Chapter-3

Materials and Methods

MATERIALS AND METHODS

3.1. Animals

Adult Charles Foster albino rats (150 ± 10 g) and Swiss albino mice (20 ± 5 g), of both sexes were obtained from Central Animal House of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India (Registration Number: 542/AB/CPCSEA, dated 22-01-2002), 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 (Pranav Agro Industries Ltd., Sangali, India) 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/11-12/CAEC/325, dated 30-11-2011).

3.2. Plant Extract and Andrographolide

Standardised hydro-methanolic *Andrographis paniculata* leaves extract (AP; KalmCold™, 32.20 %, w/w andrographolide) and andrographolide (99.0 % pure by HPLC) were generously supplied by Natural Remedies Pvt. Ltd., Bangalore, India. The plant leaves were collected in the month of March and identified as *Andrographis paniculata* (Burm. F.) Wall. Ex Nees by in-house botanist at R&D Centre of Natural Remedies Pvt. Ltd., Bangalore, India, and a voucher herbarium specimen (No: NR582) was kept in the R&D Centre of Natural Remedies Pvt. Ltd., Bangalore, India. Extraction procedure and analytical methods used for standardising AP and isolation of andrographolide were described in details elsewhere (Chandrasekaran *et al.*, 2009; Chandrasekaran *et al.*, 2010). Briefly, the coarse ground leaves of *Andrographis paniculata* was refluxed with methanol

in stainless steel jacketed extractor for 3 h. The extraction procedure as above was repeated two more times and the liquid extracts from each extraction step was separately subjected to distillation under vacuum (at <55 °C) until a thick paste with a total solid content of 40–50 % (w/w) was obtained. Thick paste obtained from the three extraction steps were mixed and dried under vacuum (<65 °C) to get lumps of the extract. The extract lumps were then milled and sieved (#40) to get a uniform powdered extract of *Andrographis paniculata*. Water was added to the marc in the extractor, and the contents were refluxed for 3 h. The liquid extract was drained from the extractor into a concentrator and was subjected to distillation under vacuum (at <75 °C) until the total solid content in the liquid reached about 15-20 % (w/v). The concentrated liquid was then spray dried to get the water extract of *Andrographis paniculata*. The alcohol and water extracts were then analyzed for the content of active constituents and blended (ratio 2:1) to get KalmCold™ with the required levels of active constituents by HPLC system consisting of quaternary pump with UV detector, auto injector and column with class LC 10A software (Shimadzu, Model LC 2010 A, Japan). The analytical method was validated for specificity, linearity, precision, accuracy and range of quantification. The extract was subjected to liquid-liquid partitioning between ethyl acetate and water. The ethyl acetate layer was repeatedly chromatographed over silica gel using combination of hexane:ethyl acetate and chloroform:methanol. Purity of the isolated compounds was determined by high performance liquid chromatography (HPLC).

3.3. Other Chemicals and Reference Drugs

a) Streptozotocin: Sigma-Aldrich, India and HiMedia, India

b) Nicotinamide: SD Fine-Chemical Ltd., India

c) Plasma glucose and cholesterol kit: Span Diagnostics Ltd., India

d) Insulin ELISA kit: DRG Diagnostics, Germany

e) Glibenclamide, Atorvastatin and Diazepam: Cipla Ltd., India

f) Imipramine: Sun Pharmaceutical Industries Ltd., India

g) Piracetam: Torrent Pharmaceuticals Ltd., India

h) *Withania somnifera* root extract: Natural Remedies Pvt. Ltd., India

i) Norepinephrine, Serotonin and Dopamine: Sigma-Aldrich, USA

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

3.4. Animal Grouping and Drug Administration

Experimental groups consisting of both sex animals (n=6) per group were used unless stated otherwise, whereupon the animals were randomly allotted to different experimental groups. *Andrographis paniculata* extract was suspended in 0.3% carboxymethylcellulose (CMC) for once daily per-oral administrations. Andrographolide was macerated with Tween 80 (0.2%) and suspended in 0.2% aqueous agar for daily administrations. Control groups (negative control) were similarly treated with vehicle only. Standard drug treated group for respective study was exercised parallel as positive control. Body weight was recorded on daily basis. Except when stated, each experiment was conducted with different groups of experimental animals.

3.5. Experimental Methods

3.5.1. Pilot Study

3.5.1.1. Pilot experiments with Andrographis paniculata extract: In these exploratory experiments, the effect of single and repeated daily (5, 7 and 10 days) doses of the extract (25, 50, 100, 200, 400, 600 and 800 mg/kg/day, p.o.) were quantified in four well standardised pharmacological models described in the following to establish three graded doses (minimum, intermediate and ceiling dose of observed effects) for further experimental 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 (Zethof *et al.*, 1994).

(b) Ring test: After 60 min of treatment individual mouse from each group was placed on a steel ring (diameter 5.5 cm) fixed to a steel stand at a height of 16 cm. The assay was performed by lifting-up the mouse by its tail and placing the animal gently across the wire ring. During the following 5 min, the sum of the times during which the mouse remains motionless is noted to the nearest second. The criterion for immobility is the absence of all voluntary body movements except for those associated with breathing. Lack of snout and whisker movements provides an easily recognizable endpoint for the onset of immobility and serves to make the test reasonably objective. The test was repeated at 90 min and 120 min interval after treatment of extract. Immobility was expressed as an 'immobility index', defined as that percentage of the total time spent on the ring during which the animal remains motionless (Pertwee, 1972).

Immobility index = Immobility period (sec)/ Total time spent on ring (sec) x 100

(c) Inverted screen test: This test is also known as the wire mesh test or horizontal screen test. During test, a mouse from each group after 60 min of treatment was put upon the screen (15x15 cm wire mesh) elevated at 40 cm above the floor. The screen is then slowly rotated by 180° (Coughenor *et al.*, 1977), and grading system for the inverted screen test was scored as follow: 0 = the mouse climbs to the top; 1 = the mouse fails to reach the top but holds onto the screen; and 2 = the mouse falls from the screen during 1 min of the test duration (Maxwell *et al.*, 1993).

