Diabetes mellitus occurs as a result of the decline in cell membrane sensitivity and is greatly exacerbated by the consumption of refined carbohydrates, lack of exercise and obesity. In India, high occurrence of the disease is noted in urban population. Management of diabetes mellitus is considered a global problem and successful treatment is yet to be discovered. Modern medicines which offer variety of effective treatment options can have several side effects like hepatotoxicity, flatulence, abdominal pain and hypoglycemia (Meenakshi et al., 2012).

Alternate systems of medicine like Ayurveda, Siddha, Homeopathy and Unani are widely used in India for treatment and cure of many diseases. Herbal drugs are popular for their safety and efficacy. Scientific investigations are needed to prove the efficacy of these wonder drugs to make them reach a wider population. One of the emerging trends in the management of diabetes mellitus is exploring the medicinal plants / natural products for potent inhibitors of carbohydrate metabolizing enzymes alpha-amylase and alpha-glucosidase which would help in the regulation of blood glucose level. Many medicinal plants with antidiabetic potential that are used in traditional medicine are being studied for alpha-amylase and alpha-glucosidase properties (Kathirvel et al., 2012 and Bhat et al., 2008). Saraca indica and Polyalthia longifolia are two such medicinal plants being used in the preparation of many ayurvedic formulations. The present study is aimed at determining their efficacy on the treatment of diabetes mellitus by testing for the inhibition of α-amylase, α-glucosidase, antioxidant potential, cytotoxicity studies in vera cell-lines and L6 cell-lines, in vivo studies for testing acute toxicity using Swiss albino mice and antidiabetic potential using niacinamide-streptozotocin (NIA-STZ) induced diabetic Wistar rats as model system, isolation and characterization of...
antidiabetic and antioxidant principles using various techniques. The experimental design conducted in the present study was carried out in four phases.

**Phase I**

**3.1 Screening and Quantification of Selected Phytochemicals and Inorganic Elements in the Bark of *Saraca indica* and *Polyalthia longifolia***

**3.1.1 Collection and Processing of Plant material**

The barks of *Saraca indica* and *Polyalthia longifolia* were collected from Central Institute for Medicinal Plants Heritage (CIMH), Kanjikode, Kerala and authenticated by the botanist in Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore and Voucher specimen is deposited at IFGTB, Coimbatore. Samples collected were shade dried, pulverised by mechanical grinder, made into powder and stored in air tight container.

**3.1.2 Identification and Quantification of Selected Phytochemicals in the bark of *Saraca indica* and *Polyalthia longifolia***

*Saraca indica* and *Polyalthia longifolia* barks were collected, shade dried, then ground to a fine powder and stored at room temperature.

**3.1.2.1 Phytochemical Screening**

Qualitative analysis of phytochemicals viz., alkaloids, phenols, tannins, flavonoids, saponins, steroids and terpenoids was performed in the bark of *Saraca indica* and *Polyalthia longifolia* by standard procedures (Harborne, 1973 and Trease and Evans, 1989).

**Test for Alkaloids**

1.2 g of iodine and 2.0 g of potassium iodide were dissolved in 5 mL of sulphuric acid and the solution was diluted to 100 mL. 10 mL of the extracts of *Saraca indica* and *Polyalthia longifolia* barks were acidified by adding 1.5% v/v of HCl and a few drops of Wagner's reagent. Formation of yellowish brown precipitate confirmed the presence of alkaloid.

**Test for Phenols**
Neutral ferric chloride reagent was prepared and added to the extracts. The appearance of blue colour showed the presence of phenols.

**Test for Tannins**

The test solutions of the extracts were treated with few drops of lead acetate solution. The formation of white precipitate indicated the presence of tannins.

**Test for Flavonoids**

Magnesium ribbon was added to the extracts and hydrochloric acid was added along the sides of the test tube. A deep blue colour showed the presence of flavonoids.

**Test for Saponins**

5 mL of the extracts were taken in different tubes and added a pinch of sodium bicarbonate. The mixture was shaken vigorously and kept for 3 min. A honeycomb like froth indicated the presence of saponins.

**Test for Terpenoids**

2 mL of extract was added to 2 mL of acetic anhydride and concentrated H$_2$SO$_4$. Formation of bluish green rings indicated the presence of terpenoids.

### 3.1.2.2 Quantification of Selected Phytochemicals

Phenols, tannins and flavonoid compounds present in various medicinal plants are reported to possess antioxidant properties and can act as potent inhibitors of carbohydrate metabolizing enzymes (Mannan *et al.*, 2014).

**Phenols**

The total phenol content was determined by the method of Mallick and Singh (1980). Phosphomolybdic acid and phosphotungstic acid present in the Folin-Ciocalteau reagent is reduced by phenolic hydroxyl group to develop a blue color in alkaline condition which can be measured at 660 nm. To 0.1 mL of ethanolic extract of *Saraca indica* and *Polyalthia longifolia* added 0.5 mL of diluted Folin Ciocalteau reagent. Incubated for 10 min and added 1.0 mL of 20 % Na$_2$CO$_3$ solution. Mixed thoroughly, incubated for 30 min at 37°C and made up the volume to 5 mL with...
distilled water. Cooled and measured the absorbance at 660 nm. Pyrocatechol (5-25 µg) standards were treated in a similar way. From the standard graph calculated the amount of polyphenols and expressed as mg of phenols per g of the sample.

**Tannins**

The tannin content of the bark was determined with Folin Denis reagent using tannic acid as standard by the method of Schanderl (1970). Tannin-like compounds reduce phosphotungstic-phosphomolybdic acids in alkaline solution to produce a highly coloured blue solution which can be measured at 660 nm. Weighed 0.1 g of the powdered bark material of *Saraca indica* and *Polyalthia longifolia* separately in two conical flasks, added 25 mL alcohol and heated gently by boiling for 30 min. Centrifuged at 2000 rpm for 20 min and collected the supernatant in 100 mL volumetric flask and made up the volume with alcohol. Pipetted out 0.2 mL of the bark extracts to different test tubes and made up the volume to 2.0 mL with distilled water. Added 0.5 mL of Folin-Denis reagent, 1 mL of 35% sodium carbonate solution and diluted to 10 mL with water. Mixed well and read the absorbance at 700 nm after 30 min. Tannic acid standards (5-25 µg) were treated in a similar way. From the standard graph calculated the amount of tannins and expressed as mg of tannins per g of the sample.

**Flavonoids**

Flavonoids were estimated by the method of Cameron *et al.* (1943). Flavonoids react with vanillin to produce a coloured product, which can be measured spectrophotometrically. A portion of the ground bark sample of *Saraca indica* and *Polyalthia longifolia* were weighed out and extraction was carried out in two steps, first with methanol : H₂O (9:1) and secondly with methanol : H₂O (1:1). At each step, sufficient solvent was added, to make liquid slurry and the mixture was left for 6-12 hours. Filtration of the extracts was carried out rapidly by using a glass wool plug in the neck of a filter funnel. The two extracts were then combined and evaporated to about 1/3rd of the original volume. The resultant aqueous extract was cleared of low polarity contaminants such as fats, terpenes and pigments by
extraction (in a separating funnel) with hexane. This was repeated several times and the extracts combined. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated. An aliquot of the extracts was pipetted into different test tubes and evaporated to dryness followed by the addition of 4.0 mL vanillin reagent (1% vanillin in 70% conc. H₂SO₄) and heated for 15 min in a boiling water bath. Catechin standard (25-100 µg) was also treated similarly. Measured the absorbance at 360 nm and calculated the concentration of flavonoids present.

**Terpenoids**

The estimation of terpenoids in the barks was performed using linalool, a monoterpenene as standard. The reaction is based on the brick red precipitation that is formed due to the reaction of terpenoids with the reaction mixture containing sulphuric acid and the precipitate that was dissolved in methanol was determined spectroscopically at 538 nm (Ferguson, 1956; Indumathi et al., 2014). The method was followed with slight modifications as described. 1 g of the bark samples were homogenized in ice-cold methanol, centrifuged at 4000 g for 15 min at room temperature and collected the supernatant in a fresh 2 mL microfuge tube. Added 1.5 mL chloroform in each 2 mL microcentrifuge tube, followed by 200 µL of sample supernatant. For the standard curve 200 µL of linalool solution in methanol was taken and mixed with 1.5 mL chloroform, serial dilution was done to get a concentration of 1 mg / 200 µL. Vortexed the sample mixture thoroughly and after 3 minutes added 100 µL of concentrated sulphuric acid (H₂SO₄) to each 2 mL microcentrifuge tube by placing the tubes in ice. The assay tube was incubated at room temperature for 1.5 h - 2 h in dark. For standard solution (linalool) incubation time was 5 minutes and during incubation time the microcentrifuge tubes were not disturbed. At the end of incubation time a reddish brown precipitation was formed in each assay microcentrifuge tube, which was carefully removed by gently decanting the supernatant reaction mixture. Added 1.5 mL of 95% (v/v) methanol & vortexed thoroughly until all the precipitation dissolved in methanol completely and transferred the sample to cuvette (95% (v/v) methanol was used as blank) and read
the absorbance at 538 nm. Calculated total terpenoids concentration in the bark samples as linalool equivalents.

