CHAPTER-3

MATERIALS AND METHODS

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3.1. Animals

Adult Charles Foster albino males rats (150 ± 10 g) and Swiss albino mice (20 ± 5 g), of either sex were procured from Central Animal House of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India (Registration number: 542/AB/CPCSEA) and six randomly selected animals were assigned to each experimental group used in the study unless otherwise specified. They were housed in groups of six in polypropylene cages (260×190×135 mm for mice & 400×250×155 mm for rats) and maintained in ambient temperature of 25 ± 1 °C and 45-55 % relative humidity, with a 12:12 hr light/dark cycle. They were supplied with commercial food pellets and water ad libitum unless otherwise stated, and were acclimatized to laboratory conditions for one week before subjecting them to experimental conditions. Behavioural experiments were conducted between 09.00 to 14.00 hr and 'Principles of laboratory animal care' (NIH publication number 85-23, revised in 1985) guidelines were always followed. Prior approval from the Central Animal Ethical Committee of Banaras Hindu University, Varanasi, India, was obtained for the study protocols (Letter No. Dean/2014/CAEC/602, dated 30-05-2014 is enclosed as appendix-1).

3.2.Plant extracts and chemicals: The analytically characterized four different *Curcuma longa* extracts (CLE-1H: contains total curcuminoids 21.84% w/w by HPLC), (CLE-2B: contains total curcuminoids 18.83% w/w by HPLC), (CLE-3R: contains total curcuminoids 95.49% w/w by HPLC), (CLE-4M: contains total curcuminoids 16.6% w/w by HPLC) quantified to contain curcumin, demethoxycurcumin and bisdemethoxycurcumin (17.93 %, 3.39 %, 0.52 % w/w); (15.45 %, 2.92%, 0.46 % w/w); (77.94%, 15.03 %, 2.52 % w/w); (13.88%, 2.46 %, 0.26 % w/w)

respectively (certificates of analysis are enclosed as appendix-2, 3, 4 and 5), were generously supplied by Natural Remedies Private Limited, Bengaluru, India. CLE-1H, CLE-2B and CLE-3R were prepared by blending 95% total curcuminoids with water extract of *Curcuma longa* with the help of acetone solvent, methanol extract of *Curcuma longa* with the help of acetone and water solvent (also added β-cyclodextrin) and methanol extract of *Curcuma longa* with the help of hexane and acetone solvent respectively. The slurry was dried by using vacuum tray dryer (VTD) and finally passed through 40#. However, CLE-4M is bioavailable marketed formulation. Purity of isolated compound was determined by Shimadzu High Performance Liquid Chromatographic System LC 2010CHT with UV & PDA detector in combination with Class LC solution software. The analytical method was validated for specificity, linearity, precision, accuracy and range of quantification. HPLC conducted at temperature 30 ± 1^{o}C via column C18 (250 x 4.6 mm) 5 µ with flow rate 1.0 ml/min. Mixture of 40 volumes of tetra-hydrofuran and 60 volumes of buffer (0.1% citric acid solution in HPLC water was taken as mobile phase. Pure curcumin was isolated from the extract by using column chromatography. For such purposes, temperature of the column (Kromasil C18) oven was maintained on 27 ± 1^{o}C with flow rate 1.5 ml/min. 0.140 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 ml HPLC grade water with 0.5 ml of orthophosphoric acid in 1000 ml water was taken as mobile phase. Curcuminoids contents of *Curcuma longa* extracts were quantified by using method USP 37-NF32 for the presence of total curcuminoids and the same method was used for assessing the purity of the curcumin sample used. The turmeric oil (TO) sample used in this study was purchased from Soulflower handmade factory, Mumbai, India. Three major constituents of TO quantified by Gas Chromatography were Turmeron-I (8.22 % w/w), ar-Turmeron (30.83 % w/w) Turmeron-II (5.03% w/w).

