Chapter 4: 

*Materials and Methods*
MATERIALS AND METHODS

Wistar rats and Swiss albino-mice of either sex were used. They were housed in animal house provided with 12-h light/dark cycle with free access to water and food. All experiments were conducted in accordance with National Institute of Health guidelines and Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals (Zimmerman, 1983) on the welfare of experimental animals and the study protocol was approved by Institutional Ethics Committee.

4.1. Induction of Diabetes: Streptozotocin (STZ) Models: In the recent year the model of STZ-induced diabetes in the rodents has been increasingly used in an attempt to provide information on underlying process, and to evaluate potential therapies. STZ is an antibiotic extracted from *streptomyces acromogenes* and is diabetogenic due to a selective cytotoxic action upon pancreatic beta-cells (Rakieten *et al*., 1963). The glucosamine - nitrosourea compound, STZ is taken up into the insulin producing β-cells of the langerhan’s via the GLUT-2 glucose transporter.

Animals were weighed and injected with STZ, a diabetogenic agent, 200 mg/kg, i.p. for the mice and 65 mg/kg, i.p. for the rats in a single dose for each species (Rakieten *et al*., 1963). The mortality rate in the single dose STZ-method was found to be very high despite adequate water and food supply (Weiss, 1982). Therefore, the multiple low dose STZ (MLDS) (STZ 40 mg/kg × 5 days in mice and STZ 20 mg/kg × 4 days in rat) method was used to induce diabetes, as previously described (Like and Rossini, 1976; Stosić-Grujičić, 2001). Challenges with sub-lethal dose of STZ initiate a cascade of cytokines mediated diabetes similar to IDDM in human (Herold *et al*., 1996; Luki *et al*., 1991). Two weeks were allowed for destruction of pancreatic beta cells and development of IDDM.
4.2. Measurement of Serum Glucose: Serum glucose levels were measured using commercially available Kit in blood sample obtained from the tail vein or capillary retro-orbital sinus. Only mice and rats with serum glucose concentration greater than 200 mg/dl (average 300 mg/dl) were considered as diabetic and used in this study. All efforts were made to minimize suffering and the number of animals used.

4.3. Experimental design

The animals were divided into following groups and each group comprised of 6-8 animals.

**Group I:** Animals received saline or vehicle served as control. Tail flick and paw withdrawal reflexes was noted 30 min after administration of citrate buffer on different day’s i.e.0th, 7th, 14th, 21th, 28th and 35th day. Serum/urinary nitrite and fasting glucose levels were noted once weekly of citrate buffer administration.

**Group II:** Rats/mice in group 2 received intra-peritoneal injection of streptozotocin (STZ) at a dose of 20 mg/kg i.p x 4 days for rats and 40 mg/kg, i.p x 5 days for mice (multiple low dose STZ model) to induce experimental diabetes respectively, animals with serum glucose levels of more than 200 mg/dl, e weeks after administration of STZ were considered as diabetic and used in the present study.

**In Group III:** The age-matched non-diabetic, diabetic and splenectomised diabetic mice, were administered either saline or morphine (4 & 8mg/kg, s.c.), [D-Ala2,N-Me Phe4,Gly-ol5]enkephalin )(DAMGO, 2 µg), [D-Pen2,5]enkephalin (DPDPE, 5µg, i.t), oxycodone (5 & 10 mg/kg,i.p).
In Group IV: The age-matched non-diabetic, diabetic and splenectomised diabetic mice, were administered either saline or Indomethacin (5 & 10 mg/kg, i.p), ibuprofen (30 mg/kg, i.p), LSA (200 & 400 mg/kg, i.v) and dipyrone (250 & 500 mg/kg, i.v).

In Group V: The age-matched non-diabetic, diabetic and splenectomised diabetic mice, were administered either saline or cannabis ethanolic extract (25, 50 & 100 mg/kg, p.o), cannabinoid agonist Win 55,212-2 (2 & 4 mg/kg, i.v) and tail flick and paw withdrawal reflex was noted 30 min after administration of these drugs.

