MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 – Plant material

The fresh roots of *Momordica cymbalaria* (MC) were collected from Gadag district, Karnataka, identified and authenticated by Dr.Sreenath, Department of Botany, Bangalore University, Bangalore. A specimen sample of the same was preserved in the herbarium of the Department of Botany, Bangalore University, Bangalore, with the voucher no. 18122003 for future reference.

The fresh fruits of *Momordica dioica* (MD) were collected from Shimoga district, Karnataka, identified and authenticated by Dr. Gajendra Rao, Survey Officer, Regional Research Institute, Bangalore. A specimen sample of the same was preserved in the herbarium section at RRI, Bangalore, as RRCBI, Acc No.1693 for future reference.

3.1.2 – Preparation of plant extract

Ethanolic extract of MC: The roots of *Momordica cymbalaria* were isolated, chopped into small pieces and dried under shade at room temperature for seven days. The dried roots were powdered and passed through the sieve (coarse 10/44). The dried, powered roots of Momordica *cymbalaria* were extracted with 95% v/v ethanol for 25 cycles using soxhlet extractor. The combined extracts were concentrated at 40° C to obtain dark brownish yellow residue. The yield obtained from the above process was found to be 21.5 % w/w. The phytochemical test indicated the presence of alkaloids, flavonoids, saponins, phytosterols and glycosides (Khandelwal, 2003) (Table19.1).

Ethanolic extract o f MD: The fruits of *Momordica dioica,* Roxb were, chopped into small pieces and dried under shade at room temperature. The dried fruits were powdered and passed through the sieve (coarse10/40). The dried powdered fruits of *Momordica dioica,* Roxb were extracted with 95%v/v ethanol using soxhlet extractor. The combined extracts were concentrated at 40°C to obtain dark brownish yellow residue. The yield obtained from the above process was found

to be 14% w/w. The phytochemical test indicated the presence of alkaloids, flavonoids, saponins, phytosterols and glycosides (Khandelwal, 2003) (Table19.1).

Saponin fraction of MC: Methanolic extract of roots *Momordica cymbalaria* was dissolved in hot distilled water and partitioned between water saturated n-butanol and water layer, organic layer (n-butanolic layer) was separated and evaporated to get residue. This n-butanolic residue was dissolved in methanol and poured ET_2O to obtain flocculent precipitate. This precipitate was separated by means of filter paper and washed with excess of ET_2O and dried to yield crude fraction of saponins(1).The phytochemical test indicate the presence of saponins-steroidal glycosides(Table19.1).

Saponin fraction of MD: Methanolic extract of fruits of Momordica dioica was dissolved in hot distilled water and partitioned between water saturated n-butanol and water layer, organic layer (n-butanolic layer) was separated and evaporated to get residue. This n-butanolic residue was dissolved in methanol and poured ET_2O to obtain flocculent precipitate. This precipitate was separated by means of filter paper and washed with excess of ET_2O and dried to yield crude fraction of saponins.The phytochemical test indicate the presence of saponins-steroidal glycosides (Table19.1).

3.1.3 – Drugs and Chemicals

i. Streptozotocin, Histamine (Sigma Labs Ltd. U.S.A)
ii. Metformin, Silymarin, Lovastatin and Gemfibrozil (Micro labs); Insulin (Torrent Pharmaceuticals Ltd).
iii. Standard kits for Glucose, Cholesterol, Triglycerides, HDL, Creatinine, AST, ALT, ALP estimation were obtained from Span Diagnostics Ltd. Surat. Standard reagents like Anthrone reagent, Nitro blue tetrazolium, Ellman's reagent were obtained from ICN chemicals Ltd. Serum insulin was estimated using the ADVIA Centaur (IRI) and Ready Pack, of Bayer of corporation, Mumbai.
iv. Cholesterol (LOBA, Mumbai); Thiobarbituric acid, Triton WR 1339,reduced glutathione,(Himedia, Mumbai); Indometacin (IPCA, Mumbai) Sodium arsenate, Trichloro acetic acid (NICE chemicals Pvt Ltd), Ferric

chloride(ROLEX chemical industries); Formalin, Hydrogen peroxide, Methanol(S. D. Fine Chemicals, Mumbai), Ethanol(Govt of Karnataka).

v. Sodium hydroxide, sodium carbonate, sodium bicarbonate, magnesium, chloride, sodium chloride, potassium chloride, calcium chloride, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, carbon tetrachloride, chloroform, ether, hydrochloric acid and con sulphuric acid(S. D. Fine Chemicals, Mumbai).

All the chemicals and reagents used in the entire study were of analytical grade.

3.1.4 - Instruments:

Shimadzu double beam UV –visible spectrophotometer

UGO BASIL two channels recorder

BIPOAC DATA acquisition system

LE-5002, LETICA Blood pressure recorder

Isolated organ bath system aerated with carbogen gas with temperature controlled circulation water bath

SIGMA cooling centrifuge

Tissue homogeniser

Heating water bath

Analytical balances

3.1.5 – Animals

Albino Wister rats of either sex weighing 150 to 250 gms were housed seperately in groups of two in an ambient temperature of 25±1°C. White New Zealand rabbits of either sex weighing 1.2 to 1.5 kgs were housed individually in an ambient temperature of 25±1°C.Animals had free access to food (Amrut rat and mice feed, Pranav agro industries Ltd. Sangli, India) and water. Animals were deprived of all food but not water 4 hours before all acute experiments. All the animals were acclimatized for 10 days at normal laboratory condition before starting the experiment. The experiments were carried out during the light period (08.00-16.00 h).The institutional animal ethics committee approved the protocol of all the animal studies.

3.2 Methods:

The following experiments were conducted on the crude extracts of MC and MD and their respective saponins fractions.

3.2.1 Experimental Design

3.2.1.1 Acute Oral Toxiciry Test

Effect on Metabolic disorders

3.2.1.2 Antidiabetic activity in streptozotocin induced Type 1 diabetes

3.2.1.3 Insulin sensitizing activity in fructose diet induced hyperinsulinemia

3.2.1.4 Antihyperlipidemic activity in atherogenic diet fed rats

3.2.1.5 Antihyperlipidemic activity in high cholesterol diet fed rabbits

Effect on cardiovascular disorders

3.2.1.6 Acute antihypertensive activity in normotensive rats

3.2.1.7 Chronic antihypertensive activity in high fructose diet induced hypertensive rats by non invasive (tail cuff) method.

3.2.1.8 Cardioprotective activity in isoproterenol induced MI in rats

Effect on isolated Tissues

3.2.1.9 In vitro effect on rat's aortic strip

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Effects on Antioxidant parameters in various pathological models

3.2.1.11 Hepatic antioxidant parameters in Carbon tetrachloride induced hepatic injury in rats

3.2.1.12. Cardiac antioxidant parameters in streptizotocin induced Type 1 diabetes

3.2.1.13. Cardiac antioxidant parameters in Isiproterenol induced myocardial infarction in rats

Effect on Reproductive parameters

3.2.1.14. Antifertility activity in rats

3.2.1.15 Antiovulatory activity in rats

3.2.1.16 Abortifacient activity in rats

3.2.1.17. Effect of male reproductive system in rats

Phytochemical investigations

3.2.1.18. Phytochemical Investigation

3.2.1.19 HPTLC Fingerprinting

Oral Toxicity Test

3.1.1 Acute Oral Toxicity Test

The acute oral toxicity test was performed according to the OPPTS (Office of Prevention, Pesticides and Toxic Substances) guidelines following the Up and Down procedures.The rats were acclimatized for 5 days and fasted overnight, food but not water was withheld.Animals were weighed;limit and main tests were performed.The limit test for MD or MC or saponins fractions of MC or MD was first carried out at 5000mg/kg for one animal and if animal did not survive,main test was performed. If the animal survived two or more animals were dosed; if both the animals survived the test was terminated and $1/10^{th}$ of 5000mg/kg waas taken as the dose for experiments. The main test was performed with an initial dose of 175mg/kg.The following sequence was followed: 175, 550, 1750, and 5000mg/kg.First one animal was dosed with 175mg/kg. If animal died a much lower dose was tested .If animal survived, then two or more animals were dosed, after 48 hours observation of the first animal.But if survived,then the main test was terminated and the $1/10^{th}$ of that dose was taken as the dose for experiment(OPPTS, 2002)

