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# The Antihyperalgesic Activity of Lacosamide and Amitriptyline in Combination in Diabetes Induced Neuropathy

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**Keywords:** Diabetic Neuropathy, Streptozotocin, Neuropathic pain, Tricyclic antidepressants, Lacosamide

**ABSTRACT**

The term diabetes mellitus describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long term damage, dysfunction and failure of various organs. Diabetes mellitus is a major and growing health problem in most countries and an important cause of prolonged ill health and early death. Diabetes has now become a global public health burden with worldwide incidence of 5% in the general population. The long term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. One of the most common diabetes-related complication is diabetic neuropathy which affects nearly 50% to 66% of all patients with diabetes and affects individuals with both type 1 and type 2 diabetes. Tricyclic antidepressants are considered the first-line choice of treatment for chronic pain associated with diabetic neuropathy. Neuropathic pain generally responds more quickly than depression to tricyclic antidepressants and often with one third to one half the dosage administered for depression. Lacosamide is a novel chemical entity with anticonvulsant and analgesic properties that is being developed to treat epilepsy and neuropathic pain conditions.

## INTRODUCTION

Diabetes mellitus is a major and growing health problem in most countries and an important cause of prolonged ill health and early death [1].Diabetes has now become a global public health burden with worldwide incidence of 5% in the general population. In USA, which ranks third after India and China in the prevalence of diabetes, the growth rate is expected to be much smaller: from 13.9 million in 1995 to 21.9 in 2025.

The rise in number of people with diabetes is expected to be fast in Pakistan, Indonesia, Egypt and Mexico, and somewhat slow in Japan [2]. The countries with the largest number of diabetic people in the year 2025 will be India, China and United States [3]. The number of adults with diabetes in the world will rise from 135 million in 1995 to 300 million in the year 2025. Recent studies of geographical and ethnical influences have shown that people of Indian origin are highly prone to diabetes [4]. There are more than 30 million people with diabetes mellitus in India and the incidence is increasing. Also, there are many patients in the community with undiagnosed diabetes.

The number of adults suffering from diabetes in India is expected to increase three-fold, from 19.4 million in 1995 to 57.2 million in 2025 [5].

Diabetes is being projected as the World's main disabler and killer in the next 25 years. The real burden of the disease is however due to its associated complications which lead to increased morbidity and mortality. The long term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease.

One of the most common diabetes-related complication is diabetic neuropathy which affects nearly 50% to 66% of all patients with diabetes and affects individuals with both type 1 and type 2 diabetes [6].The morbidity and mortality rates with diabetic neuropathy are highest as compared with other complications of diabetes [6]. Overall, approximately 10% of the patients with diabetes

experience persistent pain from neuropathy. The pain can be ongoing, spontaneous or hyperalgesic i.e., increased response to painful stimulus.

Tricyclic antidepressants are considered the first-line choice of treatment for chronic pain associated with diabetic neuropathy. Neuropathic pain generally responds more quickly than depression to tricyclic antidepressants and often with one third to one half the dosage administered for depression. Its antidepressant effect is due to inhibition of reuptake of serotonin and / or nor epinephrine by neuronal membranes.

Lacosamide is a novel chemical entity with anticonvulsant and analgesic properties that is being developed to treat epilepsy and neuropathic pain conditions. Lacosamide has shown efficacy in many animal models of chronic pain and in several short and long term Phase II/III clinical trials in humans with diabetic neuropathic pain. The mechanism of action of lacosamide used to treat neuropathic pain is that it selectively enhances sodium channel slow inactivation without affecting fast inactivation and may modulate collapsin-response mediator protein 2. [7,8,9].

Hence, the present study was undertaken to evaluate the effectiveness of combination therapy of amitriptyline and lacosamide in the management of diabetes induced neuropathy.

**AIM AND OBJECTIVES**

To evaluate the individual and synergistic effect of drug combinations i.e., Amitriptyline and Lacosamide in Diabetes induced neuropathic pain using different behavioural study models.

1. Evaluation of Anti-hyperalgesic activity of Amitriptyine and Lacosamide in Diabetes induced neuropathy by estimating the level of neurotransmitters:

- serotonin
- norepinephrine
- glutamate
- GABA

## MATERIALS AND METHODS

**Table no.1 List of Chemicals and Apparatus/ Instruments**

| Sr.No. | Name of Chemicals | Apparatus/ Instrument |
|---|---|---|
| 1 | Ethyl Alcohol (95%) | Cooling Centrifuge |
| 2 | Streptozotocin, GABA | Tissue homogenizer |
| 3 | lacosamide | UV- Visible Spectrophotometer |
| 4 | Amitriptyline | Micropipette |
| 5 | Hydrochloric acid | Hot air oven |
| 6 | Ninhydrin reagent | Eddy's hot plate |
| 7 | Glutamic acid, Standard Serotonin | Rotaroad |
| 8 | Phenol, Glucose kit | Beam walk |

## EXPERIMENTAL ANIMAL

**Rats**: Wistar rats weighing 180 – 250gm were obtained from Animal House of Pharmacology Dept. Appasaheb Birnale College of Pharmacy, Sangli. Animals of either sex were housed under standard laboratory conditions of temperature 22 ± 3°C and relative humidity of 44-56 % with free access to standard pellet diet and water ad libitum. Laboratory animal handling and experimental procedures were performed according to Animal Ethical guidelines provided by Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA, New Delhi, India).

## EXPERIMENTAL DESIGN

**Table no.2 Experimental Design**

| GROUP | TREATMENT | PHARMACOLOGICAL EVALUATION |
|---|---|---|
| **I (NS)** | Normal Saline | ➢ **Behavioural methods:**<br>○ PawHeat-Hyperalgesia (Hot Plate Test)<br>○ Hot water tail immersion test<br>○ Cold water tail immersion test<br>○ Tail Flick<br>○ Rota rod method<br>○ Beam walk<br>➢ ***In-vitro* evaluation models:**<br>○ Serotonin<br>○ Noradrenaline<br>○ Glutamate<br>○ GABA |
| **II (STZ)** | Streptozotocin (70 mg/kg, i.p.) | |
| **III (STZ+Ami-ST)** | Streptozotocin (70 mg/kg,i.p.) +Amitriptyline (15mg/kg, i.p.) | |
| **IV (STZ+Ami-T)** | Streptozotocin (70 mg/kg,i.p.) +Amitriptyline (30mg/kg, i.p.) | |
| **V (STZ+Laco-ST)** | Streptozotocin (70 mg/kg,i.p.) +Lacosamide (10 mg/kg i.p.**)** | |
| **VI (STZ+Laco-T)** | Streptozotocin (70 mg/kg,i.p.) +Lacosamide (30 mg/kg i.p.**)** | |
| **VII (STZ+Ami-ST + Laco-T)** | Streptozotocin (70 mg/kg,i.p.) +Amitriptyline (15 mg/kg i.p.) + Lacosamide (30 mg/kg i.p.**)** | |
| **VIII (STZ+Ami-T +Laco-ST)** | Streptozotocin (70 mg/kg,i.p.) +Amitriptyline (30 mg/kg i.p.) + Lacosamide (10 mg/kg i.p.**)** | |

**INDUCTION OF EXPERIMENTAL DIABETES**

All the rats were fasted 48hrs before the administration of Streptozotocin. Diabetes was induced in rats by intra-peritoneal injection of streptozotocin dissolved in 0.1M sodium citrate buffer pH4.5 at the dose of 70mg/kg body weight.

After the injection free access to food and water was done. The animals were allowed to drink 5% glucose solution overnight to overcome hypoglycaemic shock. The development of diabetes was confirmed after 48hrs of Streptozotocin injection.

The blood was collected from the retro orbital sinus and serum was seperated out to determine the blood glucose level using semi-autoanalyser.

The animals having fasting blood glucose level more than 200mg/dl were considered as diabetic rats and used for the experimentation. Diabetic animals were grouped five days after induction of diabetes.

**TREATMENT PATTERN AFTER THE INDUCTION OF DIABETES:-**

**Table no. 3. Treatment pattern after the induction of diabetes**

| Sr. No. | Groups | Dose | Route of administration | No. of animal per group | Day of Treatment after STZ administration |
|---|---|---|---|---|---|
| 1 | Normal Saline | 2 ml/kg | i.p. | 6 | - |
| 2 | Control (STZ) | 70 mg/kg | i.p. | 6 | - |
| 3 | STZ+Ami-ST | 70mg/kg +15mg/kg | i.p. | 6 | 29$^{th}$ day |
| 4 | STZ+Ami-T | 70mg/kg+ 30mg/kg | i.p. | 6 | 29$^{th}$ day |
| 5 | STZ+Laco-ST | 70mg/kg+ | i.p. | 6 | 29$^{th}$ day |

| | | | | | |
|---|---|---|---|---|---|
| | | 10mg/kg | | | |
| 6 | STZ+Laco-T | 70mg/kg+ 30mg/kg | i.p. | 6 | 29th day |
| 7 | STZ+Ami-ST + Laco-T | 70mg/kg+15 mg/kg + 30mg/kg | i.p. | 6 | 29th day |
| 8 | STZ+Ami-T + Laco-ST | 70 mg/kg + 30mg/kg+ 10mg/kg | i.p. | 6 | 29th day |

- **Biochemical estimation of serotonin (5-HT), norepinephrine (NE) (Guha.D et.al,2007) [19]:**

The animals were sacrificed by cervical dislocation and brain tissues were dissected out, washed in ice cold saline (4°C) and homogenized in 10 ml acidified butanol. Homogenate (4 ml) was mixed with 10 ml 10 per cent heptane and 5ml 0.001 N HCl and then shaken for 5 min and centrifuged at 200 *g* for 10 min. Acid layer (4.5 ml) was eluted and mixed with 200 mg alumina and 1 ml of 2M sodium acetate. The mixture was shaken for 5 min and centrifuged at 200 *g* for 10 min.

