Chapter: 4

Materials and Methods

4.0 MATERIALS AND METHODS

4.1 Plant materials:
The selection of plant species for this study was based on their traditional use for diabetes treatment, the information being culled from published sources and traditional healers. The plant parts bark, seeds, leaves and roots of *Acacia nilotica* were selected for the present studies.

4.2 Collection, identification and authentification of plant parts:
The plant parts (bark, seeds, leaves and roots) of *A. nilotica* L. (AN) were collected from medicinal garden of Modern Institute of Pharmaceutical Sciences, Madhya Pradesh, India and authentified by Dr. Sanjay Vyas, Prof. Department of Botany, Government Holkar Science College (An Autonomous Institute and Centre of Excellence) Indore, M.P., and voucher specimen number (AN/2008-2009/01) were preserved in institute department for future reference.

4.3 Preparation of extracts:
The extraction yield of the extracts from plants species is highly depends on the solvent polarity, which determines both qualitatively and quantitatively the extracted compounds. Ethanol, and water are the most widely used solvent for the extraction because of their low toxicity and high extraction yield with the advantage of modulating the polarity of the solvent by using mixtures at different ratios (Jackson et al., 1996).
The seeds and leaves were shade dried and barks and roots were cut into small fragments and then shade dried. The plant parts were dried to control temperature, humidity and damage of active constituents (WHO, 2003).
Then dried plant material of seeds, barks and roots was powdered individually by using grinder except leaves which were manually grinded and defatted with petroleum ether, separately. Defatted 500 gm of each powder was extracted by 95% ethanol in a soxhlet apparatus for 72h as well as for aqueous extract 500 gm of each powder was extracted...
with chloroform water I.P. by cold maceration followed by concentrated in a rotator evaporator under reduced pressure at temperature 40-50°C and then lyophilized to get a dry residue.

The percentage yields of the extracts were calculated with reference to air dried powder. Some part of the total extract was used for qualitative and quantitative phytochemical investigation and rest of the extract was used for preliminary pharmacological screening.

4.4 Qualitative and quantitative phytochemical screening of various extracts of AN:

4.4.1 Qualitative phytochemical screening of various extracts of AN:
AN bark alcoholic extracts (ANB. Alc. Ext.), bark aqueous extracts (ANB. Aq. Ext.), seed alcoholic extract (ANS. Alc. Ext.), seed aqueous extract (ANS. Aq. Ext.), leaves alcoholic extract (ANL. Alc. Ext.), leaves aqueous extract (ANL. Aq. Ext.), root alcoholic extract (ANR. Alc. Ext.), root aqueous extract (ANR. Aq. Ext.) were subjected to qualitative chemical analysis to detect the presence of various phytoconstituents. The various tests and reagents used are given below.

4.4.1.1 Tests for carbohydrates:

Preparation of test solution: The test solution was prepared by dissolving the test extract in water.

Molish’s test: Test solution with few drops of Molish’s reagent and concentrated \( \text{H}_2\text{SO}_4 \) added slowly from the sides of the test tube. Formation of violet ring at the junction of two liquids indicates the presence/absence of carbohydrates.

Fehling’s test: The test solution when heated with equal volume of Fehling’s A and B solutions, formation of brick red precipitate, indicates the presence/absence of reducing sugars.

Benedict’s test: The test solution mixed with Benedict’s reagent in equal quantity and heat in boiling water bath for five min formation of green, yellow or red color indicates the presence/absence of reducing sugars.
4.4.1.2 Tests for proteins and amino acids:

*Preparation of test solution:* The test solution was prepared by dissolving the extract in water.

*Biuret test:* To the test solution, 4% sodium hydroxide and few drops of 1% copper sulphate solution was added, development of violet or pink color indicates the presence/absence of protein.

*Million’s test:* To the test solution, Million’s reagent was added and warm on a heated water bath formation of red color indicates the presence/absence of protein.

*Xanthoproteic test:* To the test solution, one ml of concentrated sulphuric acid was added, white precipitate is formed, which turns into yellow and on addition of sodium hydroxide formation of orange color precipitate indicates the presence/absence of protein.

4.4.1.3 Tests for glycosides:

*Preparation of test solution:* The test solution was prepared by dissolving extract in the water and alcohol.

*Test for cardiac glycosides:*

*Legal’s test:* To the test solution, 1ml of pyridine and 1ml of alkaline sodium nitroprusside solution was added formation of blood red color indicates the presence/absence of cardiac glycosides.

*Keller-Killiani test (test for deoxy sugars):* To the test solution, glacial acetic acid, few drops of FeCl₃ and concentrated H₂SO₄ solution was added formation of reddish brown color at the junction of two liquid layers and upper layer turns blue indicates the presence/absence of deoxy sugars.

*Test for anthraquinone glycosides:*

*Borntrager’s test:* Test solution was boiled with few ml of dilute sulphuric acid for five min and then filtered. To the cool filtrate equal volume of chloroform was added, and shake for few minutes. Lower layer was separated and then treated with half of its volume of dilute ammonia solution. Formation of rose pink to red color in
ammonical layer indicates the presence of the presence/absence of anthraquinones glycosides.

4.4.1.4 Test for flavonoids:

*Preparation of test solution:* The test solution was prepared by dissolving extract in the alcohol.

*Shinoda test:* To the test solution, few drops of concentrated HCl and few pieces of magnesium turnings was added formation of pink scarlet, crimson red or occasionally green to blue color indicates the presence/absence of flavonoids.

*Alkaline reagent test:* To the test solution, few drops of sodium hydroxide solution were added, intense yellow color was formed which turns to colorless on addition of few drops of dilute HCl indicates the presence/absence of flavonoids.

4.4.1.5 Test for saponins (*Foam test*):
Test extract was dissolved in water, separately and shake for few minutes, formation of two cm layer of foam (stable for at least 15 min), indicate the presence/absence of saponins.

4.4.1.6 Test for alkaloids:

*Preparation of test solution:* The test solution was prepared by dissolving the extracts in the dilute hydrochloric acid, solution was filtered. The filtrate was then subjected to following tests for the detection of presence of alkaloids:

*Mayer’s test:* Test solution treated with Mayer’s reagent (Potassium Mercuric iodide solution), cream colored precipitate formation indicates the presence/absence of alkaloids.

*Hager’s test:* Test solution treated with Hager’s reagent (Saturated picric acid solution), yellow colored precipitate formation indicates the presence/absence of alkaloids.

*Dragendorff’s test:* Test solution treated with Dragendorff’s reagent (Potassium bismuth iodide solution) reddish brown colored precipitate formation indicates the presence/absence of alkaloids.
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**Wagner's test:** Test solution treated with Wagner's reagent (Iodine-potassium iodide solution) brown colored precipitate formation indicates the presence/absence of alkaloids.