In Group VI: The age-matched non-diabetic, diabetic and splenectomised diabetic mice, were administered either saline or neurosteroids i.e testosterone (2.5 & 5 mg/kg, i.p), allopregnanolone (2.5 & 5.0 mg/kg,i.p), dehydroepiandrosterone (10 mg/kg, i.p) and progesterone (2 & 4 mg/kg, i.p) In all group 2-4, the drugs or vehicle were administered for two weeks, starting after 3rd week of STZ injection and withdrawal reflexes was noted once a day for two weeks.

Group-VII: Cyclosporine – A (12.5 & 25 mg/kg, i.p), Thalidomide (25& 50 mg/kg,i.p), a TNF-α inhibitor, Minocycline, a glial cell inhibitor (15 mg/kg & 30 mg/kg, i.p), Pentoxifylline (cytokines inhibitors) (10 mg/kg & 20 mg/kg, i.p), L-arginine (200 mg/kg,i.p), L-NAME (15 & 30 mg/kg,i.p), and Aminoguanidine (25 & 50 mg/kg,i.p) were administered once a day for 28 days and the Dextromethorphan (5 & 10 mg/kg,i.p), Ketamine (10 mg/kg,i.v) (NMDA-receptor antagonist) and Chlerythrine (PKC-inhibitor), were administered for two weeks, starting on day 28 after STZ-injection and continue upto 42 days, and withdrawal reflexes to paw and tail were measured every week 30 min after vehicle or drug treatments.

Group-VIII; The effects on the reversal (tolerance) were investigated, NSAIDs /opioids/cannabinoid-tolerant animals were treated b.i.d. for an additional 7 -14 days
(from day 28 to day 42) and tail flick and paw withdrawal reflex was noted 30 min after administration of vehicle or the drugs.

**Group IX**: The effect of SHS was measured in both SHS of non-diabetic and SHS of diabetic (28 days) treated animals. Non-diabetic rats were administered SHS (400 µl.i.v) obtained from 28 days diabetic and non-diabetic rats, for 28 days. Tail flick and paw withdrawal reflex after administration of SHS was noted with vehicle or drug treatment on different days as described in group 1.

**Group X**: Effect of low dose naloxone/naltrexone (opioids-antagonist) and rimonabant (cannabinoid-antagonist) was tested in morphine and cannabinoid or vice-versa in tolerant mice.

**4.4. Measurement of Nociceptive latency:**

**4.4.1. Hot Plate Test:**

Mice/ Rats are brought to the testing room and allowed to acclimatize for 10 minutes before the test begins. Pain reflexes in response to a thermal stimulus are measured using Eddy and Leimbach (1953). Hot Plate methods. The hot plate test that determines response to a thermal stimulus in conscious animals is one of the most commonly used models of nociceptive pain. It provides useful data that may explain and predict higher cerebral functioning and nociceptive behaviors. The surface of the hot plate is heated to a constant temperature of 52°C, as measured by a built-in digital thermometer with an accuracy of 0.1°C and verified by a surface thermometer. In brief, each mice / rats is placed on the hot plate (25.4 cm x 25.4 cm), which is surrounded by a clear acrylic cage, and the Start/Stop button on the timer is activated. The latency to respond with either a hind paw lick, hind paw flick, or jump (whichever
comes first) is measured to the nearest 0.1 seconds by deactivating the timer when the response is observed. The mouse/rat is immediately removed from the hot plate and returned to its home cage. If a mouse does not respond within 30 (Cut off time) seconds, the test is terminated and the mouse/rat is removed from the hot plate. Animals are tested one at a time and are not habituated to the apparatus prior to testing. Each animal is tested only once. Response latencies were measured at 15 minutes intervals and the average of the results was taken.