Supernatant was taken for estimation of 5-HT and precipitate was used for estimation of NE. Supernatant was mixed with 3 volume of 10 per cent isobutanol, shaken twice with equal volume of salt saturated buffer at *p*H 10. Then 2 volumes of 10 percent heptane was added and shaken well and then the mixture was made 0.3 N with respect to HCl. This was used for estimation of 5-HT. Cold distilled water (5 ml) was added to the precipitate, shaken well and then centrifuged at 200 *g* for 3 min. Supernatant was transferred to glass stoppered centrifuged tube. 1.2 ml of freshly prepared ethylenediamine and ethylenediamine dihydrochloride mixture (7:5) was added to it and incubated at 50ºC for 40 min.

Mixture was cooled at room temperature and saturated with sodium chloride and then 4 ml 10 per cent isobutanol was added. It was centrifuged at 200 *g* for 3 min. To the precipitate 4 ml of

distilled water was added. This was taken for the estimation of NE. The fluorescence of 5-HT and NE was measured in the photoflurometer, with activation and emission wavelength set at 295 and 550 nm (for 5-HT), 320 and 385 and 485 nm (for NE).

Calibration curves for serotonin (5-HT), norepinephrine (NE):

0.01 to 0.055µg/ml and 0.1 to 0.5µg/ml solutions of serotonin (5-HT), norepinephrine (NE) were prepared in distilled water & fluorescence of 5-HT and NE was measured in the photoflurometer, with activation and emission wavelength set at 295 and 550 nm (for 5-HT), 320 and 385 and 485 nm (for NE). Graphs were plotted with concentration in µg/ml on ordinate & emission on the abscissa, the regression analysis was done using MS-Excel 2007 & R value, slope & equation in the form of y=mx+c determined for the straight line graphically obtained. The concentration of different amino acids was calculated from the regression equations of respective concentration vs. absorbance plots.

- **Biochemical estimation of Glutamate and GABA:**

Estimation of Free Amino Acids in Rat Brain:-

This method is based on the paper chromatographic methods described by the various researchers [20], modified to suit the laboratory settings here without affecting its principle, rationale & accuracy.

It was observed that use of precoated silica gel plates instead of filter paper as stationary phase, did not interfere in separation of amino acids. No change in Rf value of was GABA found in this study, as compared to that described by Udenfriend S (1950) [20].

Its also worth mentioning that the adsorption of amino acids on silica gel did not interfere in this study, provided that the silica gel (containing absorbed amino acids) which is scrapped off the plates & taken in ninhydrin solution is given heat treatment for longer duration with rigorous shaking during heating to hasten complete elution of derivatized amino acids from silica gel.

## RESULTS

### 1. Estimation of Blood Glucose level prior to drug treatment

**Table no.4 Estimation of Blood Glucose level prior to drug treatment.**

| Groups | 0th day (before STZ administration) | 3rd day | 15th day | 28 th day |
|---|---|---|---|---|
| 1 | 104.21± 0.012 | 111.23±0.032 | 116.43±0.122 | 118.23±0.154 |
| 2 | 100.03± 0.221 | 258.14±0.236## | 313.80±0.335## | 374.48±0.322## |
| 3 | 111.99± 0.232 | 267.45±0.021* | 316.13±0.155* | 376.55±0.152* |
| 4 | 112.32± 0.391 | 269.23±0.451* | 315.44±0.147* | 378.14±0.0223* |
| 5 | 118.83±0.113 | 251.14±0.117** | 288.91±0.153* | 336.76±0.122* |
| 6 | 125.74± 0.332 | 257.07±0.155* | 299.14±0.133* | 342.45±0.014* |
| 7 | 126.03± 0.412 | 258.14±0.126* | 298.01±0.175* | 348.03±0.165* |
| 8 | 128.13± 0.332 | 251.038±0.262** | 284.30±0.233** | 327.22±0.417** |

Values are Mean ± SEM and n = 6, **P < 0.01 using one way ANOVA coupled with "Dunnet`s t test". # indicate control group compared with normal **P<0.01 and *indicate other groups compared with normal group.

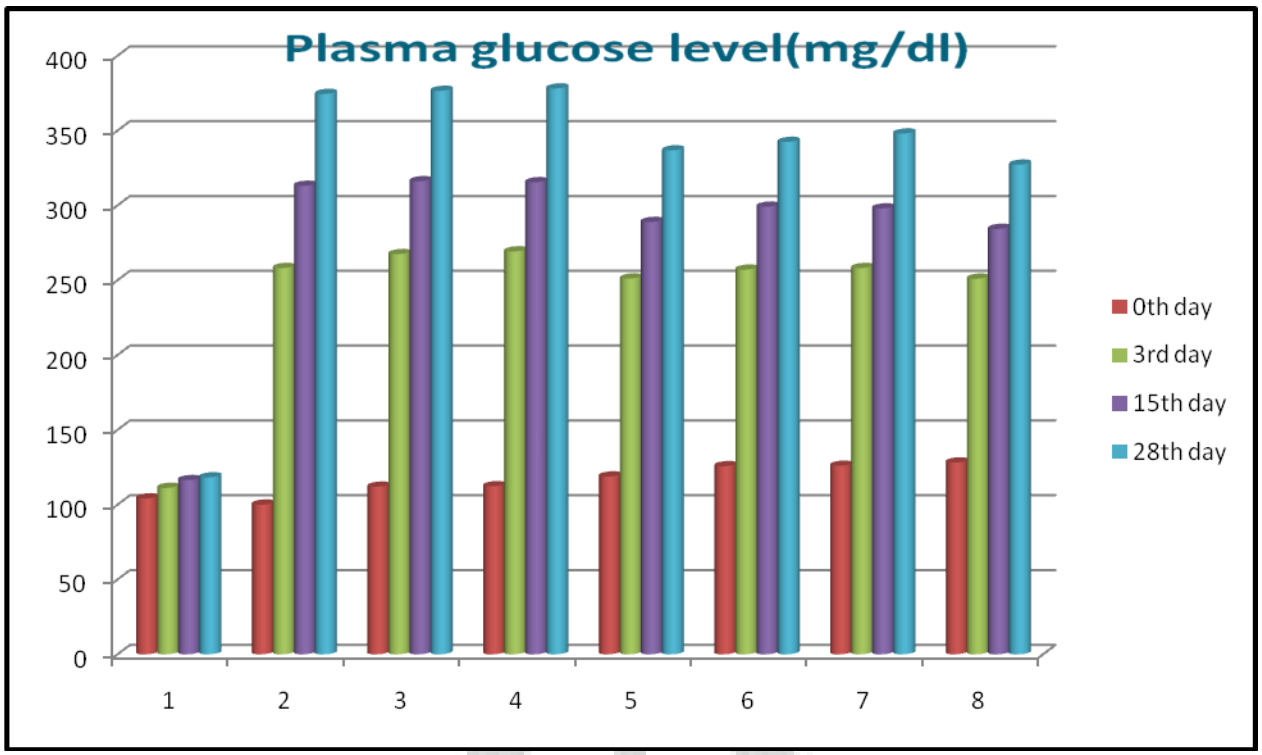


**Figure no.1 Graph of Plasma Glucose level**

**Behavioral study models:**

- **Eddy's Hot plate model**
- **Tail flick model**
- **Tail immersion model (Hot water)**
- **Tail immersion model (Cold water)**
- **Rota rod model**
- **Beam walk model**

**EDDY'S HOT PLATE MODEL**

**Table no. 5 Eddy's hot plate model**

| Sr. No. | GROUP | Paw withdrawal latency(s) | | | % increase in Reaction Time |
|---|---|---|---|---|---|
| | | 15 min | 30min | 60min | – |
| 1 | NS (2ml/kg,i.p) | 9.11±0.21 | 9.14±0.22 | 9.18±0.20 | – |
| 2 | STZ (70mg/kg,i.p) | 2.12±0.24## | 2.08±0.25## | 2.00±0.25## | – |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline (15mg/kg,i.p) | 5.23±0.40** | 5.82±0.41** | 6.33±0.42 ** | 68.40 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline (30mg/kg,i.p) | 5.56±0.36 ** | 6.12±0.38** | 7.00±0.36 ** | 71.42 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 5.12±0.42 ** | 5.24±0.42** | 6.66±0.44** | 69.96 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 5.40±0.21* * | 6.02±0.23* * | 7.66±0.26* * | 73.89 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 6.88±0.32** | 7.24±0.34** | 9.00±0.38 ** | 77.77 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 6.97±0.06 ** | 7.32±0.07** | 9.16±0.09 ** | 78.16 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on reaction time**

After four weeks of diabetes induction, the nociceptive threshold was significantly lower (p<0.01) in diabetic rats in all groups as compared with normal control.

Thermal Hyperalgesia was evident in streptozotocin treated animals since paw withdrawal latency was significantly shorter (p<0.01) than that of normal animals after four weeks of diabetes induction.

The groups treated with amitriptyline (15 mg/kg, i.p and 30 mg/kg, i.p) and lacosamide (10 mg/kg, i.p and 30 mg/kg, i.p) showed significant increase in reaction time when compared with diabetic control.

The therapeutic combination groups of amitriptyline (30 mg/kg, i.p) and lacosamide (10 mg/kg, i.p) showed much more significant (p<0.01) increase in reaction time when compared with diabetic control.

