MATERIALS AND METHODS
Nephrotic syndrome is a clinical condition produced by massive loss of urinary proteins (proteinuria) and virtually every abnormality observed during this syndrome can be traced directly or indirectly to the occurrence of proteinuria. This primary defect occurs due to the structural alterations that occur within the glomerulus. If proteinuria persists for a longer duration, it leads to hypoproteinaemia and oedema. It has been observed that diminished levels of serum proteins may stimulate the body to synthesise more proteins to compensate for the losses, which in turn leads to the synthesis of lipoproteins along with the proteins, resulting in hyperlipidaemia. Therefore, it is of utmost importance to analyse the concentrations of total protein and its fractions along with lipid profile and analytes representing kidney function in these patients so that no other complications occur in these patients and this condition can be managed in a better way.

The various techniques employed in the present study are dealt in this chapter. The methodology will be discussed in two sections:

Section I:

This section will deal with the methods involved in studying the clinico-biochemical profile of the nephrotic patients.

Section II:

This section will deal with the methodology involved in studying the efficacy of spirulina supplementation for a period of four months in nephrotic patients by analysing the various biochemical parameters.
SECTION I

1. SELECTION OF PATIENTS

The patients (n=80) were enrolled from the Special Paediatric Nephrology Clinic of Shri Sayajirao General Hospital, Vadodara, Gujarat, India with the consent of the consulting physician as well as the parents' of the patients.

2. CRITERIA FOR THE SELECTION OF PATIENTS
   a] Confirmed nephrotic syndrome patients
   b] The patient's parents' willingness to participate in the clinical trial
   c] Regular follow-up at the Special Paediatric Nephrology Clinic
   d] No apparent complications

3. SOCIO-ECONOMIC PROFILE

   This included age, education, family composition, occupation of the parent/parents and per capita income. All the information was collected through a pre-tested structured questionnaire (Appendix 3).

4. ANTHROPOMETRIC MEASUREMENTS

   i) Height measurement

       All the patients were asked to remove their footwear and any hair accessories (in the case of girls) during the height measurement. The height of the patients was measured by using a fibre-glass tape that was fixed vertically on a smooth wall perpendicular to the ground that was smooth. Height was recorded with the help of a thin scale. The readings were read from the lower edge of the scale to the nearest 0.5 cm.

   ii) Weight measurement

       The weight of the patients was recorded in their under-clothes and without footwear on an adult weighing balance. Weight was recorded to the nearest 0.25kg.
iii) Body Mass Index (BMI)

Body Mass Index was calculated as the weight (in kilograms) divided by height (in meters$^2$).

iv) Waist to hip ratio (WHR)

Waist to hip ratio was calculated as waist measurements (in centimetres) divided by hip measurements (in centimetres).

5. MEDICAL HISTORY

Detailed information was recorded by interviewing the parent's regarding the age of onset of disease, current age, duration of disease, what triggered it etc.

6. DIETARY HISTORY

24-hour dietary recall method was employed for recording the dietary history of the patients.

7. SAMPLE COLLECTION

i) Collection of blood sample

All the patients were asked to come in a fasting state (i.e. after an overnight fast of 12 hours) to the hospital in the morning, for which all parents' agreed. The venous blood was collected, sera separated and analysed for the parameters mentioned ahead.

ii) Collection of urine sample

The patients were given sterile (wide mouth) container for collecting 24-hour urine sample. The parents' were asked to discard the first urine, which the patients voided after waking up in the morning. Thereafter, all urine passed during the day and the following night was poured into the container along with the urine that was passed the next morning until the same time they had started on the previous day. If the patient wished to defecate, he/she...
was asked to empty the bladder just before defecation to avoid loss of urine.

8. BIOCHEMICAL ASSAY METHODS

The various assay methods used/employed were as following:

A] BLOOD GLUCOSE

Fasting Blood Glucose (FBG)

This was estimated by enzymatic kit (Bayers Diagnostics, Baroda, Gujarat,) following the method of GOD/POD. Glucose is oxidised by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) oxidises the chromogen 4-Aminoantipyrine to a red colour compound. Thus, peroxidase breaks hydrogen peroxide to water and oxygen. The liberated oxygen reacts with the chromogen system to from a red coloured compound. The intensity of the coloured compound developed was proportional to the glucose concentration and was measured at 505 nm (490-530nm).

