Chapter 3: MATERIALS AND METHODS
In this study under consideration, the antioxidant potential of hydro-ethanolic extracts of various parts (flowers, leaves, roots, stems and pods) of *R. communis* Linn. and the isolated compounds from the most efficient crude extract of this plant was evaluated and compared to the antioxidant effect of the standard drug Glibenclamide, in Alloxan induced, diabetic, adult, male Swiss albino mice. The potential was investigated morphologically, biochemically, histologically and relative quantitatively. Moreover, isolation and purification of principle compounds from the most effective plant part was done by thin layer and column chromatography. Thereafter, the isolated compounds were investigated for their antioxidant potential in diabetic mice. The identification and characterization of the most potent isolated compound was further undertaken by aid of spectrophotometric techniques (FTIR and GC-MS). All chemicals and reagents of analytical grade were used and purchased from Sd-fine chemicals, SIGMA, SRL, CDH, Merck, Ranbaxy, HIMEDIA and Aldorich. The plan on which research progress based is outlined as follows:

**Collection of plant and extract preparation**

**Procurement of plant material:** The leaves, flowers, pods, stems and roots of *R. communis* were procured from Agricultural Research Institute; Latitude: 26.3535° N; Longitude: 73.0331° E Mandore, Jodhpur, Rajasthan, India; and was identified and authenticated by Botanist Dr. Ishwar Singh, Associate Professor, Agricultural Research Institute, Mandore, Jodhpur, Rajasthan, India

**Drying and size reduction of plant material:** The plant parts were thoroughly washed with tap water and subjected to air drying in shade for about 2-3 weeks. The dried parts were further milled into coarse powder which was sieved out through Sieve No. 80 and preserved in air tight containers in refrigerator.

**Soxhlet Extraction** (Nagappa *et al.*, 2003): 100 grams of ground coarse powder of each plant part was packed in a filter paper thimble neatly and defatted. This was then extracted in 1 Litre of hydro-ethanol (50%- ethanol) by hot continuous percolation using soxhlet apparatus and further refluxed for 18/24 hrs at 70°C. The liquid extracts collected were evaporated with the aid of a rotary evaporator. The reduced extracts were then collected in glass petri-plates and allowed to evaporate and form semi-solid masses. These hydro-ethanolic extracts were then labeled and preserved in desiccators with CaCl₂.
Table 3.1: Hydro-ethanolic extracts of various parts of *R. communis*

<table>
<thead>
<tr>
<th>Plant Part</th>
<th>Name of the Hydro-ethanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. communis</em> Leaves</td>
<td>RCL</td>
</tr>
<tr>
<td><em>R. communis</em> Flowers</td>
<td>RCF</td>
</tr>
<tr>
<td><em>R. communis</em> Pods</td>
<td>RCP</td>
</tr>
<tr>
<td><em>R. communis</em> Stems</td>
<td>RCS</td>
</tr>
<tr>
<td><em>R. communis</em> Roots</td>
<td>RCR</td>
</tr>
</tbody>
</table>


3.1.1 Phytochemical Screening

Qualitative tests were undertaken on the hydro-ethanolic extracts of aerial parts of *R. communis* using standard protocols for the identification of phytochemicals (Sofowra, 1993; Trease and Evans, 1989; Harborne, 1973)

3.1.1.1 Test for Alkaloids:

2.5gms alcoholic extract were evaporated to dryness. 5ml of 2N HCL was added to the above residue and was heated on boiling water bath. After cooling, mixture were filtered, the filtrate were divided equally in 2 portions. First filterate was treated with Mayer’s reagent and other filterate with wagner’s reagent. Turbidity formed indicated the presence of Alkaloids.

3.1.1.2 Test for Fatty Acids:

The plant extracts were mixed with 5ml of ether. These extracts were allowed to evaporate on filter paper and then dried the filter paper. The transparency appeared on the filter paper, was indicative of the presence of fatty acids.

3.1.1.3 Flavonoids:

0.5 g of plant extract was shaken vigorously with diluted NaOH and to it HCl was added. A yellow colour solution was formed, which turned colourless was indicating the presence of flavonoids.
3.1.1.4 Tests for glycosides:
2 ml plant extract was added to 2 ml of chloroform. 2 ml of sulphuric acid was carefully added and shook gently. Occurrence of reddish brown colour suggested the presence of a steroidal ring which is the glycone portion of glycosides.

3.1.1.5 Test for Phenols:
Each extract was treated with few a drops of ferric chloride solution. Bluish-black coloration refers to the availability of phenols.

3.1.1.6 Test for Resins:
0.5g plant extract was diluted with 10ml water and shaken for 5 minutes. Turbidity formed indicated the presence of Resins.

3.1.1.7 Test for saponins:
5 ml aqueous extract and equal amount of distilled water in a test tube were stirred vigorously and warmed. Stable formation of foam was taken as a positive indication for Saponins.

3.1.1.8 Test for steroids:
2 ml of extracts of plants were stirred with 2 ml chloroform and 2 ml concentrated sulphuric acid. Red colour production in the lower chloroform layer indicated the presence of steroids.

3.1.1.9 Test for tannins:
2 ml aqueous extracts were dissolved in 2 ml distilled water and 2 or 3 drops of FeCl₃ solution were added to the mixture. Formation of a green precipitate indicated the presence of tannins.

3.1.1.10 Test for Terpenoids
(Salkowski method): 0.5g plant extracts were added to 2ml of chloroform. 3ml of
concentrated sulphuric acid was added to form a layer. A reddish brown colour formed at the interface was an indicator for the presence of terpenoids.

3.1.2 **In vitro anti-diabetic activity**

The *in vitro* anti-diabetic activity of various extracts prepared was carried out by:

3.1.2.1 α-amylose assay (Miller, 1959)

α-amylose action was measured by utilizing the DNS system. To 500 μL of plant extract 500 μL of 0.02 M sodium phosphate buffer with 6 mM sodium chloride and 0.04 units of α-amylose arrangement were included. The reaction mixture was then brooded for 10 min at 37°C. After this, 500 μL of 1% starch arrangement disintegrated in 0.02 M sodium phosphate support was included. 1.0 ml of DNSA reagent then ceased this response. The brooding of test tubes was then done in a boiling waterbath for 5 min and cooled till room temperature. After diluting the reaction mix by adding 10ml distilled water, its absorbance was noted at 540nm. The control samples were prepared similarly only without the plant extract samples.

The results were expressed as inhibition % calculated using the formula:

\[
I = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100
\]

3.1.2.2 α-glucosidase assay (Krishna Veni et al., 2011)

The plant extract with 0.2 M Tris buffer pH 8.0 and 1 ml solution of starch substrate (2 % w/v maltose or sucrose) was incubated at 37°C for 5 min. 1 ml of alpha-glucosidase enzyme (1U/ml) was added to this to start the reaction, the test tubes were incubated for 40 min at 35°C thereafter.

2 ml of 6N HCl was then added to stop the reaction. The absorbance was then measured at 540nm to estimate the intensity of the colour (18). The activity was estimated by calculating % inhibition using the same formula:

\[
I = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100
\]

3.1.3 **In vitro anti-oxidative activity**
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The assays for *in vitro* anti-oxidative potential were carried out under two headings, namely: Free radical scavenging activities (3.1.3.1-3.1.3.3) and Tests for Enzymatic antioxidant potential (3.1.3.4-3.1.3.6)

3.1.3.1 ABTS (2,2’-azinobis[3ethylbenzthiazoline]-6-sulfonic acid) radical scavenging (Roberta et al., 1999)

ABTS (7mM) with 2.45 mM potassium persulfate reacts to produce ABTS radical cations. After overnight incubation (12-16 hrs) at room temperature in dark, to the intensely coloured ABTS radical cations, 0.01 M, pH 7.4 PBS (Phosphate buffered saline) was added to dilute and to give absorbance of ~0.70 at 734 nm. ABTS solution was also added to plant extracts were to dilute them to 100 times to a total volume of 1 ml. After addition of each extract the absorbance was measured at time interval of 1 min in triplicates. The total antioxidant activities were expressed as mM trolox equivalent antioxidant capacity (TEAC).

