CHAPTER- III

METHODOLOGY

Methodology is the analysis of principles or procedures of inquiry in a particular field (Siddhu, 1996). It is the body of methods, rules and postulates employed by a discipline, a particular procedure or set of procedures. The methodology followed in the conduct of the present research entitled “ANTIOXIDANT STATUS OF SUBJECTS WITH DIABETES MELLITUS” comprises of the following sections.

3.1 Selection of Area
3.2 Selection of Sample
3.3 Selection of Tool
3.4 Selection of Methods
3.5 Formulation of the Supplement
3.6 Assessing the Quality of the Supplement
3.7 Intensive Dietary Counseling
3.8 Food Supplementation
3.9 Biochemical Analysis
3.10 Statistical Analysis of the Data

3.1. SELECTION OF AREA

Kerala’s achievements in health have been universally recognized and praised; at present the state is in a phase of health transition from that of a high child mortality/morbidity picture to that of high adult mortality/morbidity situation in just over one generation time. The health picture of the state has taken a quantum leap from that of an under developed society to that of a developed, urbanized society. The emergence of the silent epidemic of lifestyle diseases poses disastrous consequences for our state.
The area selected for the study was Thiruvananthapuram District where various ethnic groups dwell in harmony. As Thiruvananthapuram is the capital of the state the standard of living of the people in and around will be high and the probability of diseases like diabetes mellitus, coronary heart diseases and obesity exists together in many people. Moreover the availability of educated and diseased samples will be high in the district. Many studies in the state have shown that the prevalence of diabetes among a group of urban residents in Trivandrum city in Kerala is very high (Kutty et al., 1999; Soman, 2004; Mohan et al., 2007).

The first sampling units of the study were five diabetic clinics from where the permission for data collection was granted. Among the five, two were Government and three were Private Clinics. The Government centers were, Indian Institute of Diabetes located at Pulayanarkotta, which is a joint venture of Government of Kerala and World Indian Diabetes Foundation; and the Govt. Diabetic Clinic City Center located at Public Health Laboratory Compound at Pattoor. The Private clinics were the diabetic clinic of SK Hospital.
located at Edapazhanni, New Diagnostic Service located at Vazhuthacaud and the Diagnostic and Research Lab at Ulloor.

3.2. SELECTION OF SAMPLE

A population is any group of individuals that have one or more characteristics in common that are of interest to the researcher (Potti, 2000). In the present study Diabetic patients were the population. Studying the entire universe is not viable in many ways; therefore it is always convenient to pick up samples from the universe proposed to be covered by the study.

A sample consists of the individuals from the population, which is selected, for the purpose of representing the population. By observing the characteristics of the sample certain inferences can be drawn which can be generalized (Kothari, 2004). The samples were selected from the first sampling units of the study which were the diabetic clinics from the city.

As the prevalence of Type I and Type II diabetics is in the ratio of 10:90 (www.diabetic india.com, 2006) in the population, the process of sampling was carried out in such a way that both the groups are represented equally. Hence deliberate sampling was applied to collect the samples for the study. Samples of thirty years of age and above were selected alternatively from type I and type II diabetic patients who were waiting at the OP of the clinics between 7.30 am to 12 noon. This process was repeated until a sample of forty Type I and forty Type II diabetic patients comprising of a total of 80 samples from each clinic was completed to make a total of four hundred patients (N=400).

Information was collected using a pre-tested interview schedule regarding socio-economic status, dietary information, lifestyle pattern, awareness level, health and clinical status of the
patients. The truthfulness of the answers was verified by questioning and cross questioning. Anthropometric measures were taken after each interview.

To elicit the biochemical profile, in the second phase of the study a control group was also identified consisting of two hundred non-diabetic subjects of age between 40 and 50 years from the same socio-economic back ground similar to that of the type I and type II diabetic subjects. Since the sample should be true representative of population characteristic without any bias so that it may result in valid and reliable conclusions (Kothari, 2004).

Sub-sampling is a procedure by which a small, representative sample is taken from a larger sample. Good sub-sampling technique becomes important when the large sample is not homogeneous (Adèr et al., 2008). A sub-sample of 60 (15 percent of sample) was selected through convenient sampling procedure, comprising of twenty each from type I, type II and the non diabetic group. The objectives of the study were clearly explained to them and written consent was also obtained stating that he/she is convinced that there is no individual loss or benefit other than knowing their blood profile. Hence according to the willingness to participate in the study a total of sixty sub-samples (n=60) were analysed for their blood profile.