4.4.1.7 Test for steroids and triterpenoids:

**Preparation of test solution:** The test solution was prepared by dissolving the extracts in chloroform and subjected to following test

_**Libermann-Burchard’s test:** _To the test solution few drops of acetic anhydride was added, boil and cool the content. Then concentrated sulphuric acid was added from the side of the test tube, formation of brown ring at the junction of two layers and the upper layer turns green which indicates the presence of steroids and formation of deep red color indicates the presence/absence of triterpenoids.

_**Salkowski’s test:** _To the test solution few drops of concentrated sulphuric acid was added and allow to stand for some time, formation of red color in the lower layer indicates the presence of steroids and formation of yellow colored lower layer indicates the presence/absence of triterpenoids.

4.4.1.8 Tests for tannins (phenolic compounds):

**Preparation of test solution:** The test solution was prepared by dissolving extract in water and alcohol.

_**Ferric chloride test:** _To the test solution, 2-3 drops of 5% ferric chloride solution was added, formation of greenish black color indicates the presence/absence of tannins.

_**Gelatin test:** _To the test solution, few ml of 1% solution of gelatin containing 10% sodium chloride was added formation of white precipitate indicates the presence/absence of tannins.

_**Lead acetate test:** _To the test solution, few ml of 10% lead acetate was added formation of bulky white color precipitate indicates the presence of tannins (Khandelwal, 2006).
4.4.2 Quantitative phytochemical screening of various extracts of AN:

4.4.2.1 Estimation of total phenolics content:

**Principle:** Phenols react with phosphomolybdic acid in Folin-Ciocalteau’s reagent in alkaline medium to produce a blue-colored complex (molybdenum blue) which can be estimated spectrophotometrically at 760 nm.

**Procedure:** Total phenolics in alcoholic and aqueous extracts of AN were determined with Folin-Ciocalteu’s reagent. One ml of each extracts (1mg/ml in distilled water) was taken in separate volumetric flask and one ml of Folin-Ciocalteu reagent was added in both flasks. After three minute, three ml of 2% Na$_2$CO$_3$ was added. Subsequently, the mixture was shaken for two hours at room temperature and absorbance was read at 760nm and experiment was performed in triplicate (Singh et al., 2011).

\[
A = 0.001C + 0.0033
\]

Where, \(A\) is absorbance and \(C\) is concentration, \((\text{GAE})\) g/100 gm of dry matter.

GAE: Gallic acid equivalent

4.4.2.2 Estimation of total flavonoids content:

**Principle:** Total flavonoids content in alcoholic and aqueous extracts of AN was measured by aluminum chloride colorimeter assay. Flavonoids react with aluminum chloride reagent to produce a colored product which can be measured spectrophotometrically at 510nm.

**Procedure:** One gm of alcoholic and aqueous extracts of AN were macerated with 100 ml methanol (hot decoction) for one hr followed by filtration. One ml of each extracts were placed in 10 ml volumetric flask separately three ml of methanol and 0.3 ml NaNO$_2$ was added and After five minutes three ml of AlCl$_3$ was added. Two ml of 1M NaOH was added after six min and total volume was made upto ten ml with methanol. The absorbance was quantified against a blank at 510nm and the total flavonoids content was calculated using following equation (Zhinsen et al., 1999).

\[
A = 0.01069C - 0.001163
\]

Where, \(A\) = absorbance, \(C\) = flavonoids content \((\text{CE})\) g/100 gm of dry matter

CE: Catechin equivalent
4.5 Thin layer chromatography of various extracts of AN:
The extracts were subjected to thin layer chromatography for preliminary identification of the phytoconstituents.

4.5.1 Preparation of the plates: Silica gel G was taken in a glass mortar and distilled water was added to it. The mixture was stirred with glass rod until it became homogeneous. This mixture was then allowed to swell for about 15 minutes. Then an additional 15 ml of distilled water was added to it with stirring. This suspension was manually spread immediately on the thin layer chromatographic plate.

4.5.2 Drying and storage of the plates: The freshly coated plates were then air dried until the transparency of the layer had disappeared. The plates were then stacked in a drying rack and were heated in an oven for 30 min. at 110°C. The activated plates were kept in a desiccator, till required for further use.

4.5.3 Application of the sample: For applying test samples on TLC plate, glass capillaries were used, keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the samples were marked on the top of the plate to know their identity.

4.5.4 Chromatographic chamber, conditions of saturation and the development of TLC plates: Chromatographic rectangular glass chamber (16.5 cm x 29.5 cm) was used in the experiments. To avoid insufficient chamber saturation and the undesirable edge effect, a smooth sheet of filter paper approximately of 15 x 40 cm size was placed in the chromatographic chamber in a ‘U’ shape and was allowed to be soaked in the developing solvent. After being thus moistened, the paper was then pressed against the walls of the chamber, so that it adhered to the walls. The chamber was allowed to saturate for 24 hours before use. The experiments were carried out at room temperature in diffused daylight.

4.5.5 Developing solvent system: A number of developing solvent systems was tried, for each residue, and the satisfactory resolution system was noted.

4.5.6 Detector/ spraying equipment: Compressed air sprayer with a fine nozzle was used to detect the different constituents present on TLC plates. Air compressor was attached to a glass sprayer. The sprayer was filled with about 50 ml of the detecting
reagent and then used. After each spray, the sprayer was washed separately with water, chromic acid, and distilled water and then with acetone. UV chamber was also used for the substances exhibiting fluorescence nature. The results are observed at 366 nm of UV light and in day light (Mukherjee, 2002).

4.6 Free radical scavenging assays:

4.6.1 DPPH (α, α- diphenyl β-picryl hydrazyl) Free Radical Scavenging Activity:

Principle: The DPPH is stable free radical which is purple in colour and upon reaction with an antioxidant; it becomes colourless and is converted to diphenyl picryl hydrazine. It can be quantified spectrophotometrically at 517 nm to indicate the extent of DPPH scavenging activity by the plant extracts (Anandjiwala et al., 2007).

\[
\begin{align*}
\text{DPPH} & \quad \text{H} \\
\text{DPPH} & \quad \text{HN} \\
\text{DPPH} & \quad \text{NO}_2
\end{align*}
\]

Procedure: One milliliter solution of extract of different concentration (20-160 µg/ml) was added to one ml of 0.1mM DPPH solution. The contents were mixed vigorously and allowed to stand at 20°C for 30 min. The absorbance was quantified at 517nm. The experiment was performed in triplicate and IC$_{50}$ value (the concentration required to scavenge 50% DPPH free radicals) was calculated (Shirwaikar et al., 2006).

4.6.2 Nitric Oxide Scavenging Activity:

Principle: Nitric oxide (NO) is a very unstable free radical generated from sodium nitroprusside at physiological pH, which can be measured by Griess reagent. It reacts with O$_2$ to produce the stable product nitrates and nitrite through intermediates like NO$_2$, N$_2$O$_4$ and N$_3$O$_4$. In the presence of test compound, which is a scavenger, the amount of
nitrous acid will decrease. The extent of decrease will reflect the extent of scavenging, which is measured at 546 nm (Shirwaikar et al., 2006).

