6.5. PHARMACOLOGICAL SCREENING

Animals

Adult Wistar rats (180 ± 10 g) of either sex were procured from D Y Patil College of Pharmacy, Pune (Maharashtra.), India. The animals were housed in large, spacious polyacrylic cages at an ambient room temperature with 12-h light/12-h dark cycle. Rats had free access to rodent pellets diet (Hindustan Lever Ltd, Bangalore, India) and water *ad libitum*. The study was approved by the Institute Animal Ethics Committee of the D Y Patil College of Pharmacy, Pune (Maharashtra.), India and all the animal experiments were carried out according to the Committee for the Purpose of Control and Experiments on Animals (CPCSEA) guidelines. The experimental protocols were approved by Institutional Animal Ethics Committee. IAEC No 1555/PO/a/11/CPCESA The animals were acclimatized to the laboratory condition for 1 week before starting the experiment.

6.5.1. Acute Toxicity Studies (Babu et al., 2003, Parasuraman S, 2011)

Organization for Economic co-operation and Development (OECD) regulates guideline for oral acute toxicity study. It is an international organization which works with the aim of reducing both the number of animals and the level of pain associated with acute toxicity testing.

Following are the main type of guideline followed by OECD

- Guideline 420, Fixed dose procedure. (5 animals used)
- Guideline 423, Acute toxic class. (3 animals used)
- Guideline 425, Up and down method. (1 animal used)
Acute oral toxicity

Acute oral toxicity of the polyherbal formulation was carried out as per the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423. The principle involves a stepwise procedure with the use of a minimum number of animals per step to obtain sufficient information on the acute toxicity of the test substance to enable its classification. Healthy Wistar rats (3 animals/dose) of either sex were used for the experiment. Overnight fasted rats were orally fed with the polyherbal formulation (Capsule & Vati) in increasing dose levels of 5, 50, 300, and 2000 mg/kg body weight, respectively. The animals were observed for their behavioral (alertness, restlessness, irritability, and fearfulness), neurological (spontaneous activity, reactivity, touch response, pain response, and gait), and autonomic (defecation and urination) profiles continuously for 24 h. After a period of 24 h, the animals were observed for 14 days for mortality.

6.5.2. Study Design

The animals were divided into 8 groups each containing six animals. Group I served as untreated control and received 0.9 normal saline, group II served as diabetic control and others group were treated with different doses of herbal formulations (Capsule & Vati) and standard anti-diabetic drug glibenclamide (Annadurai T 2012).

6.5.2.1. Selection of Doses (Kumar et al., 2010)

For the assessment of Antidiabetic activity, two dose level were chosen in such a way that one dose was approximately one-tenth of the maximum dose during acute toxicity
studies and the other high dose was twice that of one-tenth dose (200 mg/kg, 400 mg/kg body weight)

6.5.2.2. Preparation of dosing:
The dose of 200 and 400 mg/kg of polyherbal preparation was prepared by suspending appropriate quantity of capsule & Vati in 1 % w/v CMC.

6.5.3. Oral glucose tolerance test in normal rats animals and experimental setup:
Albino rats of either sex weighing 130 – 180 g were taken. The rats were kept fasting overnight with free access to water. During experiment the animals were divided into three groups of six animals in each group. The blood sample was taken by pricking the rat’s tail. Polyherbal formulation was administered with glass syringe and microsuction canula no. 18.

Grouping of animals:
Group I Kept as negative control, i.e., neither treated with Polyherbal preparation nor standard.
Group II Treated with standard oral hypoglycemic drug, i.e., Glibenclamide (0.5 mg/kg)
Group III Treated Orally with polyherbal preparation (400 mg/kg)

6.5.3.1 Determination of Oral glucose tolerance test (OGTT) activity:
The blood glucose concentration of animals were measured at the beginning of the study. Then the rats were orally treated with 3 g/kg body weight glucose solution after 30 minutes of the product and drug treatment. The measurements were repeated after 30, 90 and 150 minutes after the glucose load (Naik SR et al. 2008, Kuttan R 1999)
6.5.4. ANTIDIABETIC STUDY