3.3. Other Chemicals and Drugs

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, LDL, AST and ALT assay kit: Arkray Healthcare Pvt. Ltd., India.

h) Metformin: Sun Pharma, India.

i) Plasma HDL assay kit: Coral Clinical System, India.

j) Norepinephrine, Serotonin and Dopamine: Sigma-Aldrich, USA.

k) Acetylcholine and butyrylcholine: Sigma-Aldrich, USA.

l) iNOS ELISA kit: LifeSpan BioSciences Inc., USA.

m) NF-κB assay kit: Cayman Chemical, USA.

n) Other chemicals and reagents used were from commercial sources.

3.4. Animal Grouping and Drug Administration

Experimental groups consisting of either sex animals (n=6) per group were used unless stated otherwise, whereupon the animals were randomly allotted to different experimental groups. *Curcuma longa* extracts were suspended in 0.3% carboxymethyl cellulose (CMC) for once daily per-oral administration. Turmeric oil was also suspended with 0.3% carboxymethyl cellulose by vortexing process. Control groups (negative control) were similarly treated with vehicle only. Standard drug treated group for respective study was exercised parallel as positive control. On all

observational days, the body weights and rectal temperatures of the animals were recorded one hour before the oral administration. All tests in observational days were conducted one hour after the drug administration.

3.5. Experimental Methods

3.5.1. Pilot Study

3.5.1.1. Pilot experiments with 95.49% curcuminoids containing *Curcuma longa* extract (CLE-3R): In these exploratory experiments, pharmacologically interesting doses, dosing regimen and effect of single and repeated daily (5, 7 and 10 days) doses of the extract (5, 20, 80, 320 mg/kg/day, p.o.) were quantified, to establish three graded doses (minimum, intermediate and ceiling dose of observed effects) for further experimental studies.

In this pilot study, equal numbers of male and female mice were allotted to each treatment group. In one set of experiment only male mice were use and in another set of experiment female mice were used. The daily oral doses of *Curcuma longa* extract (CLE-3R) 5, 20, 80 and 320 mg/kg/day and 100 mg/kg/day metformin were given for 11 consecutive days. Treatments on 1^{st}, 5^{th}, 7^{th} and 10^{th} day were given after measuring their basal rectal temperatures with a calibrated rectal thermometer (Digital Thermometer; Dr. Gene, India). All animals were subjected to a stress induced hyperthermia test only on the days 1^{st}, 5^{th}, 7^{th} and 10^{th} of the experiment. Rectal temperatures of the animals were then measured again after 10 minutes of the foot shock exposure. Temperature difference before foot shock and the after foot shock was used to quantify stress induced hyperthermia. On day 11^{th} and 12^{th} of the treatment all the experimental mice were subjected to tail suspension test and pentobarbital induced hypnosis test respectively.

3.5.1.2. Pilot experiments with four different *Curcuma longa* extracts (CLE-1H, CLE-2B, CLE-3R and CLE-4M): This pilot study was held to compare oral efficacies of four different *Curcuma longa* extracts with that of pure curcumin in male mice for their adaptogenic activity. Mice were divided in groups of six animals. CLE-1H (21.8% w/w), CLE-2B (18.8% w/w), CLE-3R (95.4% w/w), CLE-4M (16.6% w/w) and pure curcumin were taken for comparative studies. The daily oral doses of CLE-1H (21.9 mg/kg), CLE-2B (25.4 mg/kg), CLE-3R (5 mg/kg), CLE-4M (28.7 mg/kg) and pure curcumin (5 mg/kg) given for 11 consecutive days. Treatments on 1^{st}, 5^{th}, 7^{th} and 10^{th} day were given after measuring their basal rectal temperatures. All animals were subjected to a stress induced hyperthermia test only on the days 1^{st}, 5^{th}, 7^{th} and 10^{th} of the experiment. For such purposes they were individually placed in a black box with a grid floor for 1 minute. During this period the animal received five consecutive foot shocks at 10 s intervals from the grid floor, after which they were immediately placed back to their home cages. Rectal temperatures of the animals were then measured again after 10 minutes of the foot shock exposure and temperature difference were recorded before and after foot shock. On the 11^{th} day of the experiments and after 60 min of drugs administration animals were subjected to tail suspension test and one day after the last treatment animals were exposed to pentobarbital induced hypnosis test.

