CHAPTER-3
MATERIALS AND METHODS

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CHAPTER-3
MATERIALS AND METHODS

3.1. Plant material

Roots of *Kandelia rheedei* L. and Leaves of *Euphoria lopogona* L., were collected from Kothagudem forest AndraPradesh India between August and September; the plants were authenticated by the Professor. R. Venu Gopal, SR&BGNR Govt. Degree & PG College Kothagudem, Kammam Dts. A voucher specimen (SSR 2012/09/14 and SSR 2013/12/14) has been preserved in our laboratory. The plants were washed thoroughly in tap water, shade dried and powdered.

3.2. Drugs and chemicals

Streptozotecin (STZ), Alloxan, metformin and glibenclamide from Dr. Reddy's, Hyderabad and Orchid laboratories, Chennai, India respectively. All remaining chemicals were of the highest grade commercially available. GOD-POD kit, serum glutamate pyruvate transaminase (SGPT) and serum glutamate pyruvate transaminase (SGPT) were purchased from Beacon Diagnostics Ltd., Navasari, India.

3.3. Preparation of extracts

The air-dried roots of *Kandelia rheedei* Linn. (1.5 kg) and leaves of *Euphorbia lopogona* Linn. (2.5 kg) were coarsely powdered in a mixed separately. Then the coarsely powdered material of each plant was extracted with methanol by maceration for 7 days in round bottom flasks at room temperature separately. The flasks were shaken intermittently to ensure good extraction. After one week, the contents of the flasks were filtered and the filtrate of each was concentrated separately under reduced pressure. The methanolic extract of each plant was dispersed in 1 L of water separately and fractionated with toluene, ethylacetate, butan-2-one and n-butyl alcohol in succession. The solvents were removed from the fractions under reduced pressure to yield the corresponding extracts.
3.4. Maintenance of Animals

Wister rats, weighing about 150–200 g and mice weighing 22-25 g were obtained from the Mahaveer Enterprises, Bagh Ambarpet, Hyderabad (CPCSEA registration no: 146/1999/cpcsea) and the animals were kept in the animal house of Sree College of Pharmacy, Nayakula gudem, Kotha gudem, Kammam, A.P - 507020 at room temperature of 25 - 30°C and at 45 - 55% relative humidity for 12 hr, each of dark and light cycle. The animals were feed with rat pellets (Hindustan Lever Limited, Bangalore, India) and filtered water. Animal studies in the work have been strictly performed as per the Institutional Animal Ethical Committee (IAEC) constituted under the guidelines of Committee for the Purpose of Control and Supervision on experimental Animal (CPCSEA), Ministry of Environment and Govt. of India.

3.5. Acute toxicity study

Acute toxicity study was carried out according to the method described in the literature[40]. Methanolic extract of roots of Kandelia rheedei Linn (MKR) and Methanolic extract of leaves of Euphorbialopogona Linn(MEL) suspended in 5% gum acacia in doses of 5, 50, 300, 2000 mg/kg b.w. were administered orally to albino mice of either sex (22-25g). The animals were observed continuously for any change in autonomic 'or' behavioral responses for first few hours and later at 24 hours intervals for a period of 48h. At the end of this period, the mortality if any each group was noted.
Starting dose: 5 mg/kg

START

5 animals
5 mg/kg

Classify GHS Category
1 2
A B C

5 animals
50 mg/kg

A B C

5 animals
300 mg/kg

A B C

5 animals
2000 mg/kg

Classify GHS Category
2 3 4
A B C

Starting dose: 50 mg/kg

START

5 animals
5 mg/kg

Classify GHS Category
1 2 2 3
A B C

5 animals
50 mg/kg

A B C

5 animals
300 mg/kg

A B C

5 animals
2000 mg/kg

Classify GHS Category
3 4 4 5
A B C

Outcome

A ≥ 2 deaths
B ≥ 1 with evident toxicity and/or 1 death
C No evident toxicity and no death

Group size
The 5 animals in each main study group will include any animal tested at that dose level in the toxicity study.

Animal welfare override
If this dose level caused death in the toxicity study, then no further animals will be tested. Go directly to outcome A.
3.6. Evaluation of Antidiabetic activity
3.6.1. Effect of extracts on blood sugar level in euglycemic rats[41]

The procedure described by Sharma was used. Rats were fasted overnight for 18 hours and were divided into groups of six rats each. Then 0.5ml of 5% gum acacia, glibenclamide (10mg /kg, p.o), Methanolic extract of roots of Kandelia rheedei Linn (MKR) and Methanolic extract of leaves of Euphorbiaopogona Linn(MEL) were given to the group of rats in the following manner.

Group I - 5% gum acacia

Group II  glibenclamide 10 mg/kg b.w.

Group III A- MKR 100 mg/kg b.w.

Group IV A- MKR 200 mg/kg b.w.

Group V A- MKR 400 mg/kg b.w.

