4. MATERIALS AND METHODS

Materials:

Streptozotocin was purchased from Sigma Aldrich, USA. All other chemicals and reagents were purchased from local chemical supplier and they are of analytical grade.

Ethics statement:

All the collection of materials was obtained for the described field studies and is duly acknowledged. The species Phyllanthus watsonii, Orthosiphon stamineus and Melastoma malabathricum were collected and, all the species are not endangered.

Collection of the plant material:

The leaves and roots of Phyllanthus watsonii (MPH-96390, MPH-96391), roots of Orthosiphon stamineus (MPH-96415) and Melastoma malabathricum Linn (MPH-96426) were collected. Authentication of plants was carried out by taxonomist and plant specimens were preserved in our herbarium.

4.1 Preparation of the plant extract:

Leaves and roots of Phyllanthus watsonii, roots of Orthosiphon stamineus and roots of Melastoma malabathricum were shade dried and pulverized separately to a coarse powder. Each of these powders was subjected to successive solvent extraction in Soxhlet apparatus for 72 hours with the solvents in the order of increasing polarity. Then the collected concentrates were filtered and the filtrates were evaporated to dryness using vacuum evaporator under reduced pressure. The obtained extracts were freeze dried and preserved in a vacuum desiccator.
i. Petroleum ether (60°C-80°C)

ii. Chloroform

iii. Ethanol

4.2 Preliminary phytochemical studies on solvent extracts of *Phyllanthus watsonii*, *Orthosiphon stamineus* and *Melastoma malabathricum*

The extracts Petroleum ether, Chloroform and Ethanol obtained from leaves and roots of *Phyllanthus watsonii*, roots of *Orthosiphon stamineus* and roots of *Melastoma malabathricum* were screened for various phytoconstituents like alkaloids, flavonoids, steroids, glycosides, saponins, reducing sugars, phenolic compounds, proteins and amino acids.

**Chemicals and Reagents Used**

All the chemicals, reagents and solvents of analytical grade were obtained from Local supplier.

**Qualitative Analysis on solvent extracts of Leaves and roots of *Phyllanthus watsonii*, roots of *Orthosiphon stamineus* and roots of *Melastoma malabathricum***

**Acidic and Basic Compounds**

(a) To 2 ml of alcoholic extract sodium bicarbonate solution was added, effervescence indicates the acidic compound.

(b) To 2 ml of alcoholic extract, warm water was added and filtered. Filtrate tested with litmus paper, colour turns blue indicates basic compound.

**Detection of Alkaloids**

To 10 ml of the extract few drops of dilute hydrochloric acid was added and filtered. Filtrate was treated with various alkaloidal reagents.

a) **Mayer’s Test**

To 2 ml extract 1ml of Mayer’s reagent was added, formation of white or
pale yellow precipitate shows the presence of alkaloids.

b) Dragendorff’s Test

To 2 mg of the extract 5 ml of distilled water was added, 2M Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendorff’s reagent was added. Formation of orange or orange red precipitate indicates the presence of alkaloids.

c) Hager’s Test

To 1 ml extract, 1 ml Hager’s reagent was added. Formation of yellow precipitate shows the presence of alkaloids.

d) Wagner’s Test

To 1 ml extract, 1 ml Wagner’s reagent was added. Formation of reddish brown precipitate indicates the presence of alkaloids.

Detection of Flavonoids

a) Shinoda test

To 3ml of the extract, 5ml alcohol was added. To that 1ml magnesium metal and concentrated hydrochloric acid was added. Appearance of orange pink colour shows the presence of flavonoids.

b) Alkaline reagent test

To 3ml test solution 3 drops of sodium hydroxide solution was added. Formation of Intense yellow colour which turns colourless on addition of few drops of dilute acid indicates presence of flavonoids.

c) Zinc hydrochloric acid

To 3ml of the extract zinc dust was added and 1ml conc. Hydrochloric acid. Appearance of red colour after few minutes indicates presence of flavonoids.
Detection of phytosterols

To 10ml of the extract, 5 ml of chloroform was added. The chloroform layer was subjected to Libermann’s test and Salkowski test.

a) Libermann Burchard Test

The chloroform layer of extract was treated with a few drops of concentrated sulphuric acid followed by 1 ml of acetic anhydride solution. Appearance of bluish green colour solution in the extract shows the presence of phytosterols.

b) Salkowski Test

To 2 ml of chloroform layer, few drops of concentrated sulphuric acid were added. Appearance of brown ring with the extract indicates the presence of phytosterols.