Reaction sequence:

\[
\text{GOD} \quad \text{Glucose} + \text{O}_2 \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{POD} \quad \text{H}_2\text{O}_2 + \text{Phenolic compound} + 4\text{-Aminoantipyrine} \rightarrow \text{Red compound} + 2\text{H}_2\text{O}
\]

B] LIPIDS AND LIPOPROTEINS

1. Triglycerides (TG)

The evaluation of triglyceride was done with the enzymatic kit (Randox Laboratories, San Francisco, CA, USA), by GPO-PAP method. Lipase hydrolyses serum triglycerides to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to glycerol-3-phosphate (GPO) producing hydrogen peroxide. Hydrogen peroxide formed reacts with 4-aminophenazon and 4-cholorophenol in the presence of
peroxidase (POD) to produce quinoneimine, a red coloured complex that is measured at 546 nm.

Reaction sequence:

\[
\text{Lipase} \quad \text{Triglycerides} + \text{H}_2\text{O} \xrightarrow[]{} \text{Glycerol} + \text{fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow[]{} \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow[]{} \text{dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{4-aminophenazone} + \text{4-cholorophenol} \xrightarrow[]{} \text{Quinoneimine} + \text{HCl} + 4\text{H}_2\text{O}
\]

2. Total cholesterol (TC)

This was estimated by an enzymatic kit (Randox Laboratories, San Francisco, CA, USA) using the enzymatic CHOD-PAP method. Cholesterol esterase hydrolyses cholesterol esters to free cholesterol and fatty acid. The free cholesterol is then oxidised by cholesterol oxidase to cholesterol-3-one and hydrogen peroxide. Liberated hydrogen peroxide then reacts with 4-aminoantipyrine and phenol in the presence of peroxidase and forms quinoneimine, red coloured complex. The intensity of the colour produced was directly proportional to the concentration of cholesterol in the serum that was measured at 500nm.

Reaction Sequence:

\[
\text{Cholesterol esterase} \quad \text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow[]{} \text{Cholesterol} + \text{fatty acids}
\]

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

\[
\text{Peroxidase} \quad 2\text{H}_2\text{O}_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow[]{} \text{Quinoneimine} + \text{H}_2\text{O}
\]
3. High-density lipoprotein cholesterol (HDL-C)

This was measured by enzymatic kit (Randox Laboratories, San Francisco, CA, USA) using the direct HDL-C method so as to assay for the in vitro quantitative determination of HDL-C in the human serum.

The assay consists of two distinct reaction steps:

Elimination of chylomicron, VLDL-C, and LDL-C by cholesterol esterase, cholesterol oxidase and subsequently catalase.

Reaction sequence:

\[
\begin{align*}
\text{Cholesterol esterase} & \quad \text{Cholesterol ester} \rightarrow \text{Cholesterol + fatty acid} \\
\text{Cholesterol oxidase} & \quad \text{Cholesterol} \ + \text{O}_2 \rightarrow \text{Cholestenone} \ + \text{H}_2\text{O}_2 \\
\text{Catalase} & \quad 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} \ + \text{O}_2
\end{align*}
\]

Specific measurement of HDL-C after release of HDL-C by detergents in the reagent no:2*:

Reaction sequence:

\[
\begin{align*}
\text{Cholesterol esterase} & \quad \text{Cholesterol ester} \rightarrow \text{Cholesterol + fatty acid} \\
\text{Cholesterol oxidase} & \quad \text{Cholesterol} \ + \text{O}_2 \rightarrow \text{Cholestenone} \ + \text{H}_2\text{O}_2 \\
\text{Peroxidase} & \quad 2\text{H}_2\text{O}_2 + 4\text{-AA} + \text{HDAOS} \rightarrow \text{Quinone pigment} \ + 4 \text{ H}_2\text{O}
\end{align*}
\]

The intensity of the quinoneimine dye produced was directly proportional to the cholesterol concentration and was measured at 600 nm.

Composition of the reagent no:2*:

1. Goods buffer- 100 mmol/L
2. 4-Aminoantipyrine - 4 mmol/L
3. Peroxidase 4 KU/L
4. Low-density lipoprotein cholesterol (LDL-C) and Very low-density lipoprotein cholesterol (VLDL-C)

LDL-C and VLDL-C was calculated using Friedwalds formula [1972].