A comparative study on the antioxidant status of the three groups (Type I, Type II and non-diabetic) was done in this phase. Blood was analysed for sugar, cholesterol, haemoglobin, antioxidant enzymes and antioxidant vitamins. The number was limited for ease in analyzing the blood samples on the day of blood collection itself. All the laboratory experiments were done at the National Institute of Interdisciplinary Science and Technology (NIIST) formerly termed as the Regional Research Laboratory (RRL) located at Pappanamcode. Table 3.1., shows the summary of the methods adopted for the study.
Table 3.1. Summary Of The Methods Adopted-

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<th>Area of Study</th>
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<td>Diabetic Clinics Of 2 Government Hospitals</td>
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<td><strong>Phase–I</strong></td>
<td>Type I-100</td>
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<td>Survey Method</td>
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<td>Sample size (N=400)</td>
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<tr>
<td>Socio-economic Status, Life Style Pattern, Health and Clinical Data, Dietary Information, Diet and Diabetes Awareness</td>
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<td><strong>Phase–II</strong></td>
<td>Type I-20</td>
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<td>Blood Analysis- Comparison of Antioxidant Status</td>
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<td>Sub-Sample (size n=60)</td>
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<td></td>
<td>• Estimation of Glucose, Cholesterol, Hemoglobin,</td>
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<td><strong>Phase–III</strong></td>
<td>Type II-20</td>
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<td>Supplementation</td>
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<td>Micro-Sample Newly Diagnosed Diabetic Subjects (size n=20)</td>
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<td>• Dietary Analysis- Weighment Method</td>
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<td>• Formulation of the Supplement</td>
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<td>• Assessing the Acceptability &amp; Storage stability of Spirulina Biscuits</td>
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<td>• Comparison of Spirulina Biscuits with Commercial Diabetic Biscuits</td>
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<td>• Diet counseling and Dietary Stabilization</td>
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According to Lohr, (1999) the smaller the sample size, the more likely that any pattern observed in data will reflect random variation in the characteristics of samples drawn from populations, regardless of whether the research is characterised as qualitative or quantitative, we should be mindful of this when presenting findings. Having this in mind, in the third phase, for intensive supplementation study newly diagnosed type II diabetic patients of age between 40 and 50 were selected as micro samples (n=20) on the basis of convenient sampling.

Convenient sampling is a type of non-probability sampling which involves the sample being drawn from that part of the population which is close to hand, as it is readily available and convenient (Adèr et al., 2008; Saravanavel, 2004). Patients of upper middle and higher income families were purposely selected to get absolute involvement in the supplementation study; as the diet and eating habits in high and low socio economic groups vary considerably (Shahar, et al., 2005). Those taking vitamin supplementation or nutraceutical products were excluded.

Patient’s Information Sheet/ brochures were distributed to explain the procedure of the study and attain their confidence; subject’s consent form was collected with their signature so that their willingness to cooperate throughout the study was ensured.

Prior to the supplementation, the subjects were given counseling on diet and diabetics with the help of pamphlets and CD. The dietary antioxidant status was assessed through one day weighment method. After the dietary stabilization phase, supplementation started with distribution of the standardized quantity of the spirulina biscuits and 15ml of lime juice for a period of one month. Before and after supplementation the blood samples were analysed for blood sugar, haemoglobin, lipid profile, enzymatic and non-enzymatic antioxidant status.

Other recipes with spirulina were also developed and standardized for the personal daily use of the diabetic subjects as per their interest to impart variety.
3.3. SELECTION OF TOOL

An investigation requires many data gathering tools and techniques that vary in complexity, design, administration and interpretation (Lohr, 1999). Appropriate data gathering tool was devised with due reference to the objectives of the present study. An interview schedule which is defined as an oral questionnaire that permits an exchange of ideas and information is used for data collection. It is unique as it involves gathering data through direct verbal interaction between the interviewer and interviewee that he feels free to express himself fully and truthfully (Kothari, 2004).

All participating subjects gave informed consent for the present study. Various questions useful in the field of study were asked orally to the patients and their answers were jotted down at the space provided in the schedule.

Main areas covered under the study include information regarding socio-economic status, life style pattern, health and clinical data, anthropometric measurements, biochemical reports and dietary pattern. Awareness level of the subjects was measured on health, diet and diabetes. The schedule was pretested and evaluated by statisticians, doctors and also distributed among five diabetic patients and appropriate corrections were made accordingly and finalized to 45 number of questions. The model of the interview schedule is given in the Appendix 1.

3.3.1. Socio-economic Status

The interview schedule for the section Socio-economic Status aimed to collect information on age, gender, religion, domicile, marital status, educational qualification, occupation, familial and personal monthly income, family composition and expense on food, medicine and others per month.
3.3.2. Life Style Pattern

The quality of life of an individual depends on Life Style Pattern hence queries on this section aimed to elicit information on smoking habits, chewing tobacco, intake of alcohol, duration of physical activity per day or week both household activities and exercise patterns were included in this. Peace of mind is an indication of health; hence occurrence of anxiety, fear, tension, sleeplessness, worries, stress, family problems, financial problems or other family crises were incurred. Duration of work, sleep and leisure were also under the field of enquiry.