Detection of Glycosides

10ml of the extract was hydrolyzed with hydrochloric acid for few minutes on a water bath and the hydrolysate was subjected to the following tests.

a) Legal’s Test

To the hydrolysate 1 ml of the pyridine and a few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour confirms the presence of glycosides.

b) Borntrager’s Test

3ml of the hydrolysate was treated with 5ml chloroform and the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Appearance of red colour confirms the presence of glycosides.
Detection of Saponins

a) Foam Test

2ml of the extract was diluted with 20 ml of distilled water for 15 minutes. Formation of foam indicates the presence of saponin.

b) Haemolysis Test

About 2 ml of human blood was taken in the test tube. Equal quantity of ethanol extract was added. Formation of clear red liquid in the test tube indicates that the red blood corpuscles are haemolysed.

Detection of carbohydrates

10ml of extract was suspended in 5 ml of distilled water. The suspension was subjected to the following chemical tests.

a) Molisch’s Test

The extract was treated with 2-3 drops of 1 % alcoholic α-naphthol and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of purple ring between two layers indicates the presence of carbohydrates.

b) Fehling’s Test

1ml extract was treated with mixture of Fehling’s A and Fehling’s B solutions and heated for 3 minutes. Formation of red precipitate showed the presence of reducing sugar.

c) Benedict’s Test

1ml extract was treated with Benedict’s reagent and heated for 3 minutes. Formation of red precipitate shows the presence of reducing sugar.

Detection of Tannins and phenolic compounds

10ml extract was dissolved separately in minimum amount of water and
filtered. The filtrate was subjected to the following tests.

a) Ferric Chloride Test

To 2ml of the filtrate a few drops of ferric chloride solution was added. Formation of violet colour precipitate indicated the presence of phenolic compound in the extract.

b) Lead Acetate Test

To 2ml of the filtrate a few drops of lead acetate solution was added. Formation of white colour precipitate shows the presence of tannins in the extract.

c) Gelatin Test

To 2ml of the extract, 1 ml of 1 % solution of gelatin was added. Formation of white colour precipitate shows the presence of tannins in the extract.

Detection of Proteins and Amino Acids

To 2ml of the extract, 3 ml of water was added and it was subjected to the following tests.

a) Millon’s Test

1ml of the extract was treated with Millon’s reagent, boiled in water bath for 5 minutes. Appearance of pink to red colour indicates the presence of proteins.

b) Ninhydrin Test

To 1 ml of the extract, few drops of ninhydrin reagent were added. Appearance of purple colour confirms the presence of amino acids.

c) Biuret Test

To 1ml of the extract equal volume of 5 % sodium hydroxide solution and 1% copper sulphate solution was added. Appearance of violet colour shows the presence of amino acids.
Determination of Total Phenolic Content (TPC)

TPC of an extract can be evaluated with spectrophotometer method using Folin-Ciocalteu reagent. The principle of this method is the reduction ability of phenol functional group. The reduction of Folin’s reagent by phenolate ion will change its colour to be blue. The reduction of the complex will increase when the extract contain more phenolic compounds. Thus, the colour will be darker and the absorbance will be higher (Prior et al, 2005). Therefore, the presence of phenolic compound in extract determines the presence of antioxidant compound. TPC in extract was determined according to the Folin-Ciocalteu procedure employed by (Kahkonen et al, 1999). 0.3 mL of extract solution (in triplicate) was mixed with 1.5 mL of 10% Folin- Ciocalteu’s reagent (R&M) and 1.2 mL of 7.5% (w/v) of sodium carbonate solution. The mixtures were kept in dark for 30 minutes. The absorbance was read at 765 nm.

The extract will be measured using a gallic acid standard curve and equivalent was read off the straight line generated by linear regression. The gallic acid standard curve used was y=0.0125x (R²=0.9800) where y is the absorbance at 765 nm and x is the concentration of gallic acid in mg/L. TPC was expressed as mg gallic acid equivalent (GAE)/100g fresh leaves and pericarps.