3.1.3 Identification and Quantification of Inorganic Elements in the bark of Saraca indica and Polyalthia longifolia by Proton Induced X-ray Emission (PIXE)

Proton Induced X-ray Emission (PIXE) is an X-ray spectrographic technique, which can be used for the non-destructive, simultaneous analysis for the 72 inorganic elements from Sodium through Uranium on the periodic table for solid, liquid, and thin film (i.e. aerosol filter) samples. Inorganic elements were analysed and quantified in the barks of the two medicinal plants using this technique.

3.1.4 Preparation of various Solvent Extracts and Calculation of Percentage Yield

Preparation of aqueous, ethylacetate, ethanol, chloroform and hexane extracts of Saraca indica and Polyalthia longifolia barks was done by placing the bark in five parts of the respective solvents in mechanical shaker for 48 hours at 40°C with 100 revolutions per minute and filtered. Aqueous extracts were concentrated by lyophilization and other solvent extracts were concentrated by flash evaporation and the extracts were stored at 4°C. Aqueous extracts of Saraca indica and Polyalthia longifolia bark are referred as SIA, PLA; ethyl acetate extracts of Saraca indica and Polyalthia longifolia bark are referred as SIEA, PLEA; ethanol extracts of Saraca indica and Polyalthia longifolia bark are referred as SIE, PLE; hexane extracts of Saraca indica and Polyalthia longifolia are referred as SIH, PLH; chloroform extracts of Saraca indica and Polyalthia longifolia are referred as SIC and PLC respectively. The yield of extract obtained using various solvents was calculated and expressed as percentage.

Phase II

3.2 Inhibition of Alpha-amylase and Alpha-glucosidase activities, Antioxidant potential and in vitro Cytotoxicity of the Barks of Saraca indica and Polyalthia longifolia
The solvent extracts of *Saraca indica* bark - aqueous (SIA), ethyl acetate (SIEA), ethanol (SIE), chloroform (SIC), hexane (SIH) and solvent extracts of *Polyalthia longifolia* bark - aqueous (PLA), ethyl acetate (PLEA), ethanol (PLE), chloroform (PLC), hexane (PLH) were screened for inhibition of alpha-amylase, alpha-glucosidase and total antioxidant potential. From this the potent extracts (one from polar solvent and one from non-polar solvent) were used for testing the antioxidant properties in terms of various free radical scavenging properties and *in vitro* inhibition of lipid peroxidation. The extract with highest α-amylase inhibition as well as antioxidant potential was further used for studying the dose dependent inhibition of α-amylase, α-glucosidase activities, determination of *in vitro* cytotoxicity in Vero cell-lines and L6 cell-lines.

### 3.2.1 Screening of various Solvent Extracts of the Bark of *Saraca indica* and *Polyalthia longifolia* for Inhibition of Alpha-amylase and Alpha-glucosidase activities and Antioxidant potential

#### Preparation of extracts

The solvent extracts of *Saraca indica* bark - aqueous (SIA), ethyl acetate (SIEA), ethanol (SIE), chloroform (SIC), hexane (SIH) and solvent extracts of *Polyalthia longifolia* bark - aqueous (PLA), ethyl acetate (PLEA), ethanol (PLE), chloroform (PLC), hexane (PLH) were prepared by dissolving at a concentration of 1 mg/mL. Aqueous, ethyl acetate and ethanol extracts were dissolved in respective solvents while hexane and chloroform extracts were dissolved using dimethyl sulphoxide (DMSO).

#### 3.2.1.1 Inhibition of Alpha-amylase activity

The extracts were tested for alpha-amylase inhibitory potential by the method of Bernfeld, (1955) with slight modifications as described below. The reducing sugars produced by the action of α-amylase react with dinitrosalicylic acid and reduce it to a coloured product, which is measured at 540 nm. The amylase inhibitor inhibits the action of amylase that hydrolyses starch to maltose. In brief, 100 μL of various extracts (SIA, SIEA, SIE, SIC, SIH, PLA, PLEA, PLE, PLC and PLH) were allowed to react with 200 μL of porcine pancreatic α-amylase enzyme (dissolved 1 mg α-amylase / mL of 0.1 M sodium acetate buffer, pH 4.7) and
100 μL of 0.1 M sodium acetate buffer, pH 4.7. After 20 min of incubation 0.5 mL of 1% starch was added. The same was performed for the control where 200 μL of enzyme was replaced by the buffer. Incubated for 15 minutes and added 1 mL of DNS (dissolved by stirring 1 g dinitrosalicylic acid, 200 mg crystalline phenol in 100 mL of 1% NaOH solution, 50 mg sodium sulphite added at the time of use) to both the control and test. The tubes were kept in a boiling water bath for 10 minutes. At hot conditions added 0.5 mL of 40 % potassium sodium tartarate and made up the volume to 5 mL with distilled water. The absorbance was recorded at 540 nm using a spectrophotometer and the percentage of α-amylase inhibition was calculated using the formula,

\[
\text{Inhibition (\%)} = 100 \left( \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \right).
\]

Suitable reagent blank and inhibitor controls were also carried out and subtracted the absorbance values from the test value. Dose dependent variation in the α-amylase inhibition was measured using 25 μL to 200 μL of the extracts. Acarbose was used as standard inhibitor and tested in parallel at varying concentrations (10-100 µg).

3.2.1.2 Inhibition of Alpha-glucosidase activity

The extracts to be tested for their inhibitory property of α-glucosidase activity were prepared as mentioned in 3.2.1.1. The α-glucosidase inhibitory activity was determined by measuring the release of 4-nitrophenol from p-nitrophenyl α-D glucopyranoside as given by Sun et al. (1995). The assay mixtures for these experiments contained 0.3 mL of 10 mM paranitrophenyl alpha-D-glucopyranoside, 1.0 mL of 0.1 M potassium phosphate buffer, pH - 6.8, 0.2 mL of enzyme solution and 0.2 mL of various extracts (SIA, SIEA, SIE, SIC, SIH, PLA, PLEA, PLE, PLC and PLH) all in a final volume of 1.7 mL. Following an incubation time of 30 min at 37°C, the reaction was terminated by the addition of 2.0 mL of 100 mM sodium carbonate. The liberated p-nitrophenol was determined at 400 nm using spectrophotometer. The percentage inhibition rates were calculated using the formula
Inhibition (%) = 100 (Absorbance \text{Control} - \text{Absorbance \text{Test}} / \text{Absorbance Control})

Suitable reagent blank and inhibitor controls were also carried out and the absorbance values were subtracted.

3.2.1.3 Total Antioxidant potential by Ferric Reducing Antioxidant Power (FRAP) assay

The total antioxidant potential of sample was determined using ferric reducing ability of plasma (FRAP) assay of Benzie and Strain (1996) as a measure of “antioxidant power”. FRAP method is based on the reduction of the Fe$^{3+}$-tripyridyl-s-triazine (Fe$^{3+}$-TPTZ) complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm.

The various solvent extracts of the bark of \textit{Saraca indica} (SIA, SIEA, SIE, SIH, SIC) and \textit{Polyalthia longifolia} (PLA, PLEA, PLE, PLH, PLC) were screened for total antioxidant potential by FRAP method (Ferric Reduction Antioxidant Power). The reaction mixture containing different volumes of extracts were made up to a final volume of 1.5 mL to which 1.5 mL of FRAP reagent (25 mL of acetate buffer of pH 3.6, 2.5 mL of 10 mM triphenyl tetrazolium chloride (TPTZ) solution and 2.5 mL of 20 mM ferric chloride) was added and the absorbance measured at 593 nm at 37°C. A standard solution of 4 mM ascorbic acid was tested in parallel.

3.2.2 Free Radical Scavenging assays

Among the various polar solvent extracts, ethanol extract of the bark and among the non-polar solvent extracts, hexane extract of the bark of the two selected medicinal plants showed highest inhibition of alpha-amylase, alpha-glucosidase activities and total antioxidant potential. Hence they were compared for their free radical scavenging properties and inhibition of \textit{in vitro} lipid peroxidation.

Ethanol extract of \textit{Saraca indica} and \textit{Polyalthia longifolia} (SIE, PLE) were dissolved in ethanol while the hexane extract of \textit{Saraca indica} and \textit{Polyalthia longifolia} (SIH, PLH) were dissolved in DMSO to get a final concentration of 1 mg/mL. This was diluted to get various concentrations (20-100 µg/mL) in the final volume of reaction mixture. The free radical scavenging activity of the
extracts was analyzed by following the various standard in vitro radical generating model systems namely 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), superoxide anion (O₂), hydroxyl (OH) and nitric oxide (NO). In all the experiments de-ionized water served as blank and reaction mixtures without extracts served as control samples. The percentage scavenging (or) inhibition was calculated according to the following formula

\[
\text{Percentage scavenging (or) Inhibition} = \frac{(C - T)}{C} \times 100
\]

C - Absorbance of control, T - Absorbance of test. All the experiments were performed thrice and the mean values were taken.