4.4.2. Tail- Flick Test

The antinociceptive effect of analgesics was measured by tail-flick (latency) test. The tail-flick test was described initially by D’Amour and Smith (1941) and is widely used for determining the antinociceptive effect of pharmacological agents. Similar to the hotplate test, the tail-flick assay uses heat as a noxious stimulus. In contrast to the hot-plate test, in which the endpoint consists of a complex behaviour (licking of the hind paw), noxious heat stimulation of the tail produces a simple nociceptive reflex response—a flick of the tail away from the heat source, which is a spinally mediated flexion reflex. Tail-flick latency was considered as time between tail exposure to radiant heat and tail withdrawal. The intensity of radiant heat was selected so as to obtain a pretreatment latency between 2 and 4 sec. in non diabetic control animals. The maximum cut-off latency time was fixed at 12 S in analgesic treated animals. Tail-flick latency was expressed as a percentage of the maximum possible effect (% MPE).
Post treatment latency time - pretreatment latency time

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\text{MPE} = \frac{\text{Cut off time} - \text{Pretreatment latency time}}{\text{Pretreatment latency time}} \times 100
\]

Pretreatment latency refers to the control latency before drug administration, while post-treatment latency refers to the latency after drug administration. Nociceptive latency was measured at 0, 15, 30, 60 and 180 min and expressed as mean latency.

4.4.3. Assessment of Thermal Hyperalgesia (a): The pain sensitivity to thermal heat was tested according to the Hargreaves procedure (Hargreaves et al., 1988) using the Plantar test (Ugo Basile, Varese, Italy) before diabetes induction and subsequent to STZ injection by 42 days. The latency to the first sign of paw licking or withdrawal response to avoid heat pain was taken as an index of pain threshold. The withdrawal latency was averaged from at least three trials separated by a 10-min interval and the cut-off was set at 20 s to avoid tissue damage. In brief, each animal was placed in a clear Plexiglas box and hind paw was exposed to a constant beam of radiant heat through a Plexiglas surface. The time in second, from initial heat source activation until paw withdrawal was recorded. General health state of the animals has been considered.

4.4.3.1. Tail-immersion Test (b): The STZ-mediated hypersensitivity was determined by tail immersion method as previously described (Courteix et al., 1993). The rat/mouse was maintained in a mouse/rat holder, and the distal tail, approximately 1 cm of the tip, was then immersed into a water bath maintained at 46 ± 0.5°C. The tail was rapidly immersed in the bath, and the latency to respond to the heat stimulus with vigorous flexion of the tail was measured to the nearest 0.1 s with use of a manual
stopwatch. Animals were removed immediately after responding, and the tail was wiped off with a cloth. The tail was removed from the water if the animals had not reacted after 30 s (Cutt-off time) to prevent tissue damage. The withdrawal latency in the pain tests was converted to a percentage of maximum possible effect (%MPE).

4.4.3.2. Assessment of Mechanical Allodynia: The mechanical threshold for nociceptive flexion was determined by measuring the paw-withdrawal threshold elicited by stimulation of the left hind paw using Dynamic Plantar Anesthesiometer (UGP; Basile, Varese, Italy). This device generates a mechanical force that increases linearly with time (s). The maximum force applied by a Von Frey Type–Filament was set 39 g. The nociceptive threshold was defined as the force at which the animals withdrew its paw. In brief, each animal was placed in a test cage with a wire mesh floor, and the tip of a von Frey-type filament was applied to the middle of the plantar surface of the hind paw. Brisk foot withdrawals in response to von Frey type filament stimulation were recorded. Paw-withdrawal threshold was expressed as threshold level in g. Each rat/mice was trained in the paw-withdrawal test for 30 min each day for 3 days. Each time the test was repeated five times, and the mean values represented the threshold of the individuals. The decrease in pain withdrawal threshold more than 20% in STZ treated rat indicates mechanical allodynia.