3.5.1.3. Pilot experiments with turmeric oil: Based on pilot experiments with different *Curcuma longa* extracts same battery of animal models were used for evaluation of pharmacologically interesting doses and dosing regimen of turmeric oil in male mice. In this study only male mice were taken to explore the effect of turmeric oil in different pharmacologically validated animal models. Turmeric oil (1, 3, 10, 30 and 100 mg/kg/day, p.o.)

taken as test and CLE-3R (5 mg/kg/day, p.o.) were taken as reference drug for this study. Following validated battery of animal models were used for aforesaid mention pilot studies.

(a) Stress-induced hyperthermia test: After 60 min of treatment and initial rectal temperature measurement, individual mouse from each group was placed in a black box (24 x 29 x 40 cm) with a grid floor for 1 min. Electric foot shock through the grid floor (2 mA, 50 Hz of 2 ms duration) was delivered for stress induction. After 10s of their stay in the box, 5 consecutive foot shocks of 2 mA at 10 sec intervals were given through the grid floor, immediately after that the animals were placed back in their home cages, and stress-induced change in rectal temperature was quantified after 10 min stay in their home cages [T.J.J. Zethof et al., 1994].

(b) Tail suspension test: This test was used to assess depressive state of animals. In this test, individual mouse of a group was hung by its tail in 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 mouse assumes an immobile posture and the total period of immobility during 5 min observation period was recorded [L. Steru et al., 1985].

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

3.5.2. Analgesic Activity

To evaluate comparative analgesic activity pre-selected test animals were treated orally with test substances daily as suspension in 0.3% carboxymethyl cellulose for 11 consecutive days. Except

for the animals in CON-HPT group (not subjected to hot plate test), hot plate test was performed with all other groups on 1, 5th, 7th, and 10th day of the treatments (one hour after rectal temperature measurement and subsequent oral administrations). Body weight and rectal temperature of all the animals were daily recorded before oral administration.

3.5.2.1. Hot plate test: Mice were screened by placing them on a hot plate maintained at 55 ± 1 °C and recording the reaction time in seconds for forepaw licking or jumping [R.A. Turner, 1965]. Only mouse, which was reacted within 15 sec and which was not show large variation when tested on four separate occasions, each 15 min apart, was taken for the test. The time for forepaw licking or jumping on the heated plate of the analgesiometer maintains at 55 °C was taken as the reaction time. Reaction time of mice was noted on 60 min after the drug administration on day 1st, 5th, 7th, and 10th.

3.5.3. Antidepressant Activity

Six groups of rats were used in this experiment, whereupon three of them (treated with vehicle, CLE-3R and metformin) were treated with foot shock on 1st , 5th , 7th and 10th day of the experiment and the other three (treated with vehicle, CLE-3R and metformin) were not subjected to same. On the 11th day all groups of animals were treated again and subjected to forced swim test for 15 minutes as pre-training session, which was also another type of stress to the animals. On day 12 one hour after the last treatment they were subjected to the forced swimming test for 5 min test session. On day 13 no treatments were given and after recording their body weights and rectal temperatures they were sacrificed to obtain blood samples for glucose, insulin and corticosterone estimations and for estimating weight of adrenal gland, spleen, liver and heart.

3.5.3.1. Behavioural despair test: The method of P. Willner, 1984 was followed to evaluate antidepressant activity of test drugs. In short, a rat was individually placed in a cylinder (45 × 20

cm) containing 38 cm water (25 ± 2°C), so that it could not touch the bottom of the cylinder with its hind limb or tail, or climb over the edge of the chamber. Two swim sessions were given to each rat; an initial 15 min pre-test session on day 11 of drugs treatment 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 time the animal remained floating in water without struggling and making only those movements necessary to keep its head above water) during the 5 min test period was recorded.

3.5.3.2. Plasma glucose insulin and corticosterone estimation: Blood samples were withdrawn from retro orbital venous plexus on next day of last treatment after appropriate whole night fasting. Plasma was separated from blood and centrifuge at 3000 rpm (845xg), 5°C for 5 min (Compufuge CPR-30, with Rotor No. 8; REMI, India) and plasma (clear supernatant fluid) was kept in freeze till biochemical estimation. Fasting plasma glucose level was estimated by using biochemical enzyme test kit based on GOD-POD method (ERBA Diagnostics, Germany) Plasma insulin and level was estimated using Enzyme-Linked Immunosorbent Assay (ELISA) test kit. All biochemical analysis were done by using absorbance micro-plate reader (iMarkTM- Bio-Rad Laboratories, USA) according to instruction manual of respective enzyme test kit.