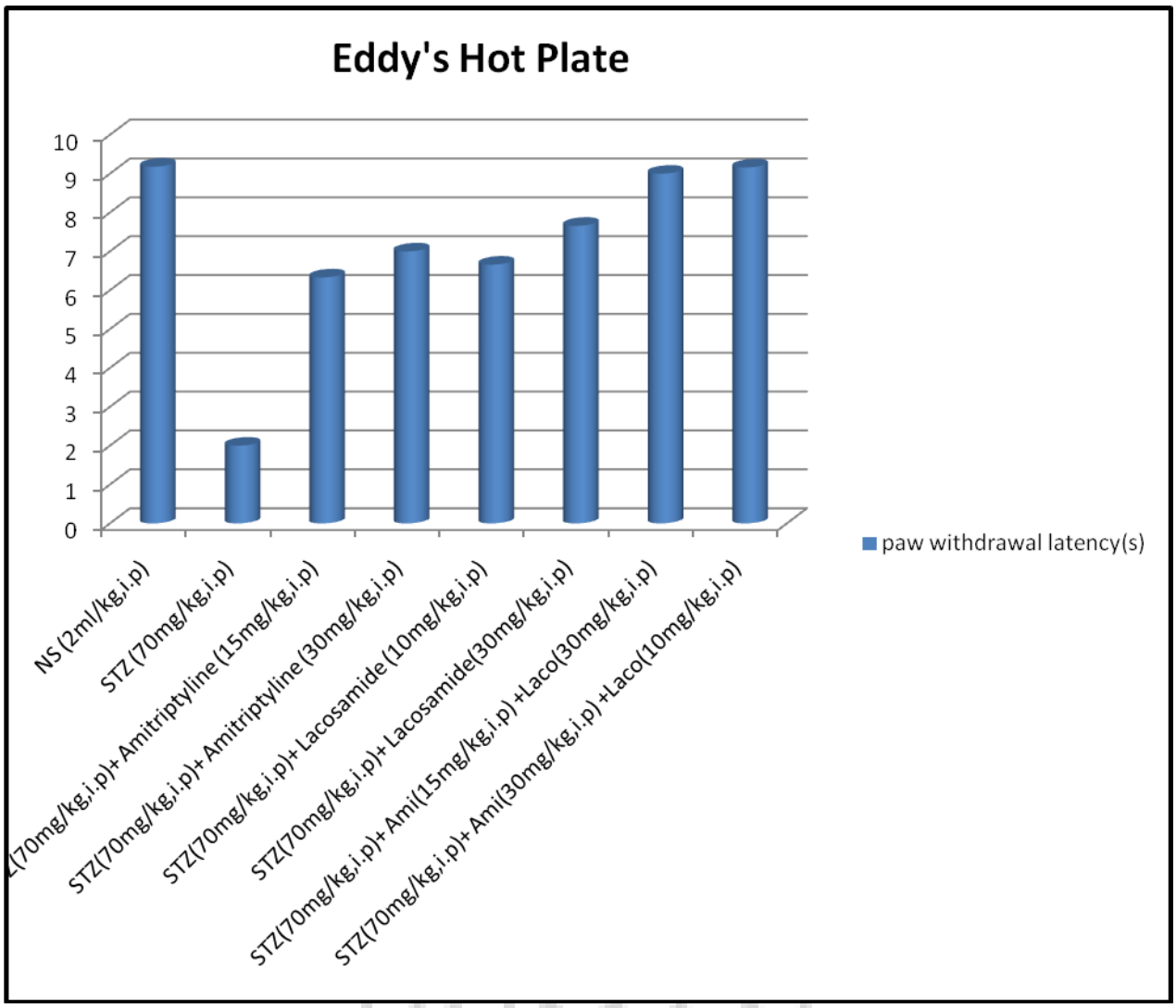


**Figure no. 2 Graph of Eddy's hot plate model**

**TAIL IMMERSION IN HOT WATER MODEL**

**Table no.6 Tail immersion in hot water model**

| Sr. No. | GROUP | Tail withdrawal latency(s) | | | % increase in Tail withdrawal latency |
|---|---|---|---|---|---|
| | | 15min | 30min | 60min | _ |
| 1 | NS (2ml/kg,i.p) | 9..11±0.30 | 9.14±0.33 | 9.16±0.31 | _ |
| 2 | STZ (70mg/kg,i.p) | 2.42±0.20## | 2.40±0.24## | 2.33±0.21## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline (15mg/kg,i.p) | 6.00±0.32** | 6.38±0.38** | 7.00±0.36** | 66.71 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline (30mg/kg,i.p) | 6.21±0.22** | 6.59±0.20** | 7.22±0.28** | 67.72 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 6.12±0.32** | 6.62±0.35** | 6.83±0.31** | 65.88 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 6.69±0.21** | 6.98±0.20** | 7.33±0.29** | 68.21 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 6.88±0.24** | 7.89±0.21** | 8.50±0.23** | 72.58 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 7.14±0.01** | 7.92±0.02** | 9.00±0.02** | 74.11 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on reaction time**

The diabetic control group showed significant decrease ($p<0.01$) in reaction time when compared with normal control. The groups treated with amitriptyline (15mg/kg,i.p and 30mg/kg,i.p) and lacosamide (10mg/kg,i.p and 30mg/kg,i.p) showed significant increase in reaction time (Tail withdrawal latency) when compared with diabetic control. The therapeutic combination groups of amitriptyline(30mg/kg,i.p) and lacosamide(10mg/kg,i.p) showed more significant ($p<0.01$) increase in reaction time (Tail withdrawal latency) when compared with diabetic control.

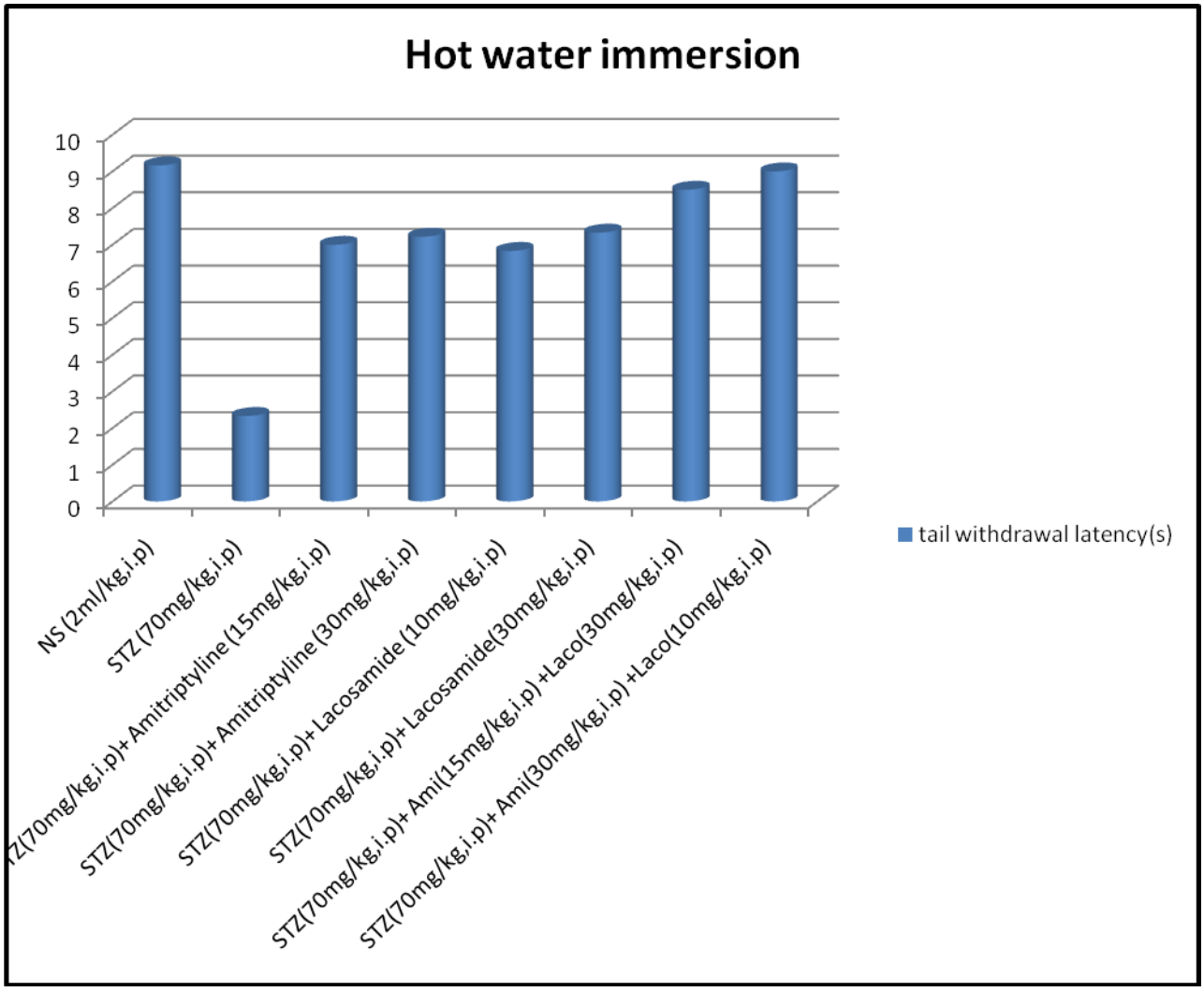


**Figure no. 3 Graph of Tail immersion in hot water model**

**TAIL IMMERSION IN COLD WATER MODEL**

**Table no. 7 Tail immersion in hot water model**

| Sr. No. | GROUP | Tail withdrawal latency(s) | | | % increase in Tail withdrawal latency |
|---|---|---|---|---|---|
| | | **15min** | **30min** | **60min** | _ |
| 1 | NS (2ml/kg,i.p) | 9.33±0.33 | 9.42±0.32 | 9.44±0.38 | _ |
| 2 | STZ (70mg/kg,i.p) | 2.57±0.21## | 2.54±0.22## | 2.50±0.24## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline (15mg/kg,i.p) | 6.25±0.02** | 6.74±0.04** | 7.00±0.01** | 64.28 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline (30mg/kg,i.p) | 6.30±0.07** | 6.87±0.02** | 7.50±0.03** | 66.66 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 6.14±0.31** | 6.56±0.30** | 6.83±0.34** | 63.39 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg ,i.p) | 6.26±0.26** | 6.77±0.21** | 7.33±0.22** | 65.89 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 6.62±0.16** | 7.32±0.18** | 8.89±0.15** | 71.87 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p)+ Laco(10mg/kg,i.p) | 6.79±0.18** | 7.86±0.12** | 9.16±0.16** | 72.70 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of

significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on reaction time:** The diabetic control group showed significant decrease (p<0.01) in reaction time when compared with normal control. The groups treated with amitriptyline(15mg/kg,i.p and 30mg/kg,i.p) and lacosamide (10mg/kg,i.p and 30mg/kg,i.p) showed significant increase in reaction time (Tail withdrawal latency) when compared with diabetic control. The therapeutic combination groups of amitriptyline (30mg/kg,i.p) and lacosamide(10mg/kg,i.p) showed much more significant (p<0.01) increase in reaction time (Tail withdrawal latency) when compared with diabetic control.