Effect on Metabolic Disorders

3.1.2 Antidiabetic activity in streptozotocin induced Type 1 diabetes

Streptozotocin was dissolved in 0.01 M cold sodium citrate buffer (pH 4.5) immediately before use. Diabetes was induced in 16 h fasted male Wistar rats (100-125g) by intraperitoneal injection of streptozotocin (65 mg/kg). Streptozotocin was dissolved in 0.01 M cold sodium citrate buffer (pH 4.5) immediately before use. The rats were then given 5% w/v glucose solution in feeding bottles for the next 24 h to prevent hypoglycemia. After 72 h, rats with marked hyperglycemic fasting blood glucose> 200 mg/dL were selected and used for the study. All the animals were allowed free access to tap water and pellet diet and maintained at room temperature in polyethylene cages.

The rats were divided into five groups consisting of six rats each.

Group 1: Vehicle (Sodium Citrate Buffer- Control).

Group 2: Streptozotocin (65mg/kg i.p.) STZ diabetic control

Group 3: Diabetic rats treated with reference standard, Insulin (6U/kg,,s.c./day) for 30 days

Group 4: Diabetic rats treated with MC (250mg/kg p.o/day/30days)

Group5: Diabetic rats treated with MC (500mg/kg p.o/day/30days)

Group6: Diabetic rats treated with MD (250mg/kg p.o/day/30days)

Group7: Diabetic rats treated with MD (500mg/kg p.o/day/30days)

Group8: Diabetic rats treated with saponin fractions of MC (87.5mg/kg p.o/day/30days)

Group9: Diabetic rats treated with saponin fractions of MC (175mg/kg p.o/day/30days)

Group10: Diabetic rats treated with saponin fractions of MD (27.5mg/kg p.o/day/30days)

Group11: Diabetic rats treated with saponin fractions of MD (55mg/kg p.o/day/30days)

Blood was collected on the 31st day by retro orbital bleeding and serum was analyzed for glucose, triglycerides, cholesterol, HDL-cholesterol, Creatinine, BUN (blood urea nitrogen), Insulin. The liver was analyzed for glycogen, mavalonate and HMG CoA (Please see page No. 107 for the details of test)

3.2.1.2 Insulin sensitizing activity in fructose rich diet (FRD) induced hyperinsulinemia

Male Wistar rats weighing 150-200gm were used in the study. The rats were randomly divided into groups.

Group 1: Rats were fed with standard rat chow (Control).

Group 2: Rats received fructose rich diet (FRD control).

Group 3: FRD + MC (250mg/kg p.o/day/15days)

Group 4: FRD + MC (500mg/kg p.o/day/15days)

Group 5: FRD + MD (250mg/kg p.o/day/15days)

Group 6: FRD + MD (500mg/kg p.o/day/15days)

Group 7: FRD + Saponin fractions of MC (87.5mg/kg p.o/day / 15days)

Group 8: FRD + Saponin fractions of MC (175mg/kg p.o/day/15days)

Group 9: FRD + Saponin fractions of MD (27.5mg/kg p.o/day / 15days)

Group 10: FRD + Saponin fractions of MD (55mg/kg p.o/day / 15days)

The FRD was prepared by using 66% fructose, 12% fat, & 22% protein (casein) (Beck-Nielsen H et al 1980). After 15 days of the start of the FRD, the extract was administered orally on a fixed time interval daily for remaining 15 days. Blood was

collected on the 31st day by retro orbital bleeding, and serum was analyzed for glucose, triglycerides, cholesterol, HDL-cholesterol Insulin and HMG CoA reductase.

3.2.1.4 Antihyperlipidemic activity in atherogenic diet (AD) fed rats

Male Wistar rats weighing 150-200gm were used in the study. The rats were randomly divided into groups of six each.

Group1: Rats were fed with standard rat chow (control).

Group 2: Rats received atherogenic diet for 26 days (AD control)

Group 3: AD + Lovastatin (6mg/kg, p.o/day/26days)

Group 4: AD + MC (250mg/kg, p.o/day/26days)

Group 5: AD + MC (500mg/kg, p.o/day/26days)

Group 6: AD + MD (250mg/kg, p.o/day/26days)

Group 7: AD + MD (500mg/kg, p.o/day/26days)

Group 8: MC (250mg/kg, p.o/day/26days)

Group 9: MC (500mg/kg, p.o/day/26days)

Group 10: MD (250mg/kg, p.o/day/26days)

Group 11: MD (500mg/kg, p.o/day/26days)

Atherogenic diet was prepared by adding coconut oil 25%contained cholesterol 1% and Cholic acid 0.5%. The atherogenic diet and the treatment were given simultaneously for 26 days. On the 27^{th} day, blood was collected by retro orbital bleeding and serum was analysed for triglyceride, cholesterol, HDL, LDL, VLDL and glucose. A portion of the aorta was fixed in formalin (10%) and subjected to histopathology studies. (Please refer page No.108 for details)

3.2.1.5 Antihyperlipidemic activity in High Cholesterol Diet (HCD) fed rabbits

White New Zealand rabbits weighing 1.5-1.8 kg were randomly divided into eight groups of six each and kept in wire mashed cages for 5 days prior dosing to allow for acclimatization to the laboratory conditions. Out of eight groups, four groups were selected randomly to induce hyperlipidemia produced by feeding hypercholesteremic diet once a day for 35 days. Hypercholesterolemia diet (HCD) contained 1% cholesterol, in 5% coconut oil and 94.5% standard laboratory diet (Brown TM et al., 1993).

The animals were divided into groups of six animals each.

Group 1: Rabbits fed with standard rabbit chow daily for 70 days (Control).

Group 2: Rabbits fed with HCD daily for 35 days and standard rabbit chow from day 36 onwards till 70th day (HCD Control).

Group 3: Rabbits fed with HCD for 35 days and saponin fraction of MC (175mg/kg p.o.) from day 36 to day 70 along with standard rabbit chow .

Group 4: Rabbits fed with HCD for 35 days and saponin fraction of MD (55mg/kg p.o.) from day 36 to day 70 along with standard rabbit chow .

Group 5: Rabbits fed with HCD for 35 days and Lovastatin (6 mg/kg/day. p.o.) from day 36 to day 70 along with standard rabbit chow.

Group 6: Rabbits fed with standard rabbit chow for 35 days and Lovastatin (6 mg/kg/day. p.o.) from day 36 to day 70 along with standard rabbit chow.

Group 7: Rabbits fed with standard rabbit chow for 35 days and saponin fraction of MC (175mg/kg p.o) from day 36 to day 70 along with standard rabbit chow.

Group 8: Rabbits fed with standard rabbit chow for 35 days and saponin fraction of MD (55mg/kg p.o) from day 36 to day 70 along with standard rabbit chow.

On the 71st day, blood was collected from the central ear artery by No. 21 pediatric needle after an over night fasting for the analysis of serum triglyceride, cholesterol, HDL, LDL and VLDL.Animals were sacrificed and liver was analysed for Hepatic HMG-CoA and mavalonate. A portion of the thoracic aorta was fixed in formalin (10%) and subjected to histopathology studies.