3.5.3.3: Organ weight: Immediately after blood collections, adrenal glands, spleen, liver and heart of the animals were dissected out, washed under slowly running tap water and weighed after removing adhered water by gently drying them on sheets of filter papers.

3.5.4. Anti-inflammatory and analgesic activity

Following procedure was used for compare the anti-inflammatory and analgesic activity of CLE-3R (10 mg/kg) and metformin (50 mg/kg). Rats were treated for nineteen consecutive days with their respective treatment .Six groups of rats were used in this experiment, whereupon three of them (treated with vehicle, CLE-3R and metformin) were injected with formalin and the other

three (treated with vehicle, CLE-3R and metformin) were injected with saline in their dorsal side of right hind paw. All animals were continuously receiving their respective daily oral treatments till the day 18th of the experiment. Meanwhile, body weights and basal rectal temperatures were also recorded on daily basis during entire experiment. Formalin or saline injections were administered to rats on the 7th experimental day. After formalin injection, a rat was placed individually in a wire cage for observations. Pain reactions was continuously (for 5 min period) counted at 10, 20, 40, 60 and 120 min and scored according to a pain scale. Meanwhile on day 7th, 11th, 13th, and 15th day of the treatment formalin induce paw edema was also measured. On day 19th all animals were subjected to hot plate test to evaluate the central analgesic activity.

3.5.4.1. Formalin test: The efficacy for central analgesic activity in animal as described previously [M. Dorazil-Dudzik et al., 2004] with some modification. Rats were injected (s.c.) with 100 µl of 12% formalin into the dorsal part of the right hind paw. After formalin injection, rats were placed individually in wire cages for observations. Pain reactions were continuously (for 5 min periods) counted at 10, 20, 40, 60 and 120 min and scored according to a pain scale. Pain-related behaviour was quantified by counting the incidence of spontaneous flinches per minute of the formalin injected paw. The paw was marked with ink at the level of the lateral malleolus and immersed in liquid up to this mark. The paw volume was measured on day 1st and after the injection of formalin by the digital plethysmometer. The edema was calculated by subtracting the initial hind paw volume of each rat.

3.5.5. Food and water intake behaviour

This experiment was held to compare food and water intake behaviour of CLE-3R and metformin in fructose fed animals. In this experiment three groups were (Vehicle, CLE-3R and metformin) maintained on normal pellet diet and normal drinking water throughout the study

period. Whereupon three groups (Vehicle, CLE-3R and metformin) were maintained on normal pellet diet and normal drinking water for initial 1st to 10th day and with normal pellet diet and 20% fructose in drinking water from 11th to 25th day of experiment. Body weights of all animals were recorded on all test days. Food and water intake in all groups was quantified during experiment on weekly basis. On the 26th day of the experiment their blood glucose, insulin, corticosterone, triglyceride, cholesterol, HDL, LDL were quantified and organ weights (adrenal glands, spleen, liver, kidney and heart) were also measured.

3.5.5.1. Fructose fed model: Rats were maintained on normal pellet diet (NPD) and normal drinking water from day 1st to day 10th and after the subsequent ten-day treatment period animals were started to receive normal pellet diet and 20 % fructose in drinking water till day 25th according to the procedure described elsewhere [G.M. Husain et al., 2011b; R. Jalal et al., 2007]. Rats showing significant weight gain compared to normal rats. The vehicle treated normal control group was maintained on NPD and normal drinking water throughout the study period. Like in other experiments, blood samples for biochemical analysis were obtained after overnight fasting.