(d) Cage-climbing test: This test was done by method described by Protais *et al.* (1976) in rats with some modifications. Apomorphine (0.50 mg/kg, s.c.; Sigma-Aldrich, USA) was administered after 60 min of extract treatment. Immediately after the injection, a rat was put into a cylindrical individual cage, 12 cm diameter and 14 cm high, with walls of vertical metal bars, 2 mm diameter and 1 cm apart, surmounted by a smooth surface. After 5 min period of exploratory behaviour, three consecutive observations were made on each animal by a

blinded observer at 10, 20 and 30 min after apomorphine injection and these three scores were averaged. This behavioural score used was as follow: 0= four paws on the floor, 1= forefeet holding the wall, and 2= four paws holding the wall.

3.5.1.2. Pilot experiments with andrographolide: Based on pilot experiments with *Andrographis paniculata* extract following validated methods were used for pilot cum dose finding study to establish three graded doses after single and repeated daily doses of andrographolide (3, 10, 30, 100 and 300 mg/kg/day, p.o.). In the pilot study, equal numbers of male and female animals were allotted to each treatment group, whereupon the vehicle treated control group consisted of 12, and the drug treated ones consisted of 6 animals each. Based on the observation made in the pilot experiment, two further confirmatory experiments using either male or female mice (6 animals per group) were conducted.

(a) Stress-induced hyperthermia: Treatments on days 1, 5, 7 and 10 were given after measuring their basal core temperatures with a calibrated rectal thermometer (Digital Thermometer; Dr. Gene, India). Tests were always conducted 60 min after the day's treatments and observations were made in a blinded manner. All animals were subjected to a foot-shock stress triggered hyperthermia test on the days 1, 5, 7 and 10 of the experiment. For such purposes they were individually placed in a black box (24 x 29 x 40 cm) with a grid floor for 1 min. During this period, the animal received five consecutive foot-shocks (2 mA, 50 Hz of 2 ms duration each) at 10 sec 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 min of the foot-shock exposure by a blinded observer (Zethof *et al.*, 1994). Calculated difference between the first and the last temperature measurements of an animal on a test day was used to quantify stress-induced hyperthermia.

(b) Pentobarbital-induced hypnosis: On the 11th day, treatments were given without prior rectal temperature measurements and 60 min before pentobarbital (40 mg/kg, i.p.) challenge. Rectal temperatures were measured on this day immediately before the pentobarbital challenge. The time necessary for

pentobarbital-induced sleep induction (loss of righting reflex) and duration of sleep recorded by a blinded observer (Ojima *et al.*, 1995).

3.5.2. General Neuropharmacological Screening

Experimental procedures described in the following were used for general neuropharmacological screening of *Andrographis paniculata* extract.

3.5.2.1. Potentiation of pentobarbital-induced hypnosis: Pentobarbital (40 mg/kg, i.p.; Loba Chemie Pvt. Ltd., India) was administered to control and drug treated rats. Onset of sleep (loss of righting reflex), and duration of sleep was measured by a blinded observer (Ojima *et al.*, 1995). The last (10th) doses of AP (50, 100 and 200 mg/kg, p.o.), or of the standard drug diazepam (5 mg/kg, p.o.) were administered 60 min before pentobarbital injection.

3.5.2.2. Spontaneous locomotor activity: The spontaneous locomotor activity in photoactometer (Techno Electronics, India) was assessed by method described by Ramanathan *et al.* (1999). The last (10th) doses of AP (50, 100 and 200 mg/kg, p.o.), or of lorazepam (1 mg/kg, p.o.), were administered 60 min before subjecting the animals to spontaneous locomotor activity test. Each rat was allowed for a period of 5 min in a square closed field arena (30x30x30 cm) equipped with six photocells in the outer wall for spontaneous locomotion. Number (N) of photocell beams interruption (locomotor activity) was recorded by means of a 6 digits resettable counter.

3.5.2.3. Maximal electroshock (MES) seizures in rats: According to this method, the supra-maximal electroshock (150 mA, 50 Hz) was given through a pair of pinnal electrodes for 0.2 sec duration using a convulsiometer (Techno Electronics, India). The hind limb extensor response was taken as the positive end point (Peterson, 1996). Albino rats were pre-screened and only those showing positive hind limb tonic extensor response were used for the experiment. At least 48 h after pre-screen, drug treatment was started for 10 consecutive days. The last (10th) doses of AP (50, 100 and 200 mg/kg, p.o.), or of phenytoin (30 mg/kg, p.o.), were administered 60 min before the MES challenge. The severity of convulsions was assessed by the duration of tonic flexion, hind limb tonic extensor (HLTE), clonus and stupor phase for each animal (in second).

3.5.2.4. Pentylenetetrazole (PTZ)-induced convulsions in mice: The mice were challenged with PTZ (80 mg/kg, i.p.; Sigma, India). The last (10th) administration of AP (50, 100 and 200 mg/kg, p.o.), or of diazepam (10 mg/kg, p.o.) was done 60 min prior to PTZ challenge. A blinded observer recorded the latency to first myoclonic jerk, percentage clonic seizure, and percentage protection (Rudzik, 1973).

3.5.3. Anti-diabesity Activity

Following tests were performed in nondiabetic, type-2 diabetic and diet-induced obese rats (high fat diet and fructose fed models):

3.5.3.1. Oral glucose tolerance test: In this test the effects of ten daily per-oral doses (50, 100 and 200 mg/kg/day) of AP and that of the standard anti-diabetic drug glibenclamide (10 mg/kg/day) in normal rats were compared. Thirty minutes after the last treatments on day 10, all animals were challenged with an oral dose of 2 g/kg of glucose. Blood glucose levels were measured immediately before glucose challenge and 30, 60 and 120 min thereafter. Hereupon blood was obtained by retro-orbital sampling technique, and an enzymatic method was used to measure glucose concentration (du Vigneaud and Karr, 1925).