Effect on cardiovascular disorders

3.2.1.6 Acute antihypertensive activity in normotensive rats by invasive method

Rats were anaesthetized with urethane (120mg/100gm) in group of 5 each. Femoral vein was canulated with a fine polythene catheter for administration of drugs. Blood pressure was recorded from left common carotid artery using pressure transducer by direct method on BIOPAC Data Acquisition system (BIOPAC MP 30 System U.S.A.) or two channels UGO Basile blood pressure recorder. After 30 minutes of stabilization, the change in blood pressure was recorded to the following drugs before and after the administration of MC or MD (10mg/kg, i.v)

i. Adr(1µg/kg)
ii. NA(1µg/kg)
iii. PE(1µg/kg)

iv. ANGII

v. Iso

vi. Ach

vii. 5-HT

viii. Histamine

3.2.1.7 Antihypertensive activity on Fructose Rich Diet (FRD) induced hypertensive rats by non invasive (tail cuff) method.

Male Wistar rats weighing 200-250gm were used in the study. The rats were randomly divided into six groups of six animals each

Group 1: Rats were fed with standard rat chow (Control)

Group 2: FRD for 5 weeks (FRD Control)

Group 3: Standard rat chow + MC (500mg/kg, p.o/day/5weeks)

Group 4: Standard rat chow + MD (500mg/kg, p.o/day/5weeks)

Group 5: FRD + MC (500mg/kg, p.o/day/5weeks)

Group 6: FRD + MD (500mg/kg, p.o/day/5weeks)

The FRD(Beck-Nelson H et al.,1980) contained 66% fructose, 12% fat, & 22% protein.To measure the arterial blood pressure using tail cuff method, rats were trained for atleast one week until the blood pressure was steadily recorded with minimal stress and restrain. The first cardiovascular parameters were discarded and the mean of five or six subsequent measurements were recorded. Cardiovascular parameters – systolic, and heart rate were measured weekly for five weeks by indirect noninvasive tail cuff method using Letica 5002 storage pressure meter (Vogal HG 1997).

3.2.1.8 Cardiopretective activity in Isoproterenol (ISO) induced myocardial infarction in rats

The rats were divided into six groups.

Group 1: Distilled water (10ml/kg p.o,) (control)

Group 2: ISO (60mg/kg, s.c.) at an interval of 24 hours for two days (ISO Control)

Group 3: MC (250 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Group 4: MC (500 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Group 5: MD (250 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Group 6: MD (500 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Twelve hours after the second injection of ISO, the animals were sacrificed by cervical decapitation, blood was collected and the heart was dissected out. The serum

was separated immediately by cold centrifugation and used for determination of myocardial infarction marker enzymes such as lactate dehydrogenase (LDH), creatinine kinase-MB fraction (CK-MB), aspartate transaminase (AST), alanine transaminase (ALT) alkaline phosphatases (ALP) along with serum uric acid, total cholesterol, triglycerides, LDL, and HDL.Heart was fixed in formalin (10%) and subjected to histopathological studies.

Effect on isolated tissues

3.2.1.9 In vitro effect of MC and MD on rat's aortic strip (Maria et al., 2001)

The rats were sacrificed by cervical dislocation; the thoracic aorta was isolated and cleaned from extraneous tissues, maintaining the tissue wet in the Krebs's solution. Two helical strips (20mm X 3mm) were cut from the aorta beginning from the end almost proximal to the heart. The endothelium was removed by rubbing with filter paper. Vascular strips were then tied with surgical threads and suspended in a jacketed tissue bath (25ml capacity) containing Krebs's medium gassed with carbogen ($5\%CO_2$ – 95% O_2).The isotonic contractions were measured using an isotonic transducer (UGO Basile 7006), connected to a "Two channel recorder Gemini" (UGO Basile 7070).After at least an hour equilibration period under an optimal tension of 2 g, the experimental procedure was carried out .The bath solution was replaced every 10 minutes.

After equilibrium period cumulative NA concentration response curve was recorded, the first one was discarded and the second one was taken as control. The tissue was allowed to equilibrate with MC or MD for 30 minutes before the generation of the third concentration response curve to NA. The concentration of the agonist was increased 2 or 3 fold at each step, with each addition made only after the response to the previous addition had attained maximal levels and remained steady. Finally the fourth CRR curve to NA was taken after 30 min repeated washings with the Krebs medium to assess for reversibility of the antagonist.NA solution contained 0.05% EDTA in 0.9% NaCl to prevent oxidation.

3.2.1.6 In vitro effect of MC and MD on rat anococcygeus muscle

A male rat was killed by blow in the head. The abdomen was opened in the mid line, the pelvis split, and the bladder and urethra removed taking care not to

damage the ventral band of muscle lying ventral to the colon. The colon was then cut through at the pelvis brim. The pelvic portion pulled forward and the connective tissues cleaned until the anococcygeus muscle comes in to the view. The ventral band was cut through and each muscle was mounted in a jacketed tissue bath (25ml capacity) containing Krebs's medium gassed with carbogen (5%CO_2 – 95% O_2).The isotonic contractions were measured using an isotonic transducer (UGO Basile 7006), connected to a "Two channel recorder Gemini" (UGO Basile 7070).After at least an hour equilibration period under an optimal tension of 1 g, the experimental procedure was carried out.The bath solution was replaced every 10 minutes.

After equilibrium period cumulative NA concentration response curve was recorded, the first one was discarded and the second one was taken as control. The tissue was allowed to equilibrate with the antagonist for 30 minutes before the generation of the third concentration response curve to NA. The concentration of the agonist was increased 3 fold at each step, with each addition made only after the response to the previous addition had attained maximal levels and remained steady. Finally the fourth CRR curve to NA was taken after a 30 min repeated washings with the Krebs medium to assess for reversibility of MC or MD.

Effect on Antioxidant parameters in various pathological models

3.2.1.11 Hapatoprotective activity in Carbon tetrachloride induced hepatic injury in rats (Recknagel RO et al., 1989)

Hepatopathy was induced in animals by administration of CCl_4 (1.25ml/kg i.p) on alternative day in liquid paraffin for 14 days. The rats were divided into 15 groups each of six animals.

Group 1: Liquid paraffin (1.0ml/kg i.p) (Control).

Group 2: CCl_4 (1.25 ml/kg, i.p/alt days/14days) (CCl_4 control)

Group 3: CCl_4+ MC (250 mg/kg p.o/day/14days);

Group IV: CCl_4 + MC (500mg/kg *p.o*/day/14days);

Group V: CCl_4+ MD (250 mg/kg *p.o*/day/14days);

Group VI: CCl_4+ MD (500 mg/kg *p.* /day/14days *o*);

Group V: CCl_4+ silymarin (100 mg/kg, *p.o*/day/14days)

At the end of the treatment, rats were sacrificed by cervical dislocation. Liver was dissected out and immediately washed immediately with ice-cold saline and a

homogenate was prepared in 0.1 N Tris HCl buffer (pH 7.4) centrifuged and analyzed for LPO, GSH, CAT and SOD.

3.2.1.12. Cardiac antioxidant parameters in Steptozotocin induced disbetic rats

Streptozotocin was dissolved in 0.01 M cold sodium citrate buffer (pH 4.5) immediately before use. Diabetes was induced in 16 h fasted male Wistar rats (100-125g) by intraperitoneal injection of streptozotocin (65 mg/kg). Streptozotocin was dissolved in 0.01 M cold sodium citrate buffer (pH 4.5) immediately before use. The rats were then given 5% w/v glucose solution in feeding bottles for the next 24 h to prevent hypoglycemia. After 72 h, rats with marked hyperglycemic fasting blood glucose> 200 mg/dL were selected and used for the study. All the animals were allowed free access to tap water and pellet diet and maintained at room temperature in polyethylene cages.

