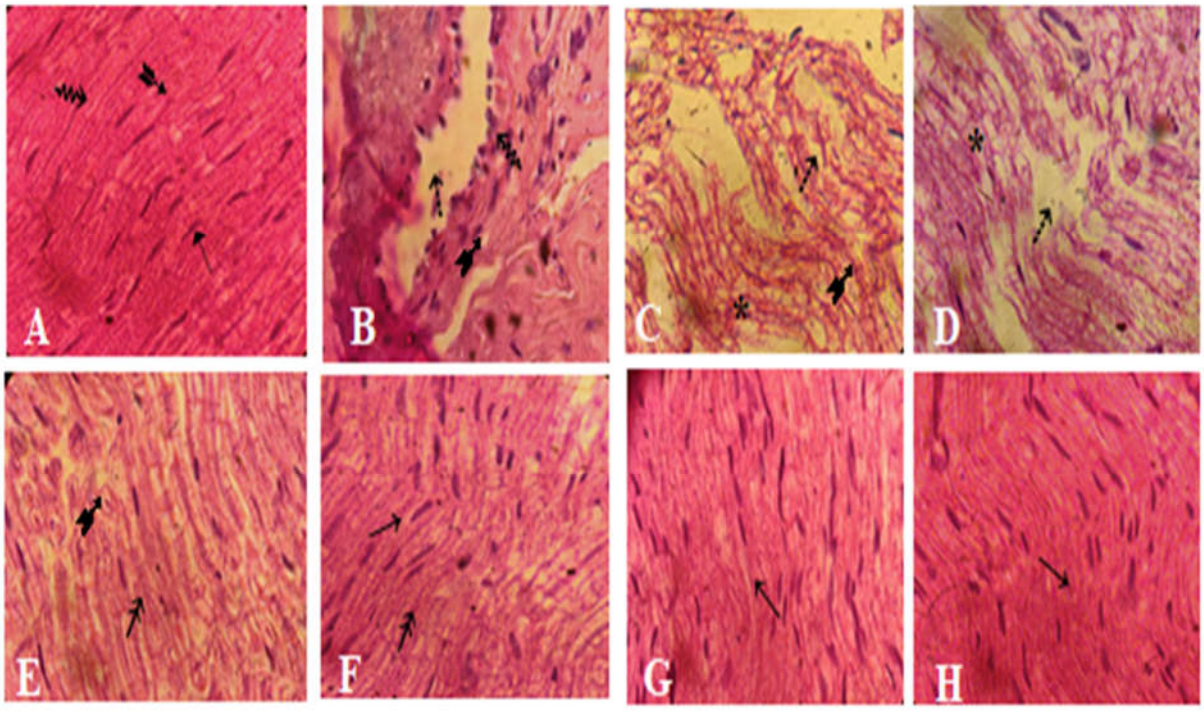

Fig.7:Effect of methyl gallate, cilnidipine and there combination on histopathological analysis of sciatic nerve in STZ induced neuropathy in rats. A, control: well-organized myelin sheath (⇝), axons (➶) and Schwann cell nuclei **(→)**. **B, C: STZ** respectively: widely separated nerve fibers **(*)**, axonal irregularity, and axonal degeneration (⇞) and mononuclear cellular infiltrations (⇝) and vacuolization(➶**). D, MG:** widely separated nerve fibers **(*)**, Mild Axonal degeneration(⇞). **E, CLN:** vacuolization (➶**)** Reduced axonal degeneration(↟). **F, MG + CLN:** well-organized myelin and axons **(→)**, slightly separated nerve fibers (↟**). G, Metformin** and **H, Pregabaline:** Well organized nerve fibers **(→)**, increased axonal regeneration with the nearly normal image such as control group.