3.5.3.2. Type-2 diabetes model: Non Insulin Dependent Diabetes Mellitus (NIDDM/type-2) was induced in overnight fasted animals by a single intra peritoneal (i.p.) injection of 65 mg/kg streptozotocin (STZ; Sigma, India), 15 min after the i.p. administration of 120 mg/kg nicotinamide (SD Fine-Chemical Ltd., India) as described by Masiello *et al.* (1998) with some modifications (Husain *et al.*, 2011c). Animals were returned to their cages and provided normal food and 10% sucrose water to minimize hypoglycemic shock. Hyperglycemia was confirmed by elevated glucose level in the blood, determined at 72 hr and then on day 7 after STZ injection (Wu and Huan, 2008). Preselected diabetic animals with blood glucose levels higher than 250 mg/dl were used in the experiments. Body weight changes of experimental animals occurring during the 10-day treatment period were recorded, thereafter they were fasted overnight for obtaining blood, liver, kidney, and pancreas samples for biochemical and

histological analysis using the methods described later. For the sake of comparison, a group treated with glibenclamide was run in parallel.

3.5.3.3. High fat fed obesity model: Rats were maintained on normal pallet diet (NPD) for one week before the start of the experiment. After one-week acclimatization, they were randomly assigned into normal control and obese groups. The normal control group was further maintained on NPD, whereas obese groups were supplied with high fat diet (HFD) for 15 days. Details of the experimental procedure used for obtaining high fat fed obese rats have been described elsewhere (Husain *et al.*, 2011b; Srinivasan *et al.*, 2005). Rats showing significant weight gain on HFD (as compared to NPD fed normal control animals) were regrouped for treatments with the vehicle or with AP (50, 100 and 200 mg/kg/day), or with atorvastatin (10 mg/kg/day). Daily treatments were given for 10 consecutive days starting on day 16 of the experiment. Body weights were recorded daily, and after the last treatment day all animals were fasted overnight for obtaining blood samples used for biochemical analysis. Average food intake by each group on day 15 and 25 of the experiment were recorded.

3.5.3.4. Fructose fed obesity model: Rats were maintained on normal pellet diet (NPD) and 20 % fructose in drinking water for 15 days according to the procedure described elsewhere (Husain *et al.*, 2011b; Jalal *et al.*, 2007). Rats showing significant weight gain compared to normal rats, were divided into different treatment groups. During the subsequent ten-day treatment period they continued to receive NPD and 20 % fructose in drinking water till day 25. The vehicle treated normal control group was maintained on NPD and normal drinking water throughout the study period. Body weights of the animals were recorded daily and the volume of drinking water consumed by each group on days 15 and 25 of the experiment were noted. Like in other experiments, blood samples for biochemical analysis were obtained after overnight fasting.

3.5.3.5. 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 (Span Diagnostic Ltd., India). Plasma total cholesterol, high-density lipoprotein-cholesterol (HDL-C), and triglycerides were estimated using biochemical enzyme test kits (Span Diagnostic Ltd., India). Low-density lipoprotein-cholesterol (LDL-C) was calculated using Friedewald's equation (Friedewald *et al.*, 1972). Plasma insulin level was estimated using Enzyme-Linked Immunosorbent Assay (ELISA) test kit (DRG Instruments GmbH, Germany). All biochemical analysis were done by using absorbance microplate reader (iMark™- Bio-Rad Laboratories, USA) according to instruction manual of respective enzyme test kit.

(a) Blood glucose: 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 catalysed 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 (Husain *et al.*, 2011c).

(b) Plasma insulin: Plasma insulin was measured by Enzyme Linked Immunosorbent Assay using micro-plate reader (iMark™- Bio-Rad Laboratories, USA) (Husain *et al.*, 2011c).

(c) 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 (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 (Husain *et al.*, 2011b).

(e) 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 dihydroxyacetone 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 (Husain *et al.*, 2011b).

(f) LDL-cholesterol: LDL-cholesterol was calculated using Friedewald's equation (Friedewald *et al.*, 1972).

3.5.3.6. Antioxidant activity: Liver, kidney and pancreas from diabetic rats were washed in ice-cold saline to remove the blood. A part of the tissue was homogenized in ice-cold Tris-HCl buffer (0.025 M; pH 7.4) to yield a 10% (w/v) homogenate. Homogenate was centrifuged at 15000×g for 30 min at 4 °C. Supernatant obtained was used for estimation of LPO, SOD and CAT activity.

(a) Protein estimation: Protein estimation was performed by the method of Lowry *et al.* (1951). Briefly, a standard curve was prepared by using Bovine serum albumin (BSA) powder. 100 µl samples/ standard was placed in respective well, 200 µl of biuret reagent was added to each well and mixed thoroughly with repeated pipeting. The mixture was then allowed to incubate at room temperature for 10-15 min and 20 µl Folin reagents were beaded in each well. Samples were mix immediately with repeated pipetting with each addition colour was allowed to develop for 30 min at room temperature. All absorbance determinations was made using an iMark™, Microplate Reader (BIO-RAD) at 655 nm and blanked on the phosphate buffer (0.1M; pH 8).

(b) Lipid peroxidation activity (LPO): Lipid peroxidation was determined by measuring the level of malondialdehyde (MDA) according to the method of Ohkawa *et al.* (1979). Briefly, 100 microliters tissue homogenate was added to

50 microliters of 8.1% sodium dodecyl sulfate, vortexed and incubated for 10 min at room temperature. Three hundred and seventy five microliters of 20% acetic acid and 375 microliters of thiobarbituric acid (0.6%) were added and placed in boiling water bath in sealed tubes for 60 min. The samples were allowed to cool at room temperature and 1.25 ml of mixture of butanol and pyridine (15:1) was added, vortexed and centrifuged at 2000×g for 5 min. Five hundred micro litres of the coloured pink layer was measured at 532 nm on a spectrophotometer using 1,1,3,3-tetra-methoxypropane as standard. LPO was expressed as nmol MDA/mg protein.