Calculation of LDL-C.

\[
LDL-C = \frac{\text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-C}}{}
\]

Calculation of VLDL-C:

\[
VLDL-C = \frac{\text{Triglycerides}}{5}
\]

5. Calculation of non-High-density lipoprotein cholesterol (Non-HDL-C)

Non-HDL-C was calculated using the following formula

\[
\text{Non-HDL-C} = \text{Total cholesterol} - \text{HDL-C}
\]

8) Serum Total lipids (TL)

The analysis of total lipids was done using the colorimetric method of Frings et al (1970). The lipids in the presence of sulphuric acid and phosphoric acid reacts with vanillin to form a chromogen which was read at 540nm.

Reaction sequence:

\[
\text{Sulphuric acid & Phosphoric acid} \rightarrow \text{Lipids + Vanillin} \rightarrow \text{Chromogen}
\]

Calculation:

\[
\text{Absorbance of test solution} \times \text{concentration of standard} = \text{mg/dl}
\]

C] APOLIPOPROTEINS

1. Apolipoprotein A1 (apo A1)

Apolipoprotein A-1 was estimated by Beckman Array Systems kit
(Brea, CA) by using the rate nephelometry. During the performance of this test, antibody to human apo A-1 is brought in contact with apo A-1 in the sample. The increase in light scatter resulting from the antigen-antibody reaction was converted to a peak rate signal, which is a function of the sample apo A-1 concentration. Following calibration, the peak rate signal for a particular assay was automatically converted to concentration units by the analyser. The Array 360 system was used for analysing the apo-A1 values.

10) Apolipoprotein B (Apo B)

Apolipoprotein B was estimated by Beckman Array Systems kit (Brea, CA) by using the rate nephelometry. During the performance of this test, antibody to human apo B was brought in contact with apo B in the sample. The increase in light scatter resulting from the antigen-antibody reaction was converted to a peak rate signal, which is a function of the sample apo B concentration. Following calibration, the peak rate signal for a particular assay was automatically converted to concentration units by the analyser. The Array 360 system was used for analysing the apo-B values.

D] TOTAL PROTEINS AND ITS FRACTIONS

1. Total Proteins

The method for the chemical analysis was based on the biuret reaction, which produces a violet complex when protein reacts with cupric ion (Cu²⁺) in an alkaline medium. The amount of colour complex formed was proportional to the amount of total protein in the sample and was measured by reflectance spectrophotometry at 540nm. The total proteins in the serum were investigated by using diagnostic kit (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY.)

Reaction sequence:

Protein + copper tartrate ------------------------^ Coloured complex

Lithium hydroxide
2. Albumin

Albumin in the sample was estimated using diagnostic kits (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY). The ALB slide is a dry, multilayered, analytical element coated on a polyester support. A 10 μl drop of patients sample was deposited on the slide and was evenly distributed by the spreading layer to the underlying layers. When the fluid penetrates the reagent layer, the bromocresol green dye diffuses to the spreading layer and binds to albumin from the sample. This binding results in a shift in wavelength of the reflectance maxima of the free dye. The colour complex formed was measured by reflectance spectrophotometry. The amount of albumin bound dye was proportional to the concentration of albumin in the sample. The coloured complex formed was measured at 630nm

Reaction sequence:
Albumin + Bromocresol green → BCG-albumin complex

3. Globulin

This was calculated by subtracting the estimated value of albumin from the total proteins values
Globulin = Total protein – Albumin

4. Albumin / Globulin ratio (A/G ratio)

This was calculated by dividing the estimated value of albumin by globulin value. A/G ratio = Albumin / Globulin

E] ANALYTES REPRESENTING KIDNEY FUNCTION

1. Creatinine

This was estimated with diagnostic kit (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY) following the two-point rate method. The vitros CREA slide is a dry, multi-layered, analytical element coated on a polyester support. A 10 μl drop of patients sample was deposited on the slide
and is evenly distributed by the spreading layer to the underlying layers. Creatinine diffuses to the reagent layer, where it is hydrolysed to creatine in the rate-determining step. The creatine is converted to sarcosine, in the presence of sarcosine oxidase, is oxidised to glycine, formaldehyde and hydrogen peroxide. The final reaction involves the peroxidase-catalysed oxidation of leuco dye to produce a coloured product. Following addition of the sample, the slide was incubated at 37°C. During the initial reaction phase, endogenous creatine in the sample was oxidised. Reflectance measurements were then made at 3.85 and 5 minutes. The change in reflectance between the two readings was proportional to the creatinine concentration in the sample and was read at 670nm.