Group VI A- MKR 800 mg/kg b.w.

Group III B-MEL 100 mg/kg b.w.

Group IV B- MEL 200 mg/kg b.w.

Group V B- MEL 400 mg/kg b.w.

Group VI B- MEL 800 mg/kg b.w.

Blood samples were collected just before and then 1, 2, 4, 6, 8, 12, and 24 h after administration of the test samples from retro-orbital plexus of each rat and were analyzed for blood glucose content by using glucose oxidase method [42].
3.6.2. Effect of extracts on blood glucose levels of streptozotocin-induced type-2 diabetic rats [43].

Streptozotocin (Sigma Aldrich Company, USA) was dissolved in citrate buffer (pH4.3) and injected to neonate rats (glucose level > 160 mg/dl) were separated and divided into groups with six animals in each group in the following manner and treated orally.

Group I served as control received 5% gum acacia

Group II received glibenclamide 10 mg/kg b.w.

Group III A- MKR 100 mg/kg b.w.

Group IV A- MKR 200 mg/kg b.w.

Group V A- MKR 400 mg/kg b.w.

Group VI A- MKR 800 mg/kg b.w.

Group III B-MEL 100 mg/kg b.w.

Group IV B- MEL 200 mg/kg b.w.

Group V B- MEL 400 mg/kg b.w.

Group VI B- MEL 800 mg/kg b.w.

Group III AF received toluene fraction of KR (TFKR) 100 mg/kg b.w.

Group IVAF received TFKR 200 mg/kg b.w.

GroupVAF received ethyl acetate faction of KR (EAFKR) 100 mg/kg b.w.

Group VI AF received EAF KR 200 mg/kg b.w.

Group VII AF received butanone fraction of KR (BNFKR) 100 mg/kg b.w.

Group VIII AF received BNFKR 200 mg/kg b.w.

Group IXAF received 1-butanol faction of KR (BLFKR) 100 mg/kg b.w.

Group XAF received BLFKR 200 mg/kg b.w.

Group III BF received touene faction of (TFER) 100 mg/kg b.w.

Group IVBF received TFEL 200 mg/kg b.w.
Group VBF received ethyl acetate faction of EL (EAFEL) 100 mg/kg b.w.

Group VI BF received EAFEL 200 mg/kg b.

Group VIIBF received butanone fraction of EL (BNFEL) 100 mg/kg b.w.

Group VIII BF received BNFEL 200 mg/kg b.w.

Group IXBF received 1-butanol faction of KR (BLFEL) 100 mg/kg b.w.

Group XBF received BLFEL 200 mg/kg b.w.

Blood samples were collected just before and then 1, 2, 4, 6, 8, 12 and 24 h after administration of the test samples from retro-orbital plexus of each rat and were analyzed for blood glucose content by using glucose oxidase method [42].

3.6.3. Assessment of activity of extracts on glucose tolerance [44]

A. Effect of methanolic extracts on glucose tolerance in healthy rats:

The antihyperglycemic effect of the extract (MKR and MEL) was assessed by improvement of glucose tolerance. Overnight fasting rats were divided into five groups (I-IV) of each consisting of 6 rats and their fasting blood glucose level was recorded. Control group (I) received vehicle (5% gum acacia) whereas II, III, and IV group received standard drug glibenclamide (10gm/kg b.w.), MKR (400 mg/kg b.w.) and MEL (400mg/kg b.w.) respectively, followed by the loading of glucose (2g/kg) to all the rats and all were administered orally. Blood samples were collected from the rats at 30min, 60min, 90min and 120min after glucose loading for determination of blood glucose levels.

B. Effect of active fractions on glucose tolerance in streptozotocin induced diabetic rats

Streptozotocin induced diabetic rats were used for the study. Over night fasted diabetic rats were divided into five groups (I-V) of each consisting of 6 rats and their fasting blood glucose level was recorded. Control group (I) received vehicle (5% gum acacia) II, III & IV group received standard drug, glibenclamide (10 mg/kg b.w.), EAFKR (100 mg/kg b.w.) EAFEL (100 mg/ kg b.w.) respectively, followed by the loading of glucose (2g/kg b.w.) to all the rats and all were administered orally.
samples were collected from the rats at 30, 60, 90, and 120 min after glucose loading for determination of blood glucose levels.

3.6.4. Effect of EAFKR and EAFEL on different parameters in Subacute study (28 days) of streptozotocin induced type 2 diabetic in rats[45,46]

Overnight fasted streptozotocin-induced type 2 diabetic rats were divided into five group of each consisting of six rats and their body weight and fasting blood glucose level, serum cholesterol, serum insulin, serum SGOT and SGPT, and total proteins were recorded. Then they were treated daily for 28 days in the following manner.

- **Group I**: as diabetic control – received 5% gum acacia.
- **Group II**: received the reference drug, glibenclamide (10 mg/kg b.w.).
- **Group III**: received metformin (250 mg/kg b.w.).
- **Group IV**: received EAFKR (100 mg/kg b.w.).
- **Group V**: received EAFEL (100 mg/kg b.w.).