DISCUSSION

Long-standing diabetes mellitus leads to microvascular and neurologic complications.[3] Among various microvascular complications, diabetic neuropathy (DN) is the most common complication.[4] First-line therapies include tricyclic antidepressants, serotonin–noradrenaline reuptake inhibitors, and anticonvulsants that act on calcium channels. Although a wide range of drugs are available, there is still a lack of specific drugs and treatment options for DN due to its complex pathogenesis, diverse clinical manifestations.[13] It is estimated that

approximately 40% of neuropathic patients are resistant to the currently available drugs.This necessitates the exploration of novel drug targets to treat neuropathic pain of various region. Polyphenols, a diverse and extensive collection of phytochemicals containing phenol rings, are multi-target agents with the highest antioxidant and anti-inflammatory effects, methyl gallate is one such kind[16] and it was found in *Terminalia myriocarpa.* Reorted activities of methyl gallate are such as anti-tumor, anti-inflammatory , anti-oxidation anti-microbial , anti-malarial.[17] Also it has anti-α-Glucosidase property and it inhibits the formation of advanced glycation end product.[42] These mechanisms suggested that methyl gallate may show protective effect in diabetic neuropathy. In the field of drug development, drug repurposing could be an interesting strategy to search new therapeutic options against neurodegenerative diseases, since it involves lower costs and time for development.[18,19] Cilnidipine is N- and L-type calcium channel blocker was approved in India in 2007 for the treatment of mild to moderate hypertension.[19] According to literature, it was found that N-type calcium channel play role in nerve pain and whenever nerve damage the α1β, α2δ1 subunit of N type calcium channel upregulated.[20] Hence by blocking this receptor neuropathy may be resolved.

Streptozotocin is a glucosamine-nitrourea derivative obtained from *Streptomyces achroogenes*, possessing cytotoxic action damaging DNA which results rapid destruction of pancreatic β cells.[43] Diabetic neuropathy is characterized by clinical features like allodynia, hyperalgesia, reduced motor nerve condition velocity, neuronal hypoxia, reduced threshold to painful stimuli, etc. Similar symptoms are exhibited by STZ induced diabetic animals.[44] hence in our investigation streptozotocin was used to induce neuropathic pain.

To predict mechanism of neuroprotection, the in-silico docking studies were performed to identify targets for methyl gallate and cilnidipine. The results indicated that calculated affinities (Kcal/mol) of methyl gallate was found to be AGE (-5.7), CaV2.2 (-5.3), GAT-1(-5.4) and for cilnidipine AGE (- 4.6), CaV2.2 (-5.9), GAT-1(- 6.1), The values exhibited good affinities for the targets, suggesting methyl gallate and cilnidipine as potential candidates for the treatment of diabetic neuropathy. Further, these targets were confirmed by performing vivo studies.

Swanston-Flatt et al., 1990, reported that increased muscle wasting and loss of tissue protein is mainly responsible for the decreased in the body weight of the diabetic rats.[45] Cellular biosynthesis and metabolism has been proven to be the underlying cause of diabetes induced

body weight loss. Body weight reduction was significantly restored in all treatment groups. In addition the combination of methyl gallate and cilnidipine treatment more significantly restored body weight. The results of present investigation were matched with the past studies evaluating naringin, a flavonoid. [46]

Kandhare et al., 2012, documented that formation of the oxidative stress due to breakdown of double strand of pancreatic islets DNA to a single strand after intraperitoneal administration of STZ caused significant elevation in blood glucose level compared with normal non diabetic rats.[47] Treatment with the metformin, methyl gallate, cilnidipine significantly restored the elevated levels of the blood glucose and thus attenuated hyperglycemia.The combination therapy of methyl gallate and cilnidipine in diabetic rats showed more significantly attenuation. The result of present investigation is in accordance with the findings of Jadhav et al., 2022.[48]

Hyperalgesia, or allodynia, is the form of nociceptive pain that manifests in inflamed tissue. reports, the validated techniques for assessing pain in laboratory animals include von frey hair, cold acetone drop test, hot plate, tail flick test.[49] In the current study, a significant reduction in mean paw withdrawal threshold, paw withdrawal latency and increased allodynia score was observed in the diabetic rats after 4 weeks of STZ injection as compared to normal non-diabetic rats. Chronic treatment with pregabalin, metformin, methyl gallate, cilnidipine showed significant amelioration of the decrease in mean paw withdrawal threshold, paw withdrawal latency and increased allodynia score in diabetic rats. The results of present investigation were matched with the past study of Dhaliwal et al., 2020.[50]