3.3.3. Health and Clinical Data

Health and Clinical data aims to elicit information on various aspects of diabetes and its management. As the duration of any disease increases, the management also becomes complex; hence to rule out the association of such factors the diabetic age and genetic inheritance was measured. Queries on other areas like system of treatment, measures taken to control diabetes like diet, exercise and drugs were asked. Diabetic complications like retinopathy, neuropathy, nephropathy, diabetic gangrene, diabetic foot, renal infections, as well as other illness were included as a major section. The prevalence of many other problems like heart disease, hyper tension, hyper cholesterolemia, asthma, arthritis, peptic ulcer, cancer, migraine and stroke were also assessed. Details of biochemical reports were also collected to understand their frequency to the clinician and medical assistance. Measurements of body proportions give sufficient information on the features of obesity, central obesity and fat distribution; hence queries on measurements of height, weight, waist and hip measures were also included in the schedule.
3.3.4. Dietary Information

Dietary information is a measure of nutritional status. This section is aimed to collect information on food habit, prescribed calorie intake, method of food preparation and consumption, special food taken during festivals and religious functions. An exhaustive list of food stuffs was prepared which contains processed, non-processed and commercial items, from which the frequency and amount of consumption of each and its expenditure is elicited to know the most commonly discarded and consumed food.

Monthly consumption frequency was elicited from a list of cereals, pulses leafy vegetables, roots and tubers, other vegetables, fruits, sea foods, egg, flesh food, milk and milk products, fats and oils, nuts and oil seeds, beverages, processed food, baked food and nutraceuticals/food supplements like spirulina are included in this section.

3.3.5. Awareness on Diet and Diabetes.

An awareness query was prepared to measure the awareness level of the subjects, which contained thirty questions related to the different aspects of diet and diabetic management. Since level of dietary awareness and diabetic management are interrelated queries included in the list were on foods to be avoided and consumed, need to test the blood sugar level, diabetic complications, obesity and exercise, nutrients in food and on personal hygiene. For carrying out the dietary counseling or education programme the knowledge was measured through the awareness schedule and the rate of awareness was assessed. After a period of three months interval, the same set of evaluation questions regarding the four main areas like Cause of diabetes, Symptoms and complications, Control and management of diabetes, Antioxidant and dietary management were reassessed by distributing it among the subjects.
3.4. SELECTION OF METHODS

The method of investigation should satisfy the objectives of study. Assessment of diet and assessment antioxidant status of the subjects is one of the prime objectives of the research, which is accomplished through Anthropometric, Biochemical and Dietary Analysis. Diet counseling was done for the correction of the dietary regimen. The other objective was to supplement an antioxidant rich food for which, formulation and standardisation of the supplement, assessing its acceptability, storage stability standardization of other feasible recipes and supplementation. The methods are explained below:

3.4.1. Anthropometric Assessment

Anthropometry is the measure of human body at various ages and level of nutritional status and it is based on the concept that an appropriate measurement should reflect any morphological variations occurring due to a significant psychological change (Rao, 1996). Anthropometry which is the science of measuring the size, weight and proportion of human body (Kathleen and Sylvia, 2004) measures the degree of fat deposition through Quetlets Index, waist-hip ratio, body frame and other significant measures.

3.4.1.1. Body Mass Index (BMI)

Dudeja et al., (2001) opine that the association of obesity with diabetes is complex and is compounded by several heterogeneous factors. BMI is directly associated with glucose intolerance. This suggests that increase in body weight, although within the ideal levels of BMI, confers a high risk to diabetes. The Quetlets Index is commonly referred to as Body Mass Index (BMI) and is a valid measurement of nutrition status that has a high co-relation with adiposity (Garrow, 2000). Body Mass Index (BMI) was calculated for
all the diabetics using the formula: Weight in Kilo Gram (kg)/Height in Meter Squares (m²).

*Measurement of Height:* - Height was measured with fiber glass tape to the nearest 0.1cm. Subjects were requested to stand without shoes and stand upright with the back against the wall, heels together and eyes directed forward (Deepa *et al.*, 2002).

*Measurement of Weight:* - Weight was measured with a standard weighing balance, which was kept on a firm horizontal surface. The scale was checked every day and calibration was done with an individual of ‘known weight’. The subjects were asked to wear light clothing; and after calibrating the standard weighing balance the body weight of all samples were recorded without shoes before breakfast after voiding (Lawler, 1993). Weight was recorded to the nearest 0.1 kg.

Assessment of body mass index was based on the recent indications of International Obesity Task Force (2005) showing that those with the corresponding BMI values ranging from Normal-18.5 to 22.9, At risk of Obesity-23 to 25, Grade I Obesity- 25 to 29.9 and Grade II Obesity- more than 30.

### 3.4.1.2. Waist Hip Ratio

Abdominal Obesity is often referred to as the waist hip ratio (WHR) which is the ratio of obesity in the upper trunk to that in the lower trunk. A high WHR is associated with increased risk of diseases.