The rats were divided into five groups consisting of six rats each.

Group 1: Administered vehicle, sodium Citrate Buffer (1ml/kg) (Control).

Group 2: Administered Streptozotocin (STZ) (65mg/kg i.p.) (diabetic control)

Group 3: Diabetic rats treated with reference standard, Insulin (6U/kg, s.c/day) for 30 days

Group 4: Diabetic rats treated with MC (250mg/kg p.o/day/30days)

Group5: Diabetic rats treated with MC (500mg/kg p.o/day/30days)

Group6: Diabetic rats treated with MD (250mg/kg p.o/day/30days)

Group7: Diabetic rats treated with MD (500mg/kg p.o/day/30days)

Group8: Diabetic rats treated with saponin fractions of MC (87.5mg/kg p.o/day / 30days)

Group9: Diabetic rats treated with saponin fractions of MC (175mg/kg p.o/day/30days)

Group10: Diabetic rats treated with saponin fractions of MD (27.5mg/kg p.o/day/30days)

Group11: Diabetic rats treated with saponin fractions of MD (55mg/kg p.o/day/30days)

After 30 days of treatment liver was isolated immediately after sacrificing, homogenized and centrifuged in ice cold saline and analysed for LPO, GSH, CAT and SOD.

3.2.2.13. Cardiac antioxidant parameters in Isoproterenol (ISO) induced myocardial infarction in rats

The rats were divided into six groups.

Group 1: Distilled water (10ml/kg p.o) (control)

Group 2: ISO (60mg/kg, s.c) at an interval of 24 hours for two days (ISO Control)

Group 3: MC (250 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Group 4: MC (500 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Group 5: MD (250 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Group 6: MD (500 mg/kg) for 45 days followed by ISO (60mg/kg, s.c)

Twelve hours after the second injection of ISO, the animals were sacrificed by cervical decapitation, heart was isolated immediately after sacrificing, homogenized and centrifuged in ice cold saline and analysed for LPO, GSH, CAT and SOD.

Antifertility activity

3.2.1.14 Antifertility activity in rats:

Antiimplantation activity was determined as described by Khanna and Chowdary (1969). Vaginal smears from each rat were monitored daily. Only rats with normal estrous cycle (Dennis EJ & Baker1979) were selected for experiment. The female rats were caged with male rat of known fertility in the ratio of 2:1 in the evening of proestrous and examined the following morning for the evidence of copulation. Rats exhibiting the copulation plug or thick clump of spermatozoa in their vaginal smear were separated and that day was designated as day one of pregnancy. Pregnant rats were divided into 15 groups containing six animals in each group to study antiimplantation, antizygotic, blastocystotoxic and early abortifacient (Hafez ES, 1970)

Group 1: Distilled water, day 1 to 7 (Control),

Group 2: MC (250 mg/kg, p.o.), day 1 to 7- Antiimplantation activity

Group 3: MC (500 mg/kg, p.o), day 1 to 7 -Antiimplantation activity

Group 4: MC (250 mg/kg, p.o), day 1 to 3 - Antizygotic activity

Group 5: MC (500 mg/kg, p.o), day 1 to 3 - Antizygotic activity

Group 6: MC (250 mg/kg, p.o), day 4 to 5 –blastocystotoxic

Group 7: MC (500 mg/kg, p.o), day 4 to 5 –blastocystotoxic

Group 8: MC (250 mg/kg, p.o), day 6 to 9 – early abortifacient

Group 9: MC (500 mg/kg, p.o), day 6 to 9 – early abortifacient

Group 10: MD (250 mg/kg, p.o), day 1 to 7- Antiimplantation activity

Group 11: MD (500 mg/kg, p.o), day 1 to 7 -Antiimplantation activity

Group 12: MD (250 mg/kg, p.o), day 1 to 3 - Antizygotic activity

Group 13: MD (500 mg/kg, p.o), day 1 to 3 - Antizygotic activity

Group 14: MD (250 mg/kg, p.o), day 4 to 5 –blastocystotoxic

Group 15: MD (500 mg/kg, p.o), day 4 to 5 –blastocystotoxic

Group 16: MD (250 mg/kg, p.o), day 6 to 9 – early abortifacient

Group 17: MD (500 mg/kg, p.o), day 6 to 9 – early abortifacient

The rats were laprotamised under light ether anesthesia and semi sterile condition on day 10 of pregnancy and the number of implantation sites were recorded.

Estrogenic and antiestrogenic activity: It was carried out by the method described by Badmi S. et al, (2003). Wistar strain female albino rats, 21 to 23 days old, weighing 35 to 45g (immature rats) were used. Animals were randomly divided into six groups consisting of 6 animals in each group.

Group 1: Distilled water (10ml/kg), (Control)

Group 2: Ethinyl estradiol (0.02mg/kg s.c/7 days.)

Group 3: MC (250 mg/kg, p.o/7days),

Group 4: MC (500 mg/kg, p.o/7days),

Group 5: MC (250 mg/kg, p.o/7days) + Ethinyl estradiol (0.02mg/kg s.c/7days)

Group 6: MC (500 mg/kg, p.o/7days) + Ethinyl estradiol (0.02mg/kg s.c/7days)

Group 7: MD (250 mg/kg, p.o/7days),

Group 8: MD (500 mg/kg, p.o/7days),

Group 9: MD (250 mg/kg, p.o/7days) + Ethinyl estradiol (0.02mg/kg s.c/7days)

Group 10: MD (500 mg/kg, p.o/7days) + Ethinyl estradiol (0.02mg/kg s.c/7days)

On the 8th day, the rats were sacrificed and the uteri were dissected out, surrounding tissues removed, blotted on filter paper and weighed quickly on a sensitive balance (Precisa, XB series). A portion of the uterine tissues from control and treated animals were fixed in formalin buffer and taken for histological examinations. The sections were examined under digital microscope (Labomed). Diameter of uterus, thickness of endometrial and height of endometrial epithelium was measured. The other portion of the uterus was homogenized with ice-cold distilled water. The homogenate was centrifuged at 3000 rpm for 15 min and the

supernatant was used for the estimation of glucose, cholesterol and alkaline phosphatase.

Progestational and antiprogestational activity:

Pregnancy maintenance test: Progestational activity was assessed by pregnancy maintenance test as described by Vogal HG & Wolfgang, (1997). Mature female SD rats were inseminated by placing with male rats overnight in the ratio of 1:2. On the 8th day of pregnancy the females were ovariectomized. Then the drug was administered as follows.

Group 1: Distilled water, from 8th to 19th day + Estradiol (0.1µg/rat/8^{th} to 19^{th} day) (Control)

Group 2: Progesterone (3mg/rat/day s.c, 8th to 19th day + Estradiol (0.1µg/rat/8^{th} to 19^{th} day) (Reference standard)

Group 3: MC (250 mg/kg, p.o./8th to 19th day) + Estradiol (0.1µg/rat/8^{th} to 19^{th} day)

Group 4: MC (500 mg/kg, p.o/8th to 19th day + Estradiol (0.1µg/rat/8^{th} to 19^{th} day)

Group 5: MD (250 mg/kg, p.o/8^{th} to 19^{th} day) + Estradiol (0.1µg/rat/8^{th} to 19^{th} day)

Group VI: MD (500 mg/kg, p.o/8th to 19th day) + Estradiol (0.1µg/rat/8^{th} to 19^{th} day)

On the 20th day, the animals were autopsied; presence or absence of implantation sites and the numbers of live embryos were recorded.