4.3 Acute toxicity studies (OECD guidelines, 423)

Animals

All the animal experiments have been carried out according to the internationally valid guidelines and they were approved by the “Institutional Animal Ethical Committee” (MRCP/IAEC/CPCSEA/PHD/3). Animals were well maintained in a restricted-access animal room with constant conditions (23 ± 2°C, 12 hour light) for one week before the experiments for acclimitisation. The animals
were fed with standard pellets diet and water *ad libitum* and maintained 12-hour light dark cycle. Prior to the experiments, the animals were fasted for 12 hour.

**Acute toxicity studies for extracts of Phyllanthus watsonii, Orthosiphon stamineus and Melastoma malabathricum**

**Principle**

Acute toxicity studies for assessing acute oral toxicity involve the identification of a dose level that causes mortality. This involves the administration of a single bolus dose of test substance to overnight fasted healthy young adult rodents by oral gavages observation for up to 15 days after dosing and recording of body weight and the necropsy of all the animals. In this method pre-specified fixed doses of the substance were used ie. 5 mg/kg, 50 mg/kg, 300 mg/kg, 2000 mg/kg and 5000 mg/kg and mortality due to these doses were observed. Group should consist of 3 animals. (Web Link).

**Experiment**

All the extracts were tested following the below protocol.

Animal: Albino mice 20-25 gm

The overnight fasted animals were weighed and were treated with a starting dose (5 mg/kg b.wt) of each extract of *P. watsonii*, *O. stamineus* and *M. malabathricum* in a step-wise procedure. The initial dose was selected as the dose expected to produce some signs of toxicity and was observed for two weeks. The toxicity doses were selected based on the OECD guidelines (Figure 4) (weblink).
4.4 *In vitro* antioxidant activity

All the extracts were tested for $\alpha$, $\alpha$-diphenyl-$\beta$-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing power (FRP) assay and Beta-carotene bleaching (BCB) assay. This study aimed to determine the *in vitro* antioxidant activity.

4.4.1 DPPH radical scavenging assay (Okawa et al, 2001)

The antioxidant activity was measured by the decrease in absorbance at 517 nm as the DPPH radical received an electron from an antioxidant compound to become a stable molecule. The antioxidant activity was expressed as IC$_{50}$ (concentration of the antioxidant required to scavenge 50% of the initial DPPH radicals) and AEAC (Ascorbic acid equivalent antioxidant capacity). The lower the IC$_{50}$ and the higher the AEAC value, the greater was the antioxidant activity as reported by Katsube *et al*, 2004. The method described by Miliauskas *et al* (2004) was employed. 1.0 ml of various dilutions of extract solutions (in triplicate) were
added to 2.0 ml of DPPH (Sigma) solution. The mixture was left in dark for 30 minutes. The absorbance was read at 517 nm with methanol as the blank. The control consisted of methanol in place of the sample. The percentage radical scavenging activity was calculated using the formula below:

\[
\text{Scavenging (\%)} = \frac{(\text{Abs Control} - \text{Abs Sample}) \times 100}{\text{Abs Control}}
\]

The results were presented as IC\textsubscript{50} and also expressed in terms of ascorbic acid equivalent antioxidant capacities (AEAC) which was calculated as follows:

\[
\text{AEAC (mg AA/100g)} = \frac{\text{IC}_{50} (\text{Ascorbic acid}) \times 105}{\text{IC}_{50} (\text{Sample})}
\]

4.4.2 Ferric reducing power (FRP) assay

The FRP method measured the ability of an antioxidant to donate electron to Fe (III) resulting in the reduction of Fe\textsuperscript{3+} or ferricyanide complex to ferrous ion Fe\textsuperscript{2+} complex, which could be monitored by the formation of Perl’s Prussian blue at 700 nm. Results were expressed as mg gallic acid equivalent (GAE)/100 g samples. The higher the FRP value, the greater was the reducing power of the extract, thus the greater the antioxidant activity. The FRP assay was carried out according to the procedure used by Juntachote and Berghofer (2005). 1.0 ml of various dilutions of extract solutions (in triplicate) were added to 2.5 ml of 0.2 M phosphate buffer with pH 6.6 and 2.5 mL of 1\% (w/v) potassium ferricyanide solution. The mixture was incubated at 50 \textdegree C for 20 minutes. Then, 2.5 mL of 10\% trichloroacetic acid were added into the mixture. An aliquot of 2.5 mL of the mixture were diluted with deionised water and 0.5 mL of 0.1\% (w/v) of iron (III) chloride were added. The absorbance was read at 700 nm after 30 minutes.
The gallic acid standard curve used was \( y=16.516x \) (\( R^2=0.9866 \)) where \( y \) is the absorbance at 700 nm and \( x \) is the concentration of gallic acid in mg/mL. The results were expressed as mg gallic acid equivalent (GAE)/100g fresh leaves or roots.