Vascular endothelial damage may be caused due to generation of superoxide and hydroxyl free radicals. This phenomenon may in turn cause activation of aldose reductase and protein kinase C and modulate pain perception.[46] SOD converts superoxide anion to H_2O_2 and thus serves as antioxidant. Furthermore, abnormalities in CAT, GSH metabolism in diabetes may also lead to the enhanced TNF-α and IL-1β production.[51] The SOD and GSH and CAT levels were significantly decreased in the sciatic nerve of diabetic animals which were restored on 4 week chronic treatment with methyl gallate and cilnidipine and the results were in parallel with investigation of Kaur et al. 2016 and Kishore et al. 2016.[52]

MDA, a marker of oxidative stress, was elevated in STZ-induced diabetic rats, leading to lipid membrane damage and nerve dysfunction. This increase in lipid peroxidation was linked to reduced antioxidant enzyme activity in peripheral nerves.[53] Treatment with methyl gallate

and cilnidipine significantly reduced MDA levels. Elevated nitric oxide (NO) in high glucose conditions leads to oxidative and nitrosative stress, forming peroxynitrite, which causes nerve degeneration, contributing to neuropathic pain.[54] Four weeks of treatment with methyl gallate and cilnidipine significantly reduced NO levels, suggesting their neuroprotective effect in diabetic rats. These results are compatible with the previous studies carried out by of Suryavanshi et al., 2020.[55]

In diabetes, the accumulation of AGEs increase glucose levels, generate free radicals, and damage tissues, contributing to nerve demyelination and dysfunction. AGE/RAGE interactions activate oxidative stress and inflammation, worsening neuropathy.[11] Methyl gallate along with cilnidipine significantly reduced AGE levels, suggesting its potential role in treating diabetic neuropathy. The results of present investigation were matched with the past studies of Alkholif et al., 2023[56] N-type calcium channels supports a role in controlling the transmission of nociceptive signal.[19] Interestingly, α1β and α2δ subunits are upregulated in neurons following tissue inflammation.[20] STZ administration elevated calcium levels, contributing to excitotoxicity and axonal cytoskeleton degranulation in diabetic neuropathy. methyl gallate and cilnidipine significantly reduced calcium levels in the sciatic nerve, preventing neuronal damage and mitigating neuropathic pain indicating role in controlling nociceptive signal transmission under pathological conditions. Our results were in accordance with investigation of Takahara et al., 2009.[30] γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the central nervous system and neuropathic pain has been attributed to decreased availability of spinal GABA, an inhibitor of spinal pain transmission. Spinal GABA depletion resulting in pain behaviour may possibly be an effect of enhanced presynaptic reuptake through upregulated GAT-1. Chronic treatment with methyl gallate and cilnidipine prevent neuronal damage *via* blocking GAT-1 expression in brain. The result of present investigation is in accordance with the findings of Yadav et al., 2015.[14]

The histoarchitecture study of methyl gallate and cilnidipine treated group indicated neuroprotective effects in sciatic nerve via reducing demyelination of peripheral nerves, cellular infiltration, axonal swelling and axonal degeneration as compared to diabetic control group. Histopathology results of the present study also showed there was slightly separated nerve fibers without any vacuolization and necrosis in nerve cell in rats treated with methyl gallate and cilnidipine. Our results were matched with previous findings of Balaha et al., 2018.[57]

CONCLUSION

In the present investigation various behavioral, biochemical and molecular effects of methyl gallate, cilnidipine and their combination were compared with standard drugs such as pregabalin and metformin. Methyl gallate and cilnidipine alone restored the elevated levels of blood glucose and partially reversed the neuropathic pain isn STZ induced diabetic rats. However, methyl gallate and cilnidipine combination not only attenuated the diabetic condition but also reversed neuropathic pain through modulation of oxidative–nitrosative stress. The current study showed scientific evidence for attenuation of oxidative stress, sprevention of nerve damage *via* improving the nerve physiology and conduction velocities by methyl gallate-cilnidipine combination in diabetic rats. This neuroprotective activity of methyl gallate and cilnidipine may be linked to inhibition of AGE synthesis, downregulation of GAT-1 exxpression, and blocking calcium channel in sciatic nerve. This seems to be novel approach to target diabetic neuropathic pain. However, further studies will vindicate to explore the exact mechanism of methyl gallate and cilnidipine antino-ciceptive effect.

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