Clauberg Assay: Progestational and antiprogestational activity was assessed in rabbits using Clauberg assay as described by Vogal HG & Wolfgang, (1997). Immature female rabbits weighing 550-650g were maintained under standard experimental conditions. The animals were grouped into groups of 6 animals each. All animals were injected with estradiol valerate (8.3µg/kg,s.c/6 days). After estrogen priming, they were treated as follows.

Group 1: Distilled water, (10ml/kg p.o/day /5 days) (Control).

Group 2: MC (250 mg/kg, p.o. /day /5 days)

Group 3: MC (500 mg/kg, p.o. /day /5 days)

Group 4: MC (250 mg/kg, p.o. /day /5 days) + norethesterone 0.75mg/kg, s.c/day /5 days)

Group 5: MC (500 mg/kg, p.o. /day /5 days) + norethesterone 0.75mg/kg s.c/day /5 days)

Group 6: MD (250 mg/kg, p.o. /day /5 days)

Group 7: MD (500 mg/kg, p.o. /day /5 days)

Group 8: MD (250 mg/kg, p.o. /day /5 days) + norethesterone 0.75mg/kg s.c/day /5 days)

Group 9: MD (500 mg/kg, p.o. /day /5 days) + norethesterone 0.75mg/kg s.c/day /5 days)

The animals were sacrificed on the 12th day.The uterus was dissected out, adherent tissues were removed, blotted on a filter paper and was preserved in the neutral formalin buffer 10% for 24 h, then dehydrated in alcohol and embedded in paraffin wax. The sections of 5μM were cut and stained with haematoxylin-eosin and examined under digital microscope (labomed).

3.2.1.15 Antiovulatory activity in rats

Vaginal smear from each rat was examined daily for 15 days, and those rats exhibited three regular cycles were included in the study. The selected rats were divided into groups of six animals each. Drugs and vehicle were started in the estrous phase and administered orally, daily for 15 days.

Group 1: Distilled water (10ml/kg p.o./day/15days) (Contro),

Group 2: MC (250 mg/kg, p.o. /day/15days)

Group 3: MC (500 mg/kg, p.o. /day/15days)

Group 4: MD (250 mg/kg, p.o. /day/15days)

Group 5: MD (500 mg/kg, p.o. /day/15days)

The 15-day treatment was to cover three regular estrous cycles. Vaginal smear from each animal was observed every morning between 9-10 A.M. On the 16th day, 24 hours after the last treatment, the animals from each group were sacrificed. Ovaries and uteri were dissected out, freed from extra deposition, and weighed on a sensitive balance (Precisa, XB series). One ovary from each animal was processed for biochemical analysis of cholesterol. The other ovary was fixed in 10% formalin buffer for histological study.

3.2.1.16 Abortifacient activity in rats

The female rats were caged with male rats of known fertility in the ratio of 2:1 in the evening of proestrous. They were examined the following morning for the presence of sperms. Rats exhibiting thick clumps of spermatozoa in the vaginal smear were separated, and that day was designated as day one of pregnancy. The pregnant rats were divided into groups of six animals each.

Group I: Distilled water (10ml/kg p.o./day/6-15 day), (Control),

Group II: MC (250 mg/kg, p.o /day/6-15 day),

Group III: MC (500 mg/kg, p.o.) /day/6-15 day),

Group IV: MD (250 mg/kg, p.o.) /day/6-15 day),

Group V: MD (500 mg/kg, p.o.) /day/6-15 day),

The extracts were administered from the 6th to the 15th day of pregnancy (period of organogenesis). The animals were laparotomised under light ether anesthesia on the 19th day of pregnancy. Both horns of the uterus were observed for the number of im-plantation sites, resorptions, and dead and alive fetuses.

3.2.1.14 Effect on male reproductive system in rats

The animals were divided into three groups of 6 animals each.

Group 1: Distilled water (10ml/kg p.o/day/60days) (control)

Group 2: MC (250mg/kg p.o/day/60days)

Group 3: MC (500mg/kg p.o/day/60days)

Group 4: MD (250mg/kg p.o/day/60days)

Group 5: MD (500mg/kg p.o/day/60days)

On the 55^{th} day the animals of all groups were subjected to matting test. Each rat in the groups was caged with 2 female rats for 6 days. Vaginal smears were then examined every morning until the presence of sperm plug. The pregnant female rats were then caged separately and on the 20^{th} day, the rats were sacrificed and number of live fetuses were recorded. On the 61st day , animals were weighed, blood was collected retro orbitaly, serum was separated and assayed for testosterone. The rats were sacrificed, testes, epididymis, seminal vesicle and ventral prostate were dissected out, freed from adhering tissue, blotted on a filter paper and weighed on a sensitive balance. The cauda epididymis was chopped in 10 ml normal saline, the aliquots of sperm suspension was filled up to 0.5 mark in WBC pipette and diluted with saline

up to 11 mark. Sperm count was done in Neubauer's chamber in WBC squares and the sperm count/ml was calculated. One of the two testes was subjected to histological examination. The other testis was homogenised and analyzed for cholesterol and ALP.

Phytochemical Investigation

3.2.1.16. Phytochemical Investigation

Tests for Alkaloids:

The ethanolic extracts were separately treated with few drops of dilute hydrochloric acid and filter. The filtrate was tested for the presence of alkaloids.

i. *Hagner's test*- Extract treated with Hagner's reagent (Picric acid solution) - yellow precipitate.

ii. *Mayer's test*- Extract treated with Mayer's reagent (Potassium mercuric iodide solution) - cream precipitate.

iii. *Dragendroff's test*- Extract treated with Dragendroff's reagent (Potassium bismuth iodide solution) - orange precipitate.

iv. *Wagner's test*- Extract treated with Wagner's reagent (Iodine-potassium solution) - reddish brown precipitate.

Tests for Carbohydrates:

Dissolve small quantities of ethanolic extracts separately with 5 ml of distilled water and filter. The filtrate was subjected to

i. Molisch's test.: Extracts treated with Molisch reagent (alpha napthol in 95% ethanol) and few drops of concentrated hydrochloric acid at the sides of the test tube, violet ring at the junction.

ii. *Fehling's test*-Extract treated with Fehling reagent (Fehling's reagent A) Copper sulphate in water and (Fehling's reagent B - Sodium potassium tartarate, red colour.

iii. *Barfoed's test*- Extract treated with Barfoed reagent (Copper acetate in water and glacial acetate), red colour.

iv. *Benedict's test-* Extract treated with Benedict reagent (Copper sulphate, sodium citrate and sodium carbonate in water), red colour.

Test for Glycosides:

The ethanolic and aqueous extracts were separately hydrolysed with dilute hydrochloric acid for few hours in a water bath and then subjected to

i. *Libermann Burchard's test-*Extract treated with chlorform in a dry test tube and few drops of glacial acetic acid and few drops of concentrated sulphuric acid at the sides of the test tube. A red colour at the junction of two layers and the upper layer shows green colour.

ii. *Legal test-* Extract treated with Disodium nitroprusside in pyridine and sodium hydroxide, red colour.

iii. *Borntrager's test-* Extract treated with diluted sulphuric acid, add solvent ether, shake and filter. To the organic layer add ammonium solution, organic layer becomes pink to red.

Test for Phytosterols:

Reflux the ethanolic extracts with alcoholic potassium hydroxide till complete saponification takes place. Dilute the saponification mixture with distilled water and extract with ether. Evaporate the ethereal extract and subject the residue to

i. *Liberman Burchard's test-* Extract treated with chlorform in a dry test tube and few drops of glacial acetic acid and few drops of concentrated sulphuric acid at the sides of the test tube. A red colour at the junction of two layers and the upper layer shows green colour.

ii. *Salkowski test-* Extract treated with equal volumes of chloroform and sulphuric acid, red or violet colour.