During the study period, the body weight of the animals, blood glucose level, and serum triglycerides were recorded after 7, 14, 21 and 28 days of treatment. Serum cholesterol, serum insulin, serum SGOT and SGPT, and total proteins were estimated after 28 days of treatment. The methodology details for estimation of the parameters used are as follows.

3.7. Estimation of BioChemical Parameters

A. Measurement of blood glucose levels

Principle:

\[
\text{Glucose +H}_2\text{O} \xrightarrow{\text{peroxidase}} \text{Gluconic acid + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4 \text{Amino Anti pyrine + P-Hydroxy benzoate} \xrightarrow{\text{peroxidase}} \text{Quinone dye glucose}
\]

is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. In a subsequent peroxidase catalyzed reaction, p-hydroxy benzoate and 4-amino antipyrine react with hydrogen peroxide to form red colored quinone complex absorbance data.
measured at 510nm using spectrophotometer are directly proportional to glucose concentrations

**Glucose Kit Constitutes Following Reagents:**

Reagent 1: Phosphate buffer 100mmol/l, pgenol 10 mmol/l, 7.0
Reagent 2: glucose oxide □10000U/L, Peroxidase □600 U/L
4-amino antipyrine 270 umol/l
Standard: Glucose (100 mg/dl)

**Preparation and Stability of Working Reagent:**

Dissolve reagent 2 in the suitable volume of reagent 1.
Stability: 1 month at 20-25°C and 3 month at 2-8°C

**Specimen:**

After collection of the blood sample, it was allowed to clot, then the serum is separated by centrifugation at 3000 rpm, for 10 min. Then the serum glucose is estimated by making following dilutions.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1 ml</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Distilled reagent</td>
<td>10 ml</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Standard</td>
<td>*</td>
<td>10ml</td>
<td>*</td>
</tr>
<tr>
<td>Sample</td>
<td>*</td>
<td>*</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Mix well incubate at 37°C for 10 min or 20-25°C for 30 min read the optical density (OD) at 510 nm.

**Calculations:**

1. Glucose concentration (mg/dl) = (OD of test /OD Of std.) +100
2. percentage blood glucose reduction = (Xdi-Xdf)/ Xdi + 100

Where Xdi = Blood glucose level, before drug treatment
Xdf = Blood glucose level, before drug treatment *

Procedure given by Glucose assay Kit (Beacon Diagnostics Ltd., Navasari, India)
B. Measurement of triglyceride levels

Triglycerides regent is used to measure triglyceride concentration by a timed end point method. Triglycerides in the sample are hydrolyzed to glycerol and free fatty acids, by action of lipase.

**Principle:**

Sequence of 3 coupled enzymatic steps using glycerol kinase (GK), glycerophosphate oxidase (GPO) and horse raddish peroxidase (HPO) causes the oxidative coupling of 3,5 di chloro-2- hydroxy benzene sulphonic acid (DHBS) with 4 - amino anti pyrine to form red quinoneimine dye. The reaction used is 1 part sample to 100 parts reagent.

1. Triglycerides
2. Glycerol + ATP \( \text{glycerol-3-phosphate} \rightarrow \text{glycerol-3-phosphate} + \text{ADP} \)
3. Glycerol-3-phospate + \( \text{O}_2 \text{GPO} \rightarrow \text{Di hydroxyl acetone} + \text{H}_2\text{O} \)
4. \( \text{H}_2\text{O} + 4 \text{ amino antipyrine} + \text{DHBS} \rightarrow \text{Quinoneimine dye} + \text{HCL} + 2\text{H}_2\text{O} \).

**Assay procedure:**

<table>
<thead>
<tr>
<th>Working reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>10μl</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Standard</td>
<td>*</td>
<td>10μl</td>
<td>*</td>
</tr>
<tr>
<td>Sample</td>
<td>*</td>
<td>*</td>
<td>10μl</td>
</tr>
</tbody>
</table>

Mix well incubate at 37º C for min or 20-25ºC for 10 min read the optical density (OD) against the blank within 60 min.
**Calculations:**

1. Triglycerides (mg/dl) = (OD of test / OD of std.) x Con. Std (mg/dl).
2. Percentage Triglycerides reduction = (Xdi - Xdf) / Xdi x 100
   Where Xdi = Triglycerides level, after drug treatment

   Procedure given by Triglycerides assay Kit (Beacon Diagnostic Ltd., Navasari, India).

**C. Measurement of cholesterol levels**

**Principle:**

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase (CHE) hydrolyses the esters. In the subsequent oxidation by cholesterol oxidase (CHO) H$_2$O$_2$ is liberated. The colorimetric indicator is quinoneimine is generation from 4-aminoantipyrine and phenol by H$_2$O$_2$ under the catalytic action of peroxidase [Trinder’s reaction ] (Hand book, 1997).