3.2.2.1 DPPH radical Scavenging activity

The effect of SIE, SIH, PLE and PLH on DPPH radical scavenging activity was estimated as per the method of Hou et al. (2001). DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine. This can be identified by the conversion of purple to light yellow colour. Aliquots containing different concentrations (20-100 μg) of ethanol extracts SIE and PLE, hexane extracts SIH and PLH were made up to 1 mL. To this 2 mL of 0.1 mM DPPH was added. In the control, 2 mL of DPPH and 1 mL of distilled water were taken. All the tubes were incubated at 37°C for 20 min. Absorbance of reaction mixtures was recorded at 517 nm.

3.2.2.2 ABTS radical Scavenging activity

The effect of SIE, PLE, SIH and PLH on ABTS scavenging activity was determined by the method of Re et al. (1999). The pre-formed radical monocation of 2, 2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) is generated by oxidation of ABTS with potassium persulfate (a blue chromogen) and is reduced in the presence of hydrogen donating antioxidants, resulting in decolourization. This assay is based on the ability of different substances to scavenge 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS⁺) radical cations. The ABTS stock reagent mixture was prepared by mixing 88 μL of 140 mM potassium persulphate with 5 mL of 7 mM ABTS stock solution (pH 7.4). The working ABTS reagent was prepared by diluting the stock solution with ethanol to give an absorbance of
0.7 ± 0.5 at 734 nm. Aliquots containing different concentrations (20-100 μg) of ethanol extract (SIE, PLE) and hexane extract (SIH, PLH) were taken separately and the final volume was made up to 1 mL with distilled water. One mL of ABTS working solution was added to the tubes and ABTS solution with equal amount of distilled water served as control. The reaction mixtures were incubated at 28°C for 30 min. Absorbance was measured at 734 nm.

3.2.2.3 Hydroxyl radical Scavenging activity

The hydroxyl radical scavenging potential of the SIE, PLE, SIE, PLH extracts were assayed by the method of Halliwell et al. (1987). The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe³⁺-ascorbate-EDTA-H₂O₂ system (the Fenton reaction). Aliquots containing different concentrations (20-100 μg) of SIE, PLE, SIH and PLH were taken in different tubes. The reaction mixture finally contained 1 mL of phosphate buffer, 100 μL of 1mM EDTA, 100 μL of 20 mM hydrogen peroxide, 100 μL of 2-deoxyribose (30 mM), 100 μL of 1mM ferric chloride and 100 μL of ascorbic acid (1mM). The tubes were incubated at 37°C for 30 min. Added 1 mL of 2.8% trichloroacetic acid followed by 1 mL of 1% thiobarbaturic acid to the tubes. Tubes were heated in a water bath maintained at 75°C for 30 min and cooled. Absorbance was measured at 534 nm and the SIE, PLE, SIH and PLH extracts were assessed for hydroxyl radical scavenging activity.

3.2.2.4 Inhibition of Nitric oxide (NO) generation

The nitric oxide scavenging potential of the SIE, PLE, SIH and PLH extracts were assayed by the method of Sreejayan and Rao (1997). Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ions which is measured colorimetrically at 546 nm. Aliquots containing different concentrations (20-100 μg) of SIE, PLE, SIH and PLH were taken in different tubes.and the volume was made up to 500 μL using distilled water. One mL of sodium nitroprusside (10 mM) was added and the tubes were incubated at room temperature for 2.5 h. To the reaction mixture, 1 mL of Greiss reagent (prepared by mixing an equal volume of 1 % sulphanilamide in 2% orthophosphoric acid with 0.1%N-(naphthyl) ethylene diamine
hydrochloride in water) was added. One mL sodium nitroprusside and 500 μL of distilled water without the extracts served as control. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitrite ions diazotize with sulphanilamide acid and couple with naphthyl ethylenediamine, forming pink colour, which was measured at 546 nm. Absorbance was recorded at 546 nm. Curcumin was used as a standard antioxidant.

3.2.2.5 Inhibition of Superoxide (SO) generation

The superoxide scavenging potential of the SIE, PLE, SIH and PLH extracts were assayed by the method of Nishimiki et al. (1972). The extent of superoxide generation was studied on the basis of inhibition in the reduction of nitroblue tetrazolium formation of the superoxide anion by the plant sample which is measured colorimetrically at 560 nm. Aliquots containing different concentrations (20-100 μg) of SIE, PLE, SIH and PLH were taken in different tubes. To the extracts added 1mL of nitroblue tetrazolium solution (156 μM in 100 mM phosphate buffer pH 7.4) and 1 mL of NADH solution (468 μM in 100 mM phosphate buffer pH 7.4). The volume was made up with distilled water and reaction started by adding 100 μL of phenazine methosulphate solution (60 μM in 100 mM phosphate buffer pH 7.4). The reaction mixture was incubated at 25°C for 5 min. Curcumin was used as a reference compound. Absorbance was measured at 560 nm and the superoxide anion scavenging activity of SIE, PLE, SIH and PLH extracts was calculated. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

3.2.2.6 Inhibition of in vitro Lipid Peroxidation

The method of Okhawa et al. (1979) was used for the determination of inhibition of lipid peroxidation. Inhibition of lipid peroxidation induced in goat liver homogenate mediated by pro-oxidant FeSO₄ by plant tissue extract was assessed by measurement of Thio Barbituric Acid Reactive substance (TBARS) at 535 nm. Aliquots containing different concentrations (20-100 μg) of SIE, PLE, SIH and PLH were taken in different tubes. Added 50 μL of 5% goat liver homogenate to the extracts followed by 50 μL of ferrous sulphate to induce oxidation and the final volume was made up to 500 μL with cold TBS (10 mM Tris, 0.15 M sodium chloride
pH 7.4). Control was prepared for each sample, containing respective extract (150 μL) and liver homogenate (50 μL) and made up to a final volume of 500 μL with cold TBS. A blank was set containing no plant extract, no liver homogenate but only ferrous sulphate and TBS. The final volume was made up to 500 μL with distilled water. A medium corresponding to 100 % oxidation was prepared by adding all constituents except the plant extracts and volume was made up to 500 μL. The experimental medium corresponding to auto oxidation contained only liver homogenate and TBS made up to final volume. All tubes were incubated at 37°C for 1 hour. After that 500 μL of 70% alcohol was added to stop the reaction. One mL of 1% TBA was added to all the tubes, followed by boiling in a hot water bath for 20 min. After cooling, the tubes were centrifuged. To the clear supernatants added 500 μL of acetone. Thiobarbituric acid reactive substance (TBARS) was measured at 535 nm and the inhibition of in vitro lipid peroxidation was calculated.

Further studies of determining dose dependent inhibition of alpha-amylase, alpha-glucosidase and determination of in vitro cytotoxicity were performed using ethanolic extracts (SIE and PLE) of the bark of Saraca indica and Polyalthia longifolia.

3.2.3 Alpha-amylase and Alpha-glucosidase Inhibitory properties of the Ethanolic extracts of Saraca indica and Polyalthia longifolia and their Mechanism of inhibition

The ethanol extracts of the two selected medicinal plants (SIE and PLE) were found to be more potent in terms of inhibition of α-amylase, α-glucosidase activities and antioxidant properties. Hence they were used for further studies.

3.2.3.1 Alpha-amylase Inhibitory potential

The α-amylase inhibitory property of SIE and PLE was determined by the method of Bernfeld, (1955) as mentioned in Section 3.2.1 with varying concentrations of SIE and PLE ranging from 25 to 200 μg, in comparison with standard acarbose (10-100 μg) and the IC₅₀ values were calculated.
3.2.3.2 Alpha-glucosidase Inhibitory potential

The α-glucosidase inhibitory activity of SIE and PLE was determined by measuring the release of 4-nitrophenol from paranitrophenyl α-D glucopyranoside as given by Sun et al. (1995) as mentioned in Section 3.2.1.2. Dose dependent variation in the α-glucosidase inhibition was measured using 25 μg to 200 μg of SIE and PLE in comparison with standard acarbose (10-100 μg) and the IC₅₀ values were calculated.

Mechanism of inhibition

The mechanism of inhibition was arrived by the use of Dixon plot and Cornish-Bowden plot. In Dixon plot the inhibitor concentration [I] was plotted against the product concentration [V], while in Cornish-Bowden plot, inhibitor concentration [I] was plotted against [S]/[V].

3.2.4 In vitro Toxicity studies of Ethanolic extracts of the Bark of Saraca indica and Polyalthia longifolia in Vero cell-lines and L6 cell-lines

Determination of Mitochondrial Synthesis by 3-(4,5 dimethyl thiazole-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) Assay:

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The assay was performed by the method of Mosmann (1983). The principle involved is the cleavage of tetrazolium salt 3 - (4, 5 dimethyl thiazole-2yl) - 2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x10⁵ cells/mL using medium containing 10% new born calf serum. To each well of the 96 well micro titreplate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and
100 µL of different drug concentrations was added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and 50 µL of MTT (2mg/mL) prepared in Hank’s balanced salt solution (HBSS) without phenol red was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 50 µL of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm.

