# CHAPTER – 3

# MATERIALS AND METHODS

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## 3.1. Plant extracts

The analytically characterized extracts of the *Withania somnifera* roots (WSR), aerial parts comprise of mainly leaves with minor components from stems and fruits (WSA), stem parts (WSS) and withanolides free *Withania somnifera* roots extract (WFWS) were generously supplied by Natural Remedies Private Limited, Bengaluru, India (certificates of analysis are enclosed as Appendix 2, 3, 4 and 5 respectively). The plant materials used for obtaining the extracts were authenticated by the CSIR-National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (certificate of plant identification is enclosed as Appendix-6). Details of the extraction and analytical procedures used for obtaining and characterizing them were also supplied by the research department of Natural remedies, and are described in short in the following.

### 3.1.1. Extraction procedure for roots and aerial parts extracts of *Withania somnifera*

The dried roots and aerial parts (leaves and flowering tops) of *Withania somnifera* were collected and extracted in the R&D facilities of Natural Remedies Private Limited, Bengaluru, India. Coarsely powdered raw materials of roots and aerial parts of *Withania somnifera* were extracted with methanol as a solvent in 1:4 (w/v) ratios under reflux at temperature 65±5°C for 1h. The liquid extract obtained was filtered. The remaining marc was again kept for reflux by adding fresh methanol in 1:3 (w/v) ratios for 1 h, this reflux procedure was repeated twice and liquid extract obtained was collected and filtered. The liquid filtrate was combined and concentrated using vacuum evaporator at temperature 55±5$^{o}$C to a thick paste. The yield obtained was found to be 9% and 10%, respectively. The marc obtained from methanolic extraction was extracted twice under reflux at temperature 85±5°C for 1h with water in 1:3 (w/v) ratios as solvent. The aqueous extract was filtered and

concentrated using vacuum evaporator at temperature 55±5$^{o}$C to a thick paste. The yield obtained was found to be 10% and 12%, respectively. The concentrated methanolic and successive water extract were mixed together in the ratio of 87:13 w/w for roots and 65:35 w/w for aerial parts and then dried in a vacuum dryer at temperature 65±5°C to obtain the final powdered extracts.

### 3.1.2. Extraction procedure for stem parts extract of *Withania somnifera*

The dried stem parts of *Withania somnifera* were collected and extracted in the R&D facilities of Natural Remedies Private Limited, Bengaluru, India. Coarsely powdered raw materials of stem parts of *Withania somnifera* was extracted with methanol in 1:4 (w/v) ratios under reflux at temperature 65±5°C for 1h. The liquid extract obtained was filtered. The remaining raw material was again kept for reflux by adding fresh methanol in 1:3 (w/v) ratios for 1 h, this reflux procedure was repeated twice and liquid extract obtained was collected and filtered. The liquid filtrate was combined and concentrated using vacuum evaporator at temperature 55±5$^{o}$C. The yield obtained was found to be 12%.

### 3.1.3. Analytical characterization of three different extracts of *Withania somnifera* (WSR, WSA and WSS)

The three different plant parts (roots, aerial parts and stem parts) extracts of *Withania somnifera* were then analytically characterized in the R&D facilities of Natural Remedies Private Limited, Bengaluru, India for their total content of withanolides by High performance liquid chromatography (HPLC) system (Shimadzu, model 2010CHT, Japan). An HPLC system consisting of a quaternary pump with vacuum degasser, thermostatted column compartment, auto sampler, UV detector and reversed-phase column Phenomenex Luna C18 (5 µ particle size, 250 X 4.6 mm) were used. The analytical method was validated for linearity, specificity, accuracy and range of quantification. For sample preparation, accurately

weighed 2 g each of *Withania somnifera* extract to 100 ml volumetric flask to this add 50 ml of methanol, sonicated for 6 min, boil on water bath for 5 min and cool to room temperature. Make up the volume to 100 ml with methanol. Sample solution was filtered using 0.2 µ membrane filter paper. The mobile phase consisted of a buffer phase (solvent A) and acetonitrile (solvent B). The buffer phase (A) was prepared by dissolving 0.136 g anhydrous potassium dihydrogen orthophosphate ($KH_2PO_4$) and 0.5 ml of o-phosphoric acid ($H_3PO_4$) in 900 ml HPLC grade water, adjusting the pH to 2.8 ± 0.05 and made up the solution to 1000 ml. The solution was filtered using 0.45 µ membrane filter and degassed in a sonicator for 3 minutes before use. The following gradient elution was used: 0 min, 5% B; in next 18 min to 45% B; increased in next 7 min to 80% B and maintained at 80% B for next 3 min; decreased the solvent B to 45% in next 7 minutes; further decreased the solvent B to 5% in next 5 minutes followed by an equilibration period of 5 min. The flow rate was 1.5 ml/min, and the injection volume was 20 µl. The column temperature was kept at 30°C. The withanolides (Withanoside IV, Withanoside V, Withaferin-A, 12-Deoxy Withastramonolide, Withanolide A and Withanolide B) were detected at 227 nm.

### 3.1.4. Extraction procedure for withanolides free *Withania somnifera* extract

The dried roots of *Withania somnifera* were collected and extracted in the R&D facilities of Natural Remedies Private Limited, Bengaluru, India. Coarsely powdered raw materials of roots of *Withania somnifera* was extracted with methanol in 1:4 (w/v) ratios under reflux at temperature 65±5°C for 1h. The liquid extract obtained was filtered. The remaining raw material was again kept for reflux by adding fresh methanol in 1:3 (w/v) ratios for 1 h, this reflux procedure was repeated twice and liquid extract obtained was collected and filtered. The liquid filtrate was combined and concentrated using vacuum evaporator at temperature 55±5$^{o}$C. The yield obtained was found to be 9%. The marc obtained from methanolic

extraction was extracted twice under reflux at temperature 85±5°C for 1h with water in 1:3 (w/v) ratios as solvent. The aqueous extract obtained was filtered and concentrated using vacuum evaporator at temperature 55±5$^{o}$C to a thick paste. The yield obtained was found to be 10%, respectively.

**3.2. Chemical, reagents and analytical kits**

a) Carboxymethyl cellulose: Central Drug House (P), New Delhi, India.
b) Streptozotocin: Sigma-Aldrich, India and HiMedia, India.
c) Nicotinamide: SD Fine-Chemical Ltd., India.
d) Plasma glucose kit: ERBA Diagnostics, Germany.
e) Insulin ELISA kit: Chemux BioSciences Inc., USA.
f) Corticosterone ELISA kit: DSI S.r.l., Italy.
g) Plasma cholesterol, triglyceride, AST and ALT assay kit: Arkray Healthcare Pvt. Ltd., India.
h) Plasma HDL assay kit: Coral Clinical System, India.
i) Norepinephrine, Serotonin and Dopamine: Sigma-Aldrich, USA.
j) Acetylcholine and butyrylcholine: Sigma-Aldrich, USA.
k) iNOS ELISA kit: LifeSpan BioSciences Inc., USA.
l) NF-κβ assay kit: Cayman Chemical, USA.
m) Other chemicals and reagents used were from commercial sources.