**4.4.3 Beta-carotene bleaching (BCB) assay** (Prieto *et al.*, 2012)

The BCB method is based on the loss of the yellow colour of \( \beta \)-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. The rate of \( \beta \)-carotene bleaching can be slowed down in the presence of antioxidants. This method evaluates the capacity of the extract to reduce the oxidative loss of \( \beta \)-carotene in a \( \beta \)-carotene linoleic acid emulsion. The procedure described by Juntachote and Berghofer; 2005 was followed. \( \beta \)-carotene (Sigma) solution were prepared by dissolving 5 mg of \( \beta \)-carotene in 50.0 ml of chloroform (R&M). 3.0 ml of an aliquot of \( \beta \)-carotene solution were added to 40.00 mg of linoleic acid (Fluka) solution and 400 mg of Tween 40 (R&M) solution. The chloroform was removed by purging with nitrogen. 100.0 mL of aerated distilled water then added into the mixture and the solution was mixed well. 3.0 ml of the aliquot of \( \beta \)-carotene and linoleic acid emulsion was mixed with 10 \( \mu \)l, 50 \( \mu \)l and 100 \( \mu \)l of the extract. The test and control (100 \( \mu \)l water) mixture were then incubated at 50\(^\circ\)C. The absorbance of the emulsion was determined at 470 nm and 700 nm. The results were calculated as the formula follows:

\[
\text{Degradation rate (DR) of } \beta \text{-carotene} = \frac{\ln \left( \frac{\text{Abs Initial}}{\text{Abs Sample}} \right)}{60}
\]

\[
\text{Antioxidant activity } (\% \text{AOA}) = \frac{(\text{DR Control} - \text{DR Sample}) \times 100}{\text{DR Control}}
\]
4.5 In vivo Antioxidant activity

Quantification of Lipid Peroxidation from Concentration of Thiobarbituric Acid Reactive Substance (TBARS), Conjugated Diene (CD), Catalase and peroxidase in Liver and Kidney was conducted for all the extracts (Zhu et al, 2009, Lim et al, 2007, Singh and Rajini, 2004). Streptozotocin induced diabetic rats were used for this study.

Male Sprague-Dawly rats were used for this research study. All the animal experiments were conducted according to the protocols approved by the Institutional Animal Ethics Committee (MRCP/IAEC/CPCSEA/PHD/3). Animals were divided into various groups of six each and were fed with standard diet and water ad libitum. They were kept in clean and dry cages and maintained in well-ventilated animal house with 12 h light-12 h dark cycle. Type 2 diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal (IP) injection of 65 mg/kg streptozotocin (STZ) was followed after 15 minutes with IP administration of 230 mg/kg nicotinamide (Szkudelski, 2012). After 72 hours hyperglycemia was confirmed and selected for the study.

Experimental Protocol:

Animals were divided into five groups of six rats each.

Group I: normal control rats received the vehicle (1% Gum acacia suspension).

Group II: Diabetic control rats received the vehicle (1% Gum acacia suspension).

Group III: Diabetic rats were administered extract (250 mg/kg b.wt/day) in 1% Gum acacia suspension by p.o. route.

Group IV: Diabetic rats were administered extract (500 mg/kg b.wt/day) in 1% Gum acacia suspension by p.o. route.

Group V: Diabetic rats were administered extract (1000 mg/kg b.wt/day) in 1% Gum
acacia suspension by p.o. route.

Each extract was tested according to the above protocol for 21 days. Only petroleum ether extract was administered using Tween 80 as additional surfactant to enable the extract miscible in the prepared suspension. Then analysis of the samples also had done as below.

All the rats were fasted for 16 hrs before experimentation, but allowed free access to water. Twenty-four hours after the last treatment animals were sacrificed. Liver and kidneys were dissected out and stored at −20°C for the quantification of the levels of the products of free radicals conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) and for the assessment of the activities of the antioxidant enzymes catalase (CAT) and peroxidase.