3.5.5.2. Biochemical estimation*:* Blood samples were withdrawn from retro orbital venous plexus on next day of last treatment after appropriate whole night fasting. Plasma was separated from blood in centrifuge at 3000 rpm (845xg), 5°C for 5 min (Compufuge CPR-30, with Rotor No. 8; REMI, India) and plasma (clear supernatant fluid) was kept in freeze till biochemical estimation. Fasting plasma glucose level was estimated by using biochemical enzyme test kit based on GOD-POD method (ERBA Diagnostics, Germany). Plasma total cholesterol, high density lipoprotein-cholesterol (HDL-C), and triglycerides were estimated using biochemical enzyme test kits (Arkray Healthcare Pvt. Ltd., India). Low-density lipoprotein-cholesterol (LDL-

C) was calculated using Friedewald's equation [W.T. Friedewald et al., 1972]. Plasma insulin level was estimated using Enzyme-Linked Immunosorbent Assay (ELISA) test kit. All biochemical analysis were done by using absorbance micro-plate reader (iMarkTM- Bio-Rad Laboratories, USA) according to instruction manual of respective enzyme test kit.

***(a) Blood glucose, insulin and corticosterone*:** Glucose is estimated by glucose oxidase/peroxidase method. Briefly, glucose is converted to gluconic acid and H_2O_2 in presence of glucose oxidase. Subsequently, in a 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. Plasma insulin and corticosterone were measured by Enzyme Linked Immunosorbent Assay using micro-plate reader (iMarkTM-Bio-Rad Laboratories, USA) [G.M Husain et al., 2011c].

(b) Total cholesterol: Total cholesterol estimation was based on the hydrolysis of cholesterol esters by cholesterol esterase to free cholesterol and fatty acids. The free cholesterol was then oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide produced was coupled with 4-aminoantipyrine and phenol, in the presence of peroxidase, to yield a chromogen with maximum absorbance at 505 nm. Absorbance of coloured dye was proportional to total cholesterol concentration present in the sample [G.M. Husain et al., 2011b].

(c) Triglycerides: For triglycerides estimation, triglycerides were hydrolysed by lipoprotein lipase to produce glycerol and free fatty acid. In the presence of glycerol kinase and ATP, glycerol was phosphorylated to glycerol-3-phosphate and ADP. Glycerol-3-phosphate was further oxidised by glycerol-3-phosphate oxidase to yield di-hydroxyacetone phosphate and

H_2O_2. H_2O_2 was then coupled with 4-aminoantipyrine and 4-chlorophenol in the presence of peroxidase to produce red quinoneimine dye. Absorbance of coloured dye was measured at 505 nm and was proportional to triglycerides concentration present in the sample [G.M. Husain et al., 2011b].

(d) HDL-cholesterol*:* For HDL-cholesterol estimation, LDL-cholesterol, VLDL cholesterol and chylomicron fractions were precipitated by addition of polyethylene glycol 6000. After centrifugation, the HDL fraction remained in the supernatant and was analysed in the same manner as mentioned in total cholesterol estimation [G.M. Husain et al., 2011b].

(e) LDL-cholesterol: LDL-cholesterol was calculated using Friedewald's equation [W.T. Friedewald et al., 1972].

3.5.6. Other pharmacological studies

Following tests were performed to compare the anxiolytic and adaptogenic activity of CLE-3R (10 mg/kg) and metformin (50 mg/kg) in stressed non-diabetic and diabetic animals. Type-2 diabetes was induced in rats by intraperitoneal administration of streptozotocin (65 mg/kg) and nicotinamide (120 mg/kg, administered 15 min after streptozotocin treatment) as described elsewhere [G.M. Husain et al., 2011a]. STZ administered rats were returned back to their cages and provided with normal food and 5% sucrose water to minimize hypoglycemic shock. Hyperglycemia was confirmed by elevated blood glucose level (more than 240 mg/dl), determined on day 3 and then on day 7 after STZ administration. Animals with blood glucose levels more than 240 mg/dl (on seventh day after streptozotocin injection) was considered as diabetic and randomly allotted to the different treatment groups.