**Procedure:** The reaction mixture comprising of 2.5 ml of different concentrations of various extracts (20-160 µg/ml) and 0.75ml of 5mM of sodium nitroprusside. The test tubes were incubated at 25°C for 5hours. After 5 hrs, 0.5ml of Griess reagent was added. For control, Griess reagent and sodium nitroprusside solution was quantified spectrophotometrically at 546nm. The experiment was performed in triplicate (Shirwaikar et al., 2006).

### 4.6.3 ABTS Scavenging Activity:

**Principle:** This method involves the scavenging of ABTS [2, 2’ azino bis (3-ethylbenzthiazoline-6-sulfonic acid) diaminonium salt] radical cation. The principle involves the reaction between ABTS and potassium persulphate to produce the ABTS radical cation, a blue green chromogen. In the presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS, the absorbance is read at 745 nm (Shirwaikar et al., 2006).

**Preparation of ABTS radical cation:** The cation was prepared by reacting ABTS solution (7mM) with 2.45mM of ammonium persulphate and mixture was allowed to stand in dark at room temperature for 12-16 hrs before use.

**Procedure:** 1.5ml of different concentration of various extract (20-160 µg/ml) was added with 0.9ml of ABTS radical cation. The absorbance was read at 745nm and all experiment was performed in triplicate (Shirwaikar et al., 2006).

### 4.6.4 Superoxide Dismutase Scavenging Activity Riboflavin–EDTA Method:

**Principle:** Phosphate buffer and riboflavin was used as superoxide generated system. The generated superoxide will react with NBT to give coloured diformazan. Diformazan being insoluble in water slowly precipitate out. Therefore, the spectral measurement must be done immediately after the reaction is carried out. In the presence of scavenger reduction of NBT occurs which measured at 560 nm (Srinivasan et al., 2007).
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**Procedure:** The reaction mixture contains one ml of sodium phosphate buffer (50mM), one ml of EDTA (12mM), 20 µg riboflavin and 0.1ml of NBT and one ml of different concentrations of various extracts (20-160 µg/ml). The absorbance was quantified at 560nm and experiment was performed in triplicate (Patel et al., 2011).

**4.6.5 Iron chelating activity**

**Principle:** It is based reduction of ferric ions by ortho-phenanthroline colour method. o-phenantroline quantitatively forms complex with Fe²⁺, which get disrupted in the presence of chelating agents. The antioxidant interfered with the formation of ferrous-o-phenantroline complex which is spectrophotometrically read at 510 nm.

**Procedure:** The reaction mixture containing one ml 0.05% o-phenantroline (w/v), two ml ferric chloride (200 µm) and two ml various concentration of the extract (20-160 µg/ml) was incubated for 10 min. The absorbance was read at 510 nm and experiment was performed in triplicate (Shirwaikar et al., 2006). The percentage scavenging was calculated by using formula.

\[
\text{% Scavenging} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100
\]

**4.7 Preliminary pharmacological study:**

Preliminary pharmacological studies involved acute oral toxicity study, oral glucose tolerance test (OGTT) and hypoglycemic activity of alcoholic and aqueous extracts of various parts of AN. and extract which showed significant results were selected for antidiabetic activity followed by fractionation.

**4.7.1 Experimental animals:**

Healthy adult wistar albino rats (150-200 gm) and swiss albino mice (25-30 gm) were used for the study. The animals were stabilized for 1 week, housed in polypropylene cages, maintained under standard conditions (12 h light and 12 h dark cycle, 25 ± 30°C ), the animals were fed with standard pellet diet and water *ad-libitum* throughout the course of the study. The animals were handled gently to avoid giving them too much stress,
which could result in an increased adrenal output. The institutional ethical committee for animal care and use approved the experimental design. The normoglycemic animals were selected for this experiment having the fasting blood glucose level around 80 mg/dL. The hyperglycemic animals were selected having fasting blood glucose concentration around 200-300 mg/dL.

4.7.2 Preparation of the dose:
The suspension of alcoholic extracts of various parts were freshly prepared in tween 80 and suspended in distilled water and glibenclamide 5 mg/kg p.o was prepared in tween 80 suspended in distilled water.

4.7.3 Acute oral toxicity study (OECD Guidelines, 2001):
**Principle:** The acute toxicity test aims at establishing the therapeutic index. The acute toxicity study was done according to OECD (Organization of Economic Co-operation and Development) guidelines 420- Fixed Dose Procedure (FDP), as in annex 2D.

**Procedure:** The suspension of alcoholic extract and aqueous extract of various parts was administrated orally to overnight fasted swiss albino mice (n=6) at dose of 2000 mg/kg b.w. respectively. The animals were observed continuously for the initial 4hrs for behavioral changes and mortality and intermittently for the next 6 h and then again at 24 h and 48 h after dosing for 14 days. The behavior parameters observed were convulsion, hyperactivity, sedation, grooming, loss of righting reflex and increased respiration.

**Selection of Doses:** In this study dose of 2000 mg kg$^{-1}$ was found to be safe, so dose 1/10th i.e. 200 mg kg$^{-1}$ was chosen for the experimentation.

4.7.4 Oral glucose tolerance test (OGTT)
**Principle:** The oral glucose tolerance test (OGTT) measures the body's ability to use main source of energy i.e. glucose. OGTT is to simplify and facilitate the diagnosis of diabetes (Luzi, 1998). This method is often referred to as physiological induction of diabetes mellitus because the blood glucose level of the animal is transiently increased with no damage to the pancreas.
Procedure: OGTT was performed according to the method reported by Sy GY, 2005. Overnight fasting normal rats were divided into ten groups of six animals per group (n=6). Group I served as control received vehicle only (Tween 80 in distill water). Group II served as standard group and received glibenclamide (5 mg kg⁻¹ p.o.) suspended in vehicle, Group III to X received 200 mg kg⁻¹ oral dose of ANB. Alc. Ext., ANB. Aq. Ext, ANS. Alc. Ext., ANS. Aq. Ext, ANL. Alc. Ext., ANL. Aq. Ext. ANR. Alc. Ext. and ANR. Aq. Ext. suspended in vehicle. All animals were loaded with glucose (1.5 g kg⁻¹, p.o.) 30 min after the extract and drug administration. The blood samples were collected by snipping tail with surgically sterilized needle. Blood glucose was determined just prior to glucose administration (0h) and 1, 2, 3 and 6h after glucose administration. Blood glucose concentration was estimated by the glucose oxidase enzymatic method, using Accu-chek Active™ Test strips in Accu-chek Active™ Test meter (Sy et al., 2005).