**3.3. Animals**

Adult Charles Foster male albino rats (150 ± 10 g) and Swiss albino mice (20 ± 5 g) of either sex were procured from Central Animal House of Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration number: 542/AB/CPCSEA). They were randomly selected and housed (six animals per cage) in polypropylene cages provided with husk bed at an ambient temperature (25±1°C) and relative humidity (50±10%) with a 12:12 h light/dark

cycle. All the animals were acclimatized to laboratory conditions for at least one week before the start of the experiments. They were always fed with standard rodent diet and water *ad libitum*. The waste in the cages was removed daily to ensure hygienic condition and maximum comfort of animals. Ethical clearance for animal experimental work was obtained from the Central Animal Ethical Committee of the University (letter number Dean/2014/CAECI/604, dated 30.05.2014 is enclosed as Appendix-1) prior to the commencement of experiments. Behavioural experiments were performed between 09.00 to 14.00 hr. All the experimental groups were always tested in parallel (i.e. on the same day of the experiment), and handled, weighed, and observed by the single blinded observer and using the same laboratory environment.

### 3.3.1. Diabetic Rats

For these experiments, preselected diabetic rats were used. Non-insulin dependent diabetes mellitus (NIDDM) was induced in male rats by a slightly modified method described by P. Masiello et al. in 1998, and often used in our laboratories [G.M. Husain et al., 2011a, A.K. Thakur et al., 2013a; 2015]. In short, rats fasted overnight were challenged by a single i.p. injection of streptozotocin (STZ; 65 mg/kg), and fifteen minutes thereafter 120 mg/kg (i.p.) nicotinamide was administered. They were then returned back to their cages and provided with normal food and 5% sucrose water to minimize hypoglycemic shock. Hyperglycemic state of an animal was confirmed by elevated blood glucose level (more than 240 mg/dl), estimated on day 3 and 7 after STZ administration. Rats with blood glucose levels more than 240 mg/dl were considered as diabetic and used for the behavioural and other tests.

## 3.4. Animal Grouping and Drug Administration

Except for diabetic rats and the animals used in experiments dealing with analgesic activities, randomly selected and experimentally naive animal groups consisting of six animals in each

group were used in all experiments. *Withania somnifera* extracts and reference drugs used were suspended in 0.3% carboxymethyl cellulose (CMC) for once daily per-oral administration. Tested doses and treatment regimens of *Withania somnifera* extracts and reference drugs were selected on the basis of our pilot cum dose-finding studies conducted earlier in our laboratories. Control groups were treated similarly with vehicle (0.3% CMC) only. During the course of an experiment, all animals were closely observed for apparent behavioural abnormalities. On all observational days, the body weights and rectal temperatures of the animals were recorded one hour before the oral administration. All tests on all observational days were conducted one hour after the drug administration.

### 3.5. Protocols of the mice experiments

#### 3.5.1. Pilot methodological cum dose-finding experiments

##### 3.5.1.1. The first pilot experiment

Five groups of six to eight weeks old male mice weighing 20±5 g and preselected for their response times in hot plate test were used in this experiment. Two of these five experimental groups, serving as stressed and non-stressed control groups, were treated (p.o) daily with the drug vehicle (0.3% CMC, 10 ml/kg/day) for eleven consecutive days, and the remaining three were similarly treated with graded oral doses of WSR (10, 20, and 40 mg/kg/day, p.o.) suspended in 0.3% CMC. Except for the animals of the non-stressed control groups, all others were subjected to foot shock stress-induced hyperthermia test on the 1st, 5th, 7th and 10th days of the treatments. Immediately thereafter, the animals of all experimental groups were subjected to hot plate test for centrally acting analgesic on each of those days. On the 11th day of the experiment, all animals of all experimental groups were subjected to tail suspension test. On the following day, i.e. the 12th and the last day of the experiment, no oral treatments were given, and animals of all experimental groups were tested for quantifying

residual treatment effects in pentobarbitone induced hypnosis test. The experimental protocol used in this and several other similar experiments is graphically summarized in **Figure 3.1**.

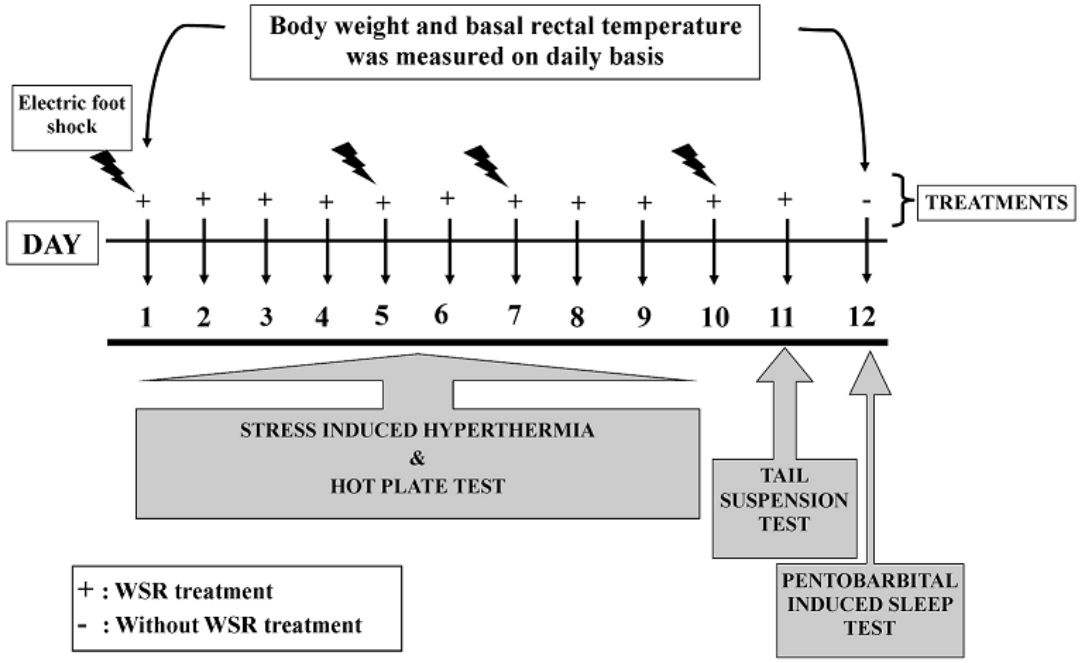


**Figure 3.1:** Summary of the experimental procedure used in the first pilot experiment.

### 3.5.1.2. The second pilot experiment

This experiment was conducted for reaffirming dose response relationship of WSR for its protective effects against stress responses and in two versions of marble burying test well standardized for their reproducibility under the environmental conditions of our laboratories. Except that the animals used in this experiment were not preselected in hot plate test (i.e. they were experimentally naive), animal groupings and treatment procedures used were the same as those used in the first pilot experiment. Like in that experiment, the animals were also subjected to foot shock stress-induced hyperthermia tests on the 1st, 5th, 7th, and 10th days of the treatments. But, in this experiment, the animals were not subjected to hot plate test on any of these days. On the 11th day of this experiment, and 60 min after the day's treatments, all animals of all groups were subjected to a standard version of marble burying test, and on

the next day, i.e. on the 12th day of the experiment, they were treated again and subjected to the two-zone version of marble burying test. The experimental protocol used in this experiment is graphically summarized in **Figure 3.2**.