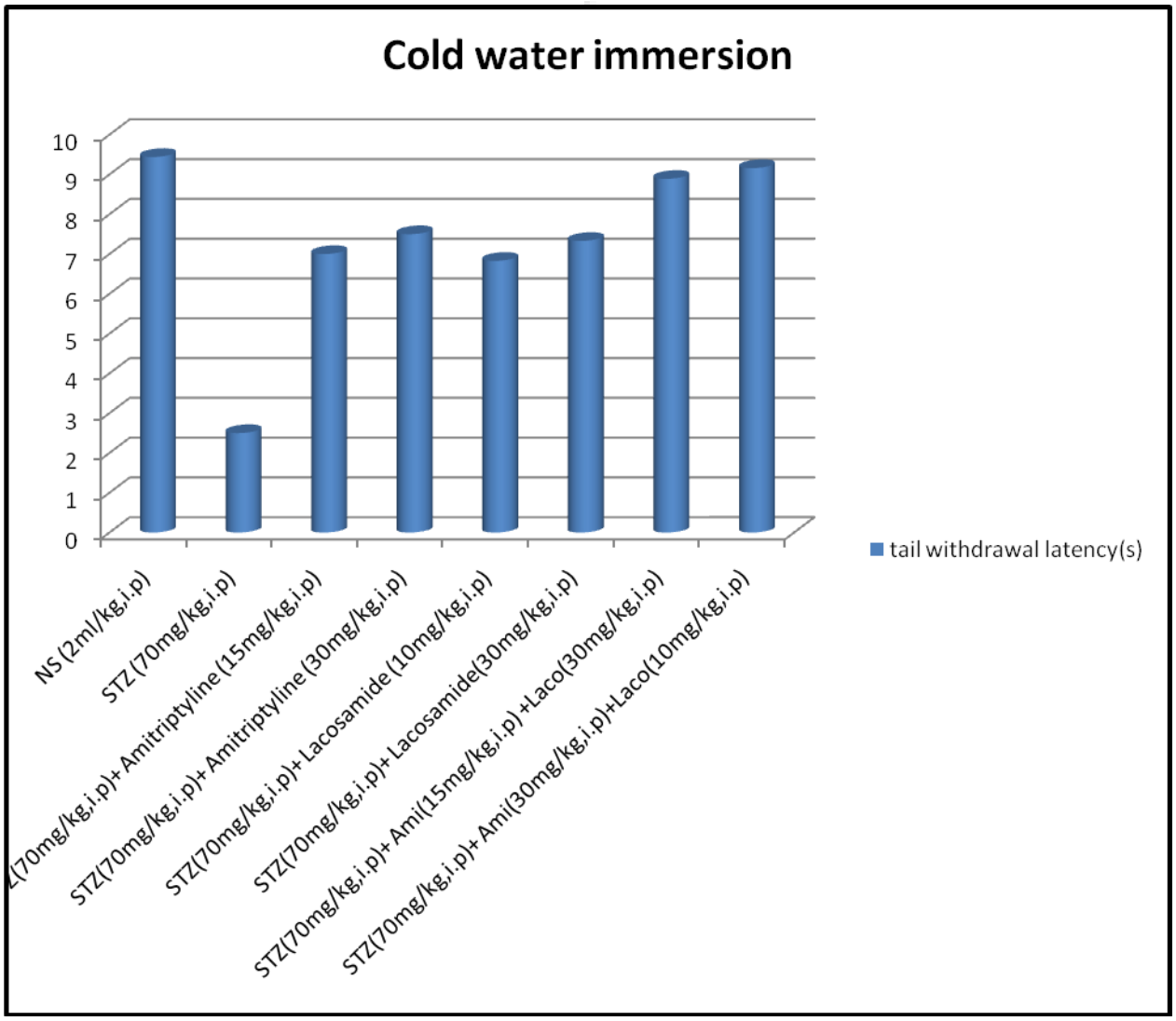


**Figure no. 4 Graph of Tail immersion in cold water model**

**BEAM WALK MODEL**

**Table no.8 Beam walk model**

| Sr. No. | GROUP | No. Of foot slips | % decrease in No. Of foot slips |
|---|---|---|---|
| 1 | NS (2ml/kg,i.p) | 2.90±0.49 | _ |
| 2 | STZ (70mg/kg,i.p) | 7.16±0.47## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline (15mg/kg,i.p) | 2.90±0.21** | 59.49 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline (30mg/kg,i.p) | 2.83±0.30** | 60.47 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 2.62±0.22** | 63.40 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 2.70±0.25** | 62.29 |
| 7 | STZ(70mg/kg,i.p)+Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 2.33±0.21** | 67.45 |
| 8 | STZ(70mg/kg,i.p)+Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 2.00±0.25** | 72.06 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on neurological score:**

The diabetic control group showed significant increase in neurological score (p<0.01) that is number of foot slips due to motor incordination when compared with normal group.

The group treated with amitriptyline(30mg/kg,i.p) showed more significant decrease in neurological score when compared with diabetic control.

The combination treated groups of amitriptyline (30mg/kg,i.p) and lacosamide (10mg/kg,i.p) showed much more significant decrease in neurological score (p<0.01) when compared with diabetic control.

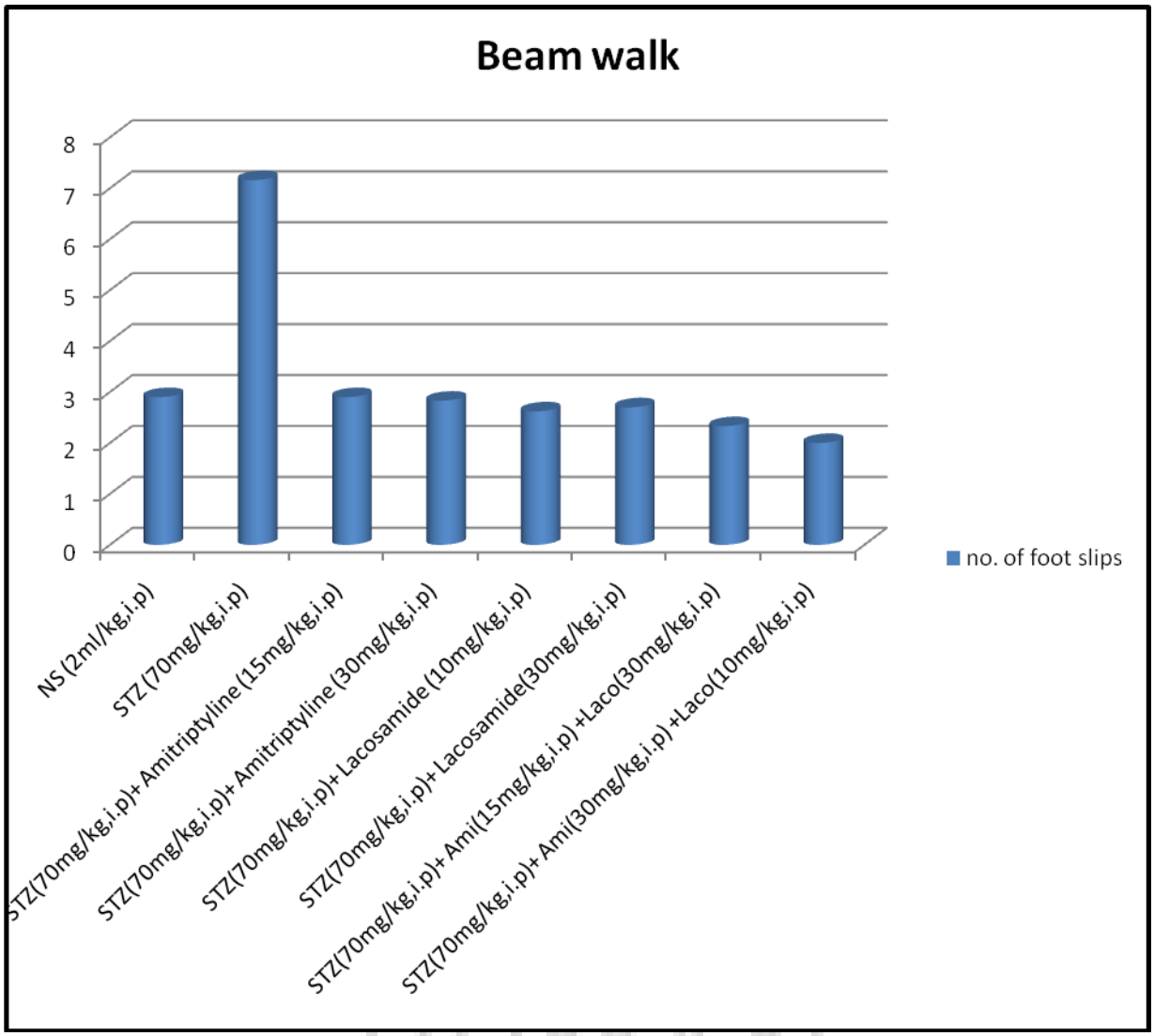


**Figure no.5 Graph of Beam walk model.**

**TAIL FLICK MODEL**

**Table no. 9 Tail flick model**

| Sr. No. | GROUP | Tail withdrawal latency(s) | | | % increase in Tail withdrawal latency(s) |
|---|---|---|---|---|---|
| | | 30min | 60min | 120min | _ |
| 1 | NS (2ml/kg,i.p) | 6.11±1.26 | 6.12±1.28 | 6.19±1.20 | _ |
| 2 | STZ (70mg/kg,i.p) | 2.21±0.31## | 1.98±0.30## | 1.83±0.34## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline(15mg/kg,i.p) | 3.24±0.04** | 4.12±0.09** | 5.00±0.08** | 63.40 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline(30mg/kg,i.p) | 3.64±0.34** | 4.49±0.32** | 5.50±0.33** | 66.72 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 3.82±0.27** | 3.98±0.20** | 4.66±0.21** | 60.72 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 3.92±0.40** | 4.25±0.44** | 5.16±0.41** | 64.53 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 4.89±0.12** | 5.22±0.18** | 5.83±0.16** | 68.61 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 5.01±0.41** | 5.56±0.40** | 6.16±0.44** | 70.29 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on reaction time:** Thermal Hyperalgesia was significantly more in case of diabetic animals as compared to the normal control (p<0.01) since the tail withdrawal latency was less in

diabetic rats than that of the normal control rats. The groups treated with amitriptyline(30mg/kg,i.p) and lacosamide (30mg/kg,i.p) showed significant increase in reaction time (Tail withdrawal latency) when compared with diabetic control. The therapeutic combination groups of amitriptyline(30mg/kg,i.p) and lacosamide(10mg/kg,i.p) showed much more significant ($p<0.01$) increase in reaction time (Tail withdrawal latency) when compared with diabetic control.