*Measurement of Waist Circumference:* - Waist circumference assess the abdominal fat accumulation which is an independent predictor of disease risks. It was measured using a non-stretchable fiber glass tape. The subjects were requested to stand erect in a relaxed position. One layer of light clothing was accepted. The distance around the smallest area below the ribcage and above the umbilicus bone is measured. Waist circumference was measured at the midpoint between the iliac crest and the lower margin of the ribs.
Waist circumference was measured to the nearest 0.5cm (Deepa et al., 2002).

Measurement of Hip Circumference: - Hip circumference was also measured using a non-stretchable fiber glass tape. The subjects were requested to stand erect in a relaxed position with their feet together. Hip girth was recorded at the greater torchanter (the widest portion of the hip) on both sides. One layer of light clothing was accepted. Hip circumference was measured to the nearest 0.5cm (Deepa et al., 2002).

The waist hip ratio was computed using the formula: Waist circumference in centimeter/Hip circumference in centimeter (ICMR, 2005). The standard ratio prescribed by American Council On Exercise Testing And Prescription, (2005) was used to classify the diabetics- High risk if WHR is >1.0 for men and if WHR is >0.85 for women; Moderately High risk if WHR is between 0.90 and 1.0 for men and is between 0.80 and 0.85 for women; and low risk if WHR is <0.09 for men and if WHR is <0.80 for women.

3.4.1.3. Mean Fat Area

Mean fat area is the difference between arm area and muscle area which is computed from the Nomogram described by Srilakshmi, (2007) who states that it is earlier published in the American Journal of Clinical Nutrition in volume 26:912(1973) where muscle area and arm area can be computed from which the mean fat area is assessed.

Measurement of Mid Upper Arm Circumference (MUAC):- Mid Upper Arm Circumference is the measure on the non-dominant arm, unless it is affected by edema, a non stretchable fiber glass tape was used to locate the midpoint of the upper arm. The midpoint is between the tip of the acromion of scapula and tip of the olecranon of the forearm bone, ulna is located with the arm flexed at the elbow and is marked with a marker pen. The tape was in complete contact with the skin surface without compressing the underlying fat. The
circumference was measured at this midpoint accurately to the nearest tenth of a centimeter and recorded (Margret and Nelson, 1998). The assumption is that the cross-section of mid-upper arm circumference contains evenly distributed adipose tissue around the area (Rao and Vijayaraghavan, 2000).

3.4.2. **Dietary Analysis.**

Dietary analysis usually begins with an interview in which the nutritionist asks questions about a person's typical food intake and uses different methods to assess typical food intake (Hammond, 2000). The dietary status was assessed through food frequency schedule, one day recall method and one day weighment method among the micro sample to assess the dietary antioxidant status.

3.4.2.1. **Food Frequency Schedule**

A food frequency schedule provides more accurate picture of a person’s typical eating patterns. The nutritionist asks how often he or she consumes certain food and how many servings of it may be consumed in a day, week or month (Hammond, 2000). An exhaustive list of food stuffs were prepared to find out the frequency of consuming different food. It provides a more accurate picture of a person’s typical eating pattern.

The food frequency schedule was aimed to collect information on the intake frequency and amount of consumption of a wide range of food stuffs like Cereals (*Raw Rice, Parboiled Rice, Ragi, Wheat whole /Flour, Rawa, Noodles, Vermicelli and other Cereal Products*). Pulses (*Sprouted Pulses non sprouted varieties*), Leafy Vegetables (*Amaranthus, Amaranth spined, Agathi, AraiKeera, Drum stick leaves and Coriander leaves, Curry Leaves, Mint leaves*), Roots and Tubers (*Tapioca, Carrot, Potato, Yam, Cola cassia, Sweet Potato and Beetroot*), Vegetables (*Ash gourd, Bitter Gourd, Brinjal, Cauliflower, Cluster beans, Cucumber, Drumstick, French Beans, Jack Tender, Ladies Finger, Plantain Green, Plantain Stem, Plantain Flower, Snake gourd, Tomato*).
Green, Mango Green, Onion Stalks, Pumpkin), Fruits (Amla, Apple, Bread Fruit, Cashew Apple, Grapes, Jack Fruit, Lemon, Mango, Water Melon, Orange, Pineapple, Plum, Passion Fruit, Tomato Ripe), Fish and Sea Foods (Herring Indian, Tuna, Mackerel, Sardine, Anchovy), Meat Egg and Poultry (Beef, Duck, Chicken, Mutton, Pork, Liver, Egg Hen, Egg Duck, Egg Quail), Milk and Milk Products (Cow’s Milk, Goat’s Milk, Buffalo’s Milk, Curd, Cheese, Butter, Panneer), Fats and Oils (Coconut Oil, Sunflower Oil, Groundnut Oil, Palm Oil, Gingely Oil, Mustard Oil, Vanaspathi, Ghee), Beverages (Carbonated Beverages, Fruit Juices, Alcohol, Wine), Tea/Coffee (With Milk/Sugar, Without Milk/Sugar), Soup (Vegetarian, Non Vegetarian), Nuts and Oilseeds (Cashew Nut, Ground Nut, Coconut, Gingely Seed, Almonds), Processed/Preserved Foods (Jam/Jelly, Pappads, Pickles), Confectionary and Bakery Items (Cake, Biscuits, Desserts), Nutraceutical Products (Health Drink, Herbal Drinks and products, Nutrient enriched products Like Spirulina).