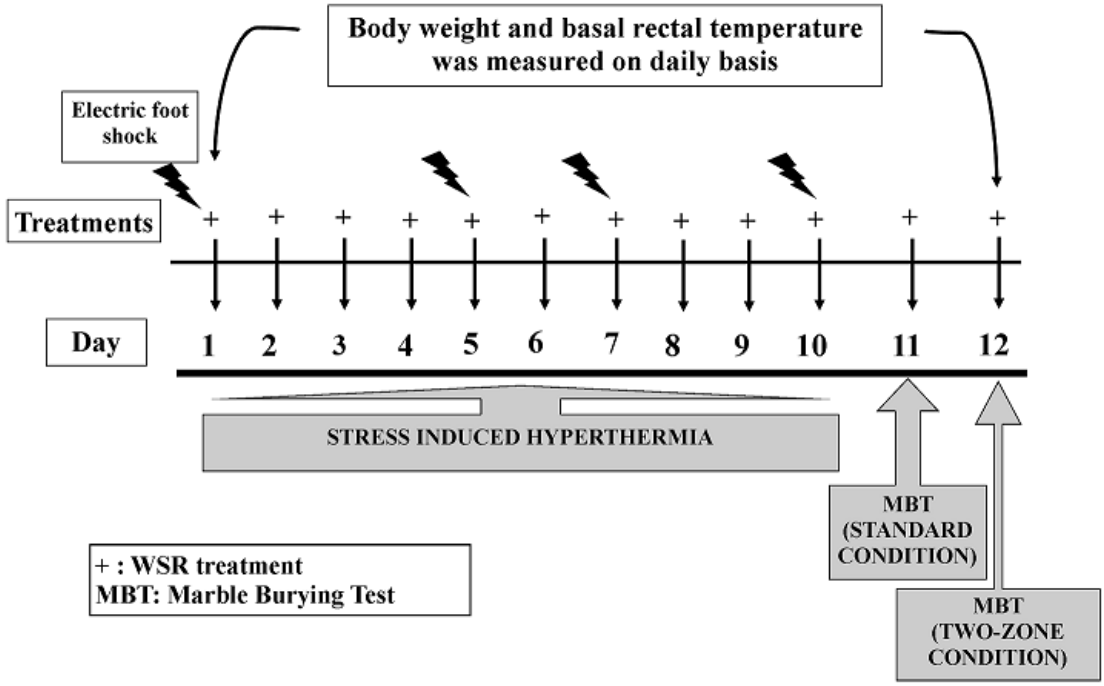


**Figure 3.2:** Summary of the experimental procedure used in the second pilot experiment.

### 3.5.2. Adaptogenic potentials of three types of *Withania somnifera* extracts

#### 3.5.2.1. The first set of comparative experiments

Using the same experimental protocol describe for the first pilot experiment (**see Figure 3.1**), these two experiments were conducted to compare the adaptogens like activity profiles of 50 mg/kg/day oral doses of three different types of *Withania somnifera* extracts (WSR, WSA, and WSS) in male or female mice with those of five reference drugs often prescribed for prevention and cure of metabolic disorders associated health problems. One of them was conducted in males and the other one in females. In both of this experiment, nine groups of six animals each were used, and all experimental groups were subjected to foot shock stress and hot plate tests on the 1st, 5th, 7th, and 10th treatment days. One of these groups treated

once daily with the vehicle served as control. Three others were similarly treated once daily for eleven consecutive days with 50 mg/kg oral doses of WSR, or WSA, or WSS. The remaining five experimental groups were also treated similarly with one of the five reference or standard drugs. These reference drugs and their daily oral doses tested were: diazepam (5 mg/kg), imipramine (15 mg/kg), metformin (50 mg/kg), nicotinic acid (1 mg/kg) and aspirin (100 mg/kg) respectively. On day 11th and 12th of the experiment, all the experimental groups were subjected to tail suspension test and pentobarbital induced hypnosis test respectively.

**3.5.2.2. The second set of experiments comparing lower dose effects of extracts**

Two experiments were conducted to compare the effectiveness of 10 mg/kg daily oral doses of WSR, WSA, and WSS. In one of the two experiments, five groups of six male mice each and the same treatment schedule and experimental protocol described in the first pilot experiment (**see Figure 3.1**) were used. In the second similar one, the experimental protocol and treatment schedule described in the second pilot experiment (**see Figure 3.2**) was used.

**3.5.3. Adaptogenic activity of WFWS, an extract devoid of withanolides**

Protocol of this experiment was quite similar to those used in the second pilot experiment (**see Figure 3.2**). The only difference was that on the last treatment day (12th day of the experiment, and immediately after the two-zone marble burying test) the animals were sacrificed (by decapitation) for obtaining blood samples used for estimating their circulating glucose, insulin and cortisol levels, and extracting and assessing their adrenal glands and spleen weights. Four of the seven experimental groups used in this experiment were treated with graded oral WFWS oral doses (3.3, 10, 33.3, and 100 mg/kg/day), and another one with 10 mg/kg daily oral doses of bactericidal and virucidal agent triethylene glycol (TEG). The treatment regimen for the two stressed and unstressed control groups of the experiment were also the same as those given to those groups of the second pilot experiment.

### 3.5.4. Effects of triethylene glycol co-administered with the root extract WSR.

Using the protocol of the second pilot experiment (**see Figure 3.2**), three separate experiments were conducted. Six groups of male mice were used in each of these experiments, and in all these experiments the animals were sacrificed on the last day of the experiment for estimating their blood glucose, insulin, and cortisol levels and organ weights (like in the experiment with WFWS described before). Doses of WSR co-administered with graded oral TEG doses (0, 4, 12, and 36 mg/kg/day) in these experiments were 1 or 3 or 10 mg/kg/day, and two vehicle treated control groups (one non-stressed and the other stressed) were also simultaneously tested in each of them.

## 3.6. Protocols of the experiments in diabetic rats.

### 3.6.1. Anti-stress activity

In this experiment, lower oral doses of the two extracts *viz*. withanolides rich *Withania somnifera* root extract (WSR) and withanolides free *Withania somnifera* extract (WFWS) was selected and compared their stress response protective potentials with standard antidiabetic metformin in stressed diabetic rats. STZ challenged diabetic rats with blood glucose levels more than 240 mg/dl were randomly assigned into five groups of six rats each and one group of non-diabetic rats was also used. Three of these six experimental groups, serving as stressed diabetic, non-stressed diabetic and non-stressed non-diabetic control groups, were daily treated (p.o) with the drug vehicle (0.3% CMC, 10 ml/kg/day) for ten consecutive days. Two other groups were similarly treated once daily for ten consecutive days either with 10 mg/kg oral doses of WSR or with WFWS and the remaining one group was also treated similarly with 50 mg/kg oral doses of metformin. Like in the mouse experiments, all the diabetic rats (except for the animals of the non-stressed control groups) were subjected to foot shock stress induced hyperthermia tests on the 1st, 5th, 7th, and 10th

treatment days of the treatments. On the last treatment day, immediately after the electric foot shock test, all the rats were sacrificed (by decapitation) for obtaining blood samples used for estimating their blood glucose, insulin and cortisol levels, and blood lipid profiles. The brain was extracted out and brain supernatants were used for quantifying the levels of nitric oxide and inducible nitric oxide synthase together with the plasma samples. The experimental protocol used in this experiment is graphically summarized in **Figure 3.3**.