3.1.3.2 DPPH (Diphenyl picryl hydrazyl) radical scavenging activity (Blois et al., 1958)

3 ml of plant extract and 1ml of 0.1 mM solution of DPPH in methanol was kept for incubation at 37°C for 30 min and absorbance was noted at 517 nm. Ascorbic acid and BHT were used as the reference materials. The inhibition percentage (I) was calculated as radical scavenging activity as follows:

\[ I = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \]

3.1.3.3 Metal ion scavenging activity (Decker et al., 1990)

To a solution of 0.1 ml of 2 mM FeCl₃5 ml plant extract was added. To this, 0.2 ml of 5 mM ferrozine solution was added and was left for 10 min at room temperature in shaking conditions. Absorbance was then measured at 562 nm. EDTA was used as a positive control.

The percentage inhibition of Ferrozine–Fe²⁺ complex was calculated using the formula:

\[ I = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \]
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3.1.3.4 Superoxide Dismutase (SOD) activity (Beauchamp and Fedovich, 1976)
To 1 ml of 125 mM Na$_2$CO$_3$, 0.4 ml of 25 μM NBT, 0.2 ml of 0.1 mM EDTA along with 0.5 ml of plant extract, 0.4 ml of 1 mM Hydroxylamine hydrochloride was added and the absorbance was measured at 5 min intervals at 560 nm. Units of SOD were, amount of enzyme needed for inhibiting the decrease in NBT by 50%. The specific activity was expressed in terms of units per mg of protein.

3.1.3.5 Catalase (CAT) activity (Chance and Maehly, 1995)
Catalase activity was calculated titrimetrically by taking 1ml plant extract along with 5 ml of 300 μM phosphate buffer (pH 6.8) and 100 μM H$_2$O$_2$ were left for 1 min at room temperature. 10 ml of 2% sulphuric acid was added to stop the reaction. KMnO$_4$ (0.01N) was utilized to titrate residual H$_2$O$_2$ till pink colour. Enzyme activity was estimated by calculating the decomposition of μM H$_2$O$_2$ per min per mg protein.

3.1.3.6 Glutathione Peroxidase (GPx) activity (Starlin and Gopalakrishnan, 2013)
Method of Rotruck as described in Starlin and Gopalakrishnan, 2013, was referred to estimate the activity of Glutathione peroxidase (GPx). A reaction mix of 0.5 ml of plant extract, 0.1 ml of 10mM sodium azide, 0.1 ml of 2.5 mM H$_2$O$_2$, 0.2 ml of 4 mM reduced glutathione, 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0) and 0.2 ml of water was prepared and incubated at 0-90 seconds successively. The reaction was stopped by adding 0.5 ml of 10% TCA and centrifuged. To 2ml of the supernatant, 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate) along with 3 ml of phosphate buffer were added such that a color was developed whose absorbance was read at 412 nm. The enzyme activity was expressed in terms of μg of glutathione utilized/min/mg protein.

Calculation of IC$_{50}$: The antiradical and antidiabetic activity of tested compounds is worked out by calculating IC$_{50}$ (concentration of a compound inhibiting the activity of a test solution by 50%) using MS Excel.

3.2 Evaluation and comparison of the antihyperglycemic and antidyslipidemic potential of hydroethanolic extracts of different parts of Ricinus communis in alloxan-induced diabetic male adult Swiss albino mice.
In the present study, the impact of standard drug Glibenclamide and all four extracts (i.e., leaves, stems, flowers and roots) of *R. communis* was investigated morphologically and biochemically on alloxan induced adult male swiss albino mice. All the chemical and reagents used in the study were of analytical grade.

### 3.2.1 Acclimitization of experimental animals and their care

Healthy male adult Swiss albino mice procured from C.C.S. Haryana Agricultural University (Hissar, India) were used in this study. Standard rat pellet diet was used for feeding animals. Prior to the experimentation, animals were acclimatized to the institutional animal house conditions for one week. The animals were caged in standard mouse plastic cages with iron bar lid, under standard laboratory conditions of light, relative humidity and temperature (23 ± 2°C). Strict hygienic conditions were maintained by sterilizing the animal house with disinfectant and all the animals were kept at identical housing conditions. The mice were daily provided with standard rat pellet feed and tap water ad libitum (Coleman *et al*., 1966).

### 3.2.2 Dose selection

The definite dose of the extract in suspension was used during the period of treatment of experimental animals. Dose fixation was carried out by stair case method on adult Swiss albino mice. None of the extract of *R. communis* exhibited lethal effect even at 3000 mg kg\(^{-1}\) body weight. Hence, one-tenth of 3000 mg kg\(^{-1}\) i.e. 300 mg kg\(^{-1}\) BW of crude extract was fixed as the dosage (Padmashali and Vaidya, 2001). Thus, the hydro-ethanolic extract of various plant parts viz., stem, root, leaves and flower will be administered orally at a dose of approximately 300mg/kg BW (Padmashali and Vaidya, 2001; Bekele, 2008; Sharma and Garg, 2012).

### 3.2.3 Experimental design

After one week of acclimatization, the experimental mice divided into 8 groups each containing 7 mice of almost equal weight. The nomenclature of the groups is as follows:
Table 3.2: Nomenclature of experimental groups

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Name of the experimental group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Non diabetic control (NC)</td>
</tr>
<tr>
<td>II</td>
<td>Alloxan induced diabetic control (DC)</td>
</tr>
<tr>
<td>III</td>
<td>Glibenclamide treated diabetic (GT)</td>
</tr>
<tr>
<td>IV</td>
<td><em>R. communis</em> Flower extract treated diabetic (RCFT)</td>
</tr>
<tr>
<td>V</td>
<td><em>R. communis</em> Leaf extract treated diabetic (RCLT)</td>
</tr>
<tr>
<td>VII</td>
<td><em>R. communis</em> Root extract treated diabetic (RCRT)</td>
</tr>
<tr>
<td>VIII</td>
<td><em>R. communis</em> Stem extract treated diabetic (RCST)</td>
</tr>
</tbody>
</table>

3.2.4 Induction of Diabetes

A single intraperitoneal injection of alloxan monohydrate with a dose of 150 mg kg\(^{-1}\) BW was sufficient to make overnight fasted experimental mice diabetic (Rao *et al.*, 1997; Szkudelski, 2001). The animals were then provided with 5% glucose solution to drink overnight to avoid drug-induced hypoglycemia. One week after alloxan injection, animals with fasting blood glucose level greater than 140 mg dl\(^{-1}\) were considered as diabetic, and were included for further study (Nagappa *et al.*, 2003; Kadnur and Goyal, 2005). These diabetic mice were kept under observation for polydipsia, polyphagia and polyuria. All experimental mice were kept and maintained under standard laboratory conditions and had free access to food (standard pellet diet) and water ad libitum.