Table 4.1: Effect of various extracts of AN on BGL of glucose loaded hyperglycemic rats

<table>
<thead>
<tr>
<th>GROUPS (n=6)</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Received vehicle only (Control)</td>
</tr>
<tr>
<td>Group-II</td>
<td>Received Glibenclamide (5 mg kg⁻¹ p.o.) (Standard drug treated)</td>
</tr>
<tr>
<td>Group-III</td>
<td>Received oral dose of 200 mg/kg bw of ANB. Alc. Ext.</td>
</tr>
<tr>
<td>Group-IV</td>
<td>Received oral dose of 200 mg/kg bw of ANB. Aq. Ext.</td>
</tr>
<tr>
<td>Group-V</td>
<td>Received oral dose of 200 mg/kg bw of ANS. Alc. Ext.</td>
</tr>
<tr>
<td>Group-VI</td>
<td>Received oral dose of 200 mg/kg bw of ANS. Aq. Ext.</td>
</tr>
<tr>
<td>Group-VII</td>
<td>Received oral dose of 200 mg/kg bw of ANL. Alc. Ext.</td>
</tr>
<tr>
<td>Group-VIII</td>
<td>Received oral dose of 200 mg/kg bw of ANL. Aq. Ext.</td>
</tr>
<tr>
<td>Group-IX</td>
<td>Received oral dose of 200 mg/kg bw of ANR. Alc. Ext.</td>
</tr>
<tr>
<td>Group-X</td>
<td>Received oral dose of 200 mg/kg bw of ANR. Aq. Ext.</td>
</tr>
</tbody>
</table>
4.7.5 Hypoglycemic activity:

**Principle:** The hypoglycemic activity is significant in the diagnosis of diabetes mellitus. It measures the ability of drug to reduce blood glucose level. This method allows for the effect of the drug to be tested in the animal with an intact pancreatic activity. The comparison may give some information regarding mechanism of action.

**Procedure:** Hypoglycemic activity was performed according to the method reported by Kesari AN, 2006. Experimental animals were divided into ten groups of six animals per group (n = 6). Group I served as a control received vehicle (Tween 80 in distill water). Group II served as standard group received glibenclamide (5 mg kg\(^{-1}\) p.o.) suspended in vehicle and Group III to X received 200 mg kg\(^{-1}\) oral dose of ANB. Alc. Ext., ANB. Aq. Ext., ANS. Alc. Ext., ANS. Aq. Ext, ANL. Alc. Ext., ANL. Aq. Ext. ANR. Alc. Ext. and ANR. Aq. Ext. suspended in vehicle. The blood glucose level of all experimental rats was determined at 0 hr (before oral administration), by snipping tail with surgically sterilized needle and then after 1, 2, 3, 4 and upto 6 h respectively (Kesari et al., 2006).

**Table 4.2:** Effect of various extracts of AN on BGL of normoglycaemic rats

<table>
<thead>
<tr>
<th>GROUPS (n=6)</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Received vehicle only (Control)</td>
</tr>
<tr>
<td>Group-II</td>
<td>Received Glibenclamide (5 mg kg(^{-1}) p.o.) (Standard drug treated)</td>
</tr>
<tr>
<td>Group-III</td>
<td>Received oral dose of 200 mg/kg bw of ANB. Alc. Ext.</td>
</tr>
<tr>
<td>Group-IV</td>
<td>Received oral dose of 200 mg/kg bw of ANB. Aq. Ext</td>
</tr>
<tr>
<td>Group-V</td>
<td>Received oral dose of 200 mg/kg bw of ANS. Alc. Ext.</td>
</tr>
<tr>
<td>Group-VI</td>
<td>Received oral dose of 200 mg/kg bw of ANS. Aq. Ext.</td>
</tr>
<tr>
<td>Group-VII</td>
<td>Received oral dose of 200 mg/kg bw of ANL. Alc. Ext.</td>
</tr>
<tr>
<td>Group-VIII</td>
<td>Received oral dose of 200 mg/kg bw of ANL. Aq. Ext.</td>
</tr>
<tr>
<td>Group-IX</td>
<td>Received oral dose of 200 mg/kg bw of ANR. Alc. Ext.</td>
</tr>
<tr>
<td>Group-X</td>
<td>Received oral dose of 200 mg/kg bw of ANR. Aq. Ext.</td>
</tr>
</tbody>
</table>
4.7.6 Antidiabetic activity

4.7.6.1 Induction of diabetes:
Overnight fasted rats were made diabetic by single intra peritoneal injection of freshly prepared solution of alloxan monohydrate in normal saline at a dose of 120 mg/kg b.w. (Viana et al., 2004). Alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, in order to stave off the hypoglycemia during the first day of alloxan administration rats were treated with 20% glucose solution (5-10 mL) orally after 6 hours for the next 24 hours. After 5 days blood samples was collected and blood glucose levels were determined to confirm diabetes. The rats with blood glucose level > 250 mg/dL were considered to be diabetic and were used in experiment (Barry et al., 1997).

Procedure: Diabetic rats were divided into five groups of six animals each (n=6). Group I served as a control received vehicle (Tween 80 in distill water). Group II, diabetic control received vehicle (Tween 80 in distill water). Group III received glibenclamide as reference drug (5 mg/kg p.o.) suspended in vehicle. Group IV and V diabetic rats received oral dose of 200 mg/kg of ANB. Alc. Ext. and ANB. Aq. Ext. All treatments were continued for 21 days.

Table 4.3: Effect of ANB. Alc. Ext. and ANB. Aq. Ext. on BGL of alloxan induced diabetic rats

<table>
<thead>
<tr>
<th>GROUPS (n=6)</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Normal rats given vehicle only (Control)</td>
</tr>
<tr>
<td>Group-II</td>
<td>Diabetic rats given vehicle only (Diabetic control)</td>
</tr>
<tr>
<td>Group-III</td>
<td>Diabetic rats given Glibenclamide (5 mg kg⁻¹p.o.) (Standard)</td>
</tr>
<tr>
<td>Group-IV</td>
<td>Diabetic rats given oral dose of 200 mg/kg bw of ANB. Alc. Ext.</td>
</tr>
<tr>
<td>Group-V</td>
<td>Diabetic rats given oral dose of 200 mg/kg bw of ANB. Aq. Ext</td>
</tr>
</tbody>
</table>

4.7.6.2 Estimation of blood glucose levels: Extracts were given orally to the animals once before food was given. The blood glucose concentrations of the animals were
determined at the beginning of the study and the measurements were repeated on at 0th, 5th, 15th and 21st day after the initial of the experiment. Blood glucose concentration was estimated by the glucose oxidase enzymatic method, using Accu-check Active™ Test strips in Accu-check Active™ Test meter (Nagappa et al., 2003).

4.7.6.3 Evaluation of changes in body weight:
The body weights of experimental animals were recorded on 0th and 21st day of the experiment (Orhan et al., 2006).