3.4.2.2. One Day Recall Method (24 Hour Recall Method)

The subject recalls food intake during the preceding 24 hours by interview, on what was eaten, how the food was prepared, how much food was eaten (Robinson and Lawler, 1993). One day recall method was administered among the type I and type II samples (N=400). Type of preparations made and raw materials used at breakfast, lunch, tea time and dinner were collected. The amount of calorie, carbohydrates, proteins, and fat in the cooked food was calculated from the nutritive value table of cooked food published by the National Institute of Nutrition, 2004 on Some Common Indian Recipes and Their Nutritive Value (Pasricha and Rebello, 2004).

3.4.2.3. Weighment Method

While using one day recall method people tend to over or under-estimate intake of certain foods (Hammond, 2000) hence weighment method was also conducted among the micro samples consisting of 20 samples. Here the weight of cooked foods was weighed using an accurate balance. Grocer’s balance with standard
weights and measures were used to weigh and a structured diet survey schedule was prepared to record the information. Details of the schedule for the weighment survey are given in Appendix 2.

According to Bamji et al., (1999) it is ideal to conduct the weighment survey for seven consecutive days to capture the true picture of diet but depending on the purpose of investigation the period of survey can be reduced or increased. Thus a day’s weighment survey was conducted were the weight of cooked food taken for consumption by the sample during breakfast, lunch, evening tea, dinner and other mid time snacks were recorded and the conversion factor on cooked to raw was used for further computation of the nutritive value. Foods are converted to nutrients using food composition table. The ingredients used in the preparation were also noted to compute the nutritive value. Carbohydrates(g), Proteins(g), Fat (g), Calories (Kcal), Vitamin C(mg), Vitamin E(mg), Beta Carotene(mg), Selenium(mg), Copper(mg), Zinc(mg) and Iron(mg) content in the diet was computed from the nutritive value table published by the National Institute of Nutrition, in the Nutritive Value of Indian Foods (Gopalan et al., 2004).

3.5. FORMULATION OF THE SUPPLEMENT

A supplement is an edible preparation acceptable in consistency suitable for age and/or disease condition containing maximum number of nutrients or a specific nutrient to be included in the daily diet of a special group of society who requires a change in the nutritional status; which can be stored in dry form and reconstituted easily. A low risk diet consisting of complex carbohydrates, adequate proteins and low fat enriched with antioxidants can be used as a therapeutic supplement in the management of various nutritional and metabolic disorders especially diabetes. The importance of antioxidants is found to be significantly high in diabetic diet therapy.
3.5.1. Selection of Raw Ingredients

The ingredients selected for the study was based on the therapeutic property, antioxidant potential, ability to soothe the disease condition, nutritive value, acceptability, digestibility, shelf-life qualities and availability. As the present study is aimed at developing a convenient antioxidant rich food; spirulina which is an ore of different antioxidants, was selected as the agent for antioxidant transport. Since ‘Biscuits’ can be stored for a longer duration without contamination, the snack selected for supplementation was ‘Spirulina Biscuits’. Ingredients selected for formulating the spirulina biscuit, contains whole wheat flour as the staple carbohydrate rather than maida or white flour due to its fiber content, spirulina the proteins supplement was selected with the aim of having 100% digestibility arising from a vegetarian source. All the ingredients were purchased from the local market except spirulina which was purchased from the Parry Nutraceuticals, located at Oonaiyur, Chennai in India. Spirulina provides 65-70 percent proteins, 8-14 percent of carbohydrates, vitamins such as β-carotene, thiamine, riboflavin, niacin, vitamin B₆, folic acid, biotin, vitamin C, vitamin E, iron, calcium and phosphorus.

3.5.2. Processing of Raw Materials

In order to develop the supplement all the ingredients were subjected to visual examination before standardization. Wheat flour was sieved and green chilies were washed well, while other ingredients like spirulina, oil, salt and baking powder were used directly after purchase for standardization of the recipe.

3.5.3. Standardization of Recipe- “Spirulina Biscuits”

A standard recipe is one of the most important and effective tools for obtaining quality food. “A standardized recipe is the one which establishes procedures that will make possible the production of a high quality food” (Drummound, 1953).
Since the supplement was formulated to feed the newly diagnosed diabetic patients, it is necessary to convert it into a recipe. It was decided to prepare spirulina biscuits. For standardization of the spirulina biscuits, each ingredient was treated separately according to the consistency of the product. The flour was measured by a single method called leveling. The measurement was repeated seven times so as to find that the weight was same all the time. The ‘preet’ measuring cup was used for this purpose. The ingredients were leveled and kept on a balance and weighed. Then the cup was weighed alone. The difference in weights gave the actual weight of the ingredients (Drummound, 1953). Electronic balance was also used to weigh the amount of spirulina so as to ensure maximum accuracy. No sugar was added as the supplement was aimed at diabetic subjects.