Study of Polyherbal Formulation (Capsule & Vati) against alloxan induced diabetes (10 days study) (Vivek et al., 2010)

In this study, Polyherbal Formulation (Capsule & Vati, 200 and 400 mg/kg b.w) were evaluated for antidiabetic activity against alloxan induced diabetes mellitus in rats. Rats were divided into 7 groups consisting of 6 rats in each group. The rats were acclimatized for a period of 7 days before starting the experiment. After overnight fasting, hyperglycaemia was induced by administering a single dose of alloxan monohydrate (125 mg/kg b.w) prepared in sterile saline to all the groups except group I which served as normal control. During this period, the animals were given free access to water. After 3 days of alloxan administration, fasting blood glucose levels of rats were checked by glucostrips. The animals having blood glucose levels > 250 mg/dl were separated and selected for further studies and then re-grouping of these hyperglycemic rats was done as per the following protocol, for studying the antidiabetic activity of Polyherbal Formulation.

Group I- Normal Control i.e., neither treated with polyherbal preparation nor with standard

Group II- Diabetic Control treated with alloxan (125 mg/kg, i.p)

Group III- Alloxan monohydrate + Glibenclamide 0.5 mg/kg, after 3rd day of the treatment with alloxan (125 mg/kg, i.p)

Group IV- Alloxan monohydrate + PHF Capsule (200 mg/kg b.w) after 3rd day of the treatment with alloxan (125 mg/kg, i.p)

Group V Alloxan monohydrate + PHF Capsule (400 mg/kg b.w) after 3rd day of the treatment with alloxan (125 mg/kg, i.p)

Group VI Alloxan monohydrate + PHF Vati (200 mg/kg b.w) after 3rd day of the treatment with alloxan (125 mg/kg, i.p)
Group VII Alloxan monohydrate + PHF Vati (400 mg/kg b.w) after 3rd day of the treatment with alloxan (125 mg/kg, i.p)

The treatment was started from the same day except normal control and diabetic control groups for a period of 10 days orally. During this period, animals in all groups had free access to standard diet and water. Blood glucose levels were estimated on 1st, 4th, 7th and 10th day of the treatment. Besides this during this study the body weight of the rats were recorded on 1st, 4th, 7th and 10th day of the treatment.

6.5.5. Anti-Diabetic screening of formulation in streptozotocin and nicotimamide induced diabetic rats

The male Wistar rats were divided into five different groups of six animals each as follows.

- Group I: Normal control
- Group II: Diabetic control
- Group III: Diabetic rats treated with polyherbal preparation (Capsule) (200 mg/kg)
- Group IV: Diabetic rats treated with polyherbal preparation (Capsule) (400 mg/kg)
- Group V: Diabetic rats treated with polyherbal preparation (Vati) (200 mg/kg)
- Group VI: Diabetic rats treated with polyherbal preparation (Vati) (400 mg/kg)
- Group VII: Diabetic rats treated with glibenclamide (0.5 mg/kg).
Diabetes was induced in overnight-fasted rats by administering single intraperitoneal (i.p.) injection of freshly prepared streptozotocin (STZ) 50 mg/kg b.w. followed by 120 mg/kg of nicotimanide (NIC) in 0.1 M citrate buffer (pH 4.5) in a volume of 0.5 ml/kg b.wt. Diabetes was confirmed in the STZ + NIC treated rats by measuring fasting blood glucose levels after 48 h of induction. After 24 h of STZ + NIC injection, the rats were given 5% w/v of glucose solution (2 ml/kg b.w.) to prevent hypoglycemic mortality. Rats with fasting blood glucose of more than 200 mg/dl were considered as diabetics and they were divided randomly into four different groups. The standard (glibenclamide) and herbal formulation were suspended in 1% w/v carboxymethyl cellulose (CMC) and administered once daily through oral gavage for 21 consecutive days. The blood samples were collected on 1st, 7th, 14th, and 21st days of the treatment, through the tail vein of rats by pricking and were immediately used for the estimation of blood glucose with a glucometer. Weekly body weight variations were monitored for all the experimental animals. (Prasad et al, 2009)