Reaction sequence:

\[
\begin{align*}
\text{Creatinine} + \text{H}_2\text{O} \xrightarrow{\text{Creatine amidino hydrolase}} \text{Creatine} \\
\text{Creatine} + \text{H}_2\text{O} \xrightarrow{\text{Sarcosine oxidase}} \text{Sarcosine} + \text{urea} \\
\text{Sarcosine} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Peroxidase}} \text{Glycine} + \text{Formaldehyde} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + \text{leucodye} \xrightarrow{} \text{Dye}
\end{align*}
\]

2. Urea

The urea estimation was done using diagnostic kit (Johnson & Johnson Clinical Diagnostics, Inc., Rochester, NY). The 'vitrous urea slide' is dry, multi-layered analytical element coated on a polyester support. A 10 μl drop of patients sample was deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Water and non-proteinaceous components then travel to the underlying reagent layer, where the urease reaction generates ammonia. The semi permeable membrane allows only ammonia to pass through to the colour-forming layer, and then it reacts with the indicator. After a fixed incubation period, the reflection density of the
dye was measured spectrophotometrically at 670nm through the polyester support using the white background of the spreading layer as a diffuse reflector.

Reaction sequence:

\[
\text{Urease} \quad \begin{array}{c}
\text{H}_2\text{NCONH}_2 + \text{H}_2\text{O} \\
\xrightarrow{\text{Urease}} \\
2\text{NH}_3 + \text{CO}_2
\end{array} \\
\text{NH}_3 + \text{ammonia indicator} \quad \Rightarrow \quad \text{Dye}
\]

F) ANALYTES MEASURED IN URINE

1. Urinary protein

This was estimated by using Glaxo diagnostic kit (Qualigens diagnostics, Vadodara, Gujarat) by using the Biuret method. Proteins bind with copper ions in an alkaline medium of the biuret reagent and produce a purple coloured complex, whose absorbance was proportional to the protein concentration and was measured on Autoanalyser at 555nm.

Calculations:

\[
\text{Urinary protein} = \frac{\text{Absorbance of test solution}}{\text{Absorbance of standard}} \times 20 \times 6 \times \text{urine vol(L)} / 100
\]

To get the results in mg/24 hour, multiply urinary protein results by total urine volume in litres.

2. Urinary Creatinine

Alkaline picrate method was used for the estimation of urinary creatinine using diagnostic kit (Chempak, Reckon Diagnostics Pvt. Ltd.) following the alkaline picrate method. Creatinine present in urine reacts with alkaline picrate to form a coloured complex. The intensity of the colour developed was proportional to the creatinine concentration and was measured photometrically at 510nm (505-530nm or green filter).
Reaction sequence:
Creatinine + alkaline picrate $\xrightarrow{\text{Creatinine picrate complex}}$

Calculations:
Urinary creatinine
\[
\frac{\text{Absorbance of test solution}}{\text{Absorbance of standard}} = \frac{X 1.5 \times \text{Urine vol (ml)}}{1000}
\]

3. Glomerular Filtration Rate (GFR)
Glomerular filtration rate was calculated using Schwartz formula (1976). The height (cm) was multiplied with 0.55 and divided by the value of serum creatinine (mg/dl).
\[
\text{GFR (ml/min/1.73m}^2) = \frac{0.55 \times \text{Ht (cm)}}{\text{Serum creatinine (mg/dl)}}
\]

4. Corrected creatinine clearance (CCrCl)
Creatinine clearance (CrCl) =
\[
\frac{\text{Urinary creatinine (mg/dl)}}{\text{Serum creatinine (mg/dl)}} \times \text{Urine volume (ml/min)}
\]
\[
\text{CCrCl} = \text{CrCl} \times \text{Wt(kgs)}^{0.426} \times \text{Ht (cms)}^{0.725} \times 0.007184
\]

STATISTICAL ANALYSIS
Tabulated mean values are expressed as Mean ± SD. Students 't' test was done for assessing the level of significance between the various variables. Regression analysis was performed to study the relationship between variables. Probability level <0.05 was considered statistically significant
The experimental plan for section I is illustrated in figure 14
ENROLMENT OF NEPHROTIC PATIENTS (N=80)