3.5.3.1. Preparation of the Spirulina Biscuits

In order to make the Spirulina biscuits, flour was sieved thrice with spirulina, salt, baking powder and garam masala to have uniformity in texture throughout the flour. To this mixture added ground green chillies and the whole mass was made into medium stiff dough with equal proportion of water and oil (1:1).

To maintain saturated/unsaturated/polyunsaturated fat ratio three types of oils were used namely, Olive oil, Safflower oil and Ground nut oil in equal proportion (1:1:1). The dough was rolled out, cut into round shape and was baked in an oven for 30 minutes at 140\textdegree\text{Celsius}. Since the biscuits were to be stored and used in future, a pinch of permitted preservatives were also added. By varying the proportion of the ingredients the correct proportion of the recipe was established. Successive trails of the recipe adopting the changes recommended from the previous trials were carried out until the product was satisfactory in all aspects. Figure 3.2., shows the preparation of spirulina biscuits in flow chart.
Fig 3.2. Flow Chart of Preparation of Spirulina Biscuits

1. DRY AND POWDERED INGREDIENTS
   {FLOUR + SPIRULINA + SALT + BAKING POWDER + GARAM MASALA}

2. SEIVED THRICE

3. ADDED GROUND GREEN CHILLIES

4. STIFF THE DOUGH WITH OIL AND WATER {1:1 RATIO}

5. BAKED IN AN OVEN {FOR 30mts AT 140°C}

6. CUT IN TO ROUND SHAPE

7. ROLLED OUT

8. KNEADED TO SOFT DOUGH
For the optimum judgment and selection of the most acceptable recipe, three variations were also done. In order to identify the acceptable proportion of spirulina in the supplement, the amount of basic ingredients like wheat flour, Garam masala and salt were kept constant whereas the proportion of spirulina was altered. Increasing and decreasing the amount of spirulina enabled to find out the maximum accepted level of spirulina that could be used for supplementation. Variation was brought about by changing the amount of spirulina where as the amount of basic ingredients were kept constant.

3.5.3.2. Development Of Other Spirulina Based Recipes

Other recipes based on spirulina were also developed and standardized for future use of the diabetic subjects as per their interest to impart variety in the daily diet.

- Breakfast/Dinner- Spirulina Uppumav, Spirulina Dosa, Spirulina Idly, Spirulina Puttu, Spirulina Idiappam and Spirulina Godhumbu Dosai.
- Refreshments- Spirulina Tea, Spirulina Bitter Gourd Juice,

3.6. ASSESSING THE QUALITY OF THE SUPPLEMENT

Quality assessment is of prime significance in the process of development of a new supplement. The acceptability of the product was assessed with special reference to nutritional significance and storage stability of the product, organoleptic quality preferences of technical experts, diabetic and non diabetic subjects.
3.6.1. Organoleptic Quality Preferences of Technical Experts

Consumer acceptability evaluation of any food product is essential before advocating or marketing the product. The acceptability of any product depends on organoleptic qualities. The acceptability of the biscuits was assessed through organoleptic evaluation in the laboratory with the selected panel of twenty judges. The judges were selected through triangle test. Ranking test and Numerical scoring tests were used to rank the samples according to the intensity of the specified characteristics (Srilakshmi, 2003). The regulation for conducting the trial was maintained as suggested by Swaminathan, (1999). From the scores the best variation or combinations were identified and was selected for further investigation.

When the quality of the food product is assessed by means of human sensory organs, the evaluation is said to be organoleptic or sensory evaluation (Srilakshmi, 2003). Colour, Texture, Flavour, Odour, Appearance, Doneness and Taste are the main criteria that determine the acceptability. The score card used to measure the Organoleptic Quality Preferences of Technical Experts for the variations of the supplement is given in Appendix 3.

3.6.2. Acceptability of the Supplement among Diabetics and Non-diabetics

The main purpose of our research is to take the technology from lab to home. To test the consumer preferences of the product, the spirulina biscuits were distributed among a group of 50 adults who were selected at random comprising of 25 diabetics and 25 non-diabetics. They were requested to mark their preferences accordingly. The Nine Point Hedonic Rating Test was used to assess the acceptability. It was used first by U.S Armed Forces for ascertaining the ‘likes’ or ‘dislikes’ for various dishes by the soldiers, so that food wastage can be avoided and psychological satisfaction of the person can be achieved. It can also be
used in research laboratories to ascertain the acceptability of new products (Swaminathan, 1999).