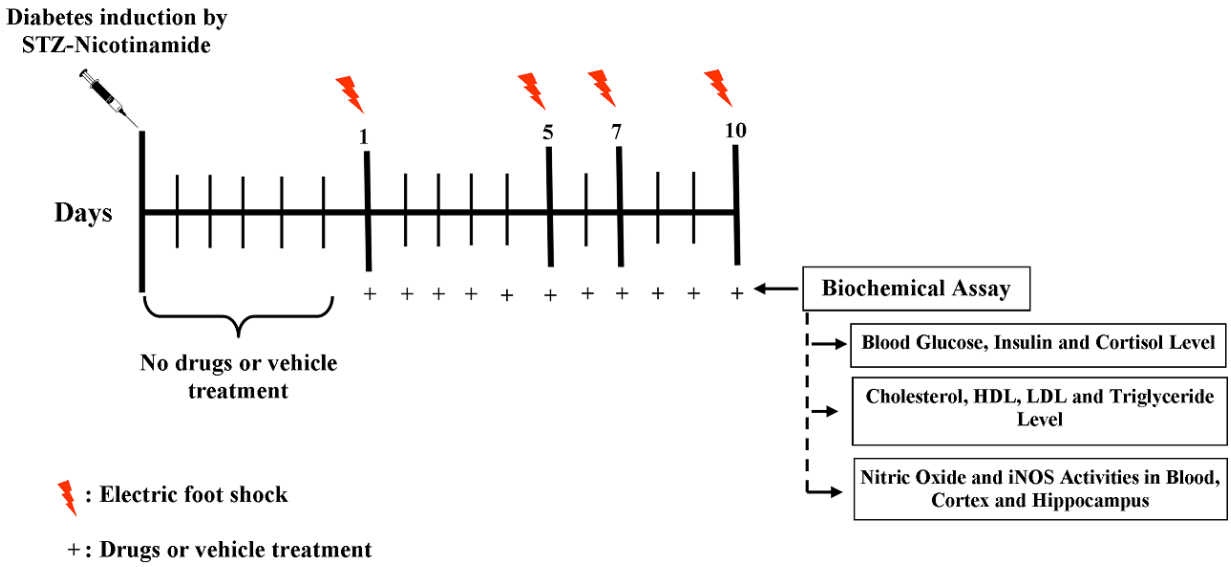


**Figure 3.3:** Summary of the experimental procedure used for comparing the anti-stress activity of two *Withania somnifera* extracts with the therapeutically used antidiabetic drug metformin in diabetic rats.

### 3.6.2. Antidepressant activity

In this experiment, the two tested extracts and metformin was used for comparing their antidepressant like effectiveness in stressed diabetic rats. As described in the previous experiment, six groups of animals were used and same treatments were given. The only difference was that the treatment was continued for eleven consecutive days. Like in that experiment, the animals (except for the animals of the non-stressed control groups) were also subjected to foot shock stress induced hyperthermia tests on the 1st, 5th, 7th, and 10th days of

the treatments. On the 11th day of this experiment and 60 min after the day's treatments, all animals of all groups were subjected to a standard version of the marble burying test, and on the next day, i.e. on the 12th day of the experiment, they were subjected to the forced swimming test. On the 12th day, immediately after the forced swimming test, all the animals were sacrificed (by decapitation) and blood and organ samples were collected. In addition to the blood glucose, insulin and cortisol levels, paraoxonase 1 (PON1) enzyme activities were also estimated in the blood samples. Liver homogenate supernatants were used for estimating their anti-oxidative status, glyoxalase-1 and PON1 enzyme activity. Similarly, brain homogenate supernatants were also used for quantifying brain anti-oxidative status and brain levels of monoamines (norepinephrine, dopamine and 5-hydroxytryptamine) and monoamine oxidases. The stomach of each rat was cut opened through their greater curvature for evaluating the stress inducing stomach ulcer. The experimental protocol used in this experiment is graphically summarized in **Figure 3.4**.

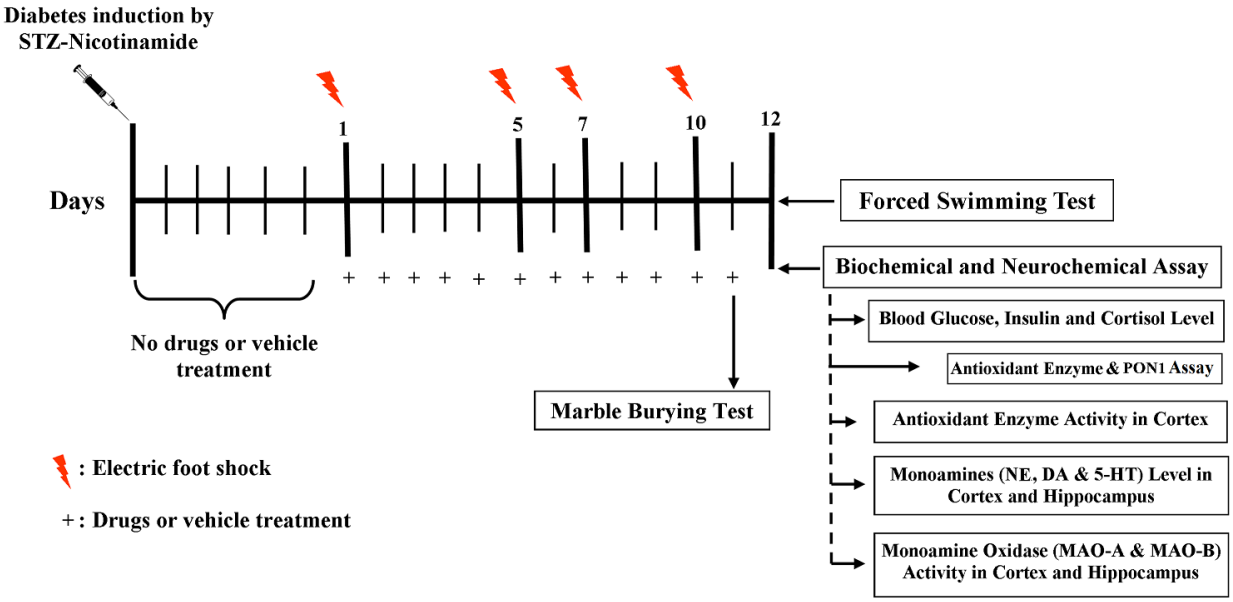


**Figure 3.4:** Summary of the experimental procedure used in diabetic rat experiment comparing anti-depressants like activities of two *Withania somnifera* extracts with metformin.