Test for Saponins:

i. *Foam test*: Dilute 1ml of ethanolic and aqueous extracts separately with distilled water to 20ml and shake in a graduated cylinder for 15 min. An one centimeter layer of foam indicates the presence of Saponins.

ii. *Haemolysis test*: 2ml of 1.8% sodium chloride solution was taken in two test tubes. To one test tube 2ml of distilled water was added and to the other 2ml

of 1% filtrate. Blood is obtained by pricking the thumb and 5 drops of blood were added to each tube, the contents were gently mixed and observed under microscope. Haemolysis occurs, indicates the presence of Saponins.

Test for Tannins:

Dilute small quantities of ethanolic and aqueous extracts separately with distilled water and subjected to

i. Ferric chloride test-Extract treated with ferric chloride solution, blue colour.

ii. *Gelatin test-* Extract treated with gelatin solution, white precipitate.

iii. *Lead acetate test-* Extract treated with lead acetate solution, yellow precipitate.

Test for Proteins and amino acids:

Dissolve small quantities of ethanolic and aqueous extracts separately with few ml of distilled water and then subjected to

i. *Million's test-* Extract treated with Million's reagent (Mercuric nitrate in nitric acid), red colour.

ii. *Biuret test-* Extract treated with sodium hydroxide and copper sulphate solution added drop wise and mixed, violet colour.

iii. *Ninhydrin test-* Extract treated with Ninhyrin reagent (Ninhydrin with alpha amino acid) and ammonium, heat, violet colour.

iv. *Sodium bicarbonate test-* Extract treated with sodium bicarbonate solution, brisk effervesces

Test for Flavonoids:

i. *Ferric chloride test-* To alcoholic extract few drops of neutral ferric chloride solution, blackish red colour.

ii. *Lead acetate test-* To alcoholic extract add lead acetate solution, yellow precipitate.

iii. *Magnesium ribbon test-* To alcoholic extract few fragments of magnesium ribbon and concentrated hydrochloric acid along the side of test tubes, Magenta colour.

iv. *Zinc- hydrochloric acid test-* To alcoholic extract, a pinch of zinc dust was added and concentrated hydrochloric acid along the side of test tubes, Magenta colour.

HPTLC Finger Printing

3.2.1.19 HPTLC Finger Printing

HPTLC fingerprinting of saponins of MC and MD was performed. Silica gel $60F_{254}$ (merck) was used as a stationary phase. Chloroform: Glacial acetic acid (9.5:0.5) was used as a mobile phase. The dried plate was scanned to visualize the migrated components under UV radiation at 254 nm, 336 and 540 ηm using Reprostar 3 with a digital camera (CAMAG).

Biochemical Methods

3.2.2. Serum Biochemical Methods

3.2.2.1 Blood glucose level:

Autospan diagnostic kit was used for the estimation of glucose (GOD/POD) following enzymatic colorimetric procedure (Nadu Rifai et al., 2001)

Reagents	Blank	Standard	Test
Sample	-	-	0.02 ml
Glucose standard, 100 mg %	-	0.02 ml	-
Working glucose reagent	1.5 ml	1.5 ml	1.5 ml
Purified water	1.5 ml	1.5 ml	1.5 ml

Mix well and incubate at 37°C for 10 minutes Read the absorbance of test and standard at 505nm against reagent blank

Calculations:-

$$\text{Glucose in mg/100ml} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

3.2.2.2 Estimation of serum insulin: The ADVIA Centaur (IRI) and Ready Pack, of Bayer of corporation diagnostic kit were used for the estimation of insulin, which follows immunoassay using two monoclonal antibodies

Reagents used:

Reagent Pack	Reagent	Volume	Ingredients
ADVIA Centur IRI Ready Pack primary reagent pack	Lite Reagent	5.0 mL/reagent pack	Monoclonal mouse anti-insulin antibody (~0.24µg/ml) labeled with acridinium ester in buffered saline with bovine serum albumin, sodium azide (<0.1%), and preservatives.

	Solid Phase	25mL/reagent pack	Monoclonal mouse anti-insulin antibody (~6.0µg/ml) covalently coupled to paramagnetic particles in buffered saline with bovine serum albumin, sodium azide (<0.1%), and preservatives.
ADVIA Centaur IRI(DIL) Ready Pack ancillary reagent pack	Insulin Diluent	10.0mL/reagent pack	Buffered saline with casein, potassium thiocyanate (3.89%), sodium azide (<0.1%), and preservatives.

Samples are free of fibrin or other particulate matter and free of bubbles. The assay is carried out on 25µL of serum at 37^0C by master curve calibration method.

3.2.2.3 Estimation of serum total cholesterol: Span diagnostic kit was used for the estimation of total cholesterol, which followed cholesterol oxidase/peroxidase (CHOD-POD) method.

Reagents used:

Sl. No.	**Reagent composition**	**Conc. in the final test mixed**
1.	Good's butter (P^H 6.7)	50mmol/l
2.	Phenol	5mmol/l
3.	4 - Aminoantipyrine	0.3 mmol/l
4.	Cholesterol esterase	≥ 200 U/l
5.	Cholesterol oxidase	≥ 100 U/l
6.	Peroxidase	≥ 3 KU/l

Procedure:

Pipetted in to test tube	**Blank**	**Standard**	**Test**
Reagent	1000 µl	1000 µl	1000 µl
Standard	-	10 µl	-
Sample	-	-	10 µl

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of reaction mixtures at 505nm against reagent blank was taken

3.2.2.4 Estimation of serum of triglycerides: Span diagnostic kit was used for estimation of triglycerides, which followed end point colorimetry enzymatic test using glycerol-3-phosphate oxidase.

Reagents used:

Sl. No.	Reagent composition	Conc. in the final test mixed
1.	Pipes butter	50mmol/l
2.	4-Chlorophenol	5mmol/l
3.	Mg^{2+}	5 mmol/l
4.	ATP	1 mmol/l
5.	Lipase	$\geq$ 5000 U/l
6.	Peroxidase	$\geq$1000 U/l
7.	Glycerol Kinase	$\geq$400 U/l
8.	Glycerol - 3- phosphate oxidase	$\geq$4000 U/l
9.	4- Aminoantigpyrine (4-AAP)	0.4 mmol/l

Procedure:

Pipetted in to test tube	Blank	Standard	Test
Reagent	1000 µl	1000 µL	1000 µl
Standard	-	10 µl	250 µl
Sample	-	-	10 µl

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of sample and standard were measured against reagent blank at 505 nm.

3.2.2.5 Estimation of serum creatinine:

Picric acid method (Jaffe reaction) was used for the estimation of serum creatinine

Reagents used:

Sl. No	Reagent composition	Conc. in the final test mixed
1	Picric acid	16 mmol/L
2	Sodium hydroxide	150 mmol/L

Procedure

	Standard	Unknown
Reagent	1.0 ml	1.0 ml
Standard	0.1 ml	-
Specimen	-	0.1 ml

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of sample and standard were measured against reagent blank at 510 nm

3.2.2.6 Estimation of serum HDL: Span diagnostic kit was used for estimation of HDL cholesterol, which followed Cholesterol oxidase / peroxidase (CHOD-POD) method

Procedure

0.5ml of serum was taken into test tube and 0.5ml of precipitating reagent was added, mixed well and kept at room temperature for 15min. Centrifuged for 15 min at 4000 rpm.