4.7.6.4 Collection of serum and tissue samples
At the end of experimental period, rats were deprived of food overnight and sacrificed by diethyl ether anesthesia. Blood was collected by heart puncture. The Blood samples of each animal were taken and allowed to clot for 45 minutes at room temperature and serum was separated by centrifugation at 4500rpm for 10 min. The serum samples were collected and left standing on ice and analyzed for biochemical parameters including insulin and lipids. The tissues (liver, pancreas and kidney) were excised and transferred into ice-cold containers for biochemical estimations and histopathology.

4.7.6.5 Estimation of serum insulin levels:
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.

**Principle:** The ADVIA Centaur Insulin assay is a two-site sandwich immunoassay using direct chemiluminescent technology, which uses constant amounts of two antibodies. The first antibody, in the Lite Reagent, is a monoclonal mouse anti-insulin antibody labeled with acridinium ester. The second antibody, in the Solid Phase, is a monoclonal mouse anti-insulin antibody, which is covalently coupled to paramagnetic particles (Mishra et al., 2010).
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### Reagents used:

<table>
<thead>
<tr>
<th>Reagent Pack</th>
<th>Reagent</th>
<th>Volume</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADVIA Centaur IRI Ready Pack</td>
<td>Lite</td>
<td>5.0 mL/reagent pack</td>
<td>Monoclonal mouse anti-insulin antibody (~0.24μg/ml) labeled with acridinium ester in buffered saline with bovine serum albumin, sodium azide (&lt;0.1%), and preservatives.</td>
</tr>
<tr>
<td>IRI Ready Pack primary reagent pack</td>
<td>Reagent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid Phase</td>
<td>25mL/reagent pack</td>
<td></td>
<td>Monoclonal mouse anti-insulin antibody (~6.0μg/ml) covalently coupled to paramagnetic particles in buffered saline with bovine serum albumin, sodium azide (&lt;0.1%), and preservatives.</td>
</tr>
<tr>
<td>ADVIA Centaur IRI(DIL) Ready Pack ancillary reagent pack</td>
<td>Insulin Diluent</td>
<td>10.0mL/reagent pack</td>
<td>Buffered saline with casein, potassium thiocyanate (3.89%), sodium azide (&lt;0.1%), and preservatives.</td>
</tr>
</tbody>
</table>

### Assay Summary:

- **Sample type**: Serum
- **Sample volume**: 25μL
- **Calibrator**: IRI
- **Sensitivity and assay range**: 0.1 – 300 mU/L
**Chapter 4: Materials and Methods**

**Assay and Procedure:** Samples are free of fibrin or other particulate matter and free of bubbles.

**Reaction parameters:**
- **Reaction type:** Immunoassay
- **Detection:** As relative light units (RLUs)
- **Temperature:** 37°C
- **Measurement:** By Master curve calibration method

**Sensitivity and assay range:** The ADVIA Centaur insulin assay measures insulin concentrations up to 300mU/L with a minimum detectable concentration of 0.1 mU/L. Analytical sensitivity is defined as the concentration of insulin that corresponds to the RLUs that are two standard deviations greater than the mean RLUs of 20 replicate determinations of the insulin zero standard. ADVIA Centaur reports insulin results in: mU/L

**4.7.6.6 Estimation of lipids in serum:**
Serum was analyzed for serum total cholesterol (TC), total glycerides (TG), high density lipoprotein (HDL) were estimated using standard enzymatic colorimetric kits (Span diagnostics Ltd., India) (Bagri et al., 2009), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were calculated using formula.

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

**Principle:** The enzyme, cholesterol esterase catalyzed hydrolysis of cholesterol esters to free cholesterol and fatty acid molecules. Then free cholesterol gets oxidized in the presence of cholesterol to form cholesten-3-one and \( \text{H}_2\text{O}_2 \). Liberated \( \text{H}_2\text{O}_2 \) reacts with phenol and 4 AAP in presence of peroxidase to form red colored quinoneimine complex the intensity of which was measured at 505 nm (Allain et al., 1974; Roschlau et al., 1974)
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**Cholesteryl ester hydrolase**

\[
\text{Cholesterol ester + } \text{H}_2\text{O} \xrightarrow{\text{Cholesteryl ester hydrolase}} \text{Cholesterol + Fatty acid}
\]

The 3-OH group of cholesterol is then oxidized to ketone in oxygen requiring reaction catalyzed by cholesterol oxidase.

**Cholesterol oxidase**

\[
\text{Cholesterol + O}_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-3-one + H}_2\text{O}_2
\]

\(\text{H}_2\text{O}_2\) one of the reaction products is measured in a peroxidase catalyzed reaction that forms a dye.

**Peroxidase**

\[
2\text{H}_2\text{O}_2 + \text{Phenol + 4 AAP} \xrightarrow{\text{Peroxidase}} 4\text{H}_2\text{O} + \text{Quinoneimine dye}
\]

**Reagents used**

<table>
<thead>
<tr>
<th>Reagent composition</th>
<th>Concentration in the final test mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good’s butter (pH 6.7)</td>
<td>50mmol/l</td>
</tr>
<tr>
<td>Phenol</td>
<td>5mmol/l</td>
</tr>
<tr>
<td>4 - Aminoantipyrine</td>
<td>0.3 mmol/l</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td>(\geq 200) U/l</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>(\geq 100) U/l</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>(\geq 3) KU/l</td>
</tr>
</tbody>
</table>

**Standard:** The concentration of standard glucose used was 200mg dl

**Assay & Procedure:** Fresh clear and unhaemolysed serum was used for the estimation.

**Reaction parameters:**

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>505 nm</td>
</tr>
<tr>
<td>Optical path</td>
<td>1 cm</td>
</tr>
</tbody>
</table>
Chapter 4: Materials and Methods

Temperature: 37°C
Measurement: Against reagent blank

Summary of assay details:

<table>
<thead>
<tr>
<th>Pipetted into test tube</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

The reaction mixtures were mixed well and incubated for 10 min at 37°C. The absorbance of reaction mixtures at 505nm against reagent blank was taken. The absorbance was measured by using spectrophotometer.

Serum total Cholesterol (mg/dl) = \( \frac{\text{Absorbance of test} \times \text{Concentration of standard}}{\text{Absorbance of Standard}} \)

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

**Principle:** The enzyme, lipoprotein lipase catalyzes hydrolysis of TGs to glycerol and FAs. Glycerol then is phosphorylated in an ATP-requiring reaction catalyzed by glycerokinase. The formed glycerophosphate is oxidized to dihydroxyacetone and \( \text{H}_2\text{O}_2 \) in a glycerophosphate oxidase catalyzed reaction. \( \text{H}_2\text{O}_2 \) then reacts with 4-AAP and 4-chlorophenol under the catalytic influence of peroxidase to form coloured quinoneimine complex, the intensity of which was measured at 505nm (Fossati, et al., 1982; Trinder et al., 1969).