Cholesterol ester + H$_2$O $\xrightarrow{\text{CHE}}$ Cholesterol + Fatty acid H$_2$O$_2$

Cholesterol+O$_2$ $\xrightarrow{\text{CHO}}$ Cholesterol -3- one + H$_2$O$_2$

2 H$_2$O$_2$+4-Aminoantipyrine + Phenol $\xrightarrow{\text{POD}}$ Quinoneimine+4H$_2$O

**Assay procedure:**

- Wavelength : 550nm
- Light path : 1 cm
- Temperature : 20 - 25°C/37°C
- Measurement : Against blank
<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1ml</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10μl</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Standard</td>
<td>*</td>
<td>10μl</td>
<td>*</td>
</tr>
<tr>
<td>Sample</td>
<td>*</td>
<td>*</td>
<td>10μl</td>
</tr>
</tbody>
</table>

Mix well incubate at 37° C for min or 20-25° for 10 min read the optical density (OD) against the blank within 60 min.

Calculations:

3. Triglycerides (mg/dl) = (OD of test /OD of std.) x Con. Std (mg/dl).

4. Percentage Triglycerides reduction = (Xdi-Xdf)/ Xdi x 100
   Where Xdi = Triglycerides level, after drug treatment

Procedure given by Triglycerides assay Kit (Beacon Diagnostic Ltd., Navasari, India).

D. Estimation of serum insulin levels by chemiluminescence assay

i. Instrument description: Automated chemiluminescence System (ACS; 180, Bayer Health Care).

ii. Intended Use: For in vitro diagnostic use in the determination of insulin in serum using the ACS: 180 automated chemiluminescence system.

iii. Assay Principle: The ACS: 180 insulin assay was a two-side sandwich immunoassay using direct chemiluminescent technology, which uses constant amount of two antibodies. The first antibody in the light reagent was a monoclonal mouse antiinsulin antibody labeled with acridinium ester. The second antibody in the solid phase was a monoclonal mouse antiinsulin antibody, which was
covalently coupled to paramagnetic particles. The system automatically performs the following steps:

- 25 µl of sample was dispensed into cuvette.
- 50 µl of liq reagent was dispensed and incubated for 5 minutes at 37°C
- 250 µl of solid phase was dispensed and incubated for 2.5 minutes at 37°C
- Sample was separated, aspirated and the cuvettes were washed with reagent water.
- 300 µl of reagent 1 and reagent 2 were dispensed to initiate the Chemiluminescence.
- Results were reported according to the selected option, as described in the system operating instructions. A direct relationship exists between the amount of insulin present in the sample and the amount of relative light units (RLUs) detected by the system.

iv. Specimen collection and handling:

The following recommendations for handling and storing blood samples were furnished by the National Committee for the Clinical Laboratory Standards (NCCLS):

- Blood samples were collected as per the official recommendations.
- Samples were centrifuged at > 1000x g for 15 to 20 min.
- Tubes were kept stoppered and upright at all times.
- Serum was separated from the red blood cells and was stored 2 - 8°C for about one hour.
- The samples were sent to Vijaya diagnostic center, Hanamkonda and were analyzed for insulin.
The details reagents in the kit as described in the printed brochure are given below.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS : 180</td>
<td>2.5mL/vial</td>
<td>Monoclonal mouse anti-insulin Antibody (~0.59µg/vial) labeled with acridinium ester in buffered saline with bovine serum albumin, sodium azide (&lt; 0.1%), and preservatives.</td>
</tr>
<tr>
<td>IRS</td>
<td>12.0mL/vial</td>
<td>Monoclonal mouse anti-insulin Antibody (~75.0 µg/vial) covalently coupled to paramagnetic particles in buffered saline with bovine serum albumin, sodium azide (&lt; 0.1%), and preservatives.</td>
</tr>
<tr>
<td>SP</td>
<td>20mL/vial</td>
<td>Buffered saline with casein, potassium thiocyanate (~3.89%), sodium azide (&lt; 0.1%), and preservatives.</td>
</tr>
</tbody>
</table>

**Storage:** 2-8 °C

**Stability:** Until the expiration date on the vial or cumulative 40 hours at room temperature

**Caution:** Sodium azide react with copper and lead plumbing to form explosive metal azides. Hence during disposal, reagents were flushed with a large volume of water to prevent the buildup of azides.

**v. preparation of the reagents:**

For best results, the solid phase was thoroughly mixed by inverting the vial before each use. The bottom of the vial was inspected visually to ensure that all particles were dispersed and suspended.
vi. Assay Procedure:

The details as described in the instrument manual are given below.

1. It calibrating the ACS:180 insulin assay:
   a. Define the sample probe settingsample probe: 6 primes
   b. Start the system, this procedure takes approximately 7.5 min

2. Schedule the requested tested or profile for each sample.

3. Prepare and load insulin calibrator, if required:
   a. Prepare and low and high calibrator according to the instruction in the insulin calibrator product instructions.
   b. Dispense the low and high calibrators into samples cups labeled with the appropriate barcode labels.