4.5.1 Determination of TBARS:

The above-mentioned tissues were homogenized separately at the concentration of 50 mg mL⁻¹ in 0.1M of ice cold phosphate buffer (pH-7.4) and the homogenates were centrifuged at 10,000 rpm at 4°C for 5min individually. Each supernatant was used for the estimation of TBARS and CD levels. For the quantification of TBARS, the homogenized mixture of 0.5 mL was mixed with 0.5mL of normal saline (0.9 g % NaCl) and 2 mL of TBA-TCA mixture (0.392 g thiobarbituric acid in 75 mL of 0.25 N HCl with 15 g trichloroacetic acid). The volume of the mixture was made up to 100 mL by 95% ethanol and boiled at 100°C for 10 minutes. This mixture was then cooled at room temperature and centrifuged at 4000 rpm for 10 minutes. The whole supernatant was taken in spectrophotometer cuvette, and absorbance was read at 535 nm (Ohkawa, 1979).

4.5.2 Determination of Conjugated Diene:

Quantification of the CD was performed by a standard method (Slater, 1984).
In brief, the lipids from the homogenate were extracted with chloroform-methanol (2:1) mixture followed by centrifugation at 1000 rpm for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 mL of cyclohexane and the absorbance was noted at 233 nm to measure the amount of hydroperoxide formed.

4.5.3 Determination of Catalase activity:

The activities of catalase of the liver and kidney were measured biochemically (Beers and Sizer, 1952). For the evaluation of catalase activity, target organ of each animal was homogenized separately in 0.05 M Tris-HCl buffer solution (pH-7.0) at the tissue concentration of 50 mg mL$^{-1}$. These homogenized samples were centrifuged at 10,000 rpm at 4°C for 10 minutes. In spectrophotometric cuvette, 0.5 mL of 0.00035 M H$_2$O$_2$ and 2.5 mL of distilled water were mixed and reading of absorbance was noted at 240 nm. Supernatant of sample was added at a volume of 40 μL and the subsequent six readings were noted at 30-second interval.

4.5.4 Determination of Peroxidase activity:

The peroxidase activity was measured in the above-said tissues, according to the standard method. (Jelena, 2012) The samples were homogenized in ice-cold of 0.1 M phosphate buffer saline (pH-7.0) at the tissue concentration of 50 mg mL$^{-1}$. Next, 20 mM guiacol was mixed with 0.1 mL supernatant collected from the homogenate. In presence of 0.3 mL of 12.3 mM H$_2$O$_2$, the time was recorded for an increase in the absorbance by 0.1 at 436 nm.

4.5.5 Pancreatic Lipase (PL) Inhibition Assay

This study was conducted in vitro for all the extracts prepared in this research study.
Porcine pancreatic lipase (PPL) inhibitory activity was measured using p-nitrophenyl butyrate (PNPB) as a synthetic substrate. All plant crude extract were screened against the PPL adapted and modified from a protocol published in Bustanji et al (2011). The enzyme solutions was prepared immediately before usage by suspending crude porcine pancreatic lipase powder type II (Sigma, EC 3.1.1.3) in Tris-HCl buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 10mM MOPS, pH 7.6) to give a concentration of 5mg/mL (200units/mL) mix using a stirrer for 15 minutes. The solution was then centrifuged at 1,500 rpm for 10 minutes and the clear supernatant was recovered. The plant extract/orlistat (100mg/mL) was pre-incubated with 200 µL of PPL solution for 5 minutes at 37°C, before the addition of 5 µL PNPB substrate solution (10 mM in acetonitrile). The total reaction volume was then top up to 1mL using the Tris-HCl buffer before measured spectrophotometrically at 410nm against blank using denatured enzyme. The denatured enzyme was prepared by boiling the enzyme solution for 5 minutes. The PPL activity is related to the rate of p-nitrophenol release, which can be estimated from the slope of the linear segment of absorbance versus time profiles. The percentage of residual activity of PPL was determined for each plant crude extract by comparing the lipase activity of PPL with and without the plant crude extract. Orlistat was used as a reference drug in the assay mixture.

The activity of the negative control was checked with and without the inhibitor. The inhibitory activity (I) was calculated as accordance to the formula below:

\[ I\%=(1-(B-b)/(A-a))\times100 \]  
(Bustanji et al, 2011)

Where, A is the activity of the enzyme without inhibitor, a is the negative control without the inhibitor, B is the activity of the enzyme with inhibitor, b is the negative
control with inhibitor.