3.2.5 Treatment

All the experimental groups were treated for a period of 45 days with their respective crude herbal extract (i.e., leaves, stems, roots, flowers, pods) and standard drug Glibenclamide. The standard drug (SD) treated group was given Glibenclamide at a dose of 10 mg kg\(^{-1}\) body weight. Further, all the extracts of plant parts were provided to the respective groups once a day at the dose of 300mg/kg body weight for continuous period of 45 days. The diabetic control (DC) and the normal control (NC) groups were provided with the saline daily. After the completion of experimental period, the serum and various tissues were collected from the mice for further processing.
3.2.6.1 Collection of serum
After 45 days of treatment, serum was collected from all experimental mice for estimation of various biochemical parameters. For this, blood was collected from the retroorbital plexus. The mouse was held in a way that the skin is retracted towards the body causing the eye to protrude out. Then, the capillary was inserted into the canthus of the eye at a 45° angle to the nose into the vessels behind the eye ball. The blood was then collected in a vial. The Vial containing blood was kept undisturbed for 30 minutes in temperature ranging from 40° to 50°. After 30 minutes, the clot was punctured with sterilized needle and serum that oozed out from the clotted blood was collected in clean centrifuge tubes. It was then centrifuged at 3000 rpm for 15 minutes (REMI 9001:2000 CM-12). The serum thus obtained was used for the estimation of various serum lipid profile parameters, globulin and albumin concentration.

3.2.6.2 Preparation of tissue homogenate
After collection of serum, all the experimental mice were sacrificed by cervical dislocation to collect the various organs. Liver, pancreas and kidney selected for the study were removed aseptically from the same animals used for collection of serum. These organs were untied from adhering tissues. Thereafter, these were then given a washing with normal saline solution (0.9% NaCl), ice-cold until it was bleached of all the blood and blotted dry by folding in between the folds of filter paper sheet. Weight of all the organs was noted after drying them. Using scissors, tissues were minced into small pieces and were then homogenized in 0.2 M tris-HCl (10% w/v of homogenate) using Potter Elvehjem homogenizer. The homogenate was filtered through cheesecloth to remove any lumps that might be present. After this, the homogenate was centrifuged at 10,000 rpm, for 20 minutes, at 4°C in cooling centrifuge (Murugesh et al., 2005). Tissue homogenate supernatant of all experimental animals was used to evaluate antioxidant properties of various extracts of *R. communis*. The supernatant thus obtained was used for the estimation of total protein, superoxide dismutase, glutathione peroxidase, reduced glutathione and lipid peroxidation byproducts, Thiobarbituric acid reactive substances (TBARS).
3.2.7 Morphological Methods

Body weight is one of the important morphological parameter in diagnosing diabetes. As in type 1 diabetes, body fails to produce insulin, so the glucose absorbed from the food is not converted to glycogen. It starts eliminating along with urine. As a consequence, individuals who suffer from type 1 diabetes, lose weight in spite of having an increased appetite. However, type 2 diabetic people have a condition of insulin resistance, they are mostly obese due to improper glucose utilization. Body weights of all the animals in the experiment were measured with the help of a physical balance prior to alloxan injection i.e. before inducing diabetes, and immediately after developing diabetes. During the experimental period of 45 days, the body weight of different experimental groups treated with respective plant extracts were monitored at a regular interval of seven days. Further the changes in behavior were also noticed.

3.2.8 Biochemical Assays

Biochemical changes were analyzed in blood, serum and tissue homogenates so as to examine the alterations in various biochemical parameters due to alloxan induced diabetes and the effect of various extracts on these altered parameters.

3.2.8.1 Blood glucose level (Shan et al., 2006)

Both types of diabetes (type 1 and 2) require concerned lifelong maintenance of the homeostasis of glucose intake and insulin. Therefore, in diabetic patients regular estimation of blood glucose level is needed. For estimating the fasting blood glucose concentration, tip of the tail was wiped with 70% ethanol to make the area aseptic. Then, a very small part of the tail tip was clipped with the help of a sterilized scissor. The first drop of blood was wiped out and the next drop was placed in the groove present on the compatible test strip of one touch ultra glucometer (Johnson & Johnson Company, U.S.A) and the sugar level was recorded. The blood glucose level was measured at a regular interval of seven days during the course of experiment. Results were expressed in mg dl⁻¹.
3.2.8.2 Serum/tissue total protein (Lowry et al., 1951)

Serum protein concentration decreases during diabetes. This hypoproteinemia is attributed due to liver damage during diabetes. Hence, estimation of serum protein concentration is important for determining the extent of liver damage in diabetic individuals. Response of Folin-Ciocalteau reagent (alkaline copper) with proteins and the reduction of phosphomolybdic acid by tyrosine and tryptophan present in the protein to give a coloured complex forms the basic principle for protein estimation. The intensity of the colour depends on the amount of the aromatic amino acids present.

Standard curve for protein was prepared by pipetting out albumin solution in a series of test tubes (range 100-1000 μg) and the volume was made up to 4 ml with distilled water. 5.5 ml reagent C (alkaline mix) was added to all the tubes and the reaction mixture was incubated at room temperature for 15 minutes. 0.5 ml of folin’s reagent was then added to it and the contents were mixed rapidly. The test tubes were left for 30 minutes at room temperature. Absorbance was recorded at 650 nm. For estimating protein content, the serum sample used was collected from all experimental animals after 21 days of treatment. 0.1 ml of tissue homogenate and serum was diluted to 4 ml with distilled water. Further, it was processed in the way standard was carried out. Absorbance was recorded at 650 nm by Systronics UV VIS spectrophotometer 2201. Protein content was measured by comparing with protein standard curve. Results were expressed in gm dl⁻¹.

3.2.8.3 Lipid profile

Total Cholesterol (TC), Triglyceride (TG) and High Density Lipoprotein (HDL)-cholesterol were estimated by using respective diagnostic kits (Erba Mannheim Cholesterol kit, Transasia Biomedical Ltd; Daman) and semiautomatic analyzer (Erba Mannheim, CHEM-5 Plus V2). The protocols for the estimation of TC, TG and HDL were followed as per the instructions in the kit literature.

3.2.8.3.a Total cholesterol (Roeschläu’s method, 1974)

Cholesterol is a hydrophobic molecule that is used by animal cells as an essential part of their membranes and as a precursor to steroid hormones. It is transported in the blood as a
part of huge lipoprotein complexes such as HDL, LDL and VLDL. The level of serum cholesterol is characteristically increased in diabetes Mellitus. The estimation of total cholesterol involves a chain of enzyme catalyzed reactions in which cholesterol ester is finally converted to quinoneimine. Absorbance of quinoneimine so formed is directly proportional to cholesterol concentration in the specimen. Preparation of Reagent A and Reagent B have been given in appendix 1.2.1

For estimating serum total cholesterol 500 μl of working reagent (Reagent A) was added to all the tubes labeled as blank (B), standard (S) and test (T). 20 μl of distilled water, 20 μl of cholesterol standard (Reagent B) and 20 μl of serum (from different plant extracts treated groups) were added to respective labeled tubes. The contents were mixed well and incubated at 37˚C for 10 minutes. Blank was aspirated followed by standard and unknown. Absorbances of standard and unknown were read against blank at 505 nm using semiautomatic analyzer.

3.2.8.3.b Serum triglyceride (McGowan et al., 1983)

Lipids absorbed from the diet, thus endogenously generated from carbohydrates are together known as Triglycerides, their estimation helps in prediction and maintenance of diseases associated with hyperlipidemia including diabetes Mellitus. The estimation of cholesterol involves an array of enzymatic catalyzed reactions for the conversion of triglycerides to quinoneimine dye. The triglycerides concentration directly proportional to the intensity of chromogen (Quinoneimine) formed by the sample when measured at 505 nm. Preparation of Reagent A and Reagent B are given in appendix 1.2.2 To estimate the serum triglycerides 500 μl of working reagent (Reagent A) was added to the labeled tubes. 20 μl of distilled water added to tube labeled blank (B), 20 μl of triglyceride standard (Reagent B) added to tube labeled standard (S) and 20 μl of serum (from different extract treated groups) added to tube labeled test (T). The contents were mixed well and incubated for 10 minutes at 37˚C. Absorbance of standard and tests were read at 505 nm on semiautomatic analyzer against reagent blank.
3.2.8.3. c HDL-cholesterol (Burstein et al., 1970)

HDLs (High density lipoproteins) have a similar construction as those of LDLs but contain different proteins. It is composed of particles of different density ranging from highest concentration of proteins, lipids to various lipoproteins. They may entail loose and esterified cholesterol molecules, triglyceride units, phospholipids and apoproteins A, C and E. The transport of cholesterol between liver and body’s cells for its removal as bile is also carried out as HDL. Thus Liver is important in assimilating cholesterol. There exists an inverse relationship between HDL-cholesterol and coronary heart diseases. Contrary to LDL, high serum levels of HDL are related to decreased threat of CHD. Low concentration i.e., below 30 mg/dL is one of the risk factor for cardiac ailments.