3.5.6.1. Foot shock stress induced hyperthermia test: This test was conducted on the 1^{st}, 5^{th}, 7^{th}, and 10^{th} days of the treatments. After 60 min of oral treatments and initial rectal temperatures

measurement, an individual rat from each test group (except non stressed non diabetic rats) was placed in a black box (24 x 29 x 40 cm) with a steel grid floor for 1 min. After 10s of their stay in the box, five consecutive foot shocks (2 mA, 50 Hz of 2 ms duration) at 10 s intervals were given through the grid floor for stress induction. Immediately thereafter, the animal was placed back in its home cages, and 10 min thereafter their rectal temperatures were recorded again. Temperature difference between after foot shock and before foot shock was calculated and used as an index for stress-triggered transient hyperthermic response. Rats of the non-stressed control group were also placed in the black box for one minute but no foot shocks were given on the test days, but their rectal temperatures were measured again 10 min after they have been returned to their home cages [T.J.J. Zethof et al., 1994].

3.5.6.2. Spontaneous locomotor activity: Effect of acute stress on locomotor activity of an animal was recorded by using photoactometer. Individual rat from each test group was placed in a digital activity cage (30×30×30 cm) for a period of 10 min on 1^{st}, 5^{th}, 7^{th} and 10^{th} day of the treatments. Each animal was allowed for a period of 5 min in a square closed field arena (30x30x30 cm) equipped with 6 photocells in the outer wall for spontaneous locomotion. 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].

3.5.6.3. Elevated plus maze test: The method of S. Pellow and S.E. File 1986 was followed. The elevated plus-maze consisted of two opposite arms, 50x10 cm, crossed with two enclosed arms of the same dimension but having 40 cm high walls. The arms were connected with a central square, 10x10 cm, giving the apparatus shape of a plus sign. The maze was kept in a dimly lit room and elevated 50 cm above the floor. Rat was placed individually in center of the maze, facing an enclosed arm. Thereafter, number of entries (all four paws of the rat were in that arm)

and time spent on the open and enclosed arms were recorded during the next 5 min by a blinded observer.

3.5.6.4. Marble burying test: Individual rats from each group was placed in a polypropylene cage (30 × 23 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. After 15 min of exposure, the animals were placed back to their home cages and the number of marbles at least two-thirds cover by husk were counted [L.B. Nicolas, et al., 2006].

3.5.6.5. Forced swimming test: 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 swim trials were given to each rat; an initial 15 min pre-test session followed by a 5 min test session on the next day (i.e. on day 12 of the experiment). Period of immobility during the 5 min test period was recorded.

3.5.6.6. Organ weights and gastric ulcers: On last treatment day immediately after blood collections, spleen, adrenal glands and liver of the animals were dissected out, washed under slowly running tap water and weighed after removing adhered water by gently drying them on sheets of filter papers. Meanwhile, stomach was removed and cut along the greater curvature. The numbers of discrete ulcers in the stomach were counted with the help of a magnifying glass. 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].

3.5.7. Biochemical estimations

3.5.7.1. Plasma glucose insulin and corticosterone estimation: One hour after the last stress session, individual rat from each group was sacrificed by standard procedure (decapitation) and blood was collected by direct cardiac puncture and kept in heparin coated tubes in ice. Collected blood samples were then 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. Fasting plasma glucose level was estimated by using biochemical enzyme test kit. Plasma insulin and corticosterone level was estimated using Enzyme Linked Immunosorbent Assay (ELISA) test kit. Plasma glucose levels were quantified by using a glucose test kit, based on glucose oxidase-peroxidase (GODPOD) method (ERBA Diagnostics, Germany). Glucose, insulin and corticosterone estimations were performed by using absorbance microplate reader (iMarkTM- Bio-Rad Laboratories, California) according to instruction manual of the enzyme test kits used.

3.5.7.2. Plasma levels of glutamic oxaloacetate transaminase (GOT) and glutamic pyruvate transaminase (GTP), LPO, SOD and catalase: Plasma level of glutamic oxaloacetate transaminase (GOT) and glutamic pyruvate transaminase (GTP) were also estimated using biochemical enzyme test kits. All biochemical estimations were done by using an absorbance micro-plate reader (iMarkTM- Bio-Rad Laboratories, California, USA) according to instructions manual of biochemical and ELISA test kits.

(a) Glutamic oxaloacetate transaminase: Glutamic oxaloacetate 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 kit. In succeeding reaction, malate dehydrogenase reduced oxaloacetate to malate

with simultaneous oxidation of nicotinamide 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].