### 3.6.3. Anxiolytic activity

Using the similar treatment protocol and same stress paradigm described in the antidepressant activity this experiment was conducted to compare the anxiolytic potential of the two tested extracts with metformin in stressed diabetic rats. In this experiment, thirty minutes after the foot shock stress induce hyperthermia test on $1^{st}$, $5^{th}$, $7^{th}$ and $10^{th}$ day of the treatment, all the rats were placed individually in a photoactometer for a period of 10 min for accessing their locomotor activity (the number of movements for the first and last minutes was quantified separately). On the 11th day of the experiment and 60 min after the day's treatments, all animals of all groups were tested on elevated plus maze for their exploratory behaviour. Like in the other experiments, blood and organ samples were collected 24 hours after the last oral treatment (i.e. on the $12^{th}$ day of the experiment) for biochemical estimations. In addition to the blood glucose, insulin and cortisol levels, blood samples were also used for quantifying the anti-oxidative enzymes, plasma levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Liver homogenate supernatants were used for evaluating glyoxalase-1 enzyme activity and brain homogenate supernatants were used for estimating acetyl-cholinesterase (AChE) and butyrylcholinesterase (BChE) activities in the brains of animals. Like in the other experiment, stomach ulcer scores were also quantified in this experiment. The experimental protocol used in this experiment is graphically summarized in **Figure 3.5**.

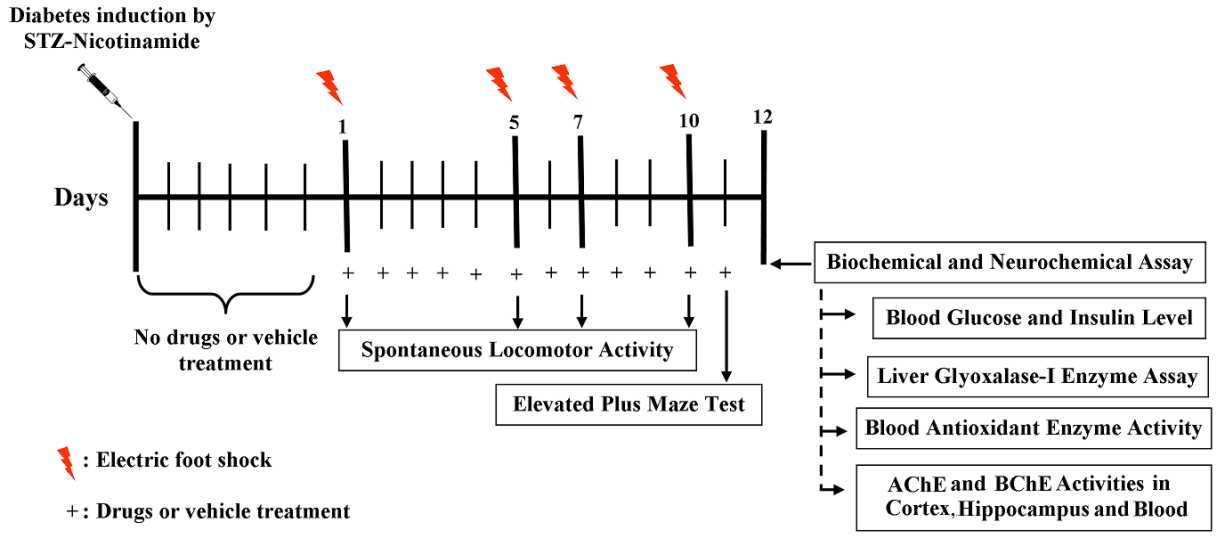


**Figure 3.5:** Summary of the experimental procedure used in the experiment in diabetic rats comparing anxiolytics like activities of metformin and two *Withania somnifera* extracts.

### 3.7. Behavioral models and tests

**3.7.1. Foot shock stress induced hyperthermia test:** The same procedure has been followed for both mice and rat experiments. The test procedure has been described elsewhere [A.K. Thakur et al. 2014a]. In short, an individual animal from a test group was placed in a black box (24 x 29 x 40 cm) with a steel grid floor for 60 sec. During this time, electric foot shock through the grid floor (2 mA, 50 Hz of 2 ms duration) was delivered for induction of stress. After 10 sec of their stay in the box, five consecutive foot shocks of 2 mA at 10 sec intervals were given through the grid floor. Immediately thereafter, the animals were placed back in their home cages and after 10 min interval, their rectal temperatures were recorded again. Numerical differences between the rectal temperature of a given animal and 10 min after the foot shock session was calculated and used as an index for stress-triggered transient hyperthermia [T.J.J. Zethof et al., 1994]. Animals of the non-stressed control group were also placed in the black box for one minute, but no foot shock stress was given and 10 min after they had been returned to their home cages their rectal temperatures were recorded again. All

core temperatures were recorded by using a calibrated rectal probe and digital thermometer (Easy Care, Mumbai, India).

**3.7.2 Hot plate reaction time in mice:** In those experiments dealing with analgesic activities, experimentally inexperienced six to eight weeks old mice were preselected by placing them individually on the hot surface of Eddy's hot plate analgesiometer maintained at 55±1°C and their reaction times were recorded [R.A. Turner, 1965]. Only mice, which responded within 15 seconds after placing them on the hot plate, and the reaction times of which did not vary enormously when tested on four separate occasions (each 15 min apart) were selected for the experiments. For preventing any thermal injury, the maximum time an animal was allowed to stay on the hot plate was 30 seconds [K.R. Paudel et al., 2007].

During the experiments, an animal from each test group was similarly placed on the hot surface of Eddy's hot plate analgesiometer maintained at 55±1°C for recording their centrally acting analgesic responses on 1st, 5th, 7th and 10th day of the treatments. This test was always conducted immediately after the second temperature measurement after foot shock tests. The latency until a mouse showed the first sign of discomfort (paw licking, hind paw lifting, or jumping) on the hot plate of the analgesiometer was recorded as its reaction time [R.A. Turner, 1965].

**3.7.3. Tail suspension test:** This test for assessing the depressive state of an animal was conducted on the 11th day of the experiment. The test procedure used has been described elsewhere in details [L. Steru et al. 1985]. In short, individual animal from each test group was hung by its tail in a head down posture by an adhesive tape placed approximately 1 cm from the tip of the tail on a horizontal wire placed 50 cm above the table floor. After initial vigorous movements, the animal assumes an immobile posture and the total period of immobility during 5 min observation period was recorded.

**3.7.4. Pentobarbital induced hypnosis test:** This test was conducted on the 12th day of the experiment on all animals in all test groups for estimating the residual effects of foot shock stress and treatments for pentobarbital induced sedation and sleep. On this day, no treatments were given, and the rectal temperatures and body weights of all the animals were recorded immediately before the pentobarbital challenge (40 mg/kg; i.p.) for sleep induction. Time taken for sleep onset (loss of righting reflex) and duration of pentobarbital induced sleep were recorded [K. Ojima et al., 1995].

**3.7.5. Marble burying tests:** In the standard version of this test, individual animal from each group was placed in a polypropylene cage [for mice test the cage size was 30 × 23 × 14 cm, and for rats the case size was 43 × 27 × 15 cm] provided with husk bed, where 12 glass marbles (colour and size of marbles were kept constant) were evenly spaced for standard marble burying condition test. In mice experiments, one day after the standard marble burying condition all the animals were tested again in the two-zones marble burying condition, whereupon 8 glass marbles (colour and size of marbles were kept constant) were evenly spaced only on one half of the cage. The numbers of marbles buried (i.e. marbles at least two-thirds cover by cage bedding husk) during 15 min (standard condition), or 30 min (two-zone condition), stay of the animal in the test cage was counted [L.B. Nicolas et al., 2006].