4.4.4. Assessment of Thermal allodynia:

In brief, each rat tail was immersed in a water bath maintained at 42 or 10 °C (a temperature that is normally innocuous in normal rats (Courteix et al., 1993) until tail withdrawal or signs of struggle were observed (cut-off time- 20 sec). As this test involves handling of the animals, one day before the experiment, the experimenter would handle the rats in the testing environment until they would sit quietly in the
hand for 20 sec (which corresponds to the cut-off time), 2 or 3 times depending on their capacity to be quiet. On the day of the experiment, rats were again handled by the experimenter for 15-20 sec above the water bath to get the rat used to the condition of the test. No rats showed aversive reaction during handling. Then, the tail of the rat was immersed into the water. The reaction time (i.e. the time necessary to observe the withdrawal of the tail from the bath) was measured 2-3 times in order to obtain two consecutive values that differed no more than 10%, and respecting an interval of at least 15 min between two measures. The tail of the rat was immediately dried with a soft cellulose paper to avoid tail cooling between two measures. A shortened duration of immersion indicates allodynia. Loss of more than 40% of the initial body weight, loss of activity, piloerection and infection were criteria that justified the removal of diabetic animals from the study. Thus, hyperalgesia and allodynia observed in STZ-induced diabetic rats was specific to hyperglycemia and was not due to the general health state of the animals.

4.4.4.1. Asssesment of Cold Allodynia: Three week after STZ treatment, cold allodynia was assessed using the acetone drop method (Choi et al., 1994). Cold allodynia was measured as the number of foot withdrawal responses after application of cold stimuli (acetone) to the plantar surface of the paw (14). In brief, acetone (0.1 mL) was applied to each hindpaw through a polyethylene (PE) 10 plastic tubing connected to a 1-mL syringe. Acetone was applied five times to each paw at intervals of 5-10 min. A brisk foot withdrawal response after the spread of acetone over the plantar surface of the paw was considered a sign of cold allodynia. Control rats either ignored the stimulus or occasionally responded with a small and brief withdrawal. Allodynic rats responded with prompt and intense paw withdrawal or escape
behaviour to acetone application. The duration of withdrawal response was recorded with an arbitrary minimum value of 0.5 s and a maximum value of 20 s.

4.5. Measurement of Lipid-peroxidation:

Lipid-peroxidation, as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was measured by using the thiobarbituric acid test of Ohkawa et al. (1979) (15). In brief, 0.2 mL of homogenate was added to 0.8% thiobarbituric acid, 8.1% sodium dodecyl sulfate (SDS) and acetic acid (20%) in distilled water. After heating for 60 min in a water bath at 95 °C, the mixture was then cooled and extracted with a mixture of n-butanol/pyridine (15:1,v:v). The absorbance of the reaction product present in the upper organic layer separated by centrifugation was measured spectrophotometrically at 532 nm.

4.6. Estimation of Antioxidant enzyme levels:

4.6.1. Superoxide dismutase (SOD): SOD was assessed by utilizing the technique of Kakkar et al. (1984). A single unit of enzyme was expressed as% inhibition of nitroblue-tetrazolium (NBT) reduction/min.

4.6.2. Catalase (CAT): CAT was assayed colorimetrically at 620 nm and expressed as ummoles of H2O2 consumed/min/mg protein as described by Sinha (1972). In brief, a mixture (1.5 ml) contained 1.0 ml of 0.01M phosphate buffer pH 7.0, 0.2 ml of tissue homogenate and 0.4 ml of 2M H2O2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

4.6.3. Reduced glutathione (GSH): GSH was estimated by the method of Ellman (1959). In brief, 10% TCA was added to the homogenate and the mixture was
centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was measured spectrophotometrically at 410 nm.