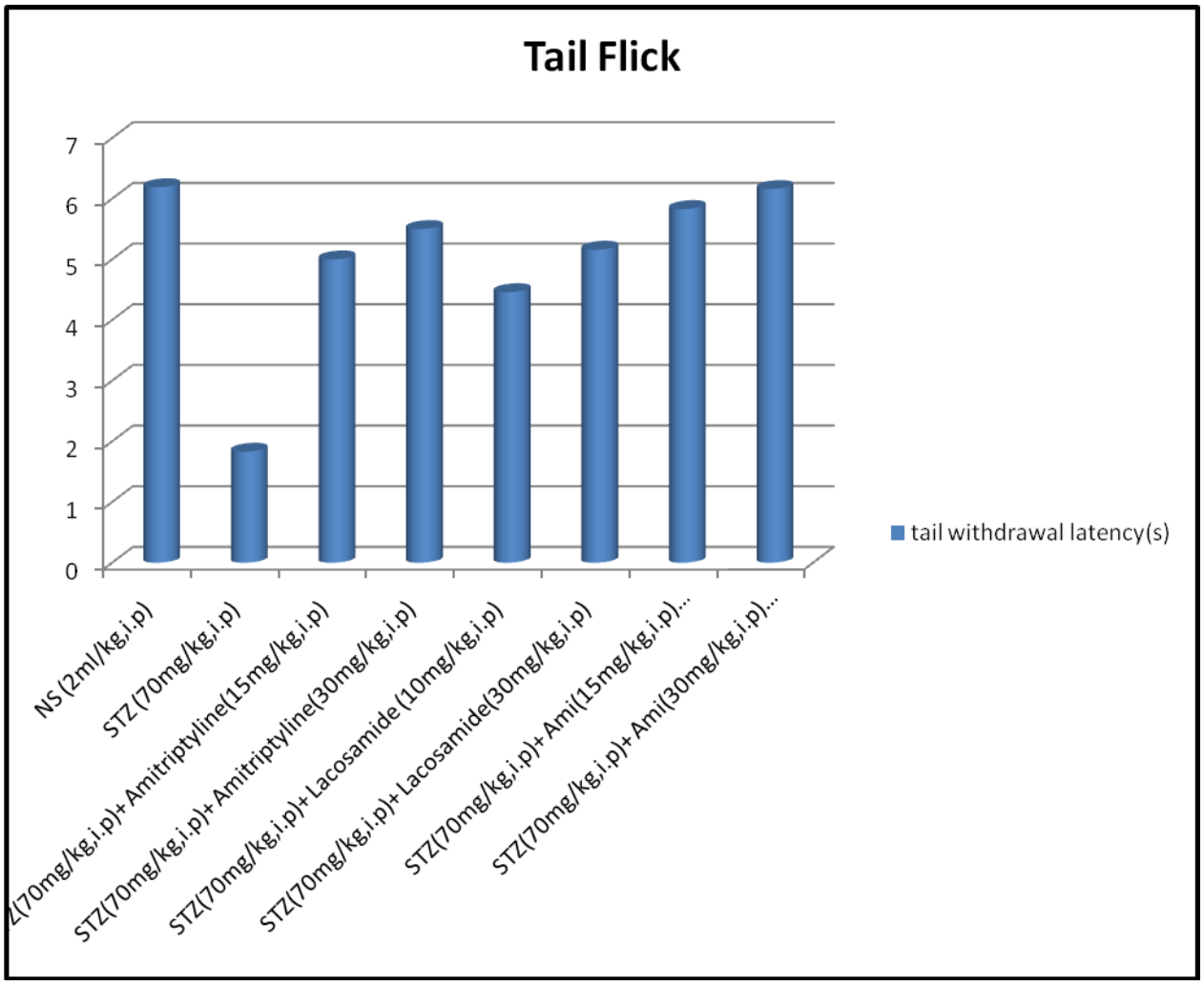


**Figure no. 6 Graph of Tail Flick Model.**

**ROTAROD MODEL**

**Table no.10 Rotarod model**

| Sr. No. | GROUP | Fall latency time(sec) | | | % increase in Fall latency time(sec) |
|---|---|---|---|---|---|
| | | **30min** | **60min** | **120min** | _ |
| 1 | NS (2ml/kg,i.p) | 92.16±2.82 | 93.12±2.84 | 93.19±2.80 | _ |
| 2 | STZ (70mg/kg,i.p) | 67.22±1.70## | 65.36±1.72## | 64.38±1.74## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline(15mg/kg,i.p) | 70.56±6.00** | 74.22±6.02** | 81. 66±6.09** | 21.16 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline(30mg/kg,i.p) | 81.34±4.27* | 82.99±4.24** | 85.33±4.21** | 24.55 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 68.87±6.41** | 73.68±6.46** | 79.16±6.48** | 18.67 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 72.21±6.15** | 79.65±6.11** | 84.66±6.10** | 23.95 |
| 7 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 88.14±2.56** | 90.32±2.58** | 92.12±2.50** | 30.11 |
| 8 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 87.23±1.41** | 89.92±1.46** | 91.00±1.44** | 29.25 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group

**Effect on fall latency time:**

After four weeks of diabetes induction muscle spindle get damage and can lead to deficits such as motor incordination. In rota rod test fall down latency time of animals on rotating rod was calculated. The diabetic control group animals showed significant decrease in fall latency time ($p<0.01$) when compared with normal group.

The groups treated with amitriptyline (30mg/kg,i.p) and lacosamide 30mg/kg,i.p) showed significant increase in fall latency time ($p<0.01$) when compared with diabetic control.

While the combination treated groups of amitriptyline (30mg/kg,i.p) and lacosamide (10mg/kg,i.p) showed more significant increase in fall latency time ($p<0.01$) when compared with diabetic control.

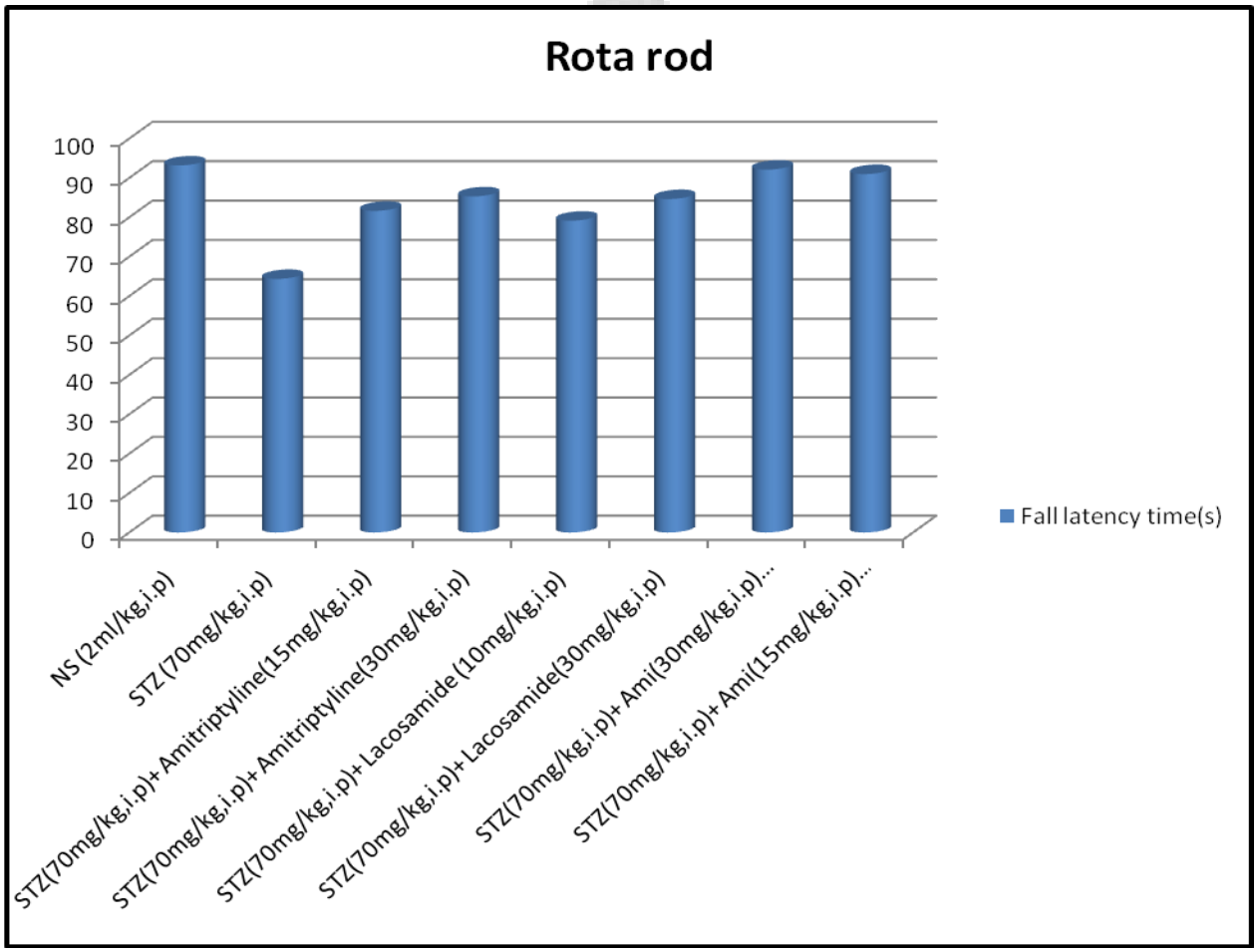


**Figure no. 7 Graph of Rotarod model**

## BIOCHEMICAL ESTIMATIONS

## SEROTONIN ESTIMATION

**Table no. 11 Serotonin estimation**

| Sr. No. | GROUP | Conc (µg/mg tissue) | % increase in conc. |
|---|---|---|---|
| 1 | NS(2ml/kg,i.p) | 0.023±0.002 | _ |
| 2 | STZ (70mg/kg,i.p) | 0.011±0.002## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline (15mg/kg,i.p) | 0.020±0.001** | 45 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline (30mg/kg,i.p) | 0.022±0.002** | 50 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide(10mg/kg,i.p) | 0.017±0.004* | 35.29 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 0.016±0.002* | 31.25 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 0.021±0.002** | 47.61 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 0.023±0.003** | 52.17 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on the level of serotonin:** The diabetic control group showed significant decrease (p<0.01) in the level of serotonin when compared with normal control.