Hedonic scale for acceptability of food products includes options to express their opinion ‘Likes Extremely’, ‘Likes Very Much’, ‘Likes Moderately’, ‘Likes Slightly’, ‘Neither Likes nor Dislikes’, ‘Dislikes Slightly’, ‘Dislikes Moderately’, ‘Dislikes Very Much’ and ‘Dislikes Extremely’. Provision to mark comments was also given. A sample of the evaluation card to test the acceptability of the spirulina biscuit among the diabetics and non-diabetics is given in the Appendix 4

3.6.3. Nutritional significance of the product

Nutritional significance is the prior and important factor in a supplement. The antioxidant potential of the product is evaluated among the three variations using the food composition table given in the Nutritive Value of Indian Foods (Gopalan et al., 2004). The major nutrients namely Calorie, protein, fat, vitamin A, vitamin E, vitamin C, β Carotene and iron were computed. Since the ingredients other than spirulina is kept constant, increase in the quantity of spirulina may also increase the antioxidant potential.

3.6.4. Comparison of Spirulina Biscuits with Commercial Diabetic Biscuits

The developed diabetic supplement was compared with popular commercially available biscuits for its nutritional significance and cost. The nutritive value of 100g of the developed diabetic supplement was calculated and compared with that of four commercial brands. The cost of 100g of the developed product was compared with cost of 100g of four commercially available biscuits designated as Brands A, B, C and D. The cost of the developed supplement was computed according to the existing market price of each ingredient used. The cost of each ingredient in Indian
Rupees was worked out by dividing total cost of the product by Edible Portion Weight (EPW).

### 3.6.5. Storage stability of the product

Storage stability of a product depends on the ability of the supplement to remain edible during the storage period without bacterial or fungal invasion and without change in colour, texture, flavour, odour, appearance and taste. Since the supplement has to be stored for future use it is always necessary to test the shelf life period/microbial activity of the spirulina biscuits. It has been suggested by Gahalawat and Sehgal, (1993) that supplementary foods should have a shelf life of six months.

For testing the shelf life of the developed supplement it was packed in poly propylene covers and was stored in an air tight container at room temperature for a period of six months. After the storage period the total microbial counts i.e. both the number of bacterial and fungal colonies was determined by Serial Dilution Technique (AOAC, 1990).

Plate count method is based on the principle that when material containing bacteria or fungus is cultured, every viable bacteria develops into a visible colony on a nutrient agar medium and every viable fungus develops into a visible colony on a Saboaurd Dextrose Agar (SDA) medium. The numbers of colonies are the same as the number of organisms contained in the sample. The detailed procedure for the estimation of total microbial count is given in Appendix 5

### 3.7. INTENSIVE DIETARY COUNSELING

Diet counseling is an ongoing process to assess dietary intake and identify areas where change is needed (Harris-Davis and Haughton, 2000). Proper diet counseling is essential for any intensive dietary studies. Irregular consumption of food is the cause of most of the life style diseases; hence the patients were given intensive one to one counseling before food supplementation.
After analyzing the data on socio economic, health, life style, clinical, dietary pattern, health, blood profile and awareness level of the subjects, topics for counseling were finalized. Thus counseling was given to the selected samples of twenty \( n=20 \) consisting of equal number of newly diagnosed male and female type II diabetic patients of age between 40 and 50 years who were selected as micro samples on the basis of convenient sampling procedure.

According to the convenience of the subjects evening time and holidays were selected for the education programme at their respective residence. An hour was allotted to each subject for face to face interaction comprising of four sessions, one per month. Counseling was given on different aspects of diet and diabetes like signs and symptoms, control and management, prevention for the causes of diabetes, need of regular health checkups, antioxidants and dietary management, importance of physical exercise, need of maintaining normal BMI and Waist Hip ratio, antioxidant property of spirulina and on spirulina based recipes.

### 3.7.1. Formulation of Educational Package

Nutrition education is the only means of solving the problems and creating awareness among the subjects. It is realized by providing information, educational materials, support and follow-up to help the individual make and maintain the needed dietary changes (Harris-Davis and Haughton, 2000).

**Lecture cum Discussion method**- Interactive lecture cum-discussion on all aspects of diet and diabetes was included with due importance to the antioxidant property of spirulina

**Information Brochure/pamphlets**- An information brochure with dietary guidelines was prepared in local language for the individual counseling which was distributed among the subjects regarding Nutrients,
Diabetes, Cause of diabetes Symptoms and complications, Control and management of diabetes, Antioxidant and dietary management, Balanced diet, Exchange list and Model diets for diabetic patients. The pamphlets were distributed among the subjects and were explained to them.

*Information CD*- A Compact Disk (CD) Power Point Presentation was developed as part of the educational package for giving awareness to the sample. Topics on nutrients, sources of nutrients, diet and diabetes, antioxidants, obesity and weight reduction were included.

*Recipe Demonstration*- During counseling the preparation of the developed supplement, spirullina biscuit was demonstrated. Other recipes developed were also discussed for incorporation in their daily menu.