(b) Glutamic pyruvate transaminase: Glutamic pyruvate transaminase or alanine transaminase estimation was performed according to the instruction manual of biochemical kit. In succeeding reaction, the lactate dehydrogenase reduced the pyruvate to lactate with simultaneous oxidation of nicotinamide 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].

(c) Lipid peroxidation (LPO) assay: 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 micro-plate absorbance reader (iMarkTM-Bio Rad Laboratories, Hercules, CA) using 1,1,3,3-tetra-methoxypropane as standard. LPO was expressed as nmol MDA/ml.

(d) Superoxide dismutase activity (SOD): The method of P. Kakkar et al., (1984) was followed for estimation of superoxide dismutase activity. 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 assay mixture contained 1.2 ml of sodium pyrophosphate buffer (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 made the total volume up to 3 ml. Reaction was started by the addition of NADH. After incubation at 30 °C for 90 sec, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The intensity of the coloured Formosan in the butanol layer was measured at 560 nm against n-butanol as blank. A system devoid of enzyme served as control. One unit of enzyme activity was defined 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.

(e) 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 (iMarkTM-Bio-Rad Laboratories, Hercules, CA). CAT activity was expressed as μM of H_2O_2 consumed/min/ml.

3.5.7.3. Antioxidant activity in liver*:* 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). Homogenate was centrifuged at 15000×g for 30 min at 4 °C. Supernatant obtained was used for estimation of Glyoxalase I enzyme activity and paraoxonase 1 enzyme activity.

(a) Protein estimation: Protein estimation was performed 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 made using microplate absorbance reader (iMarkTM Bio-Rad Laboratories, Hercules, CA) at 655 nm. Phosphate buffer (0.1M; pH 8) was used as blank.

(b) Glyoxalase 1 enzyme assay: In Glyoxalase I assay, the rate of formation of S-D-lactoylglutathione was measured spectrophotometerically at 240 nm and in 25°C temperature. The assay mixture contained the following in a final concentration and volume of 1 ml: 100mM Sodium phosphate buffer (pH 7.2), 3.5mM methylglyoxal, 1.7mM Glutathione (GSH) and 16.0mM MgSO4.7H2O. The mixture was incubated for 10 min and the reaction was started by the addition of the liver tissue homogenates (equivalent to100 µg of protein). The enzyme activity was calculated as µmole/gm protein/min of the product formed. One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 µmol of S-D-lactoglutathione/gm protein/min under the standard assay condition [J. Raju, et al., 1999].

(c) Paraoxonase 1 (PON1) enzyme assay: The paraoxonase 1 enzyme in liver homogenate 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 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM $CaCl_2$ and 4 mM phenyl acetate as the substrate. Liver tissues homogenates diluted in Tris/HCl buffer (1:3

v/v) were added, and the change in absorbance was recorded following a 20s lag time. Absorbance at 270 nm was taken every 15 s for 120 s using a UV-Visible spectrophotometer [J. Hussein et al. 2013]. The results are expressed as international Units (U)/mg of tissue protein.

3.5.7.4. Brain tissues sample: 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 hippocampus part of brain were used for brain monoamines level and monoamine oxidase enzyme assays, and frontal cortex were used for assaying antioxidant status. The clear supernatants obtained from frontal cortex and hippocampus was further used for quantifying the brain levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes and iNOS, NO and NF-κB. Protein estimation was performed 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 brain tissue pellets 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 made using microplate absorbance reader (iMark^{TM}- Bio Rad Laboratories, California, USA) at 655 nm. Phosphate buffer (0.1M; pH 8) was used as blank.

(a) Monoamines levels: Monoamine levels in hippocampus were quantified by the spectrofluorometric method described by A.S. Welch and B.L. Welch (1969). Briefly, hippocampus part of a brain was homogenized in 1.5 ml ice-cold 0.01 N HCl to which 0.1 ml 10% EDTA had been added. The homogenate was added to 25 ml n-butanol in 60 ml glass-stoppered bottle containing 4 gm NaCl. After centrifugation (3000 rpm for 8 min), 24 ml n-

butanol was decanted in a bottle containing 40 ml n-hepatne and 1.5 ml of phosphate buffer and then centrifuged (2000 rpm for 8 min). After that 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 (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 NE (400/510 nm), DA (335/380 nm) and 5-HT (295/535 nm) in spectrofluorometer (RF 1501 Spectrofluorometer; Shimadzu, Japan) and compared with the standard calibration curves prepared from respective standard amines (Sigma-Aldrich, USA).