The groups treated with amitriptyline (30mg/kg, i.p) showed significant increase in the level of serotonin when compared with diabetic control.

The therapeutic combination groups of amitriptyline (30mg/kg, i.p) and lacosamide (10mg/kg, i.p) showed much more significant ($p<0.01$) increase in the level of serotonin when compared with diabetic control.

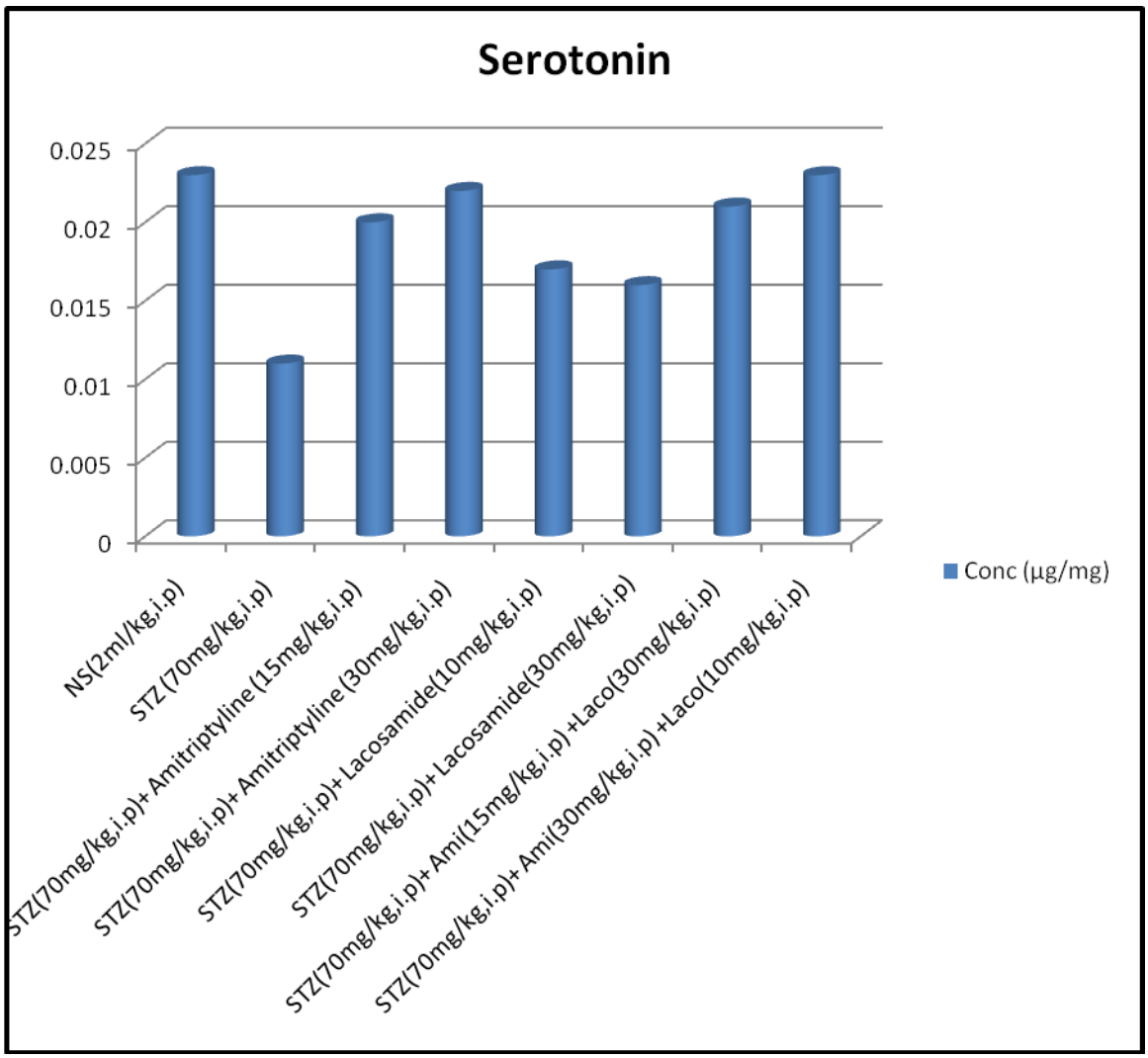


**Figure no. 8 Graph of Serotonin estimation**

**NORADRENALINE ESTIMATION**

**Table no.12 Noradrenaline estimation**

| Sr. No. | GROUP | Conc (µg/100g tissue) | % increase in conc. |
|---|---|---|---|
| 1 | NS(2ml/kg,i.p) | 0.018±0.001 | _ |
| 2 | STZ (70mg/kg,i.p) | 0.010±0.001## | _ |
| 3 | STZ(70mg/kg,i.p)+ Amitriptyline (15mg/kg,i.p) | 0.015±0.001** | 33.33 |
| 4 | STZ(70mg/kg,i.p)+ Amitriptyline (30mg/kg,i.p) | 0.016±0.004** | 37.50 |
| 5 | STZ(70mg/kg,i.p)+ Lacosamide(10mg/kg,i.p) | 0.012±0.004* | 16.66 |
| 6 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 0.013±0.002* | 23.07 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p) +Laco(30mg/kg,i.p) | 0.016±0.004** | 37.50 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p) +Laco(10mg/kg,i.p) | 0.018±0.002** | 44.44 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on the level of noradrenaline:**

The diabetic control group showed significant decrease (p<0.01) in the level of noradrenaline when compared with normal control.

The groups treated with amitriptyline (30mg/kg, i.p) showed significant increase in the level of noradrenaline when compared with diabetic control.

The therapeutic combination groups of amitriptyline (30mg/kg, i.p) and lacosamide (10mg/kg,i.p) showed much more significant ($p<0.01$) increase in the level of noradrenaline when compared with diabetic control.