4. Prepare and load the quality control samples:
   a. Prepare and quality material according to the instruction in the quality control product instructions.
   b. Dispense the quality control samples into labeled sample cups.

5. If dilution is required, dispense insulin diluents into a sample cup labeled with the appropriate barcode label and load the sample cup and load them on the sample tray.

6. Prepare the primary tubes or sample cups and load them on them on the sample tray.

7. Load the solid phase and lite reagent in adjacent positions on the reagent tray.

8. Start the System

Dilutions:

- Samples with insulin levels greater than 300 mU/L must be diluted and retested to obtain accurate results.
- Samples can be automatically diluted by the system or prepared manually.
• For automatic dilutions, ensure that insulin diluents is loaded and set the system parametric as follows:
  
  Dilution set point : < 300 m U/L
  
  Dilution factor: 2.5

• Manually dilute serum samples, when sample result exceed the linearity of the assay using automatic dilution.

• Use insulin diluents to manually dilute patient samples, and then load the diluted sample on the sample tray, replacing the undiluted sample.

• Ensure that results are mathematically corrected for dilution. If a predilution factor is entered when scheduling the test, the system automatically calculates result.

**Specificity:**

The cross reactivity of the ACS: 100 insulation assays was determined by spiking. Serum samples with the following compounds at the indicated levels. These compounds did not have a significant effect on the insulin measurement.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount added</th>
<th>Mean% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proinsulin</td>
<td>1mg/ml</td>
<td>100.8</td>
</tr>
<tr>
<td>C-Peptide</td>
<td>500ng/ml</td>
<td>95.1</td>
</tr>
<tr>
<td>Gastrin_1</td>
<td>1mg/ml</td>
<td>96.1</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1mg/ml</td>
<td>101.6</td>
</tr>
</tbody>
</table>

Interference testing was determined according to NCCLS Document EP7-P12

**Sensitivity and Assay Range:**

The ACS: 100 insulin assay measures insulin concentration 300 m U/L with a minimum detectable concentration of 0.5 m U/L. Analytical sensitivity is defined as the concentration of insulin that corresponds to the RLUₜ that are two standard
deviations greater than the mean RLUₜ of 20 replicate determination of the insulin zero standard.

**Standardization:** The ACS: 100 insulin assay is standardized against World Health Organization (WHO) 1st LR 66/304. Assigned values of calibrators are traceable to his Standardization.

**E. Estimation of SGOT**

**Intended use:** SGOT (AST) kit is intended for the *in vitro* quantitative determination of SGOT (AST) (Serum Glutamate Oxaloacetate Transaminase / Aspartate Amino Transferase) in human serum or plasma. SGOT activity is usually assayed by Reitman and Frankel (1957) colorimetric method.

**Clinical Significance:** SGOT is present in high concentrations in cells of cardiac and skeletal muscles, liver, kidney and erythrocytes. Damage to any of these tissues may increase SGOT levels. Other liver disorders like liver cirrhosis, infectious mononucleosis episodes may lead to moderate increase of AST.

**Principle:** SGOT catalyzes the transfer of the amino group from L-Aspartate (ASP) to α-ketoglutarate (α-kg) resulting in the formation of oxaloacetate (OAA) and (L-Glu). The Oxaloacetate so formed, is allowed to react with 2,4-DNPH to form 2,4-dinitro phenyl hydrazone derivative which is brown colored in alkaline medium. SGOT activity of the specimen is directly read on the calibration curve.

L+Asp+α-KG SGOT OAA + L-Glu OAA +2,4-DNPH alkaline medium 2,4 – Dinitro phenyl hydrazone.

**Reagents:**

Reagent 1. Buffered aspartate –α-ketoglutarate substrate pH 7.4

Reagent 2. 2,4-DNPH Reagent

Reagent 3. Pyruvate Standard

Reagent 4. 4N NaOH (DILUTE 1:10 When used)

**Stability:** All reagents are stable upto the given expiry date when stored at 2-8⁰C.
**Specien: Serum** (Haemolysed samples are not suitable) SGOT levels are stable for on week at 2-8° C.

**Procedre:**

Wavelength : 505 nm (490-520nm).