Serum HDL-cholesterol was estimated by using kit method in which chylomicrons, LDL and VLDL are precipitated from the serum by phospho-tungstate in the availability of divalent cations such as Mg²⁺. The HDL is left unaltered as the supernatant and is calculated using ERBA cholesterol reagent. Serum on reacting with phosphotungstate in the presence of Mg²⁺ is converted to HDL (supernatant) and LDL, VLDL and chylomicrons (precipitate). For the preparation of reagents/buffers used in protocol refer appendix 1.2.3.

For estimating HDL-cholesterol, first of all LDL, VLDL and chylomicrons were precipitated by adding 500 μl of precipitating agent (Reagent A) to 250 μl of test sample serum. The reaction mixture was mixed well and kept to wait at room temperature for 10 minutes. Then it was made to undergo centrifugation at 4000 rpm for 10 minutes to get a clear supernatant, which, in turn was taken for determining the amount of HDL-cholesterol in the samples. For this 500 μl of cholesterol working reagent was added to all the tubes, 25 μl of distilled water was added to the tube labeled blank, 25 μl of HDL standard (Reagent B) added to tube labeled standard and 25 μl supernatant added to tubes labeled test. After mixing well, the reaction mixture was incubated at 37°C. The absorbance of standard and each test was read against blank at 505 nm on semiautomatic analyzer.
3.2.8.3.d Low density lipoprotein (LDL) and Very low density lipo-protein (VLDL) cholesterol

Besides HDL, LDL and VLDL-cholesterol also transport cholesterol in blood. Low-density lipoprotein (LDL) serves primarily to carry cholesterol molecules from the liver (where they are packaged), to the body’s cells, via blood. Patients with type 2 diabetes overproduce triglyceride-rich VLDL, a response that may be caused by increased serum levels of free fatty acids and glucose. Obesity and insulin resistance also cause overproduction of VLDL.

Transportation of lipoproteins from the liver to muscle and adipose tissue through blood occurs for their activation by apo C-II to lead to the release of free fatty acids from the triacylglycerols of the VLDL. These fatty acids are then taken up by Adipocytes to resynthesize triacylglycerols, the products in intracellular lipid droplets. Myocytes on the other hand, oxidize free fatty acids primarily to supply free energy. Hepatocytes work to remove the maximum number of VLDL remnants from circulation. The loss of TG changes some VLDL to VLDL leftovers (also known as intermediate density lipoproteins, IDL), and with thereafter elimination of TG to LDL. LDL and VLDL were calculated according to the Friedevald’s equation (Kadnur and Goyal, 2005),

\[
\text{LDL-cholesterol} = \text{serum total-cholesterol} - \text{VLDL-cholesterol} - \text{HDLcholesterol}
\]

\[
\text{VLDL-cholesterol} = \frac{\text{serum triglyceride}}{5}
\]

Results are expressed in mg dl\(^{-1}\).

3.2.8.4 Hepatic glycogen content (Jayaraman, 1981)

Glucose is primarily stored as glycogen in cells. Insulin activity motivates the deposition of glucose as glycogen by enhancing the production of glycogen synthetase and lowering down the production of glycogen phosphorylase. Thus, estimation of glycogen content indicates the amount of insulin activity. Hence, depletion of hepatic glycogen storage is directly related to improper activity of insulin in diabetic patients.

Hepatic glycogen content was measured according to the anthrone-\(\text{H}_2\text{SO}_4\) method, with glucose as standard (El-Shenawy and Nabi, 2006). Carbohydrates are dehydrated by conc. \(\text{H}_2\text{SO}_4\) to form Furfural. Furfural condenses with anthrone to form a green colored
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complex, which is measured spectrophotometrically. For reagents/buffers refer appendix 1.3. To prepare standard curve, different volumes of glucose solution was pipetted out in a series of test tubes (range 10-100 μg) and the volume was made upto 1 ml with water. To each tube 4 ml of anthrone-reagent was added and mixed well. The tubes were covered and kept in boiling water bath for 10 minutes. The tubes were then cooled at room temperature and the optical density was measured at 620 nm against blank containing 1ml water and 4 ml anthrone reagent.

Glycogen was precipitated by adding twice the volume of 45% ethanol to 5 ml of homogenate and left in refrigerator for overnight. The precipitate was collected by centrifugation, dissolved in a minimal volume of water. It was then reprecipitated by adding twice the volume of 45% ethanol. The precipitate was then washed with ethanol once and then with diethyl ether. 5 ml of 2.5 N HCl was then added to the precipitate and hydrolyzed by keeping it in boiling water bath for 4 hours. It was then cooled to room temperature. 0.4 ml aliquot was taken from each tube and the volume was made up to 1 ml with distilled water. After cooling the contents on ice, 4 ml anthrone reagent was added to each tube. The reaction mixture was kept on boiling water bath for 10 minutes. After cooling the tubes optical density was read at 620 nm and the hepatic glycogen content was measured by comparing with glucose standard. Results were expressed in mg gm⁻¹ tissue.

3.2.8.4 Estimation of Tissue Total Protein Content (Lowry et al., 1951)

This method is based on the color reactions of amino acids, tryptophan and tyrosine with the Folin’s phenol reagent. By the reaction of these amino acids with phenol molybidic and phosphotungstic acid (present in Folin’s reagent) a blue color is formed which is calorimetrically estimated. This color is the result of reduction of phosphomolybidic acid and phosphotungstic acid and Biuret reaction of proteins with Cu²⁺ in alkaline medium.

0.1ml of suitably diluted sample, BSA standard, water for blank was taken in different test tubes and the final volume was made to 1.0 ml with double distilled water. 3.0 ml of Lowry’s reagent was added to all the tubes, vortexed for 30 seconds and kept the tubes at room temperature for 30 minutes. The absorbance was measured at 750 nm. The protein
concentration was calculated from standard curve made by different concentrations of BSA standard.

3.3 Comparative analysis of in vivo antioxidant potential of hydroethanolic extracts of various parts of R. communis on diabetes induced oxidative stress and the consequent histopathological changes

3.3.1 Indirect measure of free radicals - Lipid peroxidation products (TBARS) (Ohkawa et al., 1979)

The peroxidation of lipids is very often estimated by the Thiobarbituric acid (TBA) test. The method is based on the principle that when the sample is heated with TBA under acidic conditions, it gets the pink colour of various compounds that react with it. Small amount of malondialdehyde (MDA) is produced during peroxidation and produce a pink colored product on reacting with TBA. The product is readily extractable into organic solvents such as n-butanol and absorbs light at 532 nm in acidic solution. Estimation of TBARS is helpful in assessing the amount of tissue damage. Refer appendix 1.4 for preparation of reagents/buffers used.

For determining the lipid peroxidation, 0.2 ml 1.15% KCl, 0.2 ml 8.1% SDS, 1.5 ml 20% acetic acid solution and 1.5 ml aqueous TBA solution was added to 0.2 ml tissue homogenate. Volume of the reaction mixture was made up to 4 ml by adding 0.4 ml distilled water. Reaction mixture mixed thoroughly and heated at 95°C for 60 minutes. After cooling to room temperature, 5 ml of nbutanol: pyridine mixture was added to each tube and thoroughly mixed. After this, 10 mins of centrifugation was carried out at 3000 rpm on the contents of each tube. The upper, clear organic layer was separated and its absorption was measured at 532 nm on a UV-VIS spectrophotometer.