(c) Superoxide dismutase activity (SOD): SOD was measured by method of Kakkar *et al.* (1984). The inhibition of reduction of nitro blue tetrazolium (NBT) to blue coloured Formosan in presence of phenazine methyl sulphate (PMS) and NADH was measured. Briefly, 0.5 ml of tissue homogenate was diluted with 1 ml of water. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 μM PMS, 0.3 ml of 30 μM NBT, 0.2 ml of 780 μM NADH, appropriately diluted enzyme preparation and water in a total volume of 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 chromogen in the butanol layer was measured at 560 nm against *n*-butanol 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/mg protein.

(d) Catalase activity (CAT): CAT was assayed by the method of Luck (1963), wherein the decomposition of H_2O_2 being measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 - phosphate buffer (12.5 mM H_2O_2 solution, pH=7.0) and 0.05 ml of supernatant tissue homogenate (10%) and the changes in absorbance was recorded at 240 nm using Shimadzu UV-1700 spectrophotometer. Enzyme activity was calculated using the mill molar extinction coefficient of H_2O_2 (0.07). CAT activity was expressed as μM of H_2O_2 consumed/min/mg protein.

3.5.3.7. Histological examinations: Tissue slices of liver, kidney, pancreas and spleen obtained from type-2 diabetes animals were fixed in 10% formalin and embedded in paraffin wax. Sections of 5 micron thickness were made using a microtome and stained with haematoxylin-eosin (H&E). For analysis, photographs of each of the slide were taken at 100X magnification under microscope (Nikon E200- Trinocular Microscope, Japan).

3.5.4. Co-morbid Brain Disorders Associated with Type-2 Diabetes

Efficacy of *Andrographis paniculata* extract was evaluated for anti-depressant and anxiolytic like activities in nondiabetic and type-2 diabetic male rodent. *Andrographis paniculata* extract and andrographolide was evaluated for beneficial effects in memory impairment in nondiabetic and diabetic male rats. As described earlier (section **3.5.3.2**), type-2 diabetes was induced in overnight fasted animals by a single intra peritoneal (i.p.) injection of 65 mg/kg streptozotocin (STZ; HiMedia Mumbai, India), 15 min after the i.p. administration of 120 mg/kg nicotinamide (SD Fine-Chemical Ltd., Mumbai, India). Animals were returned to their cages and provided normal food and 10% sucrose water to minimize hypoglycemic shock. The elevated glucose level in the blood confirmed hyperglycemia, quantified after 72 h and 7th day after STZ injections. Only preselected diabetic animals with blood glucose levels higher than 250 mg/dl were used as diabetic animals for the behavioural test.

3.5.4.1. Antidepressant Activity

Following behavioural tests were performed in nondiabetic and diabetic rats after treatments with AP (50, 100 and 200 mg/kg/day, p.o.). Imipramine (15 mg/kg/day, p.o.) was used as standard antidepressant drug.

3.5.4.1.1. Behavioural despair test: The method of Willner (1984) was followed. 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 9 of drugs treatment followed by a 5 min test session on the next day (i.e. on day 10 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.

3.5.4.1.2. Rats learned helplessness test: The experimental procedure of Sherman *et al.* (1982) was used with some modification, and has been described earlier in details elsewhere (Kumar *et al.*, 1999). Briefly, the two parts of the test procedure were:

(a) Inescapable shock pre-treatment: One hour after per-oral treatments on the 7th day, electric foot shocks were delivered to a given rat placed in a 20 x 10 x 10 cm plexiglass chamber with cover, and with a steel grid floor for delivering foot shocks. A constant current shocker delivering 60 scrambled, randomized inescapable shocks (15 sec duration, 0.8 mA, every min) was used.

(b) Conditioned avoidance training: Avoidance training was initiated 24 h after inescapable shock pre-treatment in a jumping box. The jumping box were divided into two equal chambers (27 x 29 x 25 cm) by a plexiglass partition with a gate providing access to the adjacent compartment through a 14 x 17 cm open space in the partition. An individual animal was placed in one of the chambers of the jumping box and was allowed to habituate to the test environment for 5 min (for the first session only) and then was subjected to 30 avoidance trials (inter-trial intervals being 30 sec). During the first 3 sec of each trial, a light signal (conditioned stimulus) was presented, allowing the animals to avoid shocks. If a response did not occur within this period, a 0.8 mA shock (3 sec duration; unconditioned stimulus) was applied via the grid floor. In case no escape response occurred within this period, shock and light conditioned stimulus were terminated. Avoidance sessions performed for three consecutive days (days 8-10), and the number of escape failures (referred as no crossing response during shock delivery) were recorded.

3.5.4.1.3. Blood glucose and insulin estimation: Blood sample from animals was collected by the retro-orbital venous plexus sampling method on day 10 after performing behaviour activity in learned helplessness test. Plasma was prepared by cold centrifugation (5 °C) at 3000 rpm (845xg) for 5 min

(Compufuge CPR-30 Plus, with Rotor No. 8; REMI, India) and stored in a freezer (-20 °C) till used for biochemical estimation. Plasma glucose levels were quantified by using a glucose test kit, based on glucose oxidase-peroxidase (GOD-POD) method (Autospan Glucose test kit; Beacon Diagnostic Pvt. Ltd., Navasari, India). Plasma insulin levels were estimated by using an Enzyme Linked Immunosorbent Assay (ELISA) test kit (DRG Instruments GmbH, Germany). Both glucose and insulin estimations were performed by using absorbance micro-plate reader (iMark™- Bio-Rad Laboratories, California) according to instruction manual of the enzyme test kits used.