**3.7.6. Forced swimming test:** This test was performed on the diabetic and non-diabetic rats in antidepressant activity protocol. Individual animal from each group was subjected to the forced swimming test for 5 min test session on day 12 of the experiment. The method described by P. Willner, 1984 was followed with little modification. In brief, a rat was individually placed in a glass cylinder (45 × 20 cm) containing 40 cm water maintained at 25 ± 2°C temperature, so that animal could not touch the bottom of the cylinder with its hind

limb or tail, or climb over the edge of the cylinder. Two numbers of swim trials were given to each rat; an initial 15 min pre-test session on day 11 after the oral treatments followed by a 5 min test session on the next day (i.e. on day 12 of the experiment). Period of immobility (i.e. the total period of time the animal remained floating position in water without struggling and making only those movements necessary to keep its head above water) during the 5 min test period was recorded.

**3.7.7. Locomotor activity test:** This test was performed on the diabetic and non-diabetic rats in anxiolytic activity protocol**.** Effect of acute stress on locomotor activity of an animal was recorded by using Digital Photoactometer (Techno Electronics, Lucknow). Thirty minutes after the second temperatures measurement in foot shock stress induce hyperthermia test on day 1st, 5th, 7th and 10th of the treatments, individual rat from each test group was placed in a digital activity cage (30×30×30 cm) for a period of 10 min. During its stay inside the cage, the number of movements (photobeam counts) for the first and last minutes was quantified separately [R.A. Turner, 1972].

**3.7.8. Elevated plus-maze test:** This test was also performed on the diabetic and non-diabetic rats in anxiolytic activity protocol. The method described by S. Pellow and S.E. File, 1986 was followed with little modification. The maze had two opposite open arms and two enclosed arms having 40 cm high walls of the same dimension (50 × 10 cm). The arms were crossed with a central square, 10 × 10 cm, with an elevation of 50 cm above the floor giving the apparatus the shape of a plus sign. The maze was kept in an isolated room with dim light. Individual animal from each group was placed in the middle of the plus maze, facing a closed arm. Thereafter, the number of entries and time spent on the open and closed arms were recorded during the next 5 min [A.K. Thakur at al., 2013a].

### 3.8. Evaluation of Biochemical and neurochemical and other methods

**3.8.1. Blood collection and organ extraction:** Animals were sacrificed by decapitation and blood was collected by direct cardiac puncture and kept in heparin coated tubes in the ice. Collected blood samples were centrifuged at 1000×g (Compufuge CPR-30 Plus, with Rotor No. 8; REMI, India) for 20 min at 4°C to separate plasma from whole blood. Plasma was separated and aliquots were stored at -70°C for biochemical estimations.

Immediately after blood collections, spleen, adrenal glands, liver and brain of the animals were dissected out (when necessary), washed with normal water and weighed after removing adhered water by gently drying them on sheets of filter papers [T.M. Salman et al., 2013].

**3.8.2. Biochemical estimations:** All biochemical estimations were done by using an absorbance microplate reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA) according to instructions manual of biochemical and Enzyme Linked Immunosorbent Assay (ELISA) test kits. Plasma total cholesterol, triglycerides and high-density lipoprotein (HDL) were estimated using biochemical enzyme test kits.

***(i). Plasma glucose:*** Glucose in blood plasma was estimated by using biochemical test kit (ERBA Diagnostics, Germany) according to glucose oxidase/peroxidase method described elsewhere [G. M. Husain et al., 2011]. Briefly, glucose is converted to gluconic acid and Hydrogen peroxide ($H_2O_2$) in presence of glucose oxidase then in peroxidase catalyzed reaction, the oxygen liberated was accepted by the chromogen system to give a red coloured quinoneimine compound. The absorbance of red colour was measured at 505 nm and was directly proportional to glucose concentration.

***(ii). Plasma insulin and corticosterone:*** Plasma insulin and corticosterone levels were quantified by using ELISA test kits (Chemux BioSciences Inc., USA) and according to instructions manual of ELISA test kits.

***(iii). Total cholesterol:*** Total cholesterol was estimated by using biochemical test kit according to method described elsewhere [G.M. Husain et al., 2011b]. Briefly, the total cholesterol in plasma was determined by the hydrolysis of cholesterol esters with the help of cholesterol esterase to free cholesterol and fatty acids. The free cholesterol was oxidized via cholesterol oxidase to cholest-4-en-3-one with the immediate production of hydrogen peroxide ($H_2O_2$). The absorbance of the colored compound produced by reaction of $H_2O_2$ with phenol and 4-aminoantipyrine in the presence of peroxidase was measured by using an absorbance microplate reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA) at 505 nm. Absorbance of coloured compound was directly related to total cholesterol concentration present in the sample.

***(iv). Triglycerides:*** For plasma triglycerides estimation biochemical test kit was used. The method described in G.M. Husain et al., 2011b was followed. Briefly, in plasma triglycerides were hydrolyzed through lipoprotein lipase to form free fatty acid and glycerol. The glycerol formed was phosphorylated to glycerol-3-phosphate and adenosine diphosphate (ADP) in presence of glycerol kinase and adenosine triphosphate (ATP). Glycerol-3-phosphate was then oxidized by glycerol-3-phosphate oxidase to produced dihydroxyacetone phosphate and $H_2O_2$. Absorbance of red coloured quinoneimine resulting from the reaction of $H_2O_2$ with 4-aminoantipyrine and 4-chlorophenol in the presence of peroxidase was measured by using an absorbance microplate reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA) at 505 nm and the absorbance was directly proportional to triglycerides concentration present in the sample.

***(v). High-density lipoprotein:*** To estimate the total HDL-cholesterol concentration, LDL-cholesterol, VLDL-cholesterol and chylomicron fractions were precipitated by addition of polyethylene glycol 6000 in the plasma. After centrifugation, the HDL fraction remained in the supernatant and was analyzed as a sample for cholesterol [G.M. Husain et al., 2011b].

***(vi). Low-density lipoprotein:*** LDL-cholesterol concentration in plasma was calculated by using Friedewald's equation [W.T. Friedewald et al., 1972].

***(vii). Glutamic oxaloacetic transaminase:*** Glutamic oxaloacetic transaminase or aspartate transaminase catalyses the transamination of L-aspartate and α-Ketoglutarate to form L-Glutamate and Oxaloacetate. GOT estimation was performed according to the instruction manual of biochemical test kit (Arkray Healthcare Pvt. Ltd., India). In succeeding reaction, malate dehydrogenase reduced oxaloacetate to malate with simultaneous oxidation of nicotinamide reduced adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH was measured by monitoring the decrease in absorbance at 340 nm and was directly proportional to GOT activity in sample [H.U. Bergmeyer et al., 1978].

***(viii). Glutamic pyruvic transaminase:*** Glutamic pyruvic transaminase or alanine transaminase estimation was performed according to the instruction manual of biochemical test kit (Arkray Healthcare Pvt. Ltd., India). In succeeding reaction, the lactate dehydrogenase reduced the pyruvate to lactate with simultaneous oxidation of nicotinamide reduced adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH was measured by monitoring the decrease in absorbance at 340 nm and was directly proportional to GPT activity in sample [H.U. Bergmeyer et al., 1978].