The amount of MDA was inferred by calculating the absorbance coefficient for MDA-TBA complex, which is 1.56 X 105 cm-1M-1 and was expressed in nM TBARS mg-1 tissue protein.
3.3.2 Antioxidant assays

Enzymatic antioxidants form the first line of defense mechanism to protect the organism from reactive oxygen species (ROS) mediated oxidative damage. Following assays were performed to find out antioxidant property of various extracts.

3.3.2.1 Superoxide dismutase (EC 1.15.1.1) Dhindsa et al. (1981).

SOD is a metalloprotein, which functions mainly as first order of antioxidant enzyme for ameliorating the deleterious effect of free radicals produced during the diabetes induced oxidative stress. It neutralizes the effect of superoxide anion which is an important precursor for oxidative stress in the tissues. For reagents and buffer refer appendix 1.5.1

Inclusive in subcellular compartments of all aerobic organisms, this enzyme catalyzes the dismutation reaction of superoxide radicals into oxygen and hydrogen peroxide which is less toxic. The assay derives its basis from the O$_2$ and an electron donor (like Methionine) guided riboflavin illumination that generates superoxide anion. Superoxide radicals are responsible for the reduction of NBT to blue coloured Formazan, practically insoluble, which was measured at 560 nm. SOD by scavenging O$_2^-$ inhibits Formazan formation, reduces blue color in the assay.

$$\text{MnSOD} + \text{O}_2 \rightarrow \text{MnSOD} + \text{O}_2^-$$
$$\text{MnSOD} + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{MnSOD} + \text{H}_2\text{O}_2$$

In 100 μl of the sample (Tissue homogenate), 1.15 ml of phosphate buffer, 100 μl of NBT, 100 μl of sodium carbonate, 200 μl of methionine and 100 μl of EDTA was added. Then 700 μl of distilled water was added to the reaction mixture. 100 μl of riboflavin was then added to the reaction mixture. After that the samples were incubated in light for 60 minutes at room temperature. The SOD activity was measured at 560 nm. Results are expressed as Units min$^{-1}$ mg protein$^{-1}$.

3.3.2.2 Catalase (Luck, 1971)

Catalase activity was determined in the tissue homogenates by the method of Luck (1971). Catalase catalyses the breakdown of hydrogen peroxide (H$_2$O$_2$)
Materials and Methods

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \]

The rate of decomposition of \( \text{H}_2\text{O}_2 \) by catalase is measured spectrophotometrically at 240 nm. 3 ml of \( \text{H}_2\text{O}_2 \) – Phosphate buffer was pipetted out. To it was added 10 µl of the tissue homogenate and was mixed thoroughly. The decrease in absorbance was observed at 240 nm for 3:30 minutes after every 30 sec.

The activity of enzyme was expressed as µmoles of \( \text{H}_2\text{O}_2 \) decomposed/min/mg protein.

### 3.3.2.3 Glutathione peroxidase (GPx, EC 1.11.1.9) (Rotruck et al., 1973)

Two-thirds of the seleno-enzyme, GPx is present in the cytosol and one-third in the mitochondria of the liver. It catalyzes the mechanism in which reduced GSH detoxifies ROS such as hydrogen peroxides and lipidperoxides into water to form glutathione disulphide (GSSG). Besides protecting the tissues from free radical damage, glutathione is also known to be involved in GPx pathway that is known to be capable to decrease hydrogen peroxide and organic hydroperoxides. DTNB was used for the estimation of GSH content. DTNB is reduced by sulfhydryl groups (SH), present in reduced glutathione to form one mole of 2-nitro-5-mercaptobenzoic acid per mole of SH- present. The yellow color of nitromercaptobenzoic can be used to estimate SH- groups. Preparation of reagents/buffers refer appendix 1.5.2. Reaction mixture contained 0.1 ml tissue homogenate, 0.2 ml tris-buffer, 0.25 ml GSH, 0.25 ml sodium azide, 0.4 ml EDTA and 0.5 ml \( \text{H}_2\text{O}_2 \). The contents were incubated at 37°C for 1 minute. Thereafter, the reaction was arrested by adding 1 ml TCA. The contents were centrifuged at 5000 rpm for 15 minutes. Supernatant was assayed for GSH content by using Ellman’s reagent. For this, 1.5 ml phosphate buffer was added to 1 ml of supernatant. The tubes were vortexed properly and 0.5 ml of Ellman’s reagent was added to each tube. The yellowish colour produced was read at 412 nm and compared with the standard curve of reduced glutathione. The GSH-Px activity is expressed as µg GSH consumed min-1 mg-1 protein.

### 3.3.2.4 Reduced glutathione (GSH) (Ellman, 1959)

Glutathione is the most abundant tripeptide, non-enzymatic biological reductant which takes care of the coordination of body’s antioxidant defence issues. It is very important for a variety of detoxification processes such as removal of hydrogen peroxide,
superoxide radicals and alkoxy radicals, which are considered as a major source of oxidative stress. It is an active substrate for glutathione peroxidase and glutathione-S-transferase and is also responsible for maintenance of membrane protein thiols. For estimating GSH, proteins present in the sample were precipitated by adding sulphosalicylic acid and the GSH content was estimated by using DTNB, which is reduced by sulfhydryl groups (SH) present in reduced glutathione to form one mole of 2-nitro-5-mercaptobenzoic acid per mole of SH- present. The nitromercapto-benzoic acid has yellow colour and can be used to measure SH groups. Refer appendix 1.5.3 for preparation of reagents or buffers. The standard curve of GSH was prepared by pipetting out different concentrations of GSH solution (30-300 μg) in a series of test tubes. 1.5 ml phosphate buffer was added to each tube. The tubes were vortexed and 0.5 ml of Ellman’s reagent was added to all the tubes. The yellowish colour produced was read at 412 nm. For estimating GSH, 0.1 ml of sample was taken in a test tube. The volume was made up to 1 ml with distilled water and 1.5 ml sulphosalicylic acid was added to each tube for precipitating out the proteins present in the sample. The reaction mixture was shaken vigorously and centrifuged at 2500 rpm at 4°C for 15 minutes. To 1 ml supernatant, 1.5 ml phosphate buffer was added and the tubes were vortexed. 0.5 ml DTNB was then added to each tube. Yellow colour produced was read immediately at 412 nm. GSH content of the samples was calculated by comparing the absorbance with the standard curve produced from known GSH concentration and is expressed as mg gm⁻¹ tissue.

3.3.2.4 Serum albumin (Doumas et al., 1971).

One of the vital antioxidants in plasma is Albumin, in the part of the body continuously exposed to stress, thus, it may attribute a large part of serum antioxidant properties. Serum albumin concentration was determined by using Bromocresol green (BCG) manual method. The method is based on the principle that binding of a protein to an indicator changes its colour. Among serum proteins, only albumin binds to BCG (an indicator) in a buffered medium. This binding produces a change in the colour of BCG, which is measured colorimetrically, and the intensity of the colour formed is directly proportional to the amount of albumin present in the sample. Preparation of all
reagents/buffers used is described in Appendix 1.5.4. 4 ml of buffered BCG solution (Reagent C) was measured in all the tubes labeled unknown, standard and blank. 0.02 ml of serum (from different plant extract treated groups) was added to respectively labeled test tubes, 0.02 ml of standard albumin was added into the tube labeled standard and 0.02 ml of distilled water was added to blank. Reaction mixture was mixed properly and was allowed to stand for 5 minutes. Unknown and standard were read against blank at 630 nm. Results were expressed in mg dl\(^{-1}\).