4.6 Determination of Antihyperlipidemic activity

This study was conducted in vivo for all the extracts prepared in this research study using high fat diet fed rats.

4.6.1 Induction of Obesity and Hyperlipidemia

Animal in control group was fed with normal pellet diet (NPD) of rat chow while the other groups were fed with high fat diet (HFD) (by mixing powdered rat chow with vegetable shortening contributing to 60% of the total calories) ad libitum, respectively, for 21 days. The rats were deemed as obese based on the significant elevation of serum lipid profile which includes total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) when compared to the normal fed diet control group (Deepak et al, 2012).

4.6.2 Treatment with extracts

Treatment with selected bioactive extract (500 mg/kg) was started from 29th day and continued for 2 weeks (up to 42 days) as accordance to Birari et al, 2010. All the groups of rats were sacrificed on the 43rd day after an overnight fast, by anaesthetizing with cocktail of 100mg/kg for ketamine and 10mg/kg xylazine through intraperitoneal route. Then, the blood and liver were collected and immediately stored in 10% (v/v) formalin solution for histopathological analysis. Orlistat was used as standard drug for comparison.

4.6.3 Determination of Lipid Profile

The serum of all rats were collected on 29th day (pre-treatment) through tail vein and 43rd day (post-treatment) through cardiac puncture. The clotted blood samples were then centrifuged at 5,000 rpm for 10 minutes. The serum were then
collected, and then stored in -80°C freezer until further analysis. The total cholesterol, triglyceride, high-density lipoprotein-cholesterol concentrations, low-density lipoprotein-cholesterol concentration were determined using the method of Alam et al, 2011 using automated chemical analyzer LWC100 (Landwind, Hungary). The calibrators used in this analysis were universal calibrator Ser-T-Cal (Stanbio United States of America) and biochemistry calibrator for HDL and LDL (Qualichem, Hungary). In addition, the lipid levels were determined in skeletal muscles (Li J et al, web link). Liver samples were collected for histology study.

4.6.4 Histopathology Study

Histopathology study was done on liver samples collected (Alam et al, 2011)

Fixation of Organs

Liver tissue samples were immersed in a universal bottle containing 10% (v/v) formalin for better penetration by the fixative. The tissue was left two days at room temperature with replacement of formalin daily until the tissues hardened.

Impregnation and infiltration of tissues

Impregnation of liver tissue was done in two steps – dehydration and clearing. Dehydration step removed water within the liver tissue by sequential immersion in increasing concentrations of ethanol (50%, 70%, 80%, 95% and 100% v/v) in water for 1 hour each at room temperature; it was then followed by immersion in absolute ethanol for another hour. Clearing step immersed the tissues in a mixture of xylene and absolute ethanol (1:1 ratio v/v) for 24 hours, followed by pure xylene immersion for another 24 hours until the tissues became transparent. Tissue infiltration was done right after the clearing step, whereby the tissues were submerged into paraffin-xylene solution at gradually increasing temperatures (40°C, 50°C and 55°C) for 24 hours each. Then, liver tissues were immersed in fresh
molten paraffin for 3 days. All the procedures above were performed automatically
by Slee Mainz MTP Tissue Processor (Slee Medical GmbH, Germany).

**Tissues embedding**

A tissue mold was filled quarterly with molten paraffin, and then the organs
from the previous steps were immersed into the tissue mold. Tissue cassette was
placed on top of the tissue mold before the last dispensing of molten paraffin into the
mold. The dispensing of molten paraffin was performed using Slee Mainz
Dispensing Module MPS/P (Slee Medical GmbH, Germany).
The assembled tissue mold and cassette was left on Slee Mainz Cold Plate MPS/C
(Slee Medical GmbH, Germany) at -2°C to allow cooling and solidification of the
paraffin before displacing the embedded tissue from the tissue mold.

**Tissues sectioning**

The paraffin-embedded tissue was sectioned into 6 µm thick at an angle of
10° using a Slee Mainz semi-automatic microtome CUT 5062 (Slee Medical GmbH,
Germany). The tissue sections were placed afloat in a 45°C water-bath and were
then fished onto microscopic glass slides. The slides were dried overnight before
dewaxing and rehydration.