**3.8.3. Blood oxidative status:** The blood oxidative status of non-diabetic and diabetic rats was assessed by the methods given below:

***(i). Lipid peroxidation (LPO)*:** Lipid peroxidation was determined by measuring the level of malondialdehyde (MDA) according to the method described in H. Ohkawa et al., 1979. Briefly, 100 µl of blood plasma was added to 50 µl of 8.1% sodium dodecyl sulfate (SDS), mixed well and incubated for 10 min at 25°C temperature, to that 375 µl of 20% acetic acid and 375 µl of thiobarbituric acid (0.6%) were added and kept in boiling water bath in for 1 h.

The reaction mixture was then allowed to cool at room temperature. To the reaction mixture 1.25 ml of n-butanol and pyridine at ratio 15:1 v/v was added, vortexed and centrifuged at 2000×g for 5 min. Then this reaction allowed to developed colour at room temperature. 200 µl of the pink coloured layer was measured at 532 nm on a microplate absorbance reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA) using 1,1,3,3-tetra-methoxypropane as standard. LPO was expressed as nmol MDA/ml.

***(ii). Superoxide dismutase activity (SOD)*:** The method of P. Kakkar et al., 1984 was followed. The inhibition of reduction of nitro blue tetrazolium (NBT) to blue coloured Formosan in presence of phenazine methyl sulphate and NADH was measured. In brief, 0.5 ml of plasma sample was diluted with 1 ml of water. The reaction mixture contained sodium pyrophosphate buffer (1.2 ml; 0.025 M) maintained at pH 8.3, 0.1 ml of 186 µM phenazine methyl sulphate, 0.3 ml of 30 µM nitro blue tetrazolium, 0.2 ml of 780 µM NADH, appropriately diluted plasma sample and water to make the total volume up to 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 85±5 sec, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was mixed vigorously with n-butanol (4 ml). The intensity of the coloured Formosan compound in the butanol layer was measured at 560 nm against n-butanol as blank using microplate absorbance reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA). In control, no enzyme was used. One unit of SOD activity was calculated as the amount of enzyme that inhibited the rate of reaction by 50% in one min under the defined assay conditions. Results were expressed as units of SOD activity/ml.

***(iii). Catalase activity (CAT)*:** The method of H. Luck, 1963, was followed with little modification wherein the decomposition of $H_2O_2$ being measured at 240 nm. The reaction mixture contains 3 ml of $H_2O_2$ in phosphate buffer (12.5 mM) maintained at pH 7.0 and 0.05 ml of plasma sample. The absorbance was recorded at 240 nm using microplate absorbance

reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA). CAT activity was expressed as μM of $H_2O_2$ consumed/min/ml.

**3.8.4. Liver tissue homogenate preparation:** Liver tissues were minced and homogenized with the aid of 9 volumes of homogenizing medium containing 0.25 M sucrose with 0.12 mM dithiothreitol (DTT) and buffered with 0.02 M triethanolamine hydrochloride buffer (pH. 7.4). The clear liver supernatant was obtained by centrifugation at 4°C and was used for estimation of Glyoxalase I enzyme activity.

***(i). Protein estimation:*** Protein estimation was done by the method described in O.H. Lowry et al., 1951. Standard curve was prepared by using Bovine serum albumin (BSA) powder. In brief, 100 μl of either samples or standard was mixed with 200 μl of biuret reagent. The mixture was then allowed to incubate at room temperature for 15 min and to this mixture, 20 μl Folin reagents were added. The reaction mixture was then allowed to developed colour at room temperature for 30 min. Then the absorbance was measured using microplate absorbance reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA) at 655 nm. Phosphate buffer (0.1M; pH 8) was used as blank.

***(ii). Oxidative status of liver:*** Oxidative status non-diabetic and diabetic animal livers were assessed by the methods described in above section. Lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities were measured in the supernatants of liver tissue homogenates in duplicate by using microplate absorbance reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA). For LPO, lipid peroxidation levels in the incubated liver homogenates was quantified by measuring malondialdehyde (MDA) concentrations and expressed as nmol MDA/mg protein according to the method described by H. Ohkawa et al., 1979. Superoxide dismutase (SOD) and Catalase (CAT) activity were also measured using the standard method [P. Kakkar et al., 1984; H. Luck, 1963].

***(iii). Glyoxalase 1 Enzyme assay:*** In Glyoxalase I assay, the rate of formation of S-D-lactoylglutathione was measured spectrophotometrically at 240 nm and in 25°C temperature. The assay mixture contained the following in a final concentration and volume of 1 ml: 100 mM Sodium phosphate buffer maintained at pH 7.2, methylglyoxal (3.5 mM), Glutathione (1.7 mM) and magnesium sulfate ($MgSO_4.7H_2O$: 16.0 mM). The mixture was incubated for 10 min and the reaction was started by the addition of the liver tissue homogenates (equivalent to 100 µg of protein). The glyoxalase I activity was calculated as µmole/gm protein/min of the product formed. One unit of the enzyme was indicated as the amount of enzyme catalysing the formation of 1 µmol of S-D-lactoglutathione/gm protein/min under the standard assay condition [J. Raju et al., 1999]. Protein concentration is determined by the method of Lowry et al., 1951, using bovine serum albumin as standard.

***(iv). Paraoxonase 1 (PON1) enzyme assay:*** The paraoxonase 1 enzyme in liver homogenate and in blood plasma was measured spectrophotometrically using phenylacetate as a substrate. In this assay, paraoxonase 1 enzyme catalyzes the cleavage of phenyl acetate, resulting in phenol formation. The rate of phenol formation was measured by monitoring the increase in absorbance at 270 nm. The working reagent was consists of Tris/HCl buffer (20 mM) maintained at pH 8.0, 1 mM calcium chloride ($CaCl_2$) and 4 mM phenyl acetate as the substrate. Plasma or liver tissues homogenates diluted in Tris/HCl buffer (1:3 v/v) were added, and the change in absorbance was recorded following a 20 sec interval. Absorbance was measured at 270 nm on every 15 sec for 120 sec using a UV-Visible spectrophotometer (SHIMADZU UV 1700 spectrophotometer, Japan) [J. Hussein et al. 2013]. The results are expressed as international Units (U)/mg of tissue protein or per ml of plasma.

**3.8.5. Brain tissues tissue homogenate preparation:** Brain cortex and hippocampus was isolated from the whole brain using the protocol described by S. Spijker, 2011. They were

weighted and stored in the deep freezer at -80°C until use. The brain parts of the animals were minced separately and homogenized with the aid of homogenizing buffer medium. The clear supernatants obtained from frontal cortex and hippocampus were used for quantifying the brain anti-oxidative status, brain levels of nitric oxide and monoamines, and enzymatic activity levels of inducible nitric oxide synthase (iNOS), monoamine oxidases (MAO), acetylcholinesterase (AChE), and butyrylcholinesterase (BChE).

***(i). Brain oxidative status:*** Oxidative status non-diabetic and diabetic animal brain tissues (frontal cortex) were assessed by the methods described in above section. Lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities were quantified in the supernatants of frontal cortex in duplicate by using microplate absorbance reader.