(b) 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 resuspended in the same buffer. The MAO-A and MAO-B activity was assessed spectrophotometrically as described previously (Charles and McEwen, 1977]. Briefly, the assay mixtures contained 4 mM 5-HT and 2 mM β-PEA as specific substrates for MAO-A and B, respectively, 250 µl solution of the mitochondrial fraction and 100 mM sodium phosphate buffer (pH 7.4) up to a final volume of 1 ml. The reaction was allowed to proceed at 37 °C for 20 min, and stopped by adding 1M HCl (200 µl), the reaction product was extracted with 5 ml of butyl acetate (for MAO-A assay) and cyclohexane (for MAO-B assay), respectively. The organic phases were measured at a wavelength of 280 nm for MAO-A and 242 nm for MAO-B, respectively using Shimadzu UV/visible spectrophotometer. Blank samples was prepared by adding 1M HCl (200 µl) prior to reaction, and worked up as for the test samples.

(c) Brain anti-oxidative status: The brain tissue (frontal cortex) was homogenized in ten volumes (1:10 w/v) of 20 mM sodium phosphate buffer (pH 7.4) containing 140 mM KCl using a Teflon-glass homogenizer. The homogenates were centrifuged at 750×g for 10 min at 4 °C. Lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities were quantified in the supernatants of tissue homogenates in duplicate by using microplate absorbance reader (iMark-Bio-Rad Laboratories, Hercules, CA). For LPO levels, lipid peroxidation was quantified by measuring the level of malondialdehyde (MDA) and expressed as nmol MDA/mg protein according to the method of H. Ohkawa et al. (1979). For SOD activity, the method described by P. Kakkar et al. (1984) was followed, and the results were expressed as units of SOD activity/mg protein. For CAT activity, the standard method described elsewhere was used and expressed as μmol H_2O_2 decomposed/min/mg protein [H. Luck, 1963].

(d) Acetylcholinesterase and butyrylcholinesterase enzyme assay: The brain AChE and BChE activity was measured in 20% of clear brain homogenate (frontal cortex and hippocampus) 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 formation of thiocholine from acetylcholine iodide or butyrylthiocholine iodide in the presence of brain cholinesterase was measured for 3 min at regular intervals 30 sec using microplate absorbance reader (iMarkTM Bio-Rad Laboratories, Hercules, CA) at 415 nm [J.Z. Karasova et al. 2009]. The results were expressed as μmoles substrate hydrolysed/min/mg of tissue protein.

(e) Nitric oxide (NO) assay: The method described by B.A. Weissman, and Gross, 1998 was followed with little modifications to determine the nitric oxide level in rat brains. Frontal cortex and hippocampus part of rat brain were used for estimation of nitric oxide. 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 transferred to a clean tube. Add ice-cold Perchloric acid (PCA) 4 M to a final concentration of 1 M in the homogenate solutions, 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 supernatant 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 supernatant obtained was then mixed with 100 µl of working griess reagent NO level was quantified in the supernatants in triplicate by using microplate absorbance reader (iMarkTM BioRad, USA) at 546 nm.

(f) Inducible nitric oxide synthase (iNOS) assay: iNOS enzyme activity in frontal cortex and hippocampus of normal and stressed diabetic rat brains was measured by using ELISA test kit according to instructions manual of test kits.

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

3.6. Statistical analysis

Means ± standard error of mean (SEM) was calculated for the observed values in each experimental group. Statistical analysis was performed by one way analysis of variance

(ANOVA) followed by Student-Newman-Keuls multiple comparison test unless otherwise stated. GraphPad Prism-6 (GraphPad Software Inc., USA) and OriginPro-8 (OriginLab Corporation, USA) software were used for statistical analysis and graph preparation. P value less than 0.05 was always considered as statistically significant.