**Phase III**

### 3.3 Determination of Antidiabetic and Antioxidant potential of the Ethanolic extracts of the bark of *Saraca indica* and *Polyalthia longifolia* in Niacinamide-Streptozotocin Induced Diabetic rats

The study was approved by the Institutional Animal Ethical Clearance Committee of Karpagam University, Coimbatore (KU/IAEC/PhD126). All *in vivo* studies involved in the assessment of antidiabetic and antioxidant potential of the potent extracts were performed in Wistar rats and acute toxicity studies were performed using Swiss albino mice as per the Organization for Economic Co-operation and Development (OECD) guidelines 423.

#### 3.3.1 Determination of the Dosage of extracts for Acute Toxicity studies

Acute toxicity studies were performed with eight groups of three female mice / group. Group 1, Group 2, Group 3 and Group 4 received ethanol extract of *Saraca indica* bark (SIE) at 5 mg, 50 mg, 300 mg and 2000 mg / kg body weight (b.w.) while Group 5, Group 6, Group 7 and Group 8 received ethanol extract of *Polyalthia longifolia* bark (PLE) at 5 mg, 50 mg, 300 mg and 2000 mg/kg b.w. respectively. Special observation was made for 4 hours after administration of the extracts. Changes in locomotion, convulsion, rearing, sniffing, grooming, eye ball movement, fur changes and urination were observed. Since no mortality was observed for 14 days in acute toxicity studies, 1/5<sup>th</sup> and 1/10<sup>th</sup> of the highest dose (2000 mg/kg b.w.) were chosen
for performing hypoglycemic test, Oral Glucose Tolerance Test (OGTT) in normal rats.

3.3.2 Evaluation of Hypoglycemic Effect and Oral Glucose Tolerance in normal rats Administered with Ethanolic extracts of *Polyalthia longifolia, Saraca indica* Barks and Standard Glibenclamide

Hypoglycemic test

The overnight fasted rats were divided into six groups of six animals each. Rats of Group 1 were given vehicle only (1% CMC). Group 2 and Group 3 received SIE at 200 mg/kg b.w and 400 mg/kg b.w respectively while Group 4 and Group 5 received PLE at 200 mg/kg b.w and 400 mg/kg b.w of PLE respectively. Group 6 received the standard drug glibenclamide at 200 μg/kg b.w. Blood samples were collected at 0, 1, 2, 4 and 6 h after treatment. Blood glucose level was determined using acuchek glucometer.

Oral glucose tolerance test

Oral glucose tolerance test was carried out on overnight fasted rats. Group 1 was given vehicle only (1% CMC). Group 2 and Group 3 received SIE at 200 mg/kg b.w and 400 mg/kg b.w respectively while Group 4 and Group 5 received PLE at 200 mg/kg b.w. and 400 mg/kg b.w. of PLE respectively. Group 6 received the standard drug glibenclamide at 200 µg/kg b.w. The doses of SIE, PLE and glibenclamide were given at 12th hour, 24th hour and 48th hour after fasting and 30 min later, glucose (10 g/kg) was administered orally to all the rats. Blood samples were collected at 0, 30, 60 and 120 min and blood glucose level was determined using acuchek glucometer.

3.3.3 Antidiabetic study of the Ethanolic extracts of the Barks of *Saraca indica, Polyalthia longifolia* and Standard Glibenclamide

Grouping of animals and Administration of the extracts

Wistar rats were acclimatized to laboratory conditions for 15 days. Diabetes mellitus was induced by the following procedure. Niacinamide was injected at 120 mg/kg b.w. followed by streptozotocin (STZ) at 60 mg/kg b.w. after 15 min. 1 % glucose solution was given orally after 1 hour of induction to prevent hypoglycemic shock. Fasting blood glucose was measured after 48 hours and the animals with
blood glucose level above 200 mg/dL were taken for the study. The study was performed using seven groups each containing six animals viz., Group 1: Control (1% CMC), Group 2: Niacinamide - Streptozotocin (NIA-STZ) Group 3: STZ + Glibenclamide 200 µg/kg b.w, Group 4: STZ + SIE 200 mg/kg b.w. Group 5: STZ + SIE 400 mg/kg b.w. Group 6: STZ + PLE at 200 mg/kg b.w. and Group 7: STZ + PLE 400 mg/kg b.w. Extracts were administered orally for the treatment period of one month.

3.3.3.1 Determination of Body weight

Body weight and initial fasting blood glucose level were recorded. Body weight of the rats was determined initially and at weekly intervals during the treatment period.

3.3.3.2 Biochemical analyses

(i) Blood glucose

Blood was collected by tail nipping method and tested for glucose level initially and at weekly intervals of the treatment period using Accuchek glucometer.

Collection of Blood /Plasma / Serum and Liver for other Biochemical analyses

Blood was collected at the end of the treatment period by sino-orbital puncture for analysis of biochemical parameters, hematological parameters and their related indices. The liver of the experimental rats were removed and a portion of it was stored at - 40°C for performing the assays involving enzymic, non-enzymic antioxidants and selected enzymes of carbohydrate metabolism. The biochemical parameters were analysed using Biosystems kit in AlfaWasserma fully automated autoanalyser available in the Advanced Research Laboratory at Avinashilingam University. The instrument was calibrated using Biosystems calibrator for the analysis of various parameters. Biosystems control was used to check the proper functioning of the instrument and reliability of results. The standard range of each parameter was programmed in the instrument. Reagents for the respective estimation were placed in the specific labelled containers. In the case of protocols using two reagents, reagent 1 and reagent 2, they were mixed in the ratio of 4:1 just before use and filled in the respective containers. Care was taken to fill the reagent in the container without air bubbles and also that the maximum volume did not
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Serum samples were centrifuged at 10000 rpm for 20 min at 4°C to remove any clogging material. Serum samples and respective standards were placed in the sample cups. Cuvettes were loaded into the cuvette holder. Sample ID numbers were programmed according to their location in the sample holder and the following parameters were determined.

(ii) **Lipid profile**

Dyslipidemia characterized by elevation of plasma triglycerides (TGs), cholesterol or both, or low high-density lipoprotein cholesterol (HDL-cholesterol) level that contributes to the development of atherosclerosis is commonly observed in diabetics (Tagoe and Amo-Kodieh, 2013). It becomes essential to measure plasma / serum levels of total cholesterol, triglycerides and individual lipoproteins in the serum of experimental rats to test the therapeutic potential of the barks of the selected medicinal plants.

**Triglycerides**

Triglycerides are determined after enzymatic hydrolysis with lipases using standard procedures (Bucolo and David, 1973; Fossati and Prencipe, 1982). Peroxidase catalyzes the conversion of hydrogen peroxide, 4-aminoantipyrine and 4-chlorophenol to a purple coloured quinoneimine complex. The enzyme reagent contained piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES) 45 mmol/L, magnesium chloride (5mmol/L), 4-chlorophenol (6 mMol/L), lipase (100 U/ml), glycerol kinase (1.5 U/mL), glycerol-3-phosphate oxidase (4 U/mL), peroxidase (0.8 U/mL), 4-aminoantipyrine (0.75 mmol/L) and ATP (0.9 mmol/L), pH 7.0. In brief, 225 µL of enzyme reagent was taken and the blank was set. This was mixed with 3 µL of standard (200 mg/dL) / 3 µL of test serum samples and 15 µL of the diluent (1% sodium azide and Triton X-100 in double distilled water), mixed well, incubated for 1 min at 37°C and measured the absorbance at 505 nm. Triacylglycerol was expressed in mg/dL which was calculated by the formula

\[
A_{\text{sample}} / A_{\text{standard}} \times C_{\text{standard}}
\]

**Total cholesterol**

Serum total cholesterol was measured according to Allain *et al.* (1974). Cholesterol esterase hydrolyses the esters. Subsequent enzymatic oxidation by
cholesterol oxidase leads to the formation of hydrogen peroxide. This is converted into a coloured quinonimine in a reaction with 4-aminoantipyrine and phenol catalyzed by peroxidase. The reagent contained PIPES (35 mmol/L), sodium cholate (0.5 mmol/L), phenol (28 mmol/L), cholesterol esterase (0.2 U/mL), cholesterol oxidase (0.1 U/mL), peroxidase (0.8 U/mL), 4-aminoantipyrine (0.5 mmol/L), pH 7.0. In brief, 285 µL of enzyme reagent was taken and the blank was set. This was mixed with 4 µL of standard (200 mg/dL) / 4 µL of test serum samples and 15 µL of the diluent (1g of sodium azide and 0.5 mL of Triton X-100/ L double distilled water), mixed well, incubated for 4 min at 37°C and measured the absorbance at 505 nm.

Cholesterol content in mg/dL = \( \frac{A_{\text{sample}}}{A_{\text{standard}}} \times C_{\text{standard}} \)

**HDL cholesterol**

The level of HDL cholesterol was estimated as given by Grove, (1979) and Burstein et al. (1980). Chylomicrons, Very low density lipoproteins (VLDL) and Low density lipoproteins (LDL) are precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the High density lipoprotein (HDL) in the supernatant and the cholesterol content is determined enzymatically. HDL cholesterol precipitant reagent contained phosphotungstase 0.4 mmol/L and magnesium chloride 20 mmol/L.