3.3.2.5 Serum globulin (Lowry et al., 1951)
Globulins include gamma globulins (antibodies), a varied group of enzymes carrier/transporter proteinaceous molecules. They may be precisely divided into 4 as: gamma globulins (largest portion), beta globulins, alpha-2 globulins, and alpha-1 globulins. Antibodies are produced by mature B lymphocytes called plasma cells, while most of the other proteins in the alpha and beta fractions are made in the liver. Serum globulin is calculated by subtracting albumin from the total proteins. Results were recorded in mg dl\(^{-1}\).

3.3.3 Histopathological Method (Humason, 1979)
Liver, pancreas and kidneys of most effective crude extract treated group (regarding hypoglycemic potential) were studied for histological changes and the observed histological details were compared with those of normal control group, diabetic control group and glibenclamide treated group. The hematoxylene and Eosin preparation was followed for the histopathological study of all the experimental groups. The following steps were followed:

3.3.3.1 Fixation
After 45 days of treatment, the requisite tissues of liver, pancreas and kidney were taken out from the control and treated mice. Tissues were then washed in normal saline. About 2 mm thick pieces of tissue were fixed in bouin’s fluid for 24 hours. After this, the tissues were transferred into tubes covered with muslin cloth and washed in running water for 24 hours.
3.3.3.2 Dehydration and paraffin embedding

Tissues were dehydrated through ascending alcohol series (30%, 50%, 70%, 90% and absolute alcohol). Then tissues were put in xylene till they become translucent. Thereafter, they were embedded in paraffin at 55°C in an incubator.

3.3.3.3 Block preparation

Blocks were casted with pure paraffin wax. For this L-shaped pieces were arranged in a fashion so as to form a block and pure wax was poured into it. Now, tissue pieces with the help of forceps were inserted in the middle of the wax and wax blocks were allowed to solidify.

3.3.3.4 Trimming

The blocks were trimmed in a way that the tissue always lays at the center of the rectangular wax block. The sides of the wax block were made smooth by trimming away the wax and pressing the sides with heated scalpel. The trimmed blocks were mounted over wooden block holder. The blocks were now ready for sectioning.

3.3.3.5 Tissue sectioning

The block holder with block containing tissue was fitted in the microtome and tissue sections having a thickness of 5 microns were cut with the help of microtome. Desired length of the ribbon was taken and was put on the slide already coated with meyer’s albumin (egg albumin + glycerol). The ribbon was then stretched either by placing the slide over hot plate or by pouring lukewarm water drop by drop over the ribbon. After stretching, the slides were left for overnight.

3.3.3.6 Staining and Mounting

The stretched slides were dipped twice in xylene and then transferred to xylene: alcohol (1:1). In the next step, slides were transferred to absolute alcohol and then passed through various grades of descending alcohol series (90%, 70%, 50% and 30% for 5-10 minutes each). Then the slides were transferred to distilled water for 15 minutes. After washing with water, sections were stained by hematoxylein and to avoid over staining slides were transferred to distilled water containing 2 drops of HCl. Now the slides were processed through ascending series of alcohol up to 70% (for 5 minutes each). At this stage sections were stained with eosin for 2 minutes and checked for staining. The slides were then transferred to 90% and 100% for 5 minutes each. After 100%, slides were transferred to
xylene: alcohol and then to pure xylene for 5 minutes each. The slides were then mounted with D.P.X. and coverslip was placed over the slide carefully. The photomicrographs of the slides were recorded with the help of Microscope (Motic).

3.4 Isolation, purification and identification of active principle compounds from the extract showing the antihyperglycemic and antioxidative potential. (Satyanarayana et. al., 2006)

3.4.1 Isolation and purification
Thin Layer Chromatography (TLC) and Column Chromatography were performed for separating the principle components of the most effective treatment after in vivo studies.

3.4.1.1 Thin Layer Chromatography
A thin layer of stationary phase helps the movement of mobile phase across it owing to simple adsorption and capillary action, and thus transferring any analytes present on the layer as the mobile phase moves across the layer of stationary phase from one edge to opposite. This transportation of analytes is at a rate determined by their distribution coefficients, $K_d$ and expressed by its retardation factor, $R_F$, where $R_F = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent front from origin}}$. TLC was carried out in following steps:

3.4.1.1.a Sample Application
The crude extract of *R. communis* leaves was applied as a spot on the plate 2.0 to 2.5 cm away from the edge by means of a micropipette. The solvent was allowed for getting evaporated.

3.4.1.1.b Plate Development
Separation was carried out in a glass tank (TLC chamber) that contained the developing solvent i.e. mobile phase (Ethyl acetate: Acetone: Formic acid 9: 6: 1) to a depth of about 1.5 cm. This was then allowed to stand for at least 1 hour with a lid over the top of the tank to ensure that the atmosphere within the tank becomes saturated with solvent vapors (equilibrium). After equilibration, the lid was removed and the samples loaded TLC
plates were placed vertically in the tank in such a way that the sample spot did not touch the solvent. The plates were taken out of the TLC chamber when the mobile phase had covered two-third the length of the TLC plate and was thereafter dried.

3.4.1.1.c Analyte Detection
After drying the plates were exposed to iodine vapors by placing in a chamber that was saturated with Iodine vapors. The $R_F$ value of the 6 distinct spots that were observed was calculated.

3.4.1.2 Column Chromatography
Column chromatographic analysis was carried out in the following steps:

3.4.1.2.a Preparation of the Column:
The column was placed in a clamp stand in a vertical position. A plug of glass wool was pushed down to the bottom of the column. A slurry of silica gel prepared with the suitable solvent overnight was poured gently into the column. The stop cock was opened and some solvent was allowed to drain out. The layer of solvent was always made to cover the adsorbent; otherwise cracks would develop in the column.

3.4.1.2.b Adding the Sample to the Column:
The sample of the crude extract was dissolved in a minimum amount of appropriate solvent (methanol). The solvent was removed by placing the mixture in a rotary evaporator at a low temperature. The dry powder of the sample now obtained was placed on a piece of weighing paper and transferred to the top of the column through the funnel.

3.4.1.2.c Developing the Chromatogram:
The saturated solvent system (Ethyl acetate: Acetone: Formic acid; 9:6:0.5) was continuously added with the help of funnel to the top of the column. After this the stopcock was opened carefully. The components of the sample ran down the column forming separate bands.

3.4.1.2.d Recovering the Constituents:
Running the solvent system till all the bands were eluted out separately was continued. The different fractions were collected in different flasks and evaporated.

3.4.1.2. Analyzing the constituents:
The fractions collected were analyzed by TLC and this was referred to as Confirmatory TLC to make sure that the fractions collected revealed single spots with desired R\textsubscript{F} values.

3.4.2 Evaluation of effects of the purified principle compounds on diabetes and related oxidative stress on experimental animals.
To test the antihyperglycemic, antidyslipidemic and antioxidative activities of the two purified compounds, the animals were divided into 2 experimental groups of 7 mice each and named C1 treated (C1T) and C4 treated (C4T) respectively. Diabetes was induced in all the animals by a single intraperitoneal injection of Alloxan. Each compound was then administered orally to the alloxan induced diabetic mice at 50 mg kg\textsuperscript{-1} BW for 45 days (Sharma and Singh, 2014; Sharma and Garg, 2011). After the experimental period steps 3.2.6 to 3.3.3.vi were repeated on animals of these two groups.

3.4.3 Characterization and identification of the isolated compound with the best antidiabetic and antidyslipidemic potential:
For characterization of compounds isolated, IR, NMR, GC Mass spectroscopy and HPLC were performed.

3.4.3.1 Spectrophotometry
Infrared (IR, RKin ELMER), Gas Chromatography- Mass Spectroscopy (GC-MS) spectra were obtained for the compound that was found to be most effective in controlling hyperglycemia in alloxan-induced diabetic mice.