**Dewaxing and rehydration**

The slides were de-paraffinized by immersion in xylene followed by a
mixture of xylene and absolute ethanol (1:1 ratio v/v) for 30 minutes and 15 minutes
respectively. The slides were then immersed in decreasing concentrations of ethanol
(100%, 95%, 80% and 70%) for 10 minutes each prior to the 5 minutes immersion
in distilled water.
Staining

**Hematoxylin and Eosin (H&E) stain**

De-paraffinized slides were serially immersed in hematoxylin, distilled water, H&E decolorizing acid solution, H&E bluing solution, 70% ethanol, 90% ethanol and water for one minute for the first dip and 30 seconds for the remaining immersion. The slides were subsequently rinsed in eosin for one minute, followed by washing twice in 95% ethanol and twice in absolute ethanol. Finally, they were rinsed in xylene for one minute before air-dried.

**Mounting and analysis**

Formalin fixed paraffin embedded tissue sections were mounted with one drop of Depex mounting medium on the center of the slide before replacement of a cover slip. H&E stained slides were photographed in triplicate using Nikon Eclipse E200 microscope connected to Nikon Digital Sight DS-U2.

All slides were examined under 40 x and 100 x magnification. These were analysed.

4.7 **Assessment of Extracts for Antihyperglycemic Activity and Antioxidant activity in Streptozotocin induced Type II Diabetic Rats.**

Male Sprague-Dawly rats were used for this research study. All the animal experiments were conducted according to the protocols approved by the Institutional Animal Ethics Committee (MRCP/IAEC/CPCSEA/PHD/3). Animals were divided into various groups of six each and were fed with standard diet and water ad libitum. They were kept in clean and dry cages and maintained in well-ventilated animal house with 12 h light-12 h dark cycle. Type 2 diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal (IP) injection of 65 mg/kg streptozotocin (STZ) was followed after 15 minutes with IP administration of 230 mg/kg nicotinamide (Szkudelski, 2012). STZ was dissolved in sodium citrate buffer
(pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycaemia was confirmed by the elevated glucose levels in plasma after 72 h. The animals having blood glucose concentration of more than 180 mg/dl were selected and used for the study.

**Exploratory study:**

Animals were divided into 15 groups of each six animals and were treated with the plant extracts as shown below with single dose administration.

**Table no: 1 Treatment protocol for the NIDDM rats with the extracts**

<table>
<thead>
<tr>
<th>Groupings</th>
<th>Treatment</th>
<th>Plant name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>Treatment with 0.2% CMC in distilled water</td>
<td>----</td>
</tr>
<tr>
<td>Type 2 Diabetic control</td>
<td>Treatment with 0.2% CMC in distilled water</td>
<td>----</td>
</tr>
<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with petroleum ether extract (500 mg/kg body weight)</td>
<td><em>Phyllanthus watsonii</em> leaves</td>
</tr>
<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with chloroform extract (500 mg/kg body weight)</td>
<td><em>Phyllanthus watsonii</em> leaves</td>
</tr>
<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with ethanolic extract (500 mg/kg body weight)</td>
<td><em>Phyllanthus watsonii</em> leaves</td>
</tr>
<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with petroleum ether extract (500 mg/kg body weight)</td>
<td><em>Phyllanthus watsonii</em> roots</td>
</tr>
<tr>
<td>Type 2 Diabetic rats</td>
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</tr>
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<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with petroleum ether extract (500 mg/kg body weight)</td>
<td>Melastoma malabathricum roots</td>
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<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with chloroform extract (500 mg/kg body weight)</td>
<td>Melastoma malabathricum roots</td>
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<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with ethanolic extract (500 mg/kg body weight)</td>
<td>Melastoma malabathricum roots</td>
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<tr>
<td>Type 2 Diabetic rats</td>
<td>Treatment with Glibenclamide (1 mg/kg b.w./day)</td>
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Each extract was tested according to the above protocol. Only petroleum ether extract was administered using Tween 80 as additional additive to enable the extract missile in the prepared suspension. Then analysis of the samples also had done as below.

All the rats were fasted for 16 hr before experimentation, but allowed free access to water. All the groups of rats were given daily treatments for four weeks.
Blood glucose levels were determined at hourly intervals for 4 hours.

At the end of experiment, Blood was collected from the dorsal aorta by a syringe and the serum was separated by centrifugation at 5000 rpm for 5 minutes for the estimation of insulin using radioimmunoassay (RIA) kit for rats (Morgan and Lazarow, 1963). Insulin levels were also measured before diabetes induction and after induction of diabetes in all the groups. All the animals were sacrificed at fasting state by light ether anesthesia followed by decapitation.