Collection of baseline data

- Patient characteristics and medical profile
  - Socio-economic profile
  - General Habits
  - Medical History
  - Information regarding the medication

- Anthropometric measurements
  - Height
  - Weight
  - Body Mass Index (BMI)
  - Waist to hip ratio (WHR)

- Dietary history
  - 24-hour recall method

- Biochemical analysis
  - FBS
  - TG
  - TC
  - HDL-C
  - LDL-C
  - VLDL-C
  - Total lipids
  - Apolipoprotein A1
  - Apolipoprotein B
  - Total proteins
  - Albumin
  - Urea
  - Creatinine
  - Urinary protein
  - Urinary creatinine

STATISTICAL ANALYSIS
This section will deal with the methodology involved in studying the efficacy of spirulina supplementation for a period of four months in nephrotic patients by analysing the various biochemical parameters.

1. EXPERIMENTAL GROUP

Patients suffering from nephrotic syndrome were enrolled from the Special Paediatric Nephrology Clinic of Shri Sayajirao General Hospital, Vadodara, Gujarat, India with the consent of the consulting physician as well as the parents' of the patients.

Nephrotic syndrome cases with similar clinical conditions were chosen for the study. The enrolled patients were divided into two groups; experimental and control group.

Control group: Patients in the control group were treated only with medication and were studied for a period of four months.

Experimental group: Patients in the experimental group were supplemented with 1g/day (in two divided doses, i.e. 500mg each after lunch and dinner) of spirulina along with the prescribed drug regimen for a period of four months. The number of patients who completed the study for a period of two months included eighteen in control group and thirty-five in the experimental group. After four months of study period seventeen in the control group and thirty in the experimental group completed the study.

2. SUPPLEMENTATION

Spray dried spirulina capsules, each weighing 500 mg were procured under the bran name of ‘Recolina’. The patients belonging to the experimental group were supplemented with spirulina capsules at the level of 1g/day in two divided doses i.e. one capsule (500 mg) just before lunch and the other capsule (500 mg) just before dinner. These capsules were supplied to the patients every 15 days to assure good compliance. The patients were asked
to bring back the packing material of the capsule for cross checking. The data was collected in three parts i.e. at baseline, at two months and at four months of supplementation. Therefore, the total study period of supplementation was four months. During the study period, neither the diet nor the drug dose of the patients was altered except for the inclusion of the spirulina capsules.

3. BIOCHEMICAL ASSAY METHODS

The various biochemical parameters assayed were serum total protein, albumin, globulin, creatinine, urea and BUN. With regards to lipids; TG, TC, HDL-C, total lipids, apo A1 and apo B were analysed along with urinary protein and creatinine levels. All the analyses were done at baseline and at the end of two and four months of study period. Similar methods were used as described in section I.

4. STATISTICAL ANALYSIS

Paired 't' test was used for judging the significance of means between two related samples within each group. All tests were considered significant at p<0.05 levels.

The experimental plan of section II is depicted in figure 15
FIGURE 15
EXPERIMENTAL PLAN (SECTION II)

**Nephrotic Patients**

**Control Group**
(n=18)
[Only on Medication]

**Experimental Group**
(n=35)
[Medication + Spirulina]

2 months

Collection of mid-intervention data
- Biochemical analysis were done

4 months

Collection of post-intervention data
- Biochemical analysis were done

Statistical Analysis
SECTION I

❖ Patients suffering from nephrotic syndrome were enrolled from the Special Paediatric Nephrology Clinic of Shri Sayajirao General Hospital, Vadodara.

❖ The information regarding their socio-economic profile, general habits, dietary history and medical history was collected through a structured questionnaire.

❖ Anthropometric measurements were also measured (height, weight, BMI and WHR)

❖ Biochemical analyses such as FBG, total protein and its fractions, kidney function tests (urea, creatinine and BUN), lipid and lipoprotein fractions, total lipids, apolipoproteins and urinary protein and creatinine were done.

SECTION II

❖ The enrolled nephrotic patients were randomly divided into two groups
   1. Control group: The patients in this group were only on medication
   2. Experimental group: The patients in this group were supplemented with spirulina along with the medication for a period of four months.

❖ The biochemical estimation (serum total protein, albumin, creatinine, urea, BUN, FBG, TG, TC, total lipids, apolipoproteins and urinary protein and creatinine) were done at baseline and after two and four months of study period.

KEYPOINTS