Pipetted out 0.2 mL of serum sample and 0.5 mL of precipitant. Mixed and let to stand for 10 min at room temperature, then centrifuged for 10 min at 4000 rpm. After centrifugation the clear supernatant was separated within two hours and used for the assay. Placed 0.1 mL of supernatant, 0.1 mL of standard (15 mg/dL) in the respective sample cups from which 3 µL was mixed with 285 µL of cholesterol reagent and 15 µL of diluents, incubated at 37°C for 5 min and measured the absorbance at 500 nm.

The concentration of HDL cholesterolin mg/dL = \( \frac{A_{\text{sample}}}{A_{\text{standard}}} \times C_{\text{standard}} \times \text{Sample dilution factor} \)

**VLDL and LDL cholesterol**

The VLDL, LDL cholesterol values were computed using the formula
VLDL (mg/dL) = \frac{\text{Triglycerides}}{5} \\
LDL (mg/dL) = \text{Total cholesterol} - \frac{\text{Triglycerides} - \text{HDL}}{5}

(iii) **Assessment of Renal Function parameters**

The diabetic hyperglycemia induces elevation of plasma levels of urea, uric acid and creatinine which are considered as significant markers of renal dysfunction (El-Demerdash *et al*., 2005). Changes in serum creatinine concentration more reliably reflect changes in glomerular filtration rate (GFR) (Sharma *et al*., 2011). It is essential to determine these parameters to check the influence of hyperglycemia on renal function in the experimental rats.

**Urea**

Urea is the nitrogen containing end product of protein catabolism. It was estimated by the urease - glutamate dehydrogenase assay which is an enzymatic UV assay as described by Hallet and Cook (1971) and Tiffany *et al.* (1972). Reagent 1 (Tris 120 mmol/L of pH 7.8, 2-oxoglutarate 7 mmol/L, ADP 0.6 mmol/L, urease (6 KU/L), glutamate dehydrogenase (1 KU/L) and reagent 2 (NADH 0.25 mmol/L) were mixed in a ratio of 4:1. In brief, 300 µL of the working reagent was mixed with 3 µL of the serum sample / standard (50 mg/dL) and 15 µL of the diluents. Initial absorbance (A1) and absorbance after 60 seconds (A2) were recorded and the concentration of urea was calculated using the formula

\[
\text{Concentration of urea (mg/dL)} = \frac{A1 - A2 \ (\text{Sample}) \times C \ (\text{Standard})}{A1 - A2 \ (\text{Standard})}
\]

**Uric acid**

Uric acid was assayed by enzymatic colorimetric method with uricase and peroxidase where it is converted into allantoine by uricase as given by Steele (1969). This combines with 4-aminoantipyrine and hydrogen peroxide catalyzed by peroxidase to form coloured quinoneimine dye. The absorbance of the coloured product was measured at 530 nm. Mixed 150 µL of the working reagent (PIPES buffer (pH 7.8) at 150 mmol/L, chromogen 1.0 mmol/L, ascorbate oxidase
(100 mmol/L), peroxidase (POD) (100 mmol/L), uricase (100 mmol/L) with 3 µL of the serum sample / standard (50 mg/dL) and 15 µL of the diluent. Absorbance was recorded after incubation for 5 min and the concentration of uric acid was calculated using the formula

\[
\text{Concentration of uric acid (mg/dL)} = \frac{A_{\text{Sample}} \times C_{\text{Standard}}}{A_{\text{Standard}}}
\]

**Creatinine**

Creatinine is the catabolic product of creatinine phosphate. It was estimated by the modified Jaffe’s Kinetic method as given by Browers (1980). Picric acid in an alkaline medium reacts with creatinine to form an orange coloured complex with the alkaline picrate. Intensity of the colour formed during the fixed time is directly proportional to the amount of creatinine present in the sample. Mixed 7 µL of the serum sample / standard along with 140 µL of the reagent (picric acid 5.0 mMol/L, sodium hydroxide 150 mMol/L) and measured the initial absorbance (A₀) after 30 sec at 520 nm and (A₁) after 90 sec. Determined ΔA for standard (S) and test (T) and calculated the creatinine content by the following formula

\[
\text{Serum creatinine (mg/dL)} = \frac{\Delta A_T \times 2}{\Delta A_S} \text{ where } A_T = AT_1 - AT_0; A_S = AS_1 - AS_0
\]

**(iv) Total protein**

In diabetics, alteration in the enzymatic activities can occur. Hence measurement of change in the total protein content would help in the management of Diabetes mellitus. Total protein in the serum and liver homogenate was estimated by the method of Gornall et al., (1949). Peptide bonds of proteins react with copper ions in alkaline medium to give a violet coloured complex. The intensity of this colour is proportional to the protein concentration. The working reagent contained copper (II) acetate (6 mmol/L), potassium iodide (12 mmol/L), sodium hydroxide (1.15 mol/L) and detergent. Bovine serum albumin was used as standard. Mixed 225 µL of reagent A with 3 µL of serum sample / standard and incubated for 8 min at room temperature and the absorbance was measured at 544 nm. The protein concentration was calculated using the following formula
(v) Determination of the activities of Enzyme markers

Coronary heart disease, cerebrovascular disease and hepatic damage are major consequence of diabetes (Howard et al., 2000). This may be reflected by the elevated levels of AST, ALT and LDH due to the leakage of these enzymes from liver cytosol into blood (Ohaeri, 2001). The enzyme markers of hepatic injury, namely Aspartate amino transferase (AST) / Serum glutamate oxaloacetate transaminase (SGOT), Alanine amino transferase (ALT) / Serum glutamate pyruvate transaminase (SGPT), Alkaline phosphatase (ALP) and the marker of cardiac injury namely Lactate dehydrogenase (LDH) were measured in the serum of experimental animals.

Aspartate amino transferase (AST) / Serum glutamate oxaloacetate transaminase (SGOT)

Aspartate amino transferase (AST/GOT) was measured in the serum of experimental rats according to the method of Gella et al. (1985). AST serves as specific marker for hepatic damage. AST catalyzes the transfer of amino group from aspartate to 2-oxoglutarate, forming oxaloacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the malate dehydrogenase (MDH) coupled reaction. Mixed 4 mL of reagent A [Tris (121 mmol/L), L-aspartate (362 mmol/L), malate dehydrogenase (460 U/L), lactate dehydrogenase (660 U/L), sodium hydroxide (255 mmol/L), pH 7.8] and 1 mL of reagent B [NADH (1.3 mmol/L), 2-oxoglutarate (75 mmol/L), sodium hydroxide (148 mmol/L) and sodium azide (9.5 g/L)] before use and placed in the specific labelled container in the instrument. Mixed 150 µL of the working reagent and 20 µL of the serum sample, 30 µL of diluent in a cuvette and measured the initial absorbance and decrease in absorbance at 340 nm for 3 min. The change in absorbance per minute (ΔA/min) is expressed as U/L = ΔA/min x 1746.

Alanine amino transferase (ALT) / Serum glutamate pyruvate transaminase (SGPT)

Alanine amino transferase (ALT/SGPT) was measured in the serum of experimental rats according to the method of Gella et al. (1985). ALT catalyzes the
transfer of an amino group from L-alanine to alpha-ketoglutarate to form pyruvate and L-glutamate. Pyridoxal phosphate functions as a coenzyme in amino acid transfer; therefore addition of pyridoxal phosphate results in increased enzyme activity. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm by means of the Lactate dehydrogenase (LDH) coupled reaction. Mixed 4 mL of the reagent A [Tris (150 mmol/L), L-alanine (750 mmol/L), lactate dehydrogenase (1350 U/L), pH 7.3] and 1 mL of reagent B [NADH (1.9 mmol/L), 2-oxoglutarate (75 mmol/L), sodium hydroxide (148 mmol/L) and sodium azide (9.5 g/L)] before use. Mixed 150 µL of the working reagent and 20 µL of the serum sample, 30 µL of diluent and measured the initial absorbance and decrease in absorbance at 340 nm for 3 minutes at 1 minute interval. The change in absorbance per minute (ΔA/min) is expressed as U/L = ΔA/min x 1746.

**Alkaline phosphatase (ALP)**

Alkaline phosphatase was determined in the serum of experimental rats as per the method of Bergmeyer et al. (1976). ALP catalyzes the transfer of phosphate group from 4-nitro phenyl phosphate to 2-amino-2-methyl-1-propanol (AMP), liberating 4-nitrophenol. The rate of increase in paranitrophenol formation is directly proportional to the ALP activity. The working reagent was prepared by mixing 4 mL of reagent A (2-Amino-2-methyl-1-propanol 0.4 mol/L, zinc sulfate 1.2 mmol/L, n-hydroxy ethyl ethylene diamine triacetic acid 2.5 mmol/L, magnesium acetate 2.5 mmol/L, pH 10.4) with 1 mL of reagent B (4-nitrophenyl phosphate 60 mmol/L). Mixed 160 µL of the working reagent and 4 µL of the serum sample, 15 µL of diluent and measured the initial absorbance and decrease in absorbance for 3 min at 415 nm. Alkaline phosphatase activity was expressed as U/L = (ΔA/min x 2754).