Standard curve procedure: Take 6 dry test tubes and label them; mix and measure the absorbance of all tubes against Tube no.1. Plot the standard curve by plotting the absorbance against activity of SGOT.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity</td>
<td>0</td>
<td>28</td>
<td>57</td>
<td>97</td>
<td>125</td>
<td>150</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>0.5ml</td>
<td>0.45ml</td>
<td>0.4ml</td>
<td>0.38ml</td>
<td>0.3ml</td>
<td>0.25ml</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>--</td>
<td>0.05ml</td>
<td>0.1ml</td>
<td>0.38ml</td>
<td>0.2ml</td>
<td>0.25ml</td>
</tr>
<tr>
<td>Reagent 3</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1ml</td>
<td>0.1ml</td>
<td>0.1ml</td>
<td>0.1ml</td>
<td>0.1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Reagent 4</td>
<td>5.0ml</td>
<td>5.0ml</td>
<td>5.0ml</td>
<td>5.0ml</td>
<td>5.0ml</td>
<td>5.0ml</td>
</tr>
<tr>
<td>(1:10 diluted)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Mix and keep at Room Temperature for 20 minutes

**Test Procedure:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Mix and incubate</td>
<td>27°C for one hour</td>
<td></td>
</tr>
<tr>
<td>Reagent 2</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1 ml</td>
<td>---</td>
</tr>
<tr>
<td>Mix and Keep at Room temperature for 20 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent 4</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>(1:10 diluted)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix and measure the absorbance of Test against Blank at 505 nm.
Calculations: Read the corresponding SGOT activity from the graph.

**Normal Range:** 5-30 units/ml

**Note:**

1. Haemolysis of samples leads to the release of enzyme and hence gives erroneous results.

2. Reagent 4 is corrosive, avoid contact with skin.

3. Reagents contain azide, avoid contact with skin and mucous membrane. All the solutions after completion of the must be flushed with plenty of water.

**F. Estimations of SGPT**

**Intended Use:** SGPT (ALT) Kit is intended for the quantitative determination of SGPT (Serum Glutamate Pyruvate Transaminase/Alanine Aminotransferase) in human serum or plasma. SGPT activity is usually assayed by Retiman & Frankel (1957) colorimetric method.

**Clinical significance:** SGPT is present in high concentrations in liver and to a lesser extent in skeletal muscle, kidney and heart. SGPT levels increase significantly in case of acute viral or toxic hepatitis. Moderate increase of SGPT is associated with cirrhosis, infectious mononucleosis, liver congestion secondary to congestive heart failure and cholestatic jounce.

**Methodology:**

SGPT catalyze the transfer of the amino group from L-Alanine to α-ketoglutarate (α-KG) resulting in the formation of pyruvate and L-glutamate. The pyruvate so formed is allowed to react with 2,4-DNPH to produce 2,4-dinitrophenylhydrazone derivative which is brown colored in alkaline medium. The hydrazone derivative of α-KG. The final color developed does not obey Beer’s law, hence a calibration curve is plotted using private standard. SGPT activity of the specimen is directly read on the calibration curve.
Reagents:

Reagent 1. Buffered aspartate –α-κετογλυκορατ substrate pH 7.4
Reagent 2. 2,4-DNPH Reagent
Reagent 3. Pyruvate Standard
Reagent 4. 4N NaOH (DILUTE 1:10 When used)

Stability: All reagents are stable up to the given expiry date when stored at 2-8°C.

Specimen:

Use fresh nonhaemolysed serum or heparinized plasma.
Serum SGPT levels are stable for one week 2-8°C

Procedure:

Wave length: 505 nm (790-520 nm).

Standard Curve Procedure: Take 6 dry test tube and label them; mix and measure the absorbance of all tubes against Tube No. 1. Plot the standard curve by plotting the absorbance activity of SGPT.
Enzyme activity       0                 28             57           97             125            150
Reagent 1                0.5ml        0.45ml      0.4ml      0.38ml      0.3ml        0.25ml
Reagent 3                --           0.05ml      0.1ml      0.38ml      0.2ml        0.25ml
Reagent 2                0.5ml        0.5ml      0.5ml      0.5ml      0.5ml        0.5ml
Distilled water          0.1ml        0.1ml      0.1ml      0.1ml      0.1ml        0.1ml
Mix and keep at Room Temperature for 20 minutes
Reagent 4                5.0ml        5.0ml      5.0ml        5.0ml        5.0ml        5.0ml
(1:10 diluted)

Test procedure:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Mix and incubate at 37°C for 30 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent 2</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Mix and keep at Room Temperature for 20 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent 4</td>
<td>5.0ml</td>
<td>5.0ml</td>
</tr>
</tbody>
</table>
(1:10 diluted)

Mix and measure the absorbance of Test against Blank at 505 nm after 10 minutes

Calculations: Red the corresponding SGPT activity from the graph.
G. Estimation of total proteins

Serum total protein content was estimated by the method of Lowy et at. (1951). Proteins form chromophoric complex with phenol reagent, which was measured at 610 nm (Lowry et al., 1951).

Reagents:

1) Alkaline Reagent: 2g of sodium carbonate was added to 100 ml of 0.1N Sodium hydroxide solution.

2) Alkaline Mixture: To 100 ml of alkaline reagent 1 ml of 4% aqueous copper sulphate solution was added, this was prepared freshly.

3) Phenol reagent (Folin and Ciocalteu’s Reagent): Diluted by dissolving 0.5 ml of 4% aqueous copper before use and stored in refrigerator.