At the end of the experiment, the blood sample was withdrawn from all the experimental animals through retro-orbital plexus puncture/posterior vena cava in plain and sodium ethylene diamine tetra acetic acid (EDTA) tubes for biochemical analysis (Parasuraman S et al., 2010). Finally the animals were sacrificed by diethyl ether anesthesia, and liver and pancreatic tissues were excised and used for biochemical and pathological analysis. Part of the tissue sample was preserved in an ice-cold container for biochemical analysis and the remaining was stored in 10% formalin solution for histo-pathologic analysis.
6.5.6. Biochemical analysis

The whole blood sample was used for the estimation of glucose (One-Touch Horizon glucometer; Ortho-Clinical Diagnostics, Johnson and Johnson Company, USA), hemoglobin, and glycosylated hemoglobin (HbA$_{1c}$). The plasma sample was used for the estimation of insulin (radioimmunoassay kit; Diasorin, Italy). The serum was used for the estimation of biochemical markers such as creatinine, urea, protein, liver glycogen, total serum cholesterol, serum triglyceride, high density lipoprotein (HDL)-cholesterol, serum glutamate oxaloacetate transaminase (SGOT), and serum glutamate pyruvate transaminase (SGPT). The biochemical markers were measured using a Prietest Easylab - Biochemistry Analyser (Robonik [India] Private Limited) and the LAB-KITS enzymatic kits. The liver tissue homogenate was used for the estimation of protein and glycogen.

**6.5.6.1. Estimation of glucose:** A drop of the whole blood sample was used for measuring glucose using One-Touch Horizon glucometer, with gluco strips. (Trinder, 1969)

**6.5.6.2. Estimation of hemoglobin:** Hemoglobin in the blood was estimated by the method of Drabkin and Austin (1932). To 0.02 ml of blood, 5.0 ml of Drabkin’s reagent was added, mixed well, and allowed to stand for 10 min. The solution was read at 540 nm together with the standard (James SA, 2011).

**6.5.6.3. Estimation of HbA$_{1c}$:** Erythrocytes were washed with normal saline, lysed with 5 ml of water, and incubated at 37°C for 15 min. The contents were centrifuged, the supernatant was discarded, and 0.5 ml of saline was added. To 2 ml of aliquot, 4 ml of oxalate hydrochloride solution was added and the contents were heated at 100°C for 4 h, cooled, and precipitated with 2 ml of 40% trichloro
acetic acid (TCA). The mixture was centrifuged, and to 0.5 ml of supernatant, 0.5 ml of 80% phenol and 3 ml of concentrated sulfuric acid were added. The developed color was read at 480 nm after 30 min.

Working standards (10-50 µg) were prepared using 1% fructose solution. Then, 0.5 ml of 80% phenol and 3 ml of concentrated sulfuric acid were added to the working standards and read at 480 nm after 30 min. The concentration of HbA\textsubscript{1c} was expressed as mg/g of hemoglobin.

6.5.6.4. Estimation of protein: The liver tissue was homogenized in 20 mM Tris-HCl and used for the estimation of protein. To 0.5 ml of tissue homogenate, 0.5 ml of 10% TCA was added and centrifuged for 10 min. The precipitate was dissolved in 1.0 ml of 0.1 N NaOH. To this, an aliquot of 4.5 ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. To this reaction mixture, 0.5 ml of Folin's phenol reagent was added and the blue color developed was read after 20 min at 640 nm. A standard curve was obtained with standard bovine albumin and was used to assay the tissue protein level of enzyme activity. Values were expressed as mg/g of tissue (Waterborg HH, 2002).