**Lactate dehydrogenase (LDH)**

Lactate dehydrogenase was determined in the serum of experimental rats by the method of King (1965). LDH catalyzes the reduction of pyruvate by NADH to form lactate and NAD+. The catalytic concentration is determined from the rate of decrease of NADH measured at 340 nm. The working reagent was prepared by mixing 4 mL of reagent A (Tris 100 mmol/L, pyruvate 2.75 mmol/L and sodium chloride 222 mmol/L, pH 7.2) and 1 mL of reagent B (NADH 1.55 mmol/L and
sodium azide 9.5 g/L). Mixed 150 µL of the working reagent and 3 µL of the serum sample, 15 µL of diluent and measured the initial absorbance and decrease in absorbance at 340 nm for 3 minutes at 1 minute interval. Lactate dehydrogenase activity was expressed as U/L = (ΔA/min x 8095).

(vi) Determination of the activities of Enzymic Antioxidants

The complications associated with diabetes mellitus are often associated with the production of free radicals and oxidative stress. The indices like enzymic and non-enzymic antioxidant status are important determinants as suggested by Sultan et al. (2014) and the activities of enzymic-antioxidants namely catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase were determined in the liver homogenate of the experimental rats inorder to assess the protection rendered by SIE/PLE/glibenclamide in the diabetic rats.

Catalase (CAT)

Assay of catalase was performed using the liver homogenates by the method of Luck, (1974). Catalase (CAT) catalyzes the decomposition of H₂O₂ to water and oxygen and thereby protects the cells from oxidative damage of H₂O₂ and hydroxyl radical. Homogenized the liver sample in a homogenizer using ice cold M/15 phosphate buffer of pH 7.0 at 4°C and centrifuged. Repeated the extraction and the combined supernatants were used for the assay. Read against a control cuvette containing the liver tissue homogenate as in the test cuvette, but containing H₂O₂ free M/15 phosphate buffer, pH 7.0. Pipetted out into the test cuvette, 3 mL of H₂O₂ phosphate buffer (0.16 mL of H₂O₂ made upto 100 mL with M/15 phosphate buffer, pH 7.0). Mixed with 0.01 mL to 0.04 mL of the tissue homogenate and noted the time required for decrease in absorbance of 0.05 at 240 nm. Calculated the concentration of H₂O₂ using the extinction coefficient 0.036/µmole/mL. One unit of the enzyme is defined as the amount of the enzyme that brings about decrease in absorbance of 0.05 at 240 nm.

Superoxide dismutase (SOD)

Assay of superoxide dismutase was carried out using the liver homogenates by the method given by Kakkar et al. (1984). SOD acts as a first line of defense to
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Glutathione peroxidase (GPx)

The assay was performed using the liver homogenates as per the method given by Rotruck et al. (1973). The procedure is based on the reaction between left over glutathione in the reaction with 2, 4 dithionitro benzoic acid (DTNB) to form oxidized glutathione (GSSG). Pipetted out 0.4 mL sodium phosphate buffer (pH-7.0), 0.1 mL sodium azide (10 mM), 0.2 mL reduced glutathione, required amount of enzyme and 0.1 mL of 0.2 mM H₂O₂. The final volume was made up to 2 mL. The tubes were incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 0.5 mL of 10% TCA. To determine the residual glutathione in control, the supernatant was removed by centrifugation and to this 3 mL of phosphate buffer and 1 mL of Ellman’s reagent (19.8 mg 5,5’-dithiobisnitrobenzoic acid (DTNB) in 100 mL 0.1% sodium nitrate) were added. The blank contained only Ellman’s reagent and phosphate buffer while standard glutathione (40- 200 µg) was treated with Ellman’s reagent. Measured the absorbance of standard and liver tissue homogenate samples at 412 nm. The activities were expressed as µg of GSH consumed / min / mg liver protein.

Glutathione reductase (GR)

Assay of glutathione reductase using the liver homogenates was performed as given by Horn and Burns, (1978). GR catalyzes the conversion of oxidized...
glutathione to reduced glutathione employing NADPH as a substrate. Pipetted out 0.12 M potassium phosphate buffer of pH 7.2, 0.1 mL of 15 mM EDTA, 0.1 mL of 10 mM sodium azide, 0.1 mL of 6.3 mM of oxidized glutathione, 0.1 mL of the enzyme source and water in a final volume of 2 mL. Incubated for 3 minutes and added 0.1 mL of NADPH. The absorbance was recorded at an interval of 15 sec for 2-3 minutes. For each series of measurement, controls were carried out that contained water instead of oxidized glutathione. The enzyme activity was expressed as U/mg liver protein. One Unit is defined as number of mmoles of NADPH oxidized / minute.

(vii) Determination of Non-enzymic Antioxidants

Ascorbic acid

Ascorbic acid was determined in the liver homogenates as given by Roe and Keuther, (1953). Ascorbic acid / vitamin C is an electron donor, a reductant and a free radical scavenger. Ascorbate is converted to dehydroascorbate and reacts with 2, 4- dinitro phenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange-coloured solution that can be measured spectrophotometrically at 540 nm. The liver tissue was homogenized in 10 mL of 4 % TCA, centrifuged at 2000 rpm for 10 min. The supernatant obtained was treated with a pinch of activated charcoal, shaken well and incubated for 10 min, centrifuged and taken 0.5 mL and 1.0 mL of aliquots for the assay. The assay volumes were made upto 2.0 mL with 4 % TCA. Standard ascorbic acid corresponding to the concentrations 20 - 200 µg of ascorbate were pipetted out and the volumes were made upto 2.0 mL with 4% TCA. Added 0.5 mL of 2 % DNPH reagent (2,4 dinitrophenyl hydrazine in 9N H₂SO₄) to all the tubes, followed by 2 drops of 10 % thiourea solution. Incubated at 37°C for 3 hours. The osazones formed were dissolved in 2.5 mL of 85 % cold sulphuric acid with no appreciable rise in temperature. To the blank alone, 2, 4 dinitrophenyl hydrazine reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30 minutes at room temperature, the absorbance was read at 540 nm.

Alpha-tocopherol

Alpha-tocopherol / vitamin E, the fat soluble vitamin acts as free radical scavenger preventing lipid peroxidation of membrane lipids. It was determined in the
liver homogenates by the method given by Rosenberg, (1992). The estimation is based on Emmerie-Engel reaction based on the reduction of ferric to ferrous ions by \( \alpha \)-tocopherol, which then forms red colour with 2, 2’dipyridyl. The liver tissue (2.5 g) was weighed and added 50 mL of 0.1 N sulphuric acid slowly without shaking. Stoppered and allowed to stand overnight and shaken the flasks vigorously and filtered the content through Whatman No: 1 filter paper, discarding the initial 10-15 mL of the filtrate. Aliquots of the remaining filtrate were used for the estimation. Into stoppered centrifuge tubes labeled as test, standard and blank pipetted out 1.5 mL of the liver tissue homogenates, 1.5 mL of the standard (D,L \( \alpha \)-tocopherol, 10 mg/L absolute alcohol) (100 mg of tocopherol acetate is equivalent to 91 mg of \( \alpha \)-tocopherol) and 1.5 mL of water respectively. To the test and blank, added 1.5 mL of ethanol and to the standard, added 1.5 mL of water. Added 1.5 mL of xylene to all the tubes, stoppered, mixed well and centrifuged. Transferred 1 mL of xylene layer into another stoppered tube. Added 1 mL of 2, 2’-dipyridyl reagent (1.2 g/L n-propanol) to each tube, stoppered and mixed. Pipetted out 1.5 mL of the mixtures into spectrophotometer cuvettes and read the extinction of test and standard against the blank at 460 nm. Then, in turn, beginning with the blank, added 0.33 mL of ferric chloride solution. Mixed well and after exactly 15 minutes read the standard against the blank at 520 nm.

\[
\text{Amount of tocopherol} = \frac{\text{Reading at 520 nm} - \text{Reading at 460 nm}}{\text{Reading of standard at 520 nm}}
\]

**Reduced glutathione**

Reduced glutathione was estimated in the liver tissue homogenates by the procedure given by Moron et al. (1979). Reduced Glutathione (GSH) as measured by its reaction with DTNB (5, 5’-dithio 2 nitro benzoic acid (Ellman’s reaction) to give a compound that absorbs at 412 nm. Homogenized the liver samples in a blender with 0.2 M phosphate buffer (pH-8.0) and centrifuged. The supernatant was mixed with equal volume of 5 % TCA. The precipitated fraction was centrifuged and to 0.25 mL of supernatant, 2 mL of 0.6 mM 5, 5’-dithiobis (2-nitro benzoic acid) reagent was added. The final volume was made upto 3 mL with phosphate buffer (0.2 M, pH 8.0). The colour developed was read at 412 nm after 10 min against reagent blank. Different concentrations (10-50 µg) of standard glutathione were taken and processed as above.
for standard graph. The amount of reduced glutathione was expressed as µg of GSH / mg protein.

(viii) **Determination of *in vitro* Thiobarbituric Acid Reactive Substances (TBARS)**

The method given by Niehaus and Samuelson, (1968) was used for the determination of *in vitro* TBARS in the liver homogenates. Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid (TBA) in acidic condition, to generate a pink coloured chromophore, which was read at 535 nm. The liver homogenate was prepared in Tris-HCl buffer pH 7.5. 1 mL of the liver homogenate was treated with 2 mL of TBA-TCA-HCl reagent (15 % TCA and 0.375 % TBA in 100 mL water) and mixed thoroughly. The mixture was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged for 10 min and the supernatant was taken for measurement. The absorbance of the chromophore was read at 540 nm against the blank. A standard graph was constructed taking different concentration of working standard malondialdehyde (0-25 nm) and treated in a similar way. Values were expressed as nmoles / mg protein.