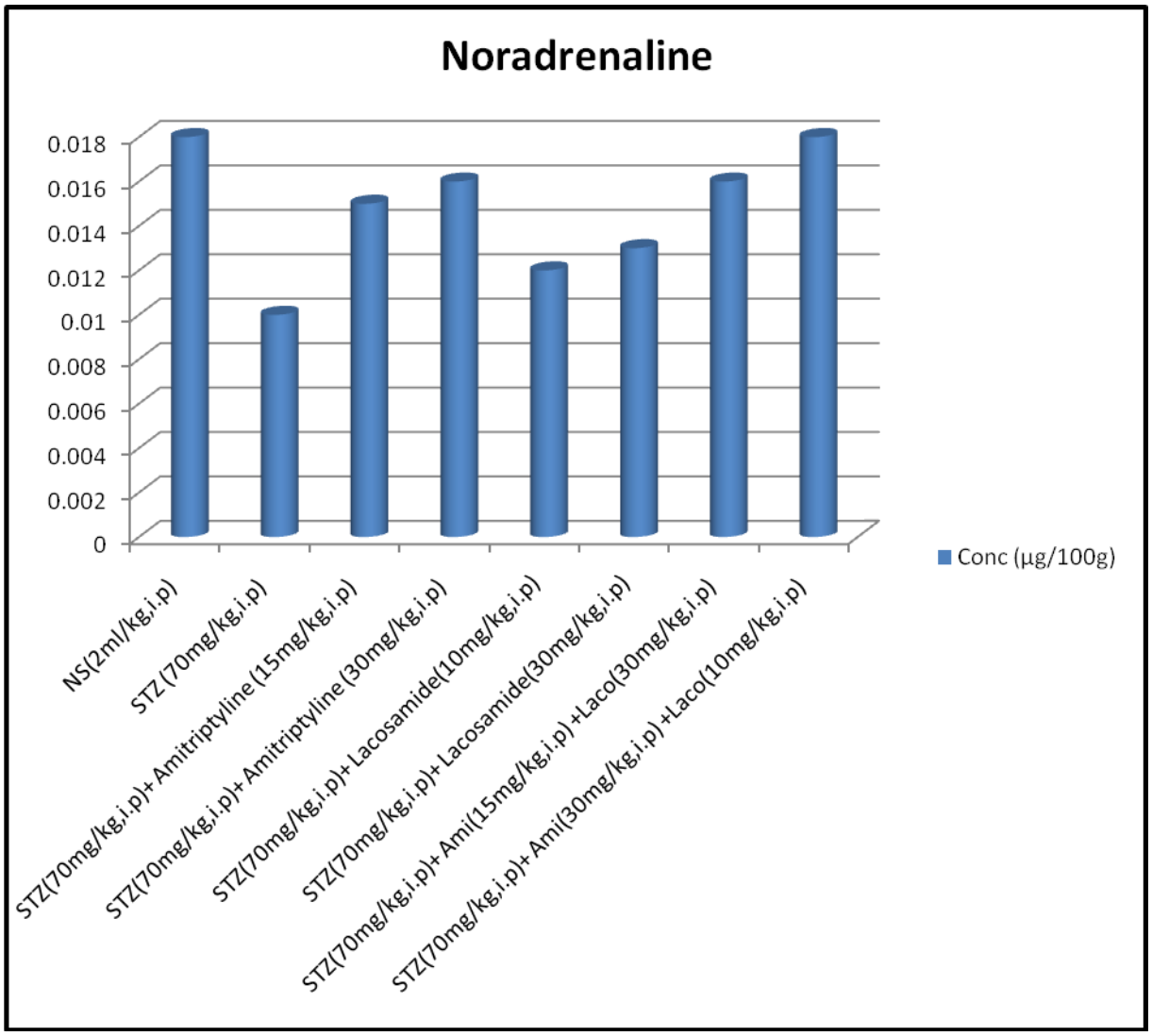


**Figure no.9 Graph of Noradrenaline estimation**

**GLUTAMATE ESTIMATION**

**Table no.13 Glutamate estimation**

| Sr. No. | GROUP | Conc (µg/mg of wet tissue) | % decrease in conc. |
|---|---|---|---|
| 1 | NS(2ml/kg,i.p) | 4.50±0.14 | _ |
| 2 | STZ (70mg/kg,i.p) | 7.26±0.11## | _ |
| 3 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 4.50±0.11** | 38.01 |
| 4 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 4.23±0.14** | 41.73 |
| 5 | STZ(70mg/kg,i.p)+ Amitriptyline(15mg/kg,i.p) | 4.72±0.15* | 34.98 |
| 6 | STZ(70mg/kg,i.p)+ Amitriptyline(30mg/kg,i.p) | 4.68±0.14* | 35.53 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p)+Laco(30mg/kg,i.p) | 4.12±0.15** | 43.25 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p)+Laco(10mg/kg,i.p) | 4.20±0.016** | 42.12 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on the level of glutamate:** The diabetic control group showed significant increase (p<0.01) in the level of glutamate when compared with normal control.The groups treated with lacosamide (30mg/kg, i.p) showed significant decrease in the level of glutamate when compared with diabetic control. The therapeutic combination groups of amitriptyline (15mg/kg, i.p) and

lacosamide (30mg/kg,i.p) showed much more significant ($p<0.01$) decrease in the level of glutamate when compared with diabetic control.

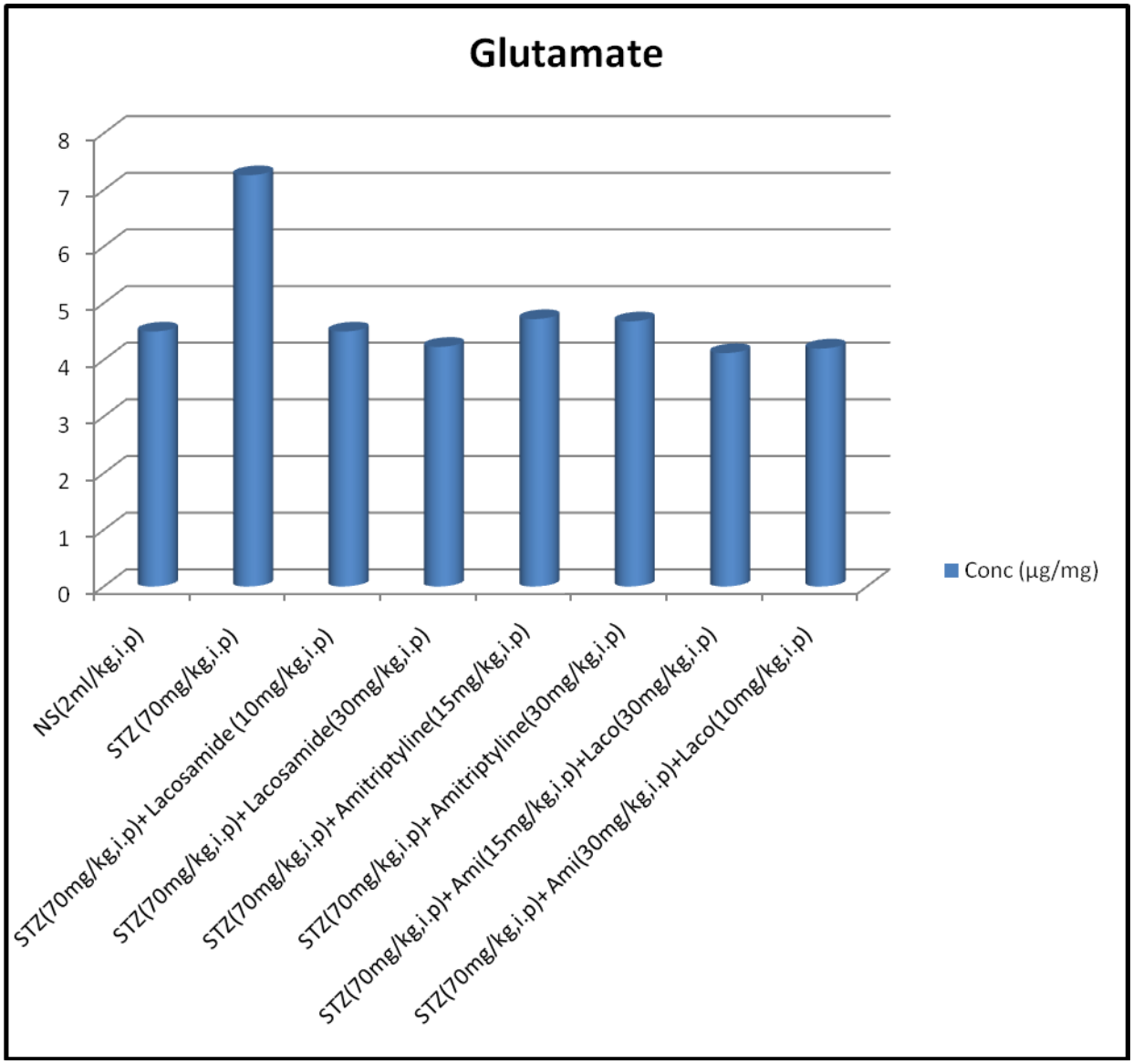


**Figure no.10 Graph of Glutamate estimation**

**GABA ESTIMATION**

**Table no.14 Gaba estimation**

| Sr. No. | GROUP | Conc (µg/mg tissue) | % increase in conc. |
|---|---|---|---|
| 1 | NS(2ml/kg,i.p) | 0.91±0.01 | _ |
| 2 | STZ (70mg/kg,i.p) | 0.43±0.02## | _ |
| 3 | STZ(70mg/kg,i.p)+ Lacosamide (10mg/kg,i.p) | 0.71±0.01** | 39.43 |
| 4 | STZ(70mg/kg,i.p)+ Lacosamide(30mg/kg,i.p) | 0.78±0.02** | 44.87 |
| 5 | STZ(70mg/kg,i.p)+ Amitriptyline(15mg/kg,i.p) | 0.52±0.02* | 17.30 |
| 6 | STZ(70mg/kg,i.p)+ Amitriptyline(30mg/kg,i.p) | 0.58±0.02* | 25.86 |
| 7 | STZ(70mg/kg,i.p)+ Ami(15mg/kg,i.p)+Laco(30mg/kg,i.p) | 0.82±0.01** | 47.56 |
| 8 | STZ(70mg/kg,i.p)+ Ami(30mg/kg,i.p)+Laco(10mg/kg,i.p) | 0.74±0.01** | 41.89 |

Statistical analysis of data was carried out by one way ANNOVA followed by Dunnett t-test. Values are expressed as mean ± SEM and n = 6, **P<0.01 is considered as criteria of significance. # indicate control group compared with normal **P<0.01 and *indicate other groups compared with control group.

**Effect on the level of GABA:**

The diabetic control group showed significant decrease (p<0.01) in the level of GABA when compared with normal control.

The groups treated with lacosamide (30mg/kg, i.p) showed significant increase in the level of GABA when compared with diabetic control. The therapeutic combination groups of amitriptyline (15mg/kg, i.p) and lacosamide (30mg/kg,i.p) showed much more significant ($p<0.01$) increase in the level of GABA when compared with diabetic control.