3.5.4.1.4. Brain tissues sample: On the 10th day after the learned helplessness test, rats were sacrificed by cerebral dislocation. Brain cortex and hippocampus was dissected out using the protocol described by Spijker (2011). They were weighted and stored in laboratory deep freezer at -80 °C until use. Hippocampus part of brain was used for brain monoamines level and monoamine oxidase enzyme assays, and its frontal cortex was used for assaying antioxidant status.

3.5.4.1.5. Monoamines levels: Monoamine levels in hippocampus were quantified by the spectrofluorometric method described by Welch and 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). 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).

3.5.4.1.6. Monoamine oxidase assay: Hippocampus mitochondrial fraction was prepared for estimating monoamine oxidase (MAO) activity (Schurr and 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.

3.5.4.1.7. 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 Ohkawa *et al.* (1979). For SOD activity, the method described by 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 (Luck, 1963). Protein estimation was performed by the method of Lowry *et al.* (1951).

3.5.4.2. Anxiolytic Activity

Following behavioural tests were performed in nondiabetic and diabetic rats after treatments with AP (50, 100 and 200 mg/kg/day, p.o.). Diazepam (5 mg/kg/day, p.o.) was used as standard anxiolytic drug.

3.5.4.2.1. Social interaction test: The method of File and Hyde (1978) was followed with some modifications. Briefly, the rats were first housed individually for 8 days during drug treatments. The apparatus used for the test was a wooden box (60x60x35 cm) with a solid floor and was placed in a dimly lit room. On day 9, the rats were placed individually in the box and given two 7.5 min familiarization sessions at 2 h interval. On day 10, after 60 min of last drugs treatments, two rats were paired on weight basis and placed in the box for 7.5 min. During this time, total time spent by the rat pair in 'social interaction', including sniffing, following, grooming, kicking, boxing, biting and crawling under or over the partner, was recorded by a blinded observer.

3.5.4.2.2. Light-dark box (LDB) test: The LDB setup consisted of two compartments; one lit compartment (40×50 cm; 350 lux; light box) and one dark compartment (40×30 cm, 70 lux; dark box). The compartments were connected via a small opening (7.5×7.5 cm) enabling transition between the compartments. At the beginning of 5 min test period, each rat was placed in the centre of the black compartment, facing the opening and (a) number of entries into the white compartment (defined as placing both forepaws into the white compartment), (b) time spent in the white compartment and (c) latency until the first entry into the white compartment were assessed by blinded observer (Henniger *et al.*, 2000; Jurek *et al.*, 2012).

3.5.4.2.3. Elevated plus maze test: The method of Pellow and 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 centre 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.

3.5.4.2.4. Blood glucose and insulin estimation: Blood sample from animals was collected by the retro-orbital venous plexus sampling method on day 10 after performing behaviour activity in elevated plus maze test. Plasma was prepared by cold centrifugation (5 °C) at 3000 rpm (845xg) for 5 minute (Compufuge CPR-30 Plus, with Rotor No. 8; REMI, India) and stored in a freezer (-20 °C) till used for biochemical estimation. Plasma glucose levels were quantified by using a glucose test kit, based on glucose oxidase-peroxidase (GOD-POD) method (Autospan Glucose test kit; Beacon Diagnostic Pvt. Ltd., Navasari, India). Plasma insulin levels were estimated by using an Enzyme Linked Immunosorbent Assay (ELISA) test kit (DRG Instruments GmbH, Germany). Both glucose and insulin estimations were performed by using absorbance micro-plate reader (iMark™- Bio-Rad Laboratories, California) according to instruction manual of the enzyme test kits used.

3.5.4.2.5. Brain anti-oxidative status: On the 10th day after the elevated plus maze test, rats were sacrificed by cerebral dislocation. Brain frontal cortex was dissected out using the protocol described by Spijker (2011). They were weighted and stored in laboratory deep freezer at -80 °C until use for assaying antioxidant 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 Ohkawa *et al.* (1979). For SOD activity, the method described by 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 (Luck, 1963). Protein estimation was performed

by the method of Lowry *et al.* (1951). Details of procedures have already described in above sections.

3.5.4.3. Nootropic Activity

Following cognition functions test was performed in nondiabetic and diabetic rats after treatments with AP (50, 100 and 200 mg/kg/day, p.o.) or andrographolide (15, 30 and 60 mg/kg/day, p.o.) for ten consecutive days. Piracetam (100 mg/kg/day, p.o.) was used as standard drug.

3.5.4.3.1. Morris water-maze task: The Morris water maze task was used extensively to investigate spatial learning and memory in rodents (Morris, 1984). The effect of AP and andrographolide on short-term memory was evaluated using the spatial working memory (Davoodi *et al.*, 2009; Ojha *et al.*, 2010). A fabricated metal semi circular pool maze (120 cm in diameter, 45 cm deep) filled with water to a depth of approximately 25 cm. The pool was divided into four equal quadrants and a platform (15 cm in diameter) was submerged 0.5 cm below the opaque surface in the centre of one of the quadrants. The pool was located in a test room and many cues external to the maze were visible from the pool (e.g., pictures, statues etc.), which was used by the rats for spatial orientation. The position of the cues was kept constant throughout the task. Animal from all groups was subjected to habituation and subsequent spatial reference memory of Morris water maze task as described below. AP, andrographolide and piracetam treated rats after habituation period was undergoes for training (days 7-9) and probe trial on day 10. A blinded observer scored escape latency during training and time spent in target quadrant during probe trial.

(a) Habituation: Twenty-four hours prior to the start of training (day 6), all the rats used in the study were habituated to the pool by allowing them to perform a 60 sec swim without the platform.