(ix) **Determination of activities of Hepatic Carbohydrate Metabolizing Enzymes**

Hepatic glucokinase / hexokinase is the most sensitive indicator of the glycolytic pathway in diabetes and its increase can increase the utilization of blood glucose for glycogen storage in the liver. The gluconeogenic enzyme glucose-6-phosphatase is a crucial enzyme of glucose homeostasis because it catalyses the ultimate biochemical reaction of both glycogenolysis and gluconeogenesis. Glucose-6-phosphate dehydrogenase activity is an important enzyme of the pentose phosphate pathway, the decrease of which results in diminished functioning of the pathway (Pari and Suman, 2010). Malate dehydrogenase and Succinate dehydrogenase are important enzymes participating in the TCA cycle and are reported to decrease under pathological states due to mitochondrial oxidative stress. To determine the efficacy of the treatment administered to diabetic rats, the enzymes were assessed in the liver homogenates.
Glucokinase

Glucokinase was assayed in the liver tissue homogenates by the method of Brandstrup et al. (1957) based on reduction of NAD+ through a coupled reaction with glucose-6-phosphate dehydrogenase. The reaction mixture in a total volume of 5.3 mL contained 1.0 mL of glucose solution (5 mM), 0.5 mL of ATP solution (0.072 M), 0.1 mL of magnesium chloride (0.05 M), 0.4 mL of sodium fluoride (0.5 M) and 2.5 mL of Tris-HCl buffer (0.01 M, pH 8.0). The mixture was pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of 2.0 mL of liver tissue homogenate; 1 mL of the reaction mixture was immediately removed to the tubes containing 1 mL of 10 % TCA which was considered as zero time. A second aliquot was removed after 30 min incubation at 37°C. The protein precipitate was removed by centrifugation and the residual glucose in the supernatant was estimated by glucose oxidase peroxidase method using Biosystems kit. Glucose after enzymatic oxidation by glucose oxidase is converted into gluconic acid and hydrogen peroxide, which reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form quinoneimine. In brief 1 mL of the reagent containing enzyme chromogen was mixed with 10 µL of sample / standard (100 mg/dL), incubated for 10 min at 37°C and read the absorbance against the blank within 30 min. The concentration of glucose was calculated using the formula

\[ C_{\text{sample}} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times C_{\text{standard}} \]  

(Glucose-6-phosphatase)

Glucose-6-phosphatase was assayed in the liver homogenates by the method of Koide and Oda (1959) based on the hydrolysis of inorganic phosphorus (Pi) from glucose-6-phosphate by the sample containing glucose-6-phosphatase. The incubation mixture containing 0.3 mL of buffer, 0.5 mL of glucose-6-phosphate and 0.2 mL of tissue homogenate was incubated at 37°C for one hour. One mL of 10 % TCA was added to terminate the enzyme activity. Centrifuged and the phosphate content of the supernatant was estimated using DiaLab Kit following the method given by Thomas (1998). In acid medium phosphate reacts with ammonium molybdate to form an yellow phosphorus molybdate complex which was measured at 340 nm. One mL of the reagent (0.4 mmol/L of ammonium molybdate, 210 mmol/L of sulphuric acid)
was treated with 10 µL of standard / 10 µL of supernatant (obtained from liver tissue after homogenizing with 10 % TCA) mixed and incubated for 5 min at 20 - 25°C and measured the absorbance of standard and sample against reagent blank within 60 min. Calculated the concentration of phosphorus according to the formula

\[
\text{Phosphorus (mg/dL) } = \frac{\Delta A_{\text{sample}} \times C_{\text{standard}} \text{ (mg/dL)}}{\Delta A_{\text{standard}}}
\]

**Glucose-6-phosphate dehydrogenase**

Glucose-6-phosphate dehydrogenase assay was performed in liver tissue homogenates as per the method of Ellis and Kirkman, (1961). The enzyme assay involves measurement of increase in absorbance when NADP reduces to NADPH. This reaction takes place when electrons are transferred from glucose-6-phosphate to NADP in the reactions catalyzed by glucose-6-phosphate dehydrogenase. Measured into a test tube 2.0 mL of triethanolamine buffer (pH 7.6), 0.1 mL of NADP and 1 mL of liver homogenate. Mixed and allowed to stand for 5 min at 25°C. Added 0.05 mL of glucose-6-phosphate (0.031 M) and after about 2 minutes read the extinction at 340 nm every minute for 5 minutes. Used a blank with tissue homogenate and buffer without NADP⁺ and glucose-6-phosphate. The glucose-6-phosphate dehydrogenase activity was measured by the initial rate of reduction of NADP⁺ at 25°C by following the increase in absorption at 340 nm. The activity of glucose-6-phosphate dehydrogenase was expressed as U/mg.

**Malate dehydrogenase (MDH)**

Malate dehydrogenase was determined in the tissue homogenates by the method of Mehler et al. (1948) measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit oxidizes one µmole of NADH per minute at 25°C and pH 7.4 under the specified conditions. In brief, the assay mixture consisted of 925 µL of PBS, 10 µL of 20 mM oxaloacetate, 20 µL of liver homogenate and 40 µL of 5 mM NADH. The change in absorbance was recorded for 5 min at an interval of 1 min. The activity was calculated using the extinction coefficient of NADH - 6.2 x 10³ m⁻¹ cm⁻¹ by the formula \( \Delta C / \text{min} = \Delta A / \text{min} \times 0.161 \times (Y/X) \) where Y is the total assay volume and X is the volume of the sample taken for the assay. The enzyme activity was expressed as U/mg protein.
**Succinate dehydrogenase (SDH)**

Succinate dehydrogenase was determined in liver tissue homogenates by following the reduction of dichlorophenol indophenol as given by King (1967). The reaction mixture contained 0.75 mL of 0.2 M phosphate buffer, 0.10 mL of 0.045 M potassium cyanide, 0.2 mL of 0.6 M succinic acid of pH 6.8, 0.1 mL of 1.5 mM dichlorophenol indophenol, 0.3 mL of 1% BSA and 0.5 mL of 9.0 mM phenazine methosulphate made upto 3.0 mL with distilled water. The reaction was initiated by the addition of 0.05 mL of tissue homogenate. The increase in absorbance was measured at 600 nm for 5 min at 30 sec interval. The enzyme activity was calculated using the formula, Enzyme activity (U) mmoles succinate oxidized/min = 0.0476 x A_{600} nm. The enzyme activity was expressed as U/mg protein.

**3.3.3.3 Hematological parameters**

Anaemia is a common pathophysiology associated with diabetes mellitus (Akindele et al., 2012). Colak et al. (2012), also reported that diabetes mellitus causes the development of hypochronic anaemia due to a fall in the iron content of the body resulting from oxidative stress associated with the condition. Hematological parameters and the related indices were reported also found to vary in diabetic animals.

Hence determination of the hematological parameters will also suggest about the efficacy of SIE/PLE/glibenclamide in restoring them to normal in the diabetic rats. Red blood corpuscular count and their indices, white blood corpuscular count and their indices, in the blood samples of the experimental animals were determined using autoanalyser.

**3.3.3.4 Histopathological analysis**

**Histopathology**

Damage caused to organs in diabetes mellitus due to metabolic alterations is well proved. Analysing the histopathology of liver, pancreas and kidney under different treatments would help to understand the potency of the selected medicinal plants in regenerating the tissues.
The rats were sacrificed by cervical dislocation and an autopsy was carried out to obtain pancreas, liver and kidney of the rats. Tissue samples were taken and preserved in 10 % formalin solution for a minimum of one hour. The organs liver, pancreas and kidney were used for histopathological analysis and pancreas was used for immunohistochemical analysis.

Histopathological analysis of the liver, pancreas and kidney of the animals were performed as given by Culling, (1979). The rats were sacrificed by cervical dislocation and an autopsy was carried out to obtain pancreas, liver and kidney of the rats. Tissue samples were taken and preserved in 10 % formalin solution for a minimum of one hour. Dehydration of the fixed tissue was done by giving three changes of acetone (each 100 mL). Cleaning of tissue from acetone was affected by three changes of xylene (each 500 mL) in a total duration of three hours. Incubation of processed tissue in melted paraffin was done by two changes for 3-4 hours in an incubator maintained at 58 - 60°C. Embedding of the tissue in paraffin wax was then done by immersing the tissue in molten paraffin and then cooling it to harden the paraffin. Sections of the paraffin embedded tissue were done using a microtome adjusted to 1-3 µ thickness. The paraffin sections were carefully taken on glass slides. The sections were then cleaned by immersing in xylene. The sections were stained with hematoxylin and aqueous eosin (1 %) and screened to evaluate the morphology and cellular composition.