3.4.3.2 High Performance Liquid Chromatography (HPLC)
HPLC of the isolated compounds was carried out to confirm its nature by comparing it against standard (Indole acetic acid). For this an isocratic HPLC (Shimadzu HPLC class VP series) with two LC-10 AT VP pumps (Shimadzu), variable wavelength programmable photodiode array detector SPD MIOA VP (Shimadzu), CTO-IOAS VP
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column oven (Shimadzu), SCL-10A VP system controller (Shimadzu) and reverse phase Luna 5 mC18 (2) Phenomenex column (250 mm x 4.6 mm) was used. The HPLC system was equipped with software class VP series version 6.1 (Shimadzu). The mobile phase components Ethyl acetate: Acetone: Formic acid; 9:6:0.5 were filtered through 0.2 µ membrane filter before use and pumped from the solvent reservoir to the column at a flow rate 1 ml min\(^{-1}\) which yielded a column backpressure of 16-165 Kgf cm\(^{-2}\). The column temperature was maintained at 27º C. 20 ml of sample was injected using Rheodyne syringe (Model 7202, Hamilton).

3.5 Analysis of gene expression of various enzymatic antioxidants.

3.5.1 Isolation of Total RNA (Chomczynski, 1993)

Total RNA from different tissues was isolated using TRIzol\(^{\circledR}\) (MRC, CAT# TR-118) as per the manufacturer’s instructions. All the centrifugation steps were performed at 4ºC, if otherwise mentioned. In general, 1 mL of TRIzol was added to 50-100 mg of tissue/approx. 1x10\(^8\) cells. The tissue was homogenized with liquid nitrogen in mortar pestle kept in ice. The homogenate (1mL) was then transferred to a sterile 2mL micro-centrifuge tube. To the homogenate, chloroform (Sigma, CAT# C2432) at the rate of 0.2 mL.mL\(^{-1}\) of TRIzol was added. The sample was mixed vigorously for 15 sec and allowed to set at room temperature for 15 min. It was then centrifuged at 12,000 x g (≈14000 rpm) in a refrigerated table top centrifuge (Mikro 22R, Hetich Zentrifugen, Germany) for 15 min. The clear aqueous phase was transferred to a fresh RNase/ DNase-free 1.5ml micro-centrifuge tube and 0.5 ml isopropanol per ml of TRIzol was added to precipitate the RNA. The contents were mixed gently and again incubated at room temperature for 10 min, followed by centrifugation at 12,000 x g for 15 min. The supernatant was discarded and the RNA pellet obtained was washed with 1 ml of 75% ethanol (prepared in nuclease free water) by centrifugation at 10000g for 2 min at 4ºC. Finally, the RNA pellet was air dried and dissolved in appropriate volume of sterile nuclease free water (sH\(_2\)O). All the RNA samples were aliquoted, stored at -70ºC till further use and named as follows:
Table 3.3: Nomenclature of isolated RNA

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL</td>
<td>Normal Liver</td>
</tr>
<tr>
<td>NK</td>
<td>Normal Kidney</td>
</tr>
<tr>
<td>DCL</td>
<td>Diabetic Control Liver</td>
</tr>
<tr>
<td>DCK</td>
<td>Diabetic Control Kidney</td>
</tr>
<tr>
<td>GTL</td>
<td>Glibenclamide Treated Liver</td>
</tr>
<tr>
<td>GTK</td>
<td>Glibenclamide Treated Kidney</td>
</tr>
<tr>
<td>RCLL</td>
<td><em>R. communis</em> Leaf Liver</td>
</tr>
<tr>
<td>RCLK</td>
<td><em>R. communis</em> Leaf Kidney</td>
</tr>
</tbody>
</table>

3.5.2 RNA quantification and purity determination

Total RNA was quantified by diluting 5 μl RNA stock solution in 995 μl of sterile DEPC-treated water (pH 8.0). The absorbance values at 260 nm and 280 nm were taken using double beam spectrophotometer (Specord 200, Analytik Jena, USA). The total RNA content of the sample was calculated using the following equation:

\[
\text{Total RNA} = \frac{(A_{260} \times 40 \times 200)}{1000}.
\]

In this equation, \(A_{260}\) is the absorbance value at 260 nm, 40 is a constant for RNA quantification, 200 is the dilution factor and division by 1000 yields μg.μl\(^{-1}\). RNA purity was determined by finding \(A_{260}/A_{280}\) ratio. The total RNA with the ratio values greater than 1.8 deemed acceptable for subsequent use and was aliquoted and stored at -70°C.

Isolated RNA samples were also analyzed by Nanodrop spectrophotometer (Bio-Tek instruments, Inc, Saint-Quentin en Yvelines, France). RNA concentration was determined at absorbance 260 nm (\(A_{260}\)). The purity of the RNA was determined from the ratio of \(A_{260}/A_{280}\).

3.5.3 cDNA synthesis

Kit used: Protoscript II- First strand cDNA synthesis kit (NEB) #E6560S

cDNA was synthesized by adding to a RNase/DNase/Pyrogen free PCR tube: 1 μg of total RNA, 1 μl of random hexamer (0.2μg/μl) and DEPC treated water up to 11 μl. The contents of the tube were mixed gently and spun briefly and were incubated at 65°C for 10 min to denature secondary structure of RNA and then for 2 min at room temperature. The reagents were further added to the tube to make the final volume to 20 μl: these were
4 µl 5X reaction buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl, 20 mM MgCl₂, 50 mM DTT), 1 µl of RNase inhibitor (20 IU), 2µl of dNTP mix (10 mM), 2µl of M-MuLV Reverse Transcriptase (200 IU). The contents were mixed gently, spun briefly and incubated in thermocycler at 25°C for 10 min, 42°C for 30 min, 95°C for 3 min and 4°C pause.

3.5.4 Primer designing for conventional and Real-Time PCR

All the primers for conventional PCR were designed using Primer 3.0 software located at Whitehead Institute for Biomedical Research, MIT for genome research and available online at [http://www.genome.wi.mit.edu](http://www.genome.wi.mit.edu) and custom synthesized by Integrated DNA Technologies (CA, USA). The primers for real time PCR analysis were designed using QuantPrime online software ([available at](http://www.quantprime.de/)). Primers were always chosen according to the following parameters, length: 18 to 25 (optimal, 25); Tm: between 57°C and 65°C (optimal, 60°C – 62°C). To determine the specificity of the primers, all sequences were compared with the sequences available in GenBank using BLAST (Basic Local Alignment Search Tool) software available at National Centre of Biotechnology (NCBI) website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The sequence homology between different species was determined by ClustalW Multiple Sequence Alignment Program (Courtesy: BCM Search Launcher and available at the website [http://searchlauncher.bcm.tmc.edu](http://searchlauncher.bcm.tmc.edu)).

3.5.5 Primer optimization by conventional gradient PCR

Optimizing the annealing temperature of PCR assay is critical for reaction specificity. After calculating the Tₘ of the primers designed, determination of the annealing temperatures empirically is required. This involved repeating the same reaction at many different temperatures.

The optimal annealing temperature for an assay was determined using a Bio-Rad thermal cycler that had a thermal gradient feature. The gradient feature allowed testing a range of temperatures above and below the calculated Tₘ of the primers simultaneously, optimizing the annealing temperature in a single experiment. The results were then analyzed using agarose gel electrophoresis. Additional bands appeared on the gel in case
of nonspecific amplification. The optimal annealing temperature was considered as the one with the highest yield with no nonspecific amplification. Additional optimization steps were undertaken if satisfactory results were not obtained at any annealing temperature in a single assay.