(b) Spatial reference memory testing: The individual rats from all the groups was placed by hand into the water facing the wall of the pool and was allowed up to 60 sec to find the hidden platform. When successful, the rat was given 20 sec on the platform to watch the spatial cues. If the animal failed to find the platform in

this time, it was guided there. The rat was allowed to stay on the platform for 20 sec. Animals had eight trials per day (separated by 10 min), for three consecutive days (days 7-9) and escape latency was recorded. The animals were returned to their home cages until the retention testing (probe trial) 24 h later on day 10. The probe trial consisted of a 60 sec free swim period without a platform in which the time spent in the target quadrant was recorded.

3.5.4.3.2. Blood glucose and insulin estimation: Blood sample from animals was collected by the retro-orbital venous plexus sampling method on day 10 after performing spatial reference memory (probe trial) testing. Plasma was prepared by cold centrifugation (5 °C) at 3000 rpm (845xg) for 5 min (Compufuge CPR-30 Plus, with Rotor No. 8; REMI, India) and stored in a freezer (-20 °C) till used for biochemical estimation. Plasma glucose levels were quantified by using a glucose test kit, based on glucose oxidase-peroxidase (GOD-POD) method (Autospan Glucose test kit; Beacon Diagnostic Pvt. Ltd., Navasari, India). Plasma insulin levels were estimated by using an Enzyme Linked Immunosorbent Assay (ELISA) test kit (DRG Instruments GmbH, Germany). Both glucose and insulin estimations were performed by using absorbance micro-plate reader (iMark™, Bio-Rad Laboratories, California) according to instruction manual of the enzyme test kits used.

3.5.4.3.3. Brain tissues sample: On the 10th day after performing spatial reference memory (probe trial) testing, rats were sacrificed by cerebral dislocation. Brain cortex and hippocampus was dissected out using the protocol described by Spijker (2011). They were weighted and stored in laboratory deep freezer at -80 °C until use. Frontal cortex was used for assaying antioxidant status. Frontal cortex and hippocampus parts of brain were used for brain acetylcholinesterase enzyme assay.

3.5.4.3.4. Brain anti-oxidative status: The tissue 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 Ohkawa *et al.* (1979). For SOD activity, the method described by 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 (Luck, 1963). Protein estimation was performed by the method of Lowry *et al.* (1951). Details of procedures have already described in above sections.

3.5.4.3.5. Acetylcholinesterase enzyme assay: This study was performed in nondiabetic and diabetic rats after performing probe trial test. The brain AChE activity was measured in 20% of brain homogenate (Prefrontal cortex and Hippocampus) in phosphate buffer (0.1M; pH 8) by the method described by Ellman *et al.* (1961) on the basis of formation of yellow colour due to the reaction of acetylthiocholine with 5,5′-dithiobis-(2-nitrobenzoic acid). The rate of formation of thiocholine from acetylcholine iodide in the presence of brain cholinesterase was measured for 3 min at regular intervals 30 sec using iMark™ Microplate Reader (BioRad, CA, USA) at 415 nm. Protein estimation was performed by the method of Lowry *et al.* (1951).

3.5.5. Other Neuropsychopharmacological Tests

Following neuropsychopharmacological behaviour tests were performed in nondiabetic and diabetic male animals after treatments with AP (50, 100 and 200 mg/kg/day, p.o.) or andrographolide (15, 30 and 60 mg/kg/day, p.o.) for ten consecutive days. As described earlier (section **3.5.3.2.**), type-2 diabetes was induced in overnight fasted animals. Only preselected diabetic animals with blood glucose levels higher than 250 mg/dl were used as diabetic animals.

3.5.5.1. Spontaneous locomotor activity: The spontaneous locomotor activity in photoactometer (Techno Electronics, India) was assessed in rats by method described by Ramanathan *et al.* (1999). The last administration of AP (50, 100 and 200 mg/kg, p.o.), andrographolide (15, 30 and 60 mg/kg/day, p.o.), or

lorazepam (1 mg/kg, p.o.) was done on day 10th, 60 min prior to spontaneous locomotor activity test. 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. Interruptions of photocell beams (locomotor activity) were recorded by means of a 6 digits resettable counter.

3.5.5.2. 5-Hydroxytryptophan (5-HTP) head twitches test in mice: The method described by Corne *et al.* (1963) was followed with some modifications. The last administration of AP (50, 100 and 200 mg/kg, p.o.), andrographolide (15, 30 and 60 mg/kg/day, p.o.), or imipramine (15 mg/kg, p.o.) was done on day 10th, 60 min prior to injection of 5-HTP (100 mg/kg, i.p.). After 5-HTP injection, number of head twitches displayed by each mouse mice were observed for 2 min at every 20 min intervals up to 1 h and averaged. The head twitch response was characterized by abrupt lateral movements, which may or may not be accompanied by body twitches and hind limb retractions.

3.5.5.3. L-Dopa potentiation test in mice: The technique used was described by Everett (1966) with some modifications. In brief, the last administration of AP (50, 100 and 200 mg/kg, p.o.), andrographolide (15, 30 and 60 mg/kg/day, p.o.), or imipramine (15 mg/kg, p.o.) was done on day 10th, 30 min prior to injection of L-Dopa (100 mg/kg, i.p.). The behaviour of the animals was rated 30, 45 and 60 min after the administration of L-dopa and averaged. The rating scale was from 0 (no any mice show hyper activity) to 6 (all six mice show hyperactive and/or jumping).

3.5.5.4. Apomorphine-induced cage-climbing test in mice: This test was done by method described by Protais *et al.* (1976) with some modifications. Apomorphine (0.50 mg/kg, s.c.) was administered in mice after 60 min of the last administration of AP (50, 100 and 200 mg/kg, p.o.), andrographolide (15, 30 and 60 mg/kg/day, p.o.), or imipramine (15 mg/kg, p.o.) on day 10th. Immediately after injection, a mouse was put into cylindrical individual cage, 12 cm diameter, and 14 cm high, with walls of vertical metal bars, 2 mm diameter and 1 cm apart, surmounted by a smooth surface. After 5 min period of exploratory behaviour, three consecutive observations were performed on each animal by a blinded

observer at 10, 20 and 30 min after apomorphine injection and these three scores were averaged. This behaviour test was scored as follows: 0= four paws on the floor, 1= forefeet holding the wall and 2= four paws holding the wall.