**Phase IV**

**3.4 Isolation and Characterization of Active Components with Alpha-amylase, Alpha-glucosidase Inhibitory properties and Antioxidant potential from the Ethanolic extracts of the Barks of Saraca indica and Polyalthia longifolia**

Isolation of active compounds with antidiabetic and antioxidant properties from the ethanolic extracts of the bark of Saraca indica and Polyalthia longifolia were performed by the following sequential steps.

- Separation of fractions from the ethanolic extracts by thin layer chromatography
- Protection of $\text{H}_2\text{O}_2$ induced DNA damage by the extracts and TLC fractions
- Subjecting the potent TLC fractions to high performance liquid chromatography (HPLC)
- Analysis of the potent HPLC peaks by Quadrupole time-of-flight mass spectrometry (QTOFMS), Nuclear magnetic resonance (NMR) spectroscopy and Fourier transform infra red (FT-IR) spectroscopy for the identification of compounds present.

**Separation of Active Components from the Ethanolic extracts of the Barks of Saraca indica and Polyalthia Longifolia by Thin Layer Chromatography**

Thin Layer Chromatography (TLC) is an easy technique for the separation, identification and characterization of unknown organic compounds. TLC was performed in silica gel coated glass plates. Placed dry, clean glassplates over a plane surface. Prepared slurry of silica gel in water in the ratio 1:2 (w/v). Stirred the slurry thoroughly for 2 min and poured into the applicator positioned on the glass plate. Coated the slurry over the glass plates at a thickness of 1 mm by moving the applicator at a uniform speed from one end to the other. Dried at room temperature for 30 - 45 min. Heated the plates in an oven at 120°C for 2 h to remove the moisture and to activate the adsorbent on the plate. Left 2.5 cm from one end of the glass plate and at least one inch equal distance from the edges. Applied the sample extracts SIE, PLE by means of a capillary tube as small spots. All spots were placed at equal distances from one end of the plate. Sample application was done repeatedly for a more concentrated sample spot.

**Separation of the Potent TLC Fractions by High Performance Liquid Chromatography (HPLC)**

High Performance Liquid Chromatography (HPLC) of the potent TLC fractions was performed in order to separate the components present in the active fractions using Reverse phase HPLC. High performance liquid chromatography (HPLC) is a versatile, robust and widely used technique for the isolation of components from natural products. HPLC can be used in both qualitative and
quantitative applications that are for both identification and quantification of components (Sasidharan et al., 2011).

Analytical HPLC was performed using the column LUNA 5 µ C18 (2 100 A) of dimension 250 x 4.60 mm, in Schimadzu HPLC instrument at the Advance Research Laboratory, Avinashilingam University, Coimbatore. Binary gradient of the solvents methanol and acetonitrile were used and method was programmed. Methanol (100%) was placed in pump A and acetonitrile (100%) in pump B. Mixing of the solvents was done automatically by the system itself according to the method in Table 6

The run was performed using a flow rate of 0.75 mL/min, with an oven temperature of 30°C, using Photo diode array (PDA) detector with the wavelength range of 190-800 nm. The run time was 15 min.

**Quadrupole Time of Flight Mass Spectral analysis of the Potent HPLC Peaks**

Quadrupole time of flight mass spectrometry (QTOF-MS) is a high mass resolution technique capable of providing specific molecular formula identification. TOF-MS can provide full-scan spectra combined with high sensitivity and accurate mass. When TOF-MS is combined with quadrupole (QTOF-MS) it provides even more structural information (Jones-Lepp and Momplaisir, 2005). It is an excellent technique to analyze multicomponents in the complex herbal extracts due to accurate mass measurement, high resolution, and ion separation (Chaudhary et al., 2011).

The chromatogram and mass spectra of the HPLC purified peaks was recorded using High resolution mass spectrometry (HRMS) (Agilent, 6538 High accurate- QTOF-LC/MS) facility available at the Department of Inorganic and Physical Chemistry, Indian Institute of Science, Bangalore. The fragmentor voltage of 175 V was used for all the samples. The potent peaks were evaporated to remove the solvents and were dissolved in 200 µL of LCMS grade methanol and from there 10 µL was injected. The flow rate was 0.3 mL / min. The raw data obtained was compared with Agilent METLIN Personal Compound database (PCD) to find the list of compounds present in the peaks.

**Nuclear Magnetic Resonance (NMR) Spectral analysis of the Potent HPLC Peaks**
Modern NMR spectroscopy has been emphasizing the application in biomolecular systems and plays an important role in structural biology. With developments in both methodology and instrumentation in the past two decades, NMR has become one of the most powerful and versatile spectroscopic techniques for the analysis of biomacromolecules, allowing characterization of biomacromolecules and their complexes up to 100 kDa.

Bruker AVANCE III 500 MHz (AV 500) multi nuclei solution NMR Spectrometer facility available at the Sophisticated Analytical Instrumentation Facility (SAIF), Indian Institute of Technology, Chennai was used for the H\(^1\) analysis of the HPLC peaks with potent alpha-glucosidase inhibition and antioxidant potential. The peaks of the active HPLC peaks collected were evaporated to dryness at room temperature to remove the solvent and were dissolved in 200 µL of deuterated methanol and the spectrum was recorded.

**Fourier Transform Infra-red (FT-IR) Spectroscopy analysis of the Potent HPLC peaks**

FT-IR plays a vital role for the identification of functional groups present in the naturally occurring substances and chemical analysis of particular analyte. The functional group present in the analyte will make vibrations of specific wave numbers. FT-IR fingerprinting provides the chemical characterization of the PTLC/HPLC isolates (Hazra et al., 2007).

The FT-IR spectra of the peaks with potent alpha-glucosidase inhibition and antioxidant potential were recorded on a Shimadzu IR Affinity-1 spectrometer with a Pike MIRacle10 ATR system at the centralized lab facility of PSG College of Arts and Science, Coimbatore.

**Molecular Docking of the Identified Compounds with selected Targets**

Protein-ligand interaction is comparable to the lock-and-key principle of enzyme-substrate interaction, in which the lock encodes the protein and the key is grouped with the ligand. The major driving force for binding appears to be hydrophobic interactions. *In silico* techniques help identifying drug target through bioinformatics tools. They can be used to explore the target structures for possible
Selection of Protein targets

*Alpha-amylase and alpha-glucosidase*

Type-2 diabetes mellitus is a chronic metabolic disorder that results from defects in both insulin secretion and insulin action. One of the recent trends in the management of type-2 diabetes involves the inhibition of carbohydrate metabolizing enzymes such as \( \alpha \)-amylase and \( \alpha \)-glucosidase. These enzyme inhibitors help in regulating the blood glucose level by delaying the process of carbohydrate digestion.

Molecular Docking using Glide

Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active-site region of the receptor. The receptor grid was generated at the receptor site bound by a ligand. The ligands were then docked to the target proteins using Glide 4.5 module of Schrödinger. Docking was done in Standard Precision Mode (SP). The docked protein and the ligands were viewed with Glide Pose Viewer. The images of the best docked poses of the ligand with the protein were saved as .jpg files.

Preparation of the Target Proteins

The protein preparation Wizard accepts a protein from its raw state (which may include missing hydrogen atoms, incorrect bond order assignments, charge states or orientations of various groups), to a state in which it is properly prepared for calculations. The selected three target proteins / receptor were prepared using the protein preparation wizard and the results were saved in .jpg format.

Preparation of the Ligand

The compounds identified through QTOF-MS and NMR were subjected to *in silico* studies for their efficacy against the selected target proteins. The target proteins were human pancreatic alpha-amylose (HPAA), human maltase-gluco amylase (HMGA) and dipeptidyl peptidase-4 (DPP-4).
The structures of eleven identified compounds pelargonidine 3,5 diglucoside, epiafzelechin, isolariciresinol, dihydrokaempferol, epicatechin, longitriol, schizandriside, lyoniside, longimide, isorhamnetin and apigenin-7-glucoside to be docked with the target proteins were obtained from NCBI-PubChem compound (http://www.ncbi.nlm.nih.gov/pubchemcompound) and were saved in a MSWord document. The structure of one compound namely petunidine gallate, was not available in the database and hence drawn using the tools available on the Maestro window of Schrodinger. The refined structure was saved as new entry in the project table.

**Ligand Preparation**

The preparation of the ligand was done using LigPrep 2.1, a module on the Maestro window of Schrödinger. LigPrep produces a number of structures for each input structure of the ligand with various ionization states, tautomers, and stereochemistry and ring conformations and eliminates molecules using various criteria including molecular weight or specified numbers and types of functional groups present. The prepared ligands can be used for docking.

**Absorption, Distribution, Metabolism and Excretion (ADME) Studies**

Compounds that interact with a target and producing stimulatory or inhibitory effect should also possess the appropriate ADME (Absorption, Distribution, Metabolism and Excretion) profile necessary to make it suitable for use as a drug. QikProp 3.0 module of Schrödinger predicts physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. The QikProp results of the twelve ligands are presented in Chapter 4.

**3.5 Statistical Analysis**

The data of biochemical estimations and *in vitro* assays in Phase I and Phase II were represented as mean ± SD / SEM values. To study the effect of varying concentrations of the extracts on the inhibition of alpha-amylase and alpha-glucosidase activities, correlation analysis was performed. The data of Phase III was subjected to one way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test.
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