3.5.6 Relative quantification
The mRNA levels of the genes were quantified by relative quantification method using the Taq PCR kit NEB #E5000S. It includes the following steps:

The primers designed for RT PCR are mentioned in table 2. The PCR was performed in a total volume of 12 µL. The reaction mixture consisted of 2 µL of cDNA, 0.2 µM primers and 5 µL of 2X SYBR mix the reaction volume was made up to 12 µL with the help of nuclear free water. For relative quantification GAPDH was used as normalizer to nullify any possible experimental error. For SOD, CAT, GPx, GSR coding regions, PCR reactions were performed by heating the contents at 94°C for 5 min (pre incubation), followed by 40 cycles of 94°C for 20 sec (denaturation), 60°C or 58°C for 15sec (annealing), 72°C for 20 sec (extension), each step was followed by a plate read for acquisition of the data. This was followed by melt curve analysis by incubation at 97°C for 1 min followed by 0.05 sec incubation for each 0.5°C rise in temperature ranging from 65°C to 95°C with continuous acquisition. The reaction was stopped by cooling the reaction mixture to 4°C.

3.5.7 Data Analysis by Relative Quantification (Livak and Schmittgen, 2001)
The quantitative (relative quantification) PCR results were analyzed with CFX manager software (BIORAD) and $2^{\Delta\Delta C_T}$ method.

Following assumptions were made to derive the formula which was used to calculate fold change is illustrated as below:

- This method assumed that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other.
- First, normalized the C_T (Threshold cycle) of the target gene to that of the reference gene, for both the test sample and the calibrator sample:

\[ \Delta C_T (\text{test}) = C_T (\text{target, test}) - C_T (\text{ref, test}) \]
\[ \Delta C_T \text{ (calibrator)} = C_T \text{ (target, calibrator)} - C_T \text{ (ref, calibrator)} \]

Where: Target was the gene of interest

Reference was the internal housekeeping gene

Calibrator was the gene selected for studying the relative expression

\[ \Delta C_T \text{ (test)} \] was equal to the difference in threshold cycles for target and reference

\[ \Delta C_T \text{ (calibrator)} \] was equal to the difference in threshold cycles for target and calibrator

- Second, normalized, the \( \Delta C_T \) of the test sample to the \( \Delta C_T \) of the calibrator:

\[ \Delta \Delta C_T = \Delta C_T \text{ (test)} - \Delta C_T \text{ (calibrator)} \]

- Finally, calculated the expression ratio \( = 2^{\Delta \Delta C_T} \)

### 3.6 Statistical Analysis

The data collected was statistically analyzed by utilizing SPSS (Version 16.0) and SigmaPlot (Version 8.0). The results with \( P<0.05 \) were taken as significant.

The statistical methods used for analyzing data in the present study are:

#### 3.6.1 Arithmetic Mean

Referred as ‘Average’ in layman language, this is the commonest method for inferring central tendency, put to use very often. It is calculated by summing up all the items and then dividing this sum by the total number of items.

\[
\overline{X} = \frac{X_1 + X_2 + X_3 + \ldots \ldots \ldots + X_n}{N} \quad \text{or} \quad \overline{X} = \frac{\Sigma X}{N}
\]

\( \overline{X} = \) Arithmetic mean

\( \Sigma X = \) Sum of all the values of the variable i.e. \( X_1 + X_2 + X_3 + \ldots \ldots \ldots X_n \)

\( N = \) Total Number of Observations.

#### 3.6.2 Standard Deviation (Pearson, 1894)

It is the most important and widely used measure of studying dispersion.

Standard deviation is also known as root mean square deviation for the reason that it is the square root of the mean of the squared deviation from the
arithmetic mean.

\[ \sigma = \sqrt{\frac{\sum x^2}{N}} \]

\[ x = (X - \bar{X}), \text{deviations of the items from the mean} \]
\[ \bar{X} = \text{Mean} \]

3.6.3 **Student's t-test** (Gossett, 1970)

It is used when the sample size is 30 or less than 30. It has following applications:

3.6.3.1. For testing the significance in the difference between the means of two independent samples. Here it is assumed that the two samples are independent i.e. the values of observations of one sample do not depend on the other.

\[ t = \frac{\bar{X}_1 - \bar{X}_2}{S \sqrt{\frac{n_1 + n_2}{n_1 n_2}}} \]

\[ \bar{X}_1 = \text{Mean of the first sample} \]
\[ \bar{X}_2 = \text{Mean of the second sample} \]
\[ n_1 = \text{Number of observations in the first sample} \]
\[ n_2 = \text{Number of observations in the second sample} \]
\[ S = \text{Combined standard deviation} \]
\[ S = \sqrt{\frac{\sum (X_1 - X_2)^2 + (X_2 - X_2)^2}{n_1 + n_2 - 2}} \]

If the calculated value of \( t > t_{0.05} \) (table value) the difference between the sample means is said to be significant at 5% level of significance, otherwise the data is said to be consistent with the null hypothesis i.e. the difference between the sample means is insignificant.
3.6.3.2. For testing the significance in the difference between the means of two dependent samples (or matched paired observations). Here the samples are dependent (or paired). Two samples are said to be dependent when the elements in one sample are related to those in the other in any significant or meaningful manner. In fact, the two samples may consist of pairs of observations made on the same object, individual, or more generally, on the same selected population elements. When samples are dependent they comprise the same number of elementary units. The test based on paired observations is defined by the following formula.

\[
t = \frac{d - \overline{d}}{\sqrt{\frac{S}{n}}} \times \sqrt{n}
\]

\[
t = \frac{d}{\sqrt{n}}
\]

\[
d = \text{the mean of the differences}
\]

\[
S = \text{standard deviation of the differences}
\]

The value of S is calculated as follows:

\[
S = \sqrt{\frac{\sum(d - \overline{d})^2}{n-1}}
\]

3.6.4 Analysis of Variance (ANOVA) (Fisher, 1920)

The Analysis of Variance frequently referred to by the contraction ANOVA is a statistical technique specially designed to test whether the means of more than two quantitative populations are equal. One-way ANOVA: In one-way classification, the data is classified according to only one criterion. The null hypothesis is:

\[
H_0 = \mu_1 = \mu_2 = \mu_3 = \ldots = \mu_k
\]
i.e., the arithmetic means of populations from which the k samples were randomly drawn are equal to one another. The alternate hypothesis is:

\[ H_1 = \mu_1 \neq \mu_2 \neq \ldots \neq \mu_k \]
i.e., all means are not equal.

**Table 3.4: Sources of variation**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS (Sum of Squares)</th>
<th>v (Degree of freedom)</th>
<th>MS (Mean square)</th>
<th>Variance ratio of F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between samples</td>
<td>SSC</td>
<td>( v_1 = c - 1 )</td>
<td>MSC = SSC/(c-1)</td>
<td>MSC/MSE</td>
</tr>
<tr>
<td>Within samples</td>
<td>SSE</td>
<td>( v_2 = n - c )</td>
<td>MSE = SSE/(c -1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>SST</td>
<td>( n -1 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SST = total sum of squares of variations
SC = sum of squares between samples (columns)
SSE = sum of squares within samples (rows)
MSC = mean sum of squares between samples
MSE = mean sum of squares within samples

By comparing the calculated value of F with the tabulated values for respective degrees of freedom at certain level of significance, the null hypothesis of the homogeneity of various treatments and various varieties may be accepted or rejected. If the calculated value of F is greater than the table value, it is considered that the difference in sample means is significant, i.e. it could not have arisen due to fluctuations of simple sampling, or in other words, samples do not come from the same population, thus rejecting the null hypothesis. On the other hand, if the calculated value of F is less than the table value, the difference is not significant and has arisen due to fluctuations of simple sampling, thus accepting the null hypothesis.
Figure 3.1 Intact and powdered forms of various parts of *R. communis*
Material and Method

Figure 3.2 Animal handling