Procedure: To 0.1 ml of serum 1 ml of alkaline mixture reagent was added and kept for 10 minutes, then 4 ml of phenol reagent was added, heated at 55°C for 5 minutes for color development and then cooled or 1 minute. Reading was taken against blank at 610 nm using spectrophotometer. The protein content was calculated from standard curve prepared with bovine serum albumin and expressed in terms of gm%.

3.8. Assessment of Antioxidant activity of the extracts

A. In vitro study by using DPPH:

For the estimation of antioxidant activity, different concentration (5, 10, 25, 50, 100 mg/ml) Of EAFKR, EAFEL and ascorbic acid in methanol were added to 100 ml of 0.2 mM methanolic solution of DPPH, mixed thoroughly and 20ml of the mixture was used for HPLC analysis. Each test was performed in triplicate, which was carried out using reverse phase C18 Column equipped with waters UV-Visible spectrophotometric detector. Isocratic elution conditions for HPLC analysis were methanol : water (85:15) monitored at 517nm [69]. The antioxidant activity was measured in terms of % inhibition of DPPH peak area. The amount of drug required to produce 50% inhibition of DPPH peak area was taken as IC50. The IC50 values computed from concentration of test extract and percent inhibition of DPPH peak area.
A. Estimation of Lipid-peroxidation product (malondialdehyde) [70]

The amount of lipid peroxidation product (malondialdehyde, MDA) present in the serum samples drawn from the streptozotocin induced diabetic control, EAFKR and EAFEL treated diabetic rats 1st and after 28 days of the study was estimated by the Thiobarbituric acid reactive substances (TBARS) method [70]. Which measures the malondialdehyde (MDA) reactive products by using High Pressure Liquid Chromatography (HPLC).

**Principle:** The reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a secondary product of lipid peroxidation, has been widely adopted as a sensitive assay method for measurement of lipid peroxidation has progressed. Since the assay procedure estimates the amount of TBA reactive substances e.g. MDA, it is also referred to as TBARS (Thiobarbituric acid reactive substance) test.

**Procedure:** To 0.5 ml of 30% trichloro acetic acid (TCA) was added to precipitate the proteins and vortexed for 30 sec. Clear supernatant was taken after centrifuging at 3000 rpm for 10 min. To the supernatant 50 μl of 1% TBA solution was heated for 1 hr at 98°C. 20 μ of the mixture, which is pink in color, was injected into HPLC. Standard graph was plotted using TEP (1,1,3,3-tetra etoxy propane).

**HPLC Conditions:**
- **MOBILE PHASE:** Methanol: Water (70:100) containing 550 μl of H₃PO₄ with 80 nM of NaOH
- **COLUMN:** Altec C18 (25 cm length 4.6 mm diameter, 5 μ size)
- **Wave length:** 532 nm
- **Temperature:** Ambient
- **Flow rate:** 1 ml / min
- **Injection volume:** 20 μl
3.9. Evaluation of EAFKR and EAFEL for Glucose uptake stimulatory activity in 3T3_L1 cells and insulin secretagogue property in RIN cells

A. Chemical and reagents

Dulbecco’s minimum essential medium (DMEM), RPMI, trypsin, sodium bicarbonate, antibiotics, dexamethasone, 1-isobutyl-3 methylxanthine (IBMX), glucose, insulin, eosin MTT, Streptozotocin and oil red “O:” were procured from Sigma Chemical (St. Louis MO, USA). Fetus Bovine Serum was obtained from Gibco, invitrogen. Triton X-100 and Dulbecco’s phosphate buffer saline were procured from Himedia (Mumbai, India.) 2-deoxy-D-glucose was purchased from American Radiolabeled Chemicals Inc., St Louis, MO, U.S.A. Insulin ELISA kit were procured from Marcodia (U.S.A), respectively.

B. Cell lines and maintenance

Mouse embryonic fibroblast 3T3-L1 and rat insulation cells RIN were obtained from the National Centre for Cell Science, Pune, India and as cultured in Dulbecco’s Minimal Essential Medium (DMEM), except RIN which was cultured in RPMI media, containing 10% FBS, 1% antibiotic – antimycological solution and 1.5 g/l sodium bicarbonate under 5% CO2 at 37°C. At semi-confluence stage, cells were sub-cultured by trypsinization with 0.25% sterilite trypsin at 1:6 split ratios approximately every 3-4 days.

C. Induction of adipogenesis

Adipogenesis was induced in the 3T3-L1 cells according to method described by Heping [71]. 3T3-L1 cells were seeded in 6-well plate at a density of 0.2 million per well and were maintained in the same conditions for 2-3 days to reach confluence. At this point (day-0) the cells were switched to differentiation medium containing DMEM, 10% FBS, 0.2511M dexamethasone, 0.25 mM 1-isobutyl-3-methylxanthine (IBMX) and 1g/ml insulin (MDI cocktail) for 3 days, with one medium change in between on day 3. Media was replaced with fresh media containing insulin and without dexamethasone and IBMX for an additional 4 days and changing the medium after every 2 days. Thereafter the cells were maintained in the original propagation
DMEM, changing medium every 2-3 days, until use. Plates where cells were >90% differentiated were used for experiments between days 9-12 post-induction.