**Reaction Scheme:**

\[
\text{Triglyceride} + 3\text{H}_2\text{O} \xrightarrow{\text{Lipase}} \text{Glycerol} + 3 \text{fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol–3-phosphate} + \text{ADP}
\]
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Glycerophosphate oxidase

\[ \text{Glycerophosphate oxidase} \rightarrow \text{DHAP} + \text{H}_2\text{O} \]

Peroxidase

\[ \text{Peroxidase} \rightarrow \text{Quinoneimine} + \text{HCl} + 4\text{H}_2\text{O} \]

Reagents used

<table>
<thead>
<tr>
<th>Reagent Composition</th>
<th>Conc. in the final test mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipes butter</td>
<td>50 mmol/l</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>Mg2+</td>
<td>5 mmol/l</td>
</tr>
<tr>
<td>ATP</td>
<td>1 mmol/l</td>
</tr>
<tr>
<td>Lipase</td>
<td>( \geq 5000 ) U/l</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>( \geq 1000 ) U/l</td>
</tr>
<tr>
<td>Glycerol Kinase</td>
<td>( \geq 400 ) U/l</td>
</tr>
<tr>
<td>Glycerol-3-phosphate oxidase</td>
<td>( \geq 4000 ) U/l</td>
</tr>
<tr>
<td>4-mimoantigpyrine (4-AAP)</td>
<td>0.4 mmol/l</td>
</tr>
</tbody>
</table>

Standard: The concentration of standard triglyceride used was 200mg/dl

Assay & Procedure: Fresh clear and unhaemolysed serum was used for the estimation.

Reaction parameters:

- Reaction type: End point
- Wave Length: 505 nm
- Optical length: 1 Cm
- Temperature: 37°C
- Measurement: Against reagent blank

Summary of assay details:

<table>
<thead>
<tr>
<th>Pipetted in to test tube</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>1000 μl</td>
<td>1000 μL</td>
<td>1000 μl</td>
</tr>
</tbody>
</table>
The reaction mixtures were mixed well and incubated for 10 min at 37°C. The absorbance of sample and standard were measured against reagent blank at 505 nm. The absorbance was measured by using a spectrophotometer (Fossati, 1982).

**Calculation:**

\[
\text{Serum Triglycerides (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard}
\]

4.7.6.6.3 **Estimation of serum high-density lipoprotein cholesterol (HDL-C):** Span diagnostic kit was used for estimation of HDL cholesterol, which followed Cholesterol oxidase/ peroxidase (CHOD-POD) method.

**Principle:** HDL-C is measured in the supernatant after the precipitation of the lipoproteins including chylomicrons, very low-density lipoproteins, low-density lipoproteins, intermediate-density lipoproteins directly from serum polyanions like phosphotungstic acid and along with MgCl₂ are added to an aliquot of serum an immediate heavy precipitation is formed. The precipitate then is sedimented by centrifugation and HDL cholesterol is measured in the clear supernatant, which is estimated by enzymatic method as described earlier in estimation serum of TC (Badimon et al., 1990).

**Assay & Procedure:** Fresh clear and unhaemolysed serum was used for the estimation.

**Standard:** The concentration of standard glucose used was 50mg/dl

**Reaction parameters:**

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>End point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>505 nm</td>
</tr>
<tr>
<td>Optical path</td>
<td>1 cm</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
</tbody>
</table>
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Measurement Against reagent blank

Summary of assay details:

1. 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.
2. Centrifuged for 15 min at 4000 rpm.
3. The clear supernatant was separated and immediately used to determine the cholesterol content as follows:

<table>
<thead>
<tr>
<th>Pipetted into test tube</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>100 μl</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant from step 3</td>
<td>-</td>
<td>-</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

The reaction mixtures were mixed well and incubated for 10 min at 37°C. The absorbance of test and standards was measured against the reagent blank at 505nm. The absorbance was measured by using a spectrophotometer (Burstein et al., 1970)

Serum HDL Cholesterol (mg/dl) = \( \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard} \)

4.7.6.4 Estimation of serum low-density lipoprotein cholesterol (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.

Calculation:
Serum LDL cholesterol = Total cholesterol - (HDL cholesterol + VLDL cholesterol)

4.7.6.5 Estimation of serum very low-density lipoprotein cholesterol (VLDL-C):
Using the data obtained including triglycerides, the VLDL cholesterol levels were calculated using empirical equation of Friede Wald (Sacks, 2006)
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Calculation:
Serum VLDL cholesterol = Triglycerides/5

4.7.6.7 Determination of the lipid peroxidation:

4.7.6.7.1 Preparation of tissue homogenates of pancreas and kidney:
The pancreas and kidney were homogenized in 0.025 M Tris–HCl buffer, pH 7.5 after centrifugation at 10,000 X g for 10 min, the clear supernatant was used for the estimation of thiobarbituric acid reactive substances (TBARS).

Principle: Malondialdehyde formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde react with thiobarbituric acid to form thiobarbituric reacting substance (TBARS) which is pink chromogen, quantified at 532 nm.

Procedure: Acetic acid 1.5ml (20%, pH 3.5), 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) was added to 0.1ml of supernatant and heated at 100°C for 60min. Mixture was cooled and five ml of n-butanol pyridine (15:1) mixture was added with one ml of distilled water and vortex vigorously. After centrifugation at 1200 x g for 10 minute, the organic layer was separated and absorbance was measured at 532 nm. It was calculated using a molar extinction coefficient of 1.56 x 10^5 M^-1 cm^-1 and expressed as nanomoles of TBARS, mg of protein. (Mukherjee et al., 2007)

Calculations were made as per the formula:

Inhibitory rate = [1 - (A_1 - A_2) / A_0] X 100

Where A_0 was the absorbance of control (without extract) and A_1 was the absorbance in the present of the extract, A_2 was the absorbance without liver homogenate

4.7.6.8 Estimation of in vivo antioxidant activity:
Preparation of Liver homogenate: Liver was rinsed in ice-cold saline to remove the blood and immediately freezeed and stored at −800°C. The liver was homogenized in 50mM phosphate buffer solution (pH 7.4) using a tissue homogenizer at 40°C. The
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homogenates were centrifuged at 15,000×g for 20 min and supernatant was used for analyses of glycogen, enzymatic and nonenzymatic antioxidants.

4.7.6.8.1 Estimation of enzymatic antioxidants:

4.7.6.8.1.1 Estimation of Superoxide Dismutase (SOD):

Principle: Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) and elemental oxygen (O$_2$) and as such provides an important defense against the toxicity of the superoxide radical. In this assay, superoxide ions (O$_2^-$) generated by xanthine oxidase (XOD) which convert xanthine to uric acid and hydrogen peroxide and nitroblue tetrazolium (NBT) to NBT-diformazan, quantified at 560 nm.