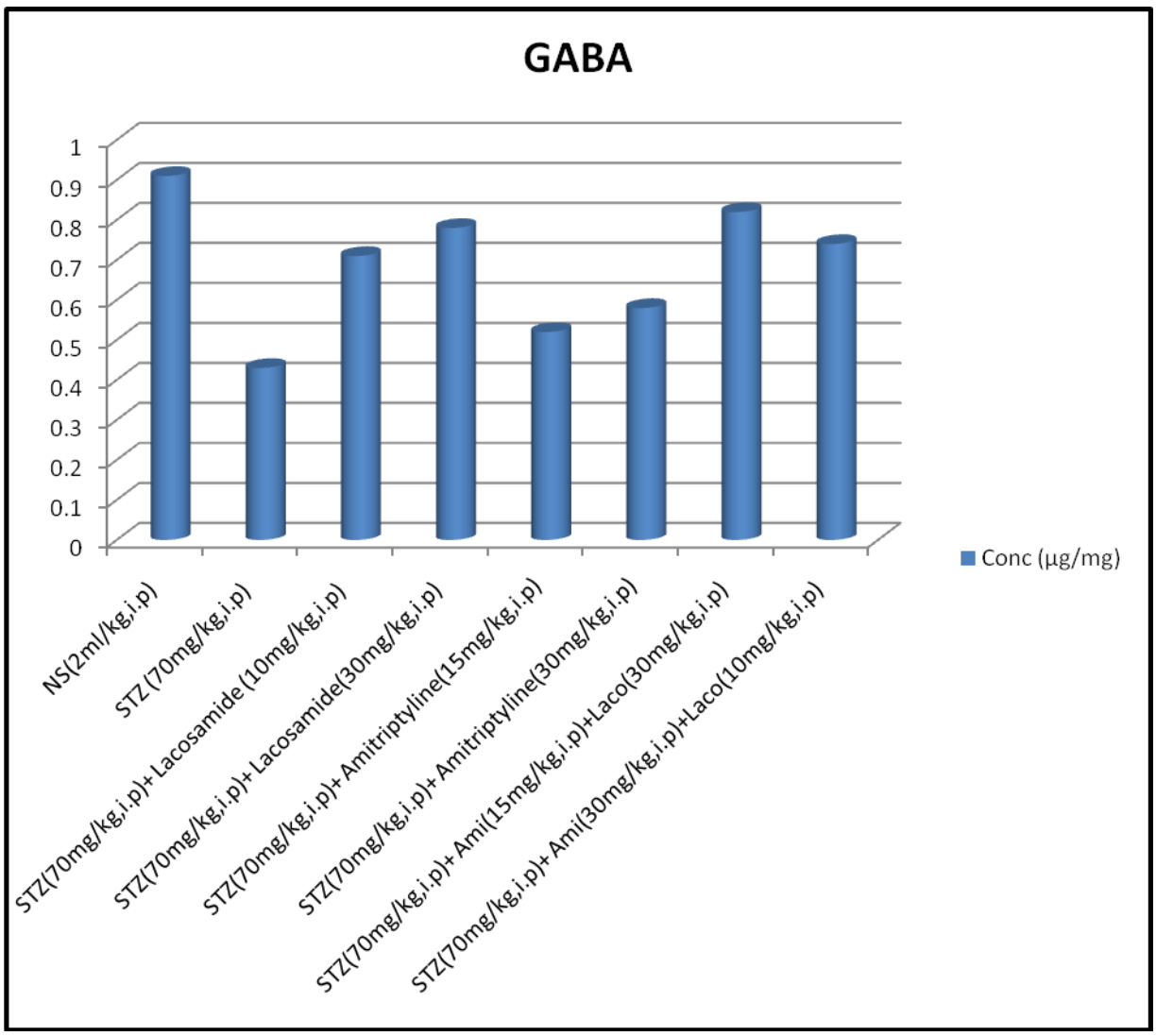


**Figure no.11 Graph of Gaba estimation**

## DISCUSSION

Diabetes is a chronic condition characterized by raised blood glucose levels. It develops when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Diabetic neuropathy encompasses a variety of forms whose impact ranges from

discomfort to death. Neuropathic pain is the most common symptom associated with diabetic neuropathy. Due to this, individuals with diabetic neuropathy were shown to have significantly lower quality of life scores. Overall, approximately 10% of patients with diabetes experience persistent pain from neuropathy [14]. Diabetic neuropathy has been defined as presence of symptoms and/or signs of peripheral nerve dysfunction in diabetics after exclusion of other causes, which may range from hereditary, traumatic, compressive, metabolic, toxic, nutritional, infectious, immune mediated, neoplastic, and secondary to other systemic illnesses. Various factors like altered lipid profile, low level of insulin cause the diabetic neuropathy (NDIC 2009). The pathogenesis of diabetic polyneuropathy is multifactorial, involving both metabolic and vascular factors. Hyperglycemia, which appears due to; (1) increased activity of the enzyme aldose reductase, decrease free nerve myoinositol causes imbalance in NADP and NAPH, (2) auto-oxidation of glucose leading to the formation of reactive oxygen species, (3) advanced glycation end-products produced by the non-enzymatic glycation of proteins and (4) the inappropriate activation of protein kinase C. These metabolic changes lead to oxidative stress and impaired mitochondrial function, with apoptosis of neurons and Schwann cells [21].

Neuropathic pain is a type of persistent pain that arises from functional changes occurring in the pain sensory system after peripheral nerve injury. Sustained or prolonged stimulation of nociceptive afferents (afferent barrage) due to tissue damage or peripheral nerve injury has been implicated in the initiation and maintenance of central neuroplastic changes [22] culminating in central neuronal hyperexcitability, possibly due to reduced inhibition of nociceptive neurons by neurotransmitters, such as serotonin and noradrenaline in both spinal and supraspinal structures [23]. The resultant state of central sensitization can produce an ongoing condition of spontaneous, persistent pain as well as an increased sensitivity to painful stimuli (hyperalgesia) or to normally non-painful mechanical or thermal stimuli (allodynia) [22]. Although the precise mechanisms involved in the pathogenesis of persistent pain states are not fully understood, there is a growing recognition that disinhibition and imbalance of serotonin and norepinephrine in endogenous pain inhibitory pathways could contribute to persistent pain mechanisms [23].Thus the neurotransmitters serotonin and norepinephrine are thought to play a significant role in attenuating persistent pain mechanisms, presumably via descending modulatory pain pathways.

Central sensitization is characterized by altered responsiveness of dorsal horn neurons, expansion of receptive fields and plasticity of neuronal connections within the pain transmitting pathways leading to increased neuronal activity at supraspinal sites and to dysfunction of the endogenous spinal and supraspinal pain inhibitory mechanisms [24]. An imbalance of the excitatory and inhibitory mechanisms within both the ascending and descending pain inhibitory pathways could ultimately lead to persistent pain [22,23,24]. Thus, restoring this balance, for example with serotonin and norepinephrine reuptake inhibitors, could be beneficial in persistent pain conditions in humans. The mechanism of action in relieving of neuropathic pain by the tricyclic antidepressants is thought to be due to the inhibition of reuptake of serotonin and norepinephrine or just norepinephrine within the central nervous system; however, other possible mechanisms of action include alpha adrenergic blockade, sodium channel effects, and NMDA receptor antagonism [25].Thus in the present investigation there was a considerable increase in level of serotonin and norepinephrine in treatment groups.

Lacosamide stood the test of time in the treatment of diabetic neuropathy. The principle mechanism of actions include sodium channel blockade, potentiation of GABA activity, calcium channel blockade, antagonism of glutamate at NMDA receptors.

Central Sensitization with NMDA Receptor Activation it involves following nerve injury damaged primary afferent neurons become spontaneously active and generate ectopic action potentials, which create a constant drive of input to the spinal cord. This initiates and maintains a hyperexcitable state of the spinal cord dorsal horn, termed ‘central sensitization’. It is associated with several changes in the dorsal horn (DH):

• Lowering of activation threshold of DH neurons

• An expansion in receptive field size

• “Wind-up”, which manifests as a progressive increase in magnitude and duration of response to repeatative painful stimuli

• Strengthening of efficacy of synaptic transmission or long term potentiation.

Activation of NMDA (N-methyl-D-aspartate) receptor is critical for the initiation and maintenance of the enhanced responsiveness of dorsal horn neurons that occurs in chronic pain

setting. In a recent study conducted in neuropathic rats, it was observed that a protein kinase A mediated NMDA receptor phosphorylation plays an important role in spinal nerve ligation induced neuropathic pain. Evidence suggests that NMDA receptor antagonists reverse established central sensitization and have a role in attenuating feature of neuropathic pain [26].

In the present study decrease in the pain threshold was observed with mildly noxious stimulus after streptozotocin induced diabetic neuropathy but this threshold was improved by using Amitriptyline and Lacosamide individually as well as in combination.

The percentage increase in reaction time in case of Eddy's hot plate shown that 78.16% increase observed in case of Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p) which was more as compared to individual groups as well as Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p) combination.

In tail immersion (Hot water) model Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p) had shown 74.11% increase in tail withdrawal latency which was more as compared to individual groups as well as Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p) combination.

In tail immersion (cold water) model Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p) had shown 72.70% increase in tail withdrawal latency which was more as compared to individual groups as well as Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p) combination.

In Beam Walk model the anti-hyperalgesic activity was evaluated by % decrease in no. of foot slips in different groups. Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p) had shown 72.06% decrease in no. of foot slips which was more as compared to individual groups as well as Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p) combination.

In Tail flick model the % increase in tail withdrawal latency observed that Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p) had shown 70.29% increase in tail withdrawal latency which was more as compared to individual groups as well as Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p) combination.

The anti-hyperalgesic activity was evaluated in case of Rota rod model by % increase in fall latency time. Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p) had shown 30.11%

increase in fall latency time which was more as compared to individual groups as well as Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p) combination.

The percentage increase in brain serotonin concentration as follows:

Amitriptyline (30mg/kg,i.p)- 50%

Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p)- 52.17%

Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p)- 47.61%

The percentage increase in brain noradrenaline concentration as follows:

Amitriptyline (30mg/kg,i.p)- 37.50%

Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p)- 44.44%

Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p)- 37.50%. Hence, serotonin and noradrenaline, play a significant role in attenuating persistent pain mechanisms, presumably via descending modulatory pain pathways.

The percentage decrease in brain glutamate concentration as follows:

Lacosamide (30mg/kg,i.p)- 41.73%

Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p)- 43.25%

Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p)- 42.12%

The percentage increase in brain GABA concentration as follows:

Lacosamide (30mg/kg,i.p)- 44.87%

Amitriptyline (15mg/kg,i.p) +Lacosamide (30mg/kg,i.p)- 47.56%

Amitriptyline (30mg/kg,i.p) +Lacosamide (10mg/kg,i.p)- 41.89%

In the present study, streptozotocin-injected rats had significantly higher blood glucose level, decreased body weight and the nociceptive threshold was significantly lower than non-diabetic rats in hot plate, tail immersion, paw pressure withdrawal tests, indicating that diabetic animals

exhibited significant thermal and mechanical hyperalgesia. This condition was reversed following the combination treatment of Amitriptyline (30mg/kg,i.p) +Lacosamide(10mg/kg,i.p).

## CONCLUSION

Considering the results obtained from the present study it can be concluded that the combination of amitriptyline and lacosamide has promising anti-hyperalgesic effect.

Combination of amitriptyline (30mg/kg,i.p) and lacosamide (10mg/kg,i.p) has shown significant neuroprotective activity in diabetic animals as compared to the individual drug treatment.

This combination also reduces dose of lacosamide hence, the dose dependent adverse effects may be reduced. Therefore, this combination might prove to be beneficial in the management of diabetes related complications namely diabetic neuropathy.

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