D. Oil Red “0” and fluorescence staining of adipocytes

Differentiated adipocytes were stained with Oil Red 0 according to the method described by Kasturi & Joshi [72] Briefly, after differentiation cells were washed twice with PBS. Incubated with 10% formalin at room temperature for 5min followed by replacement of fresh formalin and incubation for additional one hour. Formalin solution was discarded and fresh Oil Red 0 working solution (line/ml) was added to cells. After incubation for 10 min. cells were washed with excess of water and were stained with eosin (5mg/ml). After 10 min incubation, cells added again with excess of water to dry. Photography were taking TS100 microscopes after heating the cells by adding 2-3 drops of water.

E. Glucose uptake assay

Glucose uptake as determined in 12-cell plates. Hen > 90% of cells in the plates were differnetiationed into adipocytes, cells were ashed once ith securr-freee DMEM and incubated in 1 ml/well of the same for 2 h at 37°C. cells were hen hased once it Krebs-Ringer-HEPES (KRH) buffer (128Mm NacL,4.7Mm 1.25Mm CaCl₂ 1.25Mm MgS04, 10.0Mm Na2HP04, 20Mm HEPES), and incubated in 1 ml/well of the same for 2 h at 37°C. Treatment with insulin (0.5 Nm or 50Nm) was perforated in the presence and the absence of EAFKR, EAFEL at different concentrations (20, 50, 100, 200, 250 mg/ml) dissolved in KRH buffer, which all allotted to proceed for 30 mins[47]. Extracts ere first dissolved in DMSO to make a stock solution that as then further diluted into DKRH buffer. For measurement of glucose, to give a concentration of 0.2 Mm (0.5 μ /Mmol) yielding an activity of 0.1 μ/ml. After 60 min at 37°C, glucose uptake as terminated by First placing the plates on a bed of ice. The plates were washed with 3ml/well ice-cold PBS and the cells were 0.7 ml 1% Triton X-100 for 40 min at 37°C [48]. Aqua light (PerkinElmer, Netherlands) as added and tritium counts were obtained using a Hidex (Finland) liquid scintillation analyzer. Measurements were made in duplicate and corrected for specific activity. Pioglitazone was used as standard.
f. Insulin secretogogue assay

RIN cells, on attaining 75-80% confluency, were washed with rpmi-1640 medium and Pretreated for 1 h at 37°C with EAFKR, EAFEL at different concentrations (25, 50, 100, 200, 250 mg/ml) and glibenclamide (3 μM/ml) and glibenclamide (3 μM/ml). After incubation, cells were washed with KRP buffer and incubated with same buffer for another 4 h [49]. Samples were drawn at one-hour Intervals and were assayed for insulin concentration using Insulin ELISA kits (Marcodia,USA).

3.10. Evaluation of EAFKR and EAFEL for Glucose uptake studies by rat diaphragm

Glucose uptake by rate hemi-diaphragm was estimated by the methods described by Walaas and Chattopadhyay et al., with some modification [50]. Eight sets containing three numbers of graduated test tubes (n=3) each, were taken as follows:

**Group 1:** 2 ml of solution with 2% glucose.

**Group 2:** 2 ml of solution with 2% glucose and regular insulin (Nova Nordisk) 0.62 ml of 0.4 units per ml solution.

**Group 3:** 2 ml of Tyrode solution and 1.38 ml of EAFKR (0.1%)

**Group 4:** 2 ml of solution with 2% glucose and regular insulin 0.62 ml of 0.4 units per ml solution and 1.38 ml of EAFKR (0.1%).

**Group 5:** 2 ml of Tyrode solution and 1.38 ml of EAFEL (0.1%)

**Group 6:** 2 ml of solution with 2% glucose and regular insulin 0.62 ml of 0.4 units per ml solution and 1.38 ml of EAFEL (0.1%).

The volume of all the test tubes were made up to 4 ml with distilled water to match the volume of the test tubes of Group 4. Twelve albino rats were fasted overnight and killed by decapitation. The diaphragms from the same animal were not used for each group. The hemi-diaphragms were placed in test tubes and incubated for 30 min at 37°C, with a gas phase of O₂+CO₂ (95:5) with shaking at 140 cycles/min. glucose uptake of tissue was calculated as the difference between the initial and final glucose content in the incubated medium.
3.11. Statistical Analysis

All the group were statistically evaluated using one way analysis of variance (ANOVA) followed by Newman’s Keull multiple comparison test, expressed as the Mean ± SD from six rats in each group. P value of 0.05 or less was considered to be significant [51].