3.5.6. Anti-inflammatory and Analgesic Activity

Following methods were used for evaluation of anti-inflammatory and analgesic activity of AP (100, 200 and 400 mg/kg/day, p.o.) and andrographolide (30, 60 and 120 mg/kg/day, p.o.) for ten consecutive days in nondiabetic and diabetic male rodents. Indomethacin (5 mg/kg, p.o) and pentazocine (30 mg/kg, p.o) was used as standard anti-inflammatory and analgesic drug respectively. As described earlier (section **3.5.3.2.**), type-2 diabetes was induced in overnight fasted animals. Only preselected diabetic animals with blood glucose levels higher than 250 mg/dl were used as diabetic animals.

3.5.6.1. Carrageenan-induced pedal oedema in rats: Rats were injected with 0.1 ml of a 1% carrageenan solution in saline into the sub-plantar region of the left hind paw 60 min after the drug administration on day 1 and on day 10 (Winter and Porter, 1957). The paw was marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume was measured before and 1, 2, 3, 4 and 6 h after the injection of carrageenan by the mercury displacement method in plethysmography. The oedema was calculated by subtracting the initial hind paw volume of each rat.

3.5.6.2. Cotton pellet-induced granuloma in rats: Subacute inflammation was produced by cotton pellet induced granuloma in rats (Winter and Porter, 1957). Sterile cotton (50 ± 1 mg) soaked in 0.2 ml of distilled water containing penicillin (0.1 mg) and streptomycin (0.13 mg) was implanted subcutaneously bilaterally in axilla under the ether anaesthesia. Diabetic animals were hyperglyemic at the time of cotton implantation. First treatment was started on after confirmation of diabetes (7th day of STZ injection) for ten consecutive days. Cotton pellets were implanted on day 3rd of treatment and animals were sacrificed on the 10th day of treatment. The granulation tissue with cotton pellet was dried at 60°C overnight and then dry weight was taken. The weight of the cotton pellet before implantation was subtracted from the weight of the dried, dissected pellets.

3.5.6.3. Tail flick latent period: The technique used was described by Davies *et al.* (1946), using an analgesiometer (Techno, India). After 60 min of the drug administration on day 1 and on day 10, the rat was placed in a rat holder, with its tail coming out through a slot in the lid. The tail was kept on the bridge of the analgesiometer, called jacket with an electrically heated nichrome wire underneath. The tail received radiant heat from the wire, heated by passing current of 6 mA. Through the water jacket, cold water was continuously passed, so that the bridge did not get heated and tail could be conveniently placed over the bridge. The time taken for the withdrawal of the tail after switching on the current, was taken as the latent period, in sec, of 'tail flicking' response. This latent period was considered as the index of nociception. The cutoff time for determination of latent period was taken as 30 sec to avoid injury to the skin (Bhattacharya *et al.*, 1971). Three tail flick latencies were measured per rat at each time interval and the means of the tail-flick latencies was used for statistical analysis.

3.5.6.4. Hot plate reaction time in mice: 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 (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 1 and on day 10.

3.5.6.5. Formalin test in rats: The efficacy for central analgesic activity in nondiabetic and diabetic animal as described previously (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. Analgesic response or protection was indicated if both paws were resting on the floor with no obvious favouring of the injected paw.

3.5.7. Anti-stress Activity

Following tests were performed in stressed male rats after treatments with AP (50, 100 and 200 mg/kg/day, p.o.) or andrographolide (30 and 60 mg/kg/day, p.o.) for 21 consecutive days. Standardised *Withania somnifera* root extract (100 mg/kg/day, p.o.) containing 2.6% withanolides was used as standard anti-stress drug.

3.5.7.1. Chronic foot-shock stress: Stress procedure used was similar to that used in an earlier study comparing the efficacy of a *Withania somnifera* with another traditionally known medicinal herb *Panax ginseng* (Bhattacharya and Muruganandam, 2003). In short, each individual rat of the stress groups were subjected to 1-h daily foot-shocks through a grid floor in a standard conditioning chamber with the escape route closed. The duration of each unpredictable foot-shock (2 mA) and the intervals between the shocks were randomly programmed between 3 and 5 s, and 10 and 110 sec respectively.

3.5.7.2. Organ weights, gastric ulcers and plasma corticosterone levels: One hour after the last stress session on day 21, blood samples were collected from retro-orbital plexus for plasma corticosterone assay. Thereafter, the animals were sacrificed and their stomach was removed and cut along the greater curvature, and their adrenal glands and spleen were removed and weighed. The numbers of discrete ulcers in the stomach were counted with the help of a magnifying glass. The severities of ulcers were scored after adding up individual scores (Bhargava and Singh, 1981). Plasma corticosterone levels were estimated using an Enzyme Immunoassay (EIA) kit (Enzo Life Sciences; PA, USA) according to the manufacturer's instructions.

3.5.7.3. Methods used to assess stress-induced perturbations: The following methods were used to assess behavioural depression.

(a) Learned helplessness test: On day 19 of the experiment the rats were subjected to learned helpless situation, and on day 21, i.e., 48 h later, they were

subjected to avoidance test as method described by Thiebot *et al.* (1992) using in a two compartment jumping box (Techno Electronics, Lucknow, India). Failure to escape during unconditioned stimulus was assessed as 'escape' failure.

(b) Sexual behaviour: On day 21, a male rat of an experimental group was placed in a cage in a dimly lighted room for 10 min with 2 oestrinised female rats (sequentially treated with oestradiol valerate 5 μg/rat, followed 48 h later by hydroxyprogesterone 1.5 mg/rat, s.c.). The total numbers of mounts by the male rat were counted (Morishita *et al.*, 1993).