4.7. Estimation of Nitrite/Nitrate

Each rat was placed individually in metabolic cage and its urine was collected for 24 h. The animals were allowed to drink water ad libitum before the study but were denied water during 24-hr study period. Urinary, serum nitrite and tissue concentration was estimated using Greiss reagent method. In brief, 200 µl of the protein-free supernatant, 30 µl of 10% NaOH was added followed by 300 µl of Tris-HCl buffer and mixed well. To this, 530 µl of Griess reagent (0.3% N-1 [Naphthyl-ethylene-diamine-dihydrochloride] in distilled water + 3% Sulphanilamide in 1M HCl) was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm (Spectrophotometer, Beckman DU 640B, Switzerland). Nitrite concentration was calculated using standard curve for sodium nitrite. Nitrite levels in urine were expressed as amount excreted in 24 h.

4.8. Splenectomy and Preparation of Spleen Homogenate Supernatant:

Splenectomy was carried out under light anesthesia in diabetic rats and mice. After sacrificing the rats by cervical dislocation, spleen was removed and immersed in 1% Minimal Essential Media (MEM, pH = 7.8). The spleen was mashed, homogenized, and centrifuged at 3000 rpm for 15 minutes. The supernatant of spleen homogenate (SHS) was used for the study. Two fractions were made, one is heated SHS and another one is non-heated -SHS. The supernatant of the spleen homogenate (SSCH) from each diabetic rat (28 day) (0.4 ml) was injected to each recipient rat (400ul.i.v)
through tail vein. Spleen homogenate supernatant (SHS) was used in place of mononuclear spleen cells to avoid any implication of immunogenic response.

4.9. Rotarod Performance test: Rotarod test was performed to evaluate the effect of drugs on motor coordination to exclude the sedation induced analgesic and antihyperalgesic effect by the drugs used in this study.
Fig-03: Standard curve for tissue TBARS level using Malondialdehyde

Fig-04: Standard curve for tissue reduced glutathione using Glutathione (GSH)
Fig-05: Standard curve for tissue nitrite/nitrate level using Sodium nitrite

Fig-06: Standard curve for H$_2$O$_2$ using H$_2$O$_2$
Fig-07: Standard curve for tissue catalase level using catalase

Drugs and chemicals

STZ was purchased from Sigma-aldrich, USA and was dissolved in 0.1N citrate buffer. Morphine (Jackson lab), oxycodone, DPDPE and DAMGO (Sigma, USA), were dissolved in normal saline. Indometahcin and Ibuprofen were supplied by Cosmos (Ludhiana, India), as ex-gratia, and metamizole (Sigma, USA) and the acetylsalicylic acid (injectable) were purchased from Ciron Ltd, Mumbai, dissolved in appropriate vehicle and was injected intrapertonealy (i.p) or intraveneously (i.v).Win, 55,212-2 was purchased from Alexis, and was dissolved in 2.5 % DMSO. SR 141716 (rimonabant) was purchased from Lupin, India and dissolved in saline with 3% Tween 80, the obtained solution was stirred and then sonicated for preparation of stock solution.Neurosteroids i.e. progesterone, allopregnanolone, and dehydroepiandosterone were purchased from Sigma, USA. Immunomodulator i.e Cyclosporine, (Novartis), Thalidomide (Sigma, USA), Minocycline (Nidus Pharma), Pentoxyfyline (Torrent) were dissolved in appropriate vehicle and was injected i.p or i.v. Dextromethorphan (Wockhardt), Ketamine (Indus Pharma), L-NAME and
Aminoguanidine (Sigma, USA) were dissolved in normal saline and was injected i.p or i.v. Carrageenan and chlerythrine were obtained from Sigma, USA. All other chemicals and drugs are analytical grades. The solutions of the drugs were prepared freshly before use.

4.10. **Statistical analysis**: Results were expressed as means ± SD and analysed using analysis of variance (One way and Two way ANOVAs) followed by Bonferroni’s post-hoc test for multiple comparisons. Student’s *t*-test was used to compare the values from two groups. Statistical significance was fixed at *p* < 0.05. A Graphpad Prism Instate software was used as statistical tool.