The clear supernatant was separated and immediately used to determine the cholesterol content as follows:

Pipetted in to test tube	**Blank**	**Standard**	**Test**
Reagent	1000 µl	1000 µl	1000 µl
Standard	-	100 µl	-
Supernatant from step3	-	-	100 µl

The reaction mixtures were mixed well and incubated for 10 min at 37^0C. The absorbance of test and standards was measured against the reagent blank at 505nm

3.2.2.7 Estimation of serum LDL-C: Using the data obtained including total cholesterol, HDL cholesterol and VLDL, the LDL cholesterol levels were calculated using the empirical equation of Friede Wald (Sacks David B.1089).

Calculation:
Serum LDLcholesterol = Total cholesterol - (HDLcholesterol + VLDLcholesterol)

Estimation of Alanine AminoTransferase (ALT)

Components and Concentrations

R_1: TRIS	pH 7.5	100 mmol/l
L-Alanine		500 mmol/l
LDH (lactate dehydrogenase)		≥ 1200 U/l
R_2: 2-Oxoglutarate		15 mmol/l
NADH		0.18 mmol/l

Mix 4 parts of R_1 + 1 part of R_2 = Monoreagent

Mix well and aspirate immediately for measurement.

Programme the analyzer as per assay parameters.

1. Blank the analyzer with purified water.
2. Read the absorbance after 60 seconds. Repeat reading after every 30 seconds i.e upto 120 seconds at 340 nm wavelength.
3. Determine the mean absorbance change per minute.

Calculation:

ΔA/min X factor (1768) = ALT activity [IU/l]

3.2.2.8 Estimation of Serum Alkaline Phosphate (ALP)

Reagent Components:

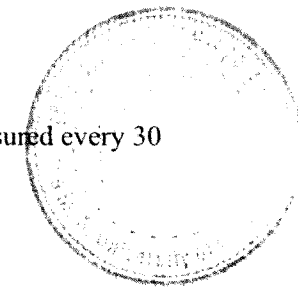
Reagent 1: AMP buffer, pH 10.3

Magnesium acetate, Zinc sulphate, Chelator

Reagent 2: PNPP substate: PNPP, Stabilizer

Preparation

	Blank	Test
Distilled water	1.0ml	----
Serum	----	20µl
Reaction solution	----	1.0ml



Approx. 30 second after mixing decreases in absorbance is measured every 30 seconds for 2 minutes.

Calculation:

Enzyme activity [U/l]: ΔA/min X 2712

3.2.2.9 Estimation of Serum Total Billirubin

Span Diagnostics Ltd reagent kits (Jendrassik & Grof Method) were used for the estimation

Reagents:

Reagent 1 (Stabilizers):		
Sodium Nitrite:	13.0ml	Sodium Nitrite, stabilizer
Reagent 2 (Preservative):		
Sulphanilic acid:	40.0ml	Sulphanilic acid HCL, preservative
Reagent 3:		
Caffien :	126.0ml	Caffiene, sodium benzoate stabilizer
Reagent 4:		
Artificial standard (5.0mg/dl):	10.0ml	Colored dye, preservative

Pipette into test tubes marked	Blank (AB_1)	Sample (AT_1)
Reagent 1	----	50 µl
Reagent 2	100 µl	50 µl
Sample	50 µl	25 µl
Working reagent3	1.0ml	1.0ml

Mix well; incubate for 5 minutes at 37^0C temperature for Total Bilirubin. Read absorbance at 546 nm. Set the analyzer to zero with purified water. Aspirate sample blank followed by the test.

Calculation:

Total Bilirubin (mg/dl) = (AT1 -AB_1) x factor

3.2.2.10 Estimation of Total Protein

Pipette in the tubes marked	Blank	Test	Standard
Serum	----	10µl	----
Total protein standard	----	----	10µl
Biuret reagent			1.0ml
	1.0ml	1.0ml	
Mixed well. Incubated at room temperature for 5 minutes and the absorbance was measured at 578 nm against reagent blank.			

Tissue Bioanalytical Methods

3.2.3. Tissue Bioanalytical Methods:

3.2.3.1: Preparation of Homogenate

The animals were sacrificed and liver, kidney and heart were isolated; their homogenate was prepared as follows

Procedure:

Kidney, liver and heart were separated and kept in cold condition, chopped with surgical scalpel in to fine slices and was chilled in cold 0.25M sucrose, quickly blotted with filter paper. The tissue was minced and homonogenized in ice cold 10mM tris HCl buffer (to pH 7.4) at a concentration of (10%w/v) with 25 strokes of light Teflon pestle of glass homogenizer at a speed of 2500rpm.Te prolonged homogenization under hypotonic condition was designed to disrupt as far as possible the ventricular structures of cells so as to release soluble proteins and leave only membrane and nonvascular matters in a sedimentable form. It was them centrifuged in cooling centrifuge at 5000RPM.The temperature was maintained at -4°C during the centrifugation. Clear supernant was separated and used for the estimation of SOD, Catalase, Glutathione and MDA.

3.2.3.2. Assay of Super oxide Dismutase (SOD)

Superoxide Dismutase was estimated using the method developed by Misra and Fridovick (1972).

Reagents:

1. Carbonate Buffer (0.5M, pH 10.2)

   16.8gms of sodium bi carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000ml with distilled water.

2. Ethylene diamine tetra acetic acid (EDTA) solution 0.49M

   1.82gms of EDTA was dissolved in 200 ml of distilled water and the volume was made up to 1000ml in distilled water

3. Hydrochloric acid (0.1N)

   8.5 ml of concentrated hydrochloric acid was mixed with 500ml of distilled water and the volume was made up to 1000ml with distilled water

4. Ephenephrine solution (3mM)

   0.99gms of epinephrine bitartarate was dissolved in 100ml of 0.1N hydrochloric acid and the volume was adjusted to 1000ml with 0.1N HCl

5. Superoxide Dismutase (SOD) standard (100U/L)

   1mg of SOD from bovine liver was dissolved in 100ml of carbonate buffer

   Procedure:

   0.5 ml o0f the homogenate was diluted with 0.5 ml of distilled water, to which 0.25ml of ice cold ethanol and 0.15 ml of ice cold chloroform were added. The mixture was mixed well using cyclo mixer for 5 minutes and centrifuged at 2500 RPM. To 0.5 ml of supernant 1.5 ml of carbonate buffer and 0.5 ml of EDTA solution was added. The reaction was initiated by the addition of 0.4 ml of ephrinephrine and the change in optical density /min was measured at 480nm against reagent blank.

   SOD activity was expressed as units/mg protein.Change in optical density per minute at 50% inhibition of epinephrine tio adrenochrome transition by the enzyme is taken as the enzyme unit.Calibration curve was prepared by using 10-125 units of SOD.

3.2.3.3. Assay of CATALASE

It was estimated by the method of Hugo Aebi as given by Colowick at al. (1984)

Reagent

1. Phosphate buffer (50mm/L; pH 7.0)
    a. Dissolve 6.81gms KH2PO4 in water and make up to 100ml
    b. Dissolve 8.9 gms NaH2PO4 2 H2O in water and make up to 100ml

    Mix solution 1 and 2 in proportion of 1:1.5 (v/v)
2. Hydrogen peroxide (30mM/L): Dilute 0.34 ml 30% Hydrogen peroxide with phosphate buffer up to 100ml.

Procedure:

To 2 ml diluted sample 1 ml of hydrogen peroxide was added to initiate the reaction. Blank was prepared by mixing 2 ml of diluted sample with 1 ml phosphate buffer. The dilution should be such that initial absorbance should be approximately 0:500. The decrease in absorbance was measured at 140nm.Catalase activity was expressed as µmoles of H_2O_2 consumed/min/mg of protein.

3.2.3.4. Reduced Glutathione

Reduced Glutathione was estimated by the method of Moran et al.