***(i). Nitric oxide (NO) assay:*** The method described by B.A. Weissman and S.S. Gross, 1998 was followed with little modifications to determine the nitric oxide level in non-diabetic and diabetic rat brains and in plasma. For brain tissue, frontal cortex and hippocampus part of rat brain were used. Briefly, 10 mg of each brain part was washed with ice-cold Phosphate buffer solution (pH 6.8). After washing the tissues were re-suspended in 100 µL of ice cold homogenizing buffer (50 mM/l Tris HCl, pH 7.4, with 2 mM/l EDTA) and homogenized with a homogenizer kept on ice. Centrifuge the tissue samples at 3000×g for 2-5 minutes at 4°C to remove any insoluble material. Collect the supernatants and blood plasma into a clean tube. Add ice-cold perchloric acid (PCA) 4 M to a final concentration of 1 M in the samples, mixed well and incubated on ice for 5 min. After incubation centrifuged the samples at 13000×g for 2 minutes at 4°C and transferred the supernatants to a fresh tube. Precipitate excess PCA by adding an equal volume of ice-cold 2 M potassium hydroxide (KOH) to the supernatants obtained and mixed briefly. After neutralization (pH 6.5 to 8), centrifuged the mixture at 13000×g for 15 minutes at 4°C, the samples obtained was then mixed with 100 µl of working

Griess reagent and NO level was quantified in the supernatants in triplicate by using microplate absorbance reader (iMark$^{TM}$ – Bio Rad, California, USA) at 546 nm.

***(iii). Inducible nitric oxide synthase (iNOS) assay:*** iNOS enzyme activity in plasma, frontal cortex and hippocampus of non-diabetic and diabetic rat brains was measured by using Enzyme Linked Immunosorbent Assay (ELISA) test kit according to instructions manual of ELISA test kits.

***(iv). Nuclear factor kappa beta (NF-κβ) assay:*** NF-κβ activity in plasma, frontal cortex and hippocampus of non-diabetic and diabetic rat brains was measured by using nuclear extraction kit (Cayman chemicals, USA) and Enzyme Linked Immunosorbent Assay (ELISA) test kit according to instructions manual of the kits.

***(v). Monoamines assay:*** Monoamine levels in hippocampus were quantified by the spectrofluorometric method described by A.S. Welch and B.L. Welch 1969. Briefly, clear homogenates obtained from hippocampus part of rat brain were mixed with 0.1 ml 10% EDTA. Then the homogenates were added to 25 ml n-butanol in 60 ml glass-stoppered bottle containing 4 gm NaCl. After centrifugation (3000 rpm for 10 min), 24 ml n-butanol was decanted in a bottle containing 40 ml n-heptane and 1.5 ml of phosphate buffer and then again centrifuged at 2000 rpm for 10 min. Aqueous layer was transferred to a clean 30 ml bottle and was acidified with 3 N HCl to pH 3.5-4.0. After adding 20 ml of peroxide free ether, the bottles were shaken for 10 min and centrifuged at 3000 rpm for 5 min. The acid-aqueous layer was taken directly from the bottom of the ether extraction bottles and was refrigerated and analyzed later for norepinephrine (NE) at 400/510 nm, dopamine (DA) at 335/380 nm and 5-hydroxytryptamine (5-HT) at 295/535 nm by using spectrofluorometer (RF 1501 Spectrofluorometer; SHIMADZU, Japan) and compared with the standard calibration curves prepared from respective standard amines.

***(vi). Monoamine oxidase assay:*** Hippocampus mitochondrial fraction was prepared for estimating monoamine oxidase (MAO) activity [A. Schurr and A. Livne, 1976]. Briefly, the mitochondrial fraction suspended in 10 volumes (1:10 w/v) of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose), was mixed at 5°C for 20 min. The mixture was centrifuged at 15000×g for 30 min and the pellets were re-suspended in the same buffer. The MAO-A and MAO-B activity was assessed spectrophotometrically as described by Charles and McEwen, (1977). The reaction mixtures contained 4 mM 5-hydroxytryptamine (5-HT) and 2 mM β-phenylethylamine (β-PEA) as specific substrates for MAO-A and MAO-B respectively to that 250 µl solution of the mitochondrial fraction and 100 mM sodium phosphate buffer (pH 7.4) were added up to a final volume of 1 ml. The reaction was allowed to proceed at 37°C for 20 min, and stopped by adding 200 µl of 1M hydrochloric acid (HCl). Then the reaction product was extracted with 5 ml of butyl acetate for MAO-A assay and cyclohexane for MAO-B assay respectively. The absorbance of organic phases was measured at 280 nm for MAO-A and at 242 nm for MAO-B using Shimadzu UV-visible spectrophotometer (SHIMADZU UV 1700 spectrophotometer, Japan). Blank samples was prepared by adding 1M HCl (200 µl) prior to reaction and worked up as for the test samples.

***(vii). Acetylcholinesterase* (AChE) *and butyrylcholinesterase* (BChE) *activities***: These activities were measured in 20% of clear brain homogenate (frontal cortex and hippocampus) and in blood plasma in phosphate buffer (0.1M; pH 8) by the method described by G.L. Ellman et al. 1961. In this assay, yellow colour was formed due to the reaction of acetylthiocholine iodide or butyrylthiocholine iodide with 5,5′-dithiobis-(2-nitrobenzoic acid). The rate of production of thiocholine from acetylcholine iodide or butyrylthiocholine iodide in the presence of tissue or plasma cholinesterase was measured for 3 min at regular intervals of 30 sec using microplate absorbance reader (iMark$^{TM}$- Bio Rad Laboratories, California, USA) at 415 nm (Karasova et al. 2009). The results were expressed as µmoles

substrate hydrolyzed/min/mg of tissue protein and µmoles substrate hydrolyzed/min/ml of blood plasma. Protein concentration is determined by the method of O.H. Lowry et al., 1951, using bovine serum albumin as standard.

**3.8.6. Stomach ulcer index:** For ulcer scoring in stomach the method described by S.K. Kulkarni, 1999 was followed. Briefly, the cardiac end of the stomach was removed from whole body and the contents of the stomach were removed. Thereafter, the stomach was cut and opened along with its greater curvature and washed slowly with normal water. After washing, stomach was spread and fixed on a glass slide for scoring ulcers (under 10X magnification). The ulcer index was evaluated according to their severity and scored as follows: 0 = Normal colored stomach, 0.5 = Red coloration, 1 = Spot ulcers, 1.5 = Hemorrhagic streaks, 2 = Ulcer > 3mm but < 5mm, 3= Ulcers >5mm [H. Govindani et al., 2012].

**3.9. Statistical analysis**

All data are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by analysis of variance (ANOVA) followed by Bonferroni post hoc test and Student-Newman-Keuls multiple comparison test wherever necessary. GraphPad Prism-5 (GraphPad Software Inc., California, USA) software was used for statistical analysis. Origin Pro 8 software (Origin Lab Corporation, Massachusetts, USA) was used for making graphs. Calculated p values less than 0.05 were always considered to represent statistically significant differences between two similar data sets obtained under similar experimental conditions.