Procedure: Assay mixture contained 0.1mL of supernatant, 1.2mL of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1mL of phenazine methosulphate (186 µm), 0.3mL of nitro blue tetrazolium, 300 µM, 0.2mL of NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30°C for 90 s, the reaction was stopped by addition of 0.1mL of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0mL of n-butanol. Colour intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm and concentration of SOD was expressed as U/mg of protein (Mukherjee, et al., 2006).

Calculation:

$$\text{SOD (U/3ml)} = \frac{(A-B) \times 100}{A \times 50}$$

Where, A=Control group, B=Treated group, Unit × 10 = Unit/ml sample solution

4.7.6.8.1.2 Estimation of Catalase:

Principle: Catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian aerobic cells containing a cytochrome system. The enzyme contains four ferrihemoprotein groups per molecule. Catalase catalyses the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen. Hydrogen peroxide is formed in the eukaryotic cell
as a by-product of various oxidase and superoxide dismutase reactions. Hydrogen peroxide is highly deleterious to the cell and its accumulation causes oxidation of cellular targets such as DNA, proteins, and lipids leading to mutagenesis and cell death. Removal of the H$_2$O$_2$ from the cell by catalase provides protection against oxidative damage to the cell.

Catalases are able to decompose H$_2$O$_2$ by different reaction pathways.

**Catalytic pathway**

\[
\text{Protein-Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Protein-Fe}^{3+} - \text{OOH} + \text{H}_2\text{O} \quad \text{(Primary Complex)}
\]

\[
\text{Protein-Fe}^{3+} - \text{OOH} + \text{H}_2\text{O}_2 \rightarrow \text{Protein-Fe}^{3+} - \text{OH} + \text{H}_2\text{O} + \text{O}_2
\]

**Per oxidative decomposition**

The primary complex can also decompose by another pathway (peroxidatic decomposition)

\[
\text{Protein-Fe}^{3+} - \text{OOH} + \text{AH}_2 \rightarrow \text{Protein-Fe}^{3+} - \text{OH} + \text{H}_2\text{O} + \text{A}
\]

**Procedure:** 0.1 ml supernatant was added to cuvette containing 1.9 ml of phosphate buffer (50 mM, pH 7.4). Reaction was started by addition of 1 ml of freshly prepared 30 mM of hydrogen peroxide. The rate of decomposition of hydrogen peroxide was measured at 240 nm. Activity of catalase was expressed as nanomole/min/litre (Mukherjee, et al., 2007).

**Calculation:**

\[
\text{Catalase (U/L)} = \frac{\text{Absorbance} \times F}{\text{Total volume (ml)} \times \text{Sample volume (ml)} / 43.6}
\]

\[
F = 0.688
\]
4.7.6.8.1.3 Estimation of Glutathione peroxidase (GPx) activity:

**Principle:** It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (Nicotinamide Adenine Dinucleotide Phosphate, reduced). The decrease in NADPH absorbance during the oxidation of NADPH to NADP+ is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions.

\[
\text{GPx} \\
\text{R-OOH + 2 GSH} \rightarrow \text{R-OH + GSSG + H}_2\text{O} \\
\text{GR} \\
\text{GSSG + NADPH + H}^+ \rightarrow 2 \text{GSH + NADP}^+ 
\]

**Procedure:** The reaction mixture consist of 400µL 0.25M potassium phosphate buffer (pH 7.0), 200 µL supernatant, 100µL GSH (10mM), 100µL NADPH (2.5mM) and 100µL Glutathione reductase (6U/ml). The reaction was started by adding 100µL Hydrogen peroxide (12mM) and absorbance measured at 366nm at 1 min interval for 5 min. using a molar extinction coefficient of 6.22 x10³ M⁻¹ cm⁻¹. Data was expressed as mU/mg of Protein.

4.7.6.8.1.4 Estimation of Glutathione-s–Transferase (GST):

**Principle:** Glutathione-S-transferase (GSTs) is a group of enzymes that are important in the detoxication of many different xenobiotics in mammals. The enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics, and thereby defend cells against the mutagenic, carcinogenic, and toxic effects of the compounds. It is an important cellular antioxidant which indirectly inhibiting the free radicals (Dengiz et al., 2007).

**Procedure:** The reaction mixture consisted of 2.75 ml of sodium phosphate buffer (pH 7.4), 0.1 ml reduced glutathione (1mM), 0.1 ml supernatant in a total volume of 3.0 ml. The change in absorbance were measured at 340 nm and the enzyme activity was expressed as nanomoles of 1- Chloro 2,4- dinitro benzene (CDNB) conjugated formed
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/min/mg protein using a molar extinction coefficient of 9.6 x10^3 M^{-1} cm^{-1} (Mukherjee, et al., 2006).

Calculation:

\[ \text{GST (U/L)} = \frac{\text{Absorbance}}{0.0096} \times 30 \times 10 \]

Where,

Molar absorption coefficient = 0.0096

Dilution factor = 10

4.7.6.8.2 Estimation of non enzymatic antioxidants activity:

4.7.6.8.2.1 Glutathione Synthetase (GSH or Reduced Glutathione):

Principle: Reduced glutathione is an important cellular antioxidant. It maintains the normal structure of red blood cells and keeps hemoglobin in the ferrous state. It is involved in detoxication process. The toxic substance (organophosphate, nitro compound) are converted into mercapturic acid. Glutathione is involved in maintaining normal brain function (Lushchak, 2012).

Procedure: To 0.01ml of supernatant, two ml of phosphate buffer (pH 8.4), 0.5ml of 5,5 di thio bis -2 nitro benzoic acid (DTNB) and 0.4ml of double distilled water was added. Mixture was vortexed and absorbance was read at 412 nm with in 15 min. The concentration of reduced glutathione was expressed as µg/mg of protein (Mukherjee, et al., 2006)

Calculation -:

\[ \text{GSH (Mole/litre)} = \frac{\text{Absorbance}}{14700} \times 50 \]

Where,

Molar absorption coefficient = 14700, Dilution factor = 50

4.7.6.9 Estimation of Glycogen in liver:

The tissue samples were rinsed twice with ice cold saline and solubilized by incubating with 30% KOH at 55°C for 30 min. After neutralization with 1N HCl, 0.2 ml of cell suspension, 0.8 ml H_2O, and two ml anthrone reagent (0.2 g anthrone/100 ml 95% H_2SO_4) were mixed on ice, incubated at 100°C for 10 min, and placed on ice. Absorbance
was measured with a spectrophotometer at a wavelength of 620 nm (Hassid and Abraham, 1957). This was expressed as milligrams of glycogen per gram tissue.

4.7.6.10 Statistical Analysis:
The data were expressed as mean ± SEM. The data of antidiabetic activity were analyzed by one way analysis of variance (ANOVA) followed by “Dunnet’s test.” p value less than 0.05 was considered as statistically significant.