Reagents

i. Trichloroacetic acid 10% (TCA): Accurately weighed 10 gms of Trichloro acetic acid was dissolved in 100 ml of distilled water
ii. Phosphate buffer (0.02M pH 8)
iii. DTNB reagent (0.6M): 60 mg of DTNB dissolved in 100 ml of 0.2M sodium phosphate pH 8
iv. Std Glutathione:Prepared by dissolving 10mg of reduced glutathione in 100 ml of distilled water

Procedure:

To 1ml of sample 1 ml of 10% TCA was added. The precipitated fraction was centrifuged and to 0.5 ml supernant. 2 ml of DTNB reagent was added. The final volume was made up to 3 ml with phosphate buffer. The color developed was read at

412 nm. The amount of glutathione was expressed as µg of GSH/mg protein reduced glutathione was used as standard.

3.2.3.5. Lipid peroxidation or Malanaldehyde formation

Malanaldehyde formation was estimated by the method of Slater and Sawyer

Reagents:

i. Thiobarbituric acid (0.67%)in 1 M tris hydrochloride ,pH-7,
ii. Trichloro acetic acid (20%)
iii. Std malanaldehyde: (0-0.25nMlo)

A stock solution containing 50 mm/ml of 1,1,3,3,tetra etoxy propane in tris hydrochloride buffer was diluted to 100 ml to get working standard 50nm malanaldehyde/ml. This was used for the preparation of calibration curve.

Procedure:

2 ml of sample was mixed with 2 ml of 20% trichloroacetic acid and kept in ice for 15 minutes. The precipitate was separated by centrifugation and 2 ml of samples of clear supernant solution were mixed with 2 ml of aqueous 0.67% thiobarbituric acid. This mixture was then heated on a boiling water bath for 10 minutes. It was then cooled in ice for 10 minutes and the absorbance was read at 535nm.The values expressed as nm of MDA formed /mg of protein. Values are normalized to the protein content of the tissue.

3.2.3.6Estimation of liver glycogen

Liver glycogen was estimated using Anthrone reagent method as described by Carroll NV et al., (1952).

Principle:

Glycogen was extracted from liver by using TCA. The solution was centrifuged and the supernatant was treated with ethanol to precipitate glycogen. The glycogen so obtained was estimated by Anthrone method. The mineral acid in Anthrone reagent breaks down glycogen into glucose units, which react with Anthrone reagent giving blue colour, the absorbance was read at 620 nm.

Anthrone reagent: 250 mg of Anthrone in 500 ml of 75% H_2So_4 .It is photosensitive and stored in dark bottles.

Glucose standard:

(a) Stock solution: 100 mg of glucose was dissolved in 100 ml of saturated benzoic acid

(b) Working standard: 5 ml of the stock solution was placed in 100 ml volumetric flask and made up to volume with saturated benzoic acid solution. 2 ml of this solution, containing 0.1 mg of glucose, was used as a standard.

Procedure:

The tissue sample was placed in an efficient blender with an appropriate volume of TCA homogenized for 3 minutes. The homogenate was poured into a suitable centrifuge tube.

The supernatant fluid was centrifuged and decanted upon an acid washed filter paper placed in a funnel draining into a graduated cylinder. The residue was quantitatively transferred to a blender with appropriate volume of TCA and homogenized again for 1 minute. The mixture was centrifuged and the supernatant fluid was poured through the same filter. Two more extractions were made in the same manner to extract better than 97.5% of the glycogen present. The desired volume was made up with 5% TCA and mixed thoroughly. The final volume contained 10 to 200 mcg of glycogen per ml. Trichloroacetic acid filtrate (1ml) was pipetted into a 15 ml centrifuge tube (in duplicate). To each tube were added 5 volumes of 95% ethanol with careful blowing to effect thorough mixing. This was checked by noting the absence of an interface. The tubes were capped with clean rubber stoppers and the tubes were placed in a water bath at 37 – 40 $^{\circ}$ C for 3 hours. After precipitation was complete, the tubes were centrifuged at 3000 rpm for 15 minutes. The clear liquid was gently decanted from the packed glycogen and the tubes were allowed to drain in an inverted position for 10 minutes.

The glycogen was dissolved by addition of 2 ml of distilled water, the water being added in a manner that will wash down the sides of the tube. If the glycogen did not dissolve instantly, the tube was agitated until the solution was complete. A reagent blank was prepared by pipetting 2 ml of water into a clean centrifuge tube. A standard was prepared by pipetting 2 ml of standard glucose solution, containing 0.1 mg of glucose into a similar tube.

At this point 10 ml portions of Anthrone reagent were delivered into each tube with vigorous but consistent blowing. The stream of Anthrone reagent was

directed into the center of the tube and was sufficient to insure good mixing. As each tube received

Anthrone reagent, it was tightly capped and placed in cold water bath.

After all tubes had reached the temperature of cold water, they were immersed in boiling Water bath to a depth a little above the level of liquid in the tubes for 15 minutes and then cooled to room temperature. The tubes and stoppers were wiped dry and the contents of each tube were transferred to a colorimeter tube and read at 620 nm after adjusting the colorimeter with reagent blank.

Calculation: The calculation of glycogen was done as follows

– (DU/DS) x 0.1 (Vol of ext / gm tissue) x 00 x 0.9 = mg of glycogen per 100 gm tissue

Where DU = Optical density of the unknown, DS = Optical density of the standard

0.1 = mg of glucose in 2 ml of standard solution, 0.9 = factor for converting glucose value to glycogen value.

3.2.3.5. HMG CoA reductase to Mavalonate ratio:

Reagents:

1. Saline: arsenate- 1 gm of sodium arsenate/ L of physiological saline.
2. Dilute perchloric acid- 50 ml/ L
3. Hydroxylamine hydrochloride reagent – 138.98 gm/ L
4. Hydroxylamine hydrochloride reagent for Mevalonate: Equal volumes of hydroxylamine hydrochloride reagent and water were mixed freshly before use.
5. Hydroxylamine hydrochloride reagent for HMG CoA - Equal volumes of hydroxylamine hydrochloride reagent and sodium hydroxide solution were mixed freshly before use.
6. Ferric chloride reagent: 5.2 gm of trichloroacetic acid (TCA) and 10 gm of ferric chloride were dissolved in 50 ml of 0.65N hydrochloric acid and diluted to 100 ml with the letter.

Procedure:

Equal volumes of fresh tissue homogenate and diluted perchloric acid were mixed, kept for 5 min and centrifuged (2000 rpm) for 10 minutes. To 1 ml of filtrate 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG Co A) was added and mixed. After 5 min, 1.5 ml of ferric chloride was added and shaken well. Readings were taken after 10 min at 540 nm using spectrophotometer against a similarly- treated saline- arsenate blank. The ratio of HMG Co A to Mevalonate was calculated. Lower ratio indicates higher enzyme activity and vice-versa.

3.2.4. Histopathological analysis: After the study period, the rats were anaesthetized with pentobarbitone sodium (30mg/kg, i.p). Heart, pancreas, aorta and kidney were perfused with chilled saline and heparin sulphate (50U/I) to remove blood and blood clots and fixed in 10%w/v formaldehyde. Paraffin blocks were prepared. Sections of µm thickness were cut on a microtome and taken on glass slide .The sections were deparafinated in xylene, down graded through different concentrations of alcohol and finally in water.The haematoxylene stained sections were stained with eosin for 2 minutes, passed through ascending grades of alcohol cleaned in xylene and mounted on Canada balsam..The stained sections were examined under Olympus BX40 pictomicroscope and photographed.

3.3 Statistical Analysis:

The results of the above estimations have been indicated in terms of ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) with Tukey-Kramer post comparison test using Graph Pad InStat version 3.00, GraphPad Software, California USA. The levels of significance was set at $P<0.05$