The extract/s which had shown significant antidiabetic activity against type II diabetic animal model was/were selected for dose dependent study. Type II diabetes was induced in rats following the above protocol stated in exploratory study.

**Experimental Protocol:**

Animals were divided into six groups of six rats each.

Group I: Diabetic control rats received the vehicle (1% Gum acacia suspension).

Group II: Diabetic rats received glibenclamide at a dose of 600 μg/kg and served as standard.

Group III: Diabetic rats were administered extract (250 mg/kg b.wt/day) in 1% Gum acacia suspension by p.o. route.

Group IV: Diabetic rats were administered extract (500 mg/kg b.wt/day) in 1% Gum acacia suspension by p.o. route.

Group V: Diabetic rats were administered extract (1000 mg/kg b.wt/day) in 1% Gum acacia suspension by p.o. route.

This study was conducted with three doses in type II diabetic rats for all the extracts selected.
4.7.1 Oral glucose tolerance test:

Oral glucose tolerance test was conducted for the selected extract/s which had shown significant antihyperglycemic effect in exploratory study.

Blood glucose levels of normal rats that fasted for at least 12 hours were determined; then, rats were orally administered with each selected extract with 500 mg/kg to different groups of rats (n=5). For the control group, an equal amount of vehicle was administered. The blood glucose level of each rat was determined immediately after treatment. Subsequently, 40% glucose was orally administered to both groups at 2 g/kg, followed by blood collection from tail veins at 30, 60, 120, and 180 minutes post-glucose administration to observe changes in blood glucose levels (Silva et al, 2002).

4.7.2 Renoprotective effect of selected extracts in streptozotocin induced Type II diabetic rats.

All the rats were NIDDM induced as per the protocol stated above in this methodology. Renoprotective activity was assessed by the below protocol for the selected plant extract or extracts.

Animals were divided into four groups of six rats each.

Group I: Normal control rats received the vehicle (1% Gum acacia suspension).

Group II: Type II diabetic control rats received the vehicle (1% Gum acacia suspension).

Group III: Type II diabetic rats received glibenclamide at a dose of 600 μg/kg and served as standard.

Group IV: Diabetic rats were administered with selected extract (500 mg/kg b.wt/day) in 1% Gum acacia suspension by p.o. route.

The treatments were continued for 90 days. Rats were placed in metabolic cages to
enable collection of urine samples. Urine samples were collected at 15 days intervals and were analyzed for glucose, albumin and total protein levels (Mhetre et al, 2013; Rajnish et al, 2011; Shiju et al, 2013; Somsuvra and Shital, 2014). After 90 days, the respective groups of rats were sacrificed by decapitation. Kidneys and pancreas were isolated and preserved in suitable conditions for further studies. Blood samples were collected same time. Blood glucose levels, insulin levels, serum creatinine and blood urea nitrogen (BUN) (Senthil et al, 2012; Wu et al, 2012; Najla, 2012; Akinnuga et al, 2014).

The kidneys were rapidly excised, weighed, and fixed in 2.5% glutaraldehyde for ultramorphologic studies. Kidney tissue samples (1 mm³) were fixed in 2.5% glutaraldehyde at 4°C for at least 2 hours. Samples were then rinsed in phosphate buffer for 30 minutes, postfixed in 1% buffered osmium tetroxide at 4°C for 2 hours, rinsed in 0.1 mol/L phosphate buffer for 10 minutes, and was dehydrated in graded ethanol. Specimens were then rinsed in propylene oxide and embedded in Epon 812 substitute. Ultrathin sections (50-70 nm) were cut, mounted on grids, and stained with uranyl acetate and lead citrate. Sections were examined and photographed using a transmission electron microscope. Photographs were taken for each kidney sample (Wei et al, 2012). Pancreatic tissue was processed and stained using Hemotoxilin and eosin as described in 4.6.4.

4.8 Statistical Analysis:
Results are expressed as the mean ± SD. Statistical comparisons were performed using Graph Pad Prism software (version 7.0). Data was evaluated for statistical significance by one-way ANOVA followed by Bonferroni’s Multiple Comparison Test for significant difference between treated and untreated groups; different doses treated. Differences in the values were considered significant at p<0.05.