4.7.6.11 Histopathological studies of pancreas, liver and kidney:
Histopathological studies of the pancreas, liver and kidney were conducted in control and diabetic control, alcoholic and aqueous extracts of bark of Acacia nilotica at a dose of 200 mg/kg for 21 day.
On day 22nd when the animals were sacrificed, the pancreas, liver and kidney of all animals from each group was excised and washed with saline solution, soaked in filter paper and transferred into 10% formalin solution for histopathological studies.

Basic steps for tissue processing:
1. **Fixing:** when a tissue is removed from the body it begins to decompose. To preserve the natural state (as nearly as possible) the tissue is immediately placed into a solution called fixative (10% formal saline) and this process is called fixing.
2. **Grossing:** selection of tissue piece for processing.
3. **Embedding:** to provide necessary hardness to cut sections, infiltration of paraffin wax is carried out. This process is called embedding, which involves
   - Removal of water by alcohol dehydration,
   - Infiltration of xylene or chloroform as a solvent for paraffin wax and
   - Introduction of wax impregnation.
4. **Block Preparation:** Embedded the tissue in paraffin wax in block moulds.
5. **Microtomy:** This section (slices) of the tissue is cut by suing equipment called microtonge. This is performed when the paraffin is solidified when the tissue is ready in form of blocks. These thin sections are prepared for staining.
6. **Staining:** The paraffin is removed from the section on the slide by drying and
then by dewaxing the section in the slide are treated first xylene and then the section are dehydrated with ethyl alcohol in decreasing concentration. Using appropriate staining methods then stain are re hydrated sections.

7. **Moulding:** After staining, the section on the slide are dehydrated by treating with ethyl alcohol in increasing concentration and finally with xylene. By placing mounting medium such as canaa balsome and by placing a cover slip in the mounting medium, the stain section on the slide are said to be mounted.

### 4.8 Solvent-solvent fractionation of ANB. Alc. Ext.:

Considering the result obtained from preliminary pharmacological screening the most active extract. ANB. Alc. Ext. was further fractionated to separate out the different fractions. Dried bark alcoholic extract was suspended in water and fractionated through solvent-solvent fractionation with ethyl acetate (3×300ml) and then n butanol (3×300ml) in a separating funnel. Fractions and remaining aqueous phase were concentrated to dryness under reduced pressure. Collected fractions F-A (Ethyl acetate fraction), F-B (n Butanol fraction) and F-C (remaining aqueous fraction) were freeze dried; percentage yield was calculated and subjected to pharmacological screening.

### 4.9 Free radical scavenging assay of fractions of ANB. Alc. Ext.:

*In vitro* free radical scavenging models includes reduction of 1, 1- diphenyl- 2- picryl hydrazyl (DPPH), nitric oxide scavenging activity, ABTS scavenging activity, superoxide dismutase scavenging activity (Riboflavin –EDTA method) and iron chelation assay were used to determine antioxidant activity of fraction as per above protocol (Shirwaikar, 2006).

### 4.10 Pharmacological screening of fractions of ANB. Alc. Ext.:

Separated fractions of biologically most active extracts were tested for acute oral toxicity and antidiabetic activity according to above methods.
4.10.1 Acute oral toxicity study:
Acute oral toxicity study of all the fractions was performed according to above method. At the conclusion of 14 day observation 1/10th of the maximum safest dose was taken for further pharmacological screening (OECD, 2001).

4.10.2 Antidiabetic activity:
Antidiabetic activity was performed in alloxan induced experimentally diabetic rats according to above method.
Group I serves as control received vehicle, Group II received single intra peritoneal injection of freshly prepared solution of alloxan monohydrate in normal saline at a dose of 120 mg/kg b.w., serves as diabetic control given vehicle only (Tween 80 in distill water), G-III received glibenclamide (5 mg/kg) suspended in vehicle. Test samples F-A (Ethyl acetate fraction), F-B (n-butanol fraction) and F-C (remaining aqueous phase) were administered orally at dose of 100 mg/kg/day in vehicle once before food was given for 21 days.

Table 4.4: Effect of fractions of ANB. Alc. Ext. on BGL of alloxan induced diabetic rats

<table>
<thead>
<tr>
<th>GROUPS (n=6)</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Normal rats given vehicle only (Control)</td>
</tr>
<tr>
<td>Group-II</td>
<td>Diabetic rats given vehicle only (Diabetic control)</td>
</tr>
<tr>
<td>Group-III</td>
<td>Diabetic rats given Glibenclamide (5 mg kg⁻¹p.o.) (Standard)</td>
</tr>
<tr>
<td>F-A</td>
<td>Diabetic rats given oral dose of ethyl acetate fraction (F-A)</td>
</tr>
<tr>
<td>F-B</td>
<td>Diabetic rats given oral dose of of n-butanol fraction (F-B)</td>
</tr>
<tr>
<td>F-C</td>
<td>Diabetic rats given oral dose of remaining aqueous fraction (F-C)</td>
</tr>
</tbody>
</table>

4.10.2.1 Evaluation of changes in body weight: The body weights of all the rats were determined on the 1st and 21st days of the experiment as per above methods.
4.10.2.2 Biochemical Analysis:

4.10.2.2.1 Estimation of serum insulin levels:
The serum insulin levels of the fractions were determined as per above procedure (Mishra et al., 2010).

4.10.2.2.2 Estimation of lipids in serum:
The serum was prepared and analyzed for the biochemical estimations: serum total cholesterol (TC), total Glycerides (TG), high density lipoprotein (HDL) were estimated using standard enzymatic colorimetric kits (Span diagnostics Ltd., India) (Bagri et al., 2009). Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were calculated using formula as per above procedures.

4.10.2.2.3 Determination of the lipid peroxidation in pancreas and kidney:
Preparation of tissue homogenates of Pancreas and kidney:
The tissues were weighted and 10% tissue homogenate was prepared with 0.025 M Tris–HCl buffer, pH 7.5. After centrifugation at 10,000 X g for 10 min, the clear supernannt was used to measure thiobarbituric acid reactive substances (TBARS). The tissue homogenate of pancreas and kidney was prepared and Lipid peroxidation was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) by using above methods.

4.10.2.2.4 Preparation of Liver homogenate and estimation of invivo antioxidants:
The liver homogenate was prepared according to above methods. Enzymatic antioxidants Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione-S-transferase (GST), and Non enzymatic antioxidants Glutathione (GSH) was estimated as per the above protocol.

4.10.2.2.5 Estimation of Glycogen in liver:
The estimation of glycogen content in liver tissue sample using above experimental protocol.
4.10.2.3 Histopathology of Pancreas, liver and Kidney of fractions:
A histopathological study was conducted to explore the effect of fractions of *Acacia nilotica* bark alloxan induced diabetic rats.

Sections of Pancreas, liver and Kidney tissues were collected in 10% formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 microns in thickness were cut and stained with hematoxylin and eosin.

The experimental details were the same as above protocol and sections were observed in trinocular microscope for changes in histoarchitecture.