(c) Behavioural despair test: On day 20, each rat was forced to swim individually for 15 min in a cylinder (height: 45; diameter: 20 cm) filled with water (25 ± 2 °C) up to 38 cm. Thereafter they were dried and returned to their home cages. On day 21, they were forced to swim again for 5-min, and the total period of immobility during the 5-min of the swimming period was recorded (Porsolt *et al.*, 1978).

3.5.7.4. Cytokines expression in blood and brain: Blood and brain samples were obtained from rats used in the behavioural despair test. Blood samples were taken from the retro-orbital plexus and frontal cortex of the brain was dissected out after sacrificing the animals. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) were used to assess the expression of cytokines (TNF-α, IL-1β and IL-10).

(a) RNA isolation: RNA was isolated from blood and brain frontal cortex using TRI Reagent™ (Sigma-Aldrich, USA) according to manufacturer's instructions (Singh *et al.*, 2012, 2013). Briefly, rat whole blood (1 ml) was mixed with 4 ml of RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) and incubated at room temperature for 10 min to lyse the red blood cells (RBCs). The remaining WBCs were washed with phosphate buffer saline (PBS) and lysed in TRI Reagent™ (Sigma-Aldrich, USA). In the case of brain, tissue was chopped and lysed with TRI Reagent™. The quality of RNA was assessed by running the RNA samples on 1% denaturating formaldehyde-agarose gel. The RNA was quantified using Nano Drop Spectrophotometer (Thermo Scientific, USA) and equal amount of total RNA from each rat was pooled for each treatment group.

(b) *cDNA preparation:* In order to avoid amplification of contaminating genomic DNA, total RNA was treated with RNase-free DNase (New England Biolabs, USA) and reverse transcribed by using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). DNase treated RNA (2μg) was reverse transcribed by using a high capacity cDNA reverse transcription kit (Applied biosystems, USA). Briefly, 10 μl of nuclease free water containing 2 μg of RNA was added to 10 μl of RT mix containing 2 μl of RT random primer (10X), 0.8 μl of dNTPs mix (100mM), 2 μl of RT buffer (10X), 1 μl of MultiScribe™ Reverse Transcriptase (50 U/μl) and 1 μl of human placental ribonuclease inhibitor (10 U/μl). The samples were then incubated at 25 °C for 10 min followed by incubation at 37 °C for 2 h. The reverse transcriptase was then inactivated by heating the reaction mixture at 85 °C for 5 min.

(c) Polymerase chain reaction (PCR): All mRNA sequences were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). The gene specific primers were designed using primer 3 software (http://fokker.wi.mit.edu/primer3) and synthesized at Integrated DNA Technologies, Inc, USA **(Table 3.1)**. The PCR was carried out in a thermal cycler (Applied Biosystems, USA) in a 15 μl final volume containing 0.5 μl of cDNA, 1.5 μ1of PCR buffer (10X), 0.15 μl of Taq polymerase (5 U/μl), 0.9 μl of $MgCl_2$ (25 mM), 0.3 μl of each forward and reverse primer (10 pM). After an initial denaturation step at 94 °C for 5 min, temperature cycling was initiated. Each cycle consisted of denaturation step at 94 °C for 30 sec., annealing at primer specific temperatures **(Table 3.1)** for 30 sec and extension at 72 °C for 30 sec. After 35 cycles, a final extension at 72 °C for 7 min was done. PCR products were separated on 2% agarose gel and visualized using ethidium bromide staining. The density of each band was measured using densitometry software provided with AlphaImager® gel documentation system (Alpha-Imager, India). Amplifications were carried out in triplicate and the relative expression of cytokine mRNA genes was determined using β-actin housekeeping gene expression as an internal control.

3.5.8. Molecular Study using HL-60 Cell-line

3.5.8.1. In-vitro cell toxicity study: Human monocytic cell line HL-60, at a density of 5 x 10^4 cells/well in a 96-well plate was used to assess the effect of AP (12, 36, 60, 120 and 360 µg/ml) and andrographolide (10, 30, 50, 100, 300 µM) on cell viability by MTT assay (van Meerloo *et al.*, 2011). The viability of HL-60 cells were measured in terms of mitochondrial metabolic activity using a colourimetric 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (water-soluble tetrazolium salt-1)- based cell proliferation assay (Millipore, USA) according to the manufacturers' instructions. Briefly, after 24 h treatment WST-1 was added to the cells and incubated for 4 h under standard culture conditions. Absorbance was measured at 450 nm using iMark™ Microplate Reader (BioRad, USA).

3.5.8.2. Cytokines and toll like receptors (TLRs) expression in HL-60 cell line: HL-60 cell line was used to assess the effect of andrographolide (10 µM) on expression of cytokines and toll like receptors. The HL-60 cells were cultured at a concentration of 1x10^6 cells/well/ml of RPMI media in a 24 well culture plate. Cytokines (TNF-α, IL-1β and IL-10) and toll like receptors (TLRs) viz., TLR3, TLR7 and TLR8 expression (Applequist *et al.*, 2002) were determination using RT-PCR method as per manufacturers' instructions.

3.5.9. 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.

Table 3.1: Primers for RT-PCR

Gene	Primers sequence	Product size (bp)	Annealing temperature (°C)
TNF-α	Sense: 5`-TCTCAAAACTCGAGTGACAAGC-3` Antisense: 5`-GGTTGTCTTTGAGATCCATGC-3`	127	60
IL-10	Sense: 5`-GAGAGAAGCTGAAGACCCTCTG-3` Antisense: 5`-TCATTCATGGCCTTGTAGACAC-3`	142	54
IL-1β	Sense: 5`-AAATGCCTCGTGCTGTCTGACC-3` Antisense: 5`-CTGCTTGAGAGGTGCTGATGTACC-3`	337	64
β-actin	Sense: 5`-TCTACAATGAGCTGCGTGTG-3` Antisense: 5`-AATGTCACGCACGATTTCCC-3`	115	52