6.5.6.5. Estimation of liver glycogen: The alkali extract of the tissue was prepared by digesting 50 mg of fresh tissue with 3 ml of 30% potassium hydroxide solution in a boiling water bath for 15 min. The tubes were cooled and a drop of 1 M ammonium acetate was added to precipitate glycogen and kept in a freezer overnight for complete precipitation. Glycogen was collected by centrifuging at 300 rpm for 20 min. Then the precipitate was dissolved by heating and again the glycogen was re-precipitated by adding alcohol and 1 M ammonium acetate and centrifuged. The final precipitate was dissolved in saturated ammonium chloride solution by heating in a boiling water bath for 5 min. Aliquots of glycogen solution
were taken up for suitable dilution and 4 ml of anthrone reagent was added by cooling the tubes in an ice bath. The tubes were shaken well, covered with marble caps, and heated in a boiling water bath for 20 min. After cooling, the absorbance was read at 640 nm against water blank treated in a similar manner. The standard glucose solution was also treated similarly. The glycogen content was calculated from the amount of glucose present in the sample by multiplying with the factor 0.91, and expressed as mg/100 g of tissue.

6.5.6.6. Estimation of lipid profile: Estimation of serum Total cholesterol, Triglyceride, and HDL-cholesterol were done by using Standard kit (Nicholas India Pvt. Ltd.) with semi-auto analyzer.

6.5.6.7. Estimation of cholesterol: Cholesterol esterase (CHE) hydrolyses cholesterol ester into cholesterol and fatty acid. Cholesterol is oxidized by the cholesterol oxidize (CHO) to choloest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce pink coloured quinoneimine dye. The intensity of colour produced is proportional to cholesterol concentration. (Allain et al., 1974)

6.5.6.8. Estimation of HDL: The LDL fractions of serum sample are precipitated using PTA and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the ester cholesterol. Then cholesterol is oxidized by cholesterol oxidase (CHO) to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) reacts with 4-aminoantipyrine and phenol to produce a red colored complex, whose absorbance is proportional to HDL-cholesterol concentration. (Izzo et al., 1981)
6.5.6.9. Estimation of LDL-Cholesterol

Estimation of LDL cholesterol was done using the Friedewald formula.

\[
LDL \text{ cholesterol} = TC - HDL - \frac{TG}{5}
\]

Normal range of total cholesterol is 150-250 mg/dl and HDL normal for Male:
30-70 mg/dl; Female: 30-80 mg/dl. (Friedewald et al., 1972)

6.5.6.10. Estimation of triglycerides: Triglycerides in the sample are hydrolyzed by microbial lipase to glycerol and free fatty acid (FFA). Glycerol is phosphorylated by adenosine 5-triphosphate (ATP) to glycerol 3-phosphate (G-3-P) in reaction catalyzed by glycerol kinase. G-3-P is oxidized to dihydroxy acetone phosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction hydrogen peroxide (H₂O₂) is produced in equimolar concentration to the level of triglycerides present in the sample. H₂O₂ reacts with 4-amoanitpyraine and ADPS in the reaction catalyzed by peroxidases (POD). The result of this oxidative coupling is a quinoneimine purple colored dye. The absorbance of this dye in solution is proportional to the concentration of triglycerides. (Bucolo, 1973)

6.5.6.11. Histopathologic analysis

Part of the liver and pancreas tissue were preserved in 10% formalin for 2 days. The liver and pancreas were dehydrated with alcohol (subsequently with 70, 80, 90%, and absolute alcohol) for 12 h each. Again the tissues were cleaned by using xylene for 15-20 min and they were subjected to paraffin infiltration in automatic tissue processing unit. The tissue blocks were prepared and the blocks were cut using microtome to get sections of thickness 5 µm. The sections were taken on a microscopic slide on which egg albumin (sticky substance) was applied and
allowed for drying. Finally, the sections were stained with eosin (acidic stain) and hemotoxylin (basic stain).

6.5.6.12. Statistical analysis

All the data were expressed as mean ± SEM. Statistical significance between the groups were tested using one-way analysis of variance (ANOVA) followed by Dunnett's t-test post-hoc test. A P less than 0.5 was considered significant.