### 3.7.2 Pre and Post Awareness Score.

The goal of nutrition counseling is to help a person make and maintain dietary changes. Before and after the education programme the knowledge was assessed through an awareness schedule and the rate of awareness was measured. After a period of three months interval, the knowledge of the subjects were tested again to find out the improvement after counseling, using the same set of schedule which was administered initially. Same set of evaluation questions regarding Cause of diabetes, Symptoms and complications, Control and management of diabetes, Antioxidant and dietary management were distributed among the subjects.

### 3.7.3 Nutrient Intake Before and After Counseling- Weighment Method

Assessment of invitro dietary analysis was done by measuring the daily food intake through weighment method, among the 20 micro samples selected for supplementation, which is the pre-supplementation phase. Cooked food was weighed using an accurate balance in the morning, noon and evening before intake. It was then converted to raw portion size and the nutrient intake was calculated. Additional food
consumed by the subjects like food brought from shops, sweets and other food accepted from friends and relatives were also noted in details before computing the nutritive value. The day selected for weighment analysis was mostly public holidays and other convenient days allotted by the subjects. Preeth’s standard measuring cup, spoons and standard kitchen scale were used for executing the weighment method. Carbohydrates(g), Proteins (g), Fat (g), Calories (k.cal), Vitamin C(mg), Vitamin E(mg), Beta Carotene(mg), Selenium(mg), Copper(mg), Zinc(mg) and Iron(mg) content of the diets were computed using the nutritive value table published by the National Institute of Nutrition 2004 on *Some Common Indian Recipes and Their Nutritive Value and Nutritive Value of Indian Foods*.

### 3.7. FOOD SUPPLEMENTATION

The preparation method of the spirulina biscuit was demonstrated during counselling. Other recipes based on spirulina developed were also discussed for incorporation in their daily menu. Each subject was given a prescribed calorie intake according to their height and weight, and sample diabetic menus. The subjects were asked not to deviate from the prescribed calorie intake from one week prior to the supplementation phase and to continue it. The dietary guidelines were reiterated several times to ensure better understanding about all the topics.

Spirulina biscuits were supplemented to the twenty \((n=20)\) newly diagnosed diabetic subjects. The subjects were asked to drink 15ml lime juice as spirulina was found lacking in vitamin C. Both spirulina-biscuits and lime juice were given to the sub-samples for a period of one month. After the supplementation period of one month the blood samples were again collected and were estimated for blood sugar, lipid profile, and antioxidant status.
3.9. BIOCHEMICAL ANALYSIS

Biochemical tests can be conducted on easily accessible body fluids such as blood and urine that help to diagnose disease at the sub-clinical stage and confirm clinical diagnosis at the disease stage as the clinical signs and symptoms being often non specific (Bamji et al., 1999). Blood samples were assessed for its glucose, lipid profile, hemoglobin level, activities of enzymatic antioxidants like Cu-Zn super oxide dismutase and catalase and non enzymatic antioxidants like Vitamin A, Vitamin E and Vitamin C of diabetic and non diabetic subjects and also among the newly diagnosed diabetic subjects before and after the supplementation. Detailed procedure of the estimation of biochemical parameters in blood is given in Appendix 6.

3.9.1. Preparation of Blood Samples.

The venous blood samples were collected in heparinised tubes between 7.30am and 9am from the diabetic and non diabetic samples. Depending on the procedure adopted 0.5ml of blood sample was stored separately from each sample and the rest were centrifuged at 3200rpm for 15 minutes and separated the plasma. The erythrocytes were carefully sampled from the bottom of the tube to minimize contamination with leucocytes, and then the precipitate was washed three times with isotonic saline solution and lysed by addition of double distilled water containing 5ml/L Triton x-100, followed by vigorous vortex-mixing and stored on ice for 10 minutes. Membrane free hemolysate was obtained by centrifugation at 10,000xg rpm for five minutes. Assay of enzymatic and non enzymatic antioxidants were done within the same day of collecting the blood samples. Enzyme assays of each sample were performed in duplicate.
3.9.2. *Estimation of Glucose in Blood Plasma*

Estimation of plasma blood glucose level was based on Glucose Oxidase-Peroxidase method (GOD/POD method), where glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide. In subsequent peroxidase catalysed reaction, the oxygen liberated is accepted by the chromogen system to give a red coloured quinine-amine compound that is stable for one hour and is read colorimetrically at 505 nm. The criteria for the diagnosis of diabetes by WHO is Fasting Plasma Glucose (FPG) ≥ 126mg/dl and 2 hours post glucose value (post prandial plasma glucose) ≥ 200mg/dl. The normal fasting plasma glucose level is 70-110mg/dl (http://www.who.int/diabetes/publications/en/).

3.9.3. *Estimation of Total Cholesterol, HDL and LDL*

Regulation of Total Cholesterol, HDL (High Density Lipoproteins) and LDL (Low Density Lipoproteins) plays a central role in disease development. According to the Kit provided by BioVision, Cholesterol Oxidase recognizes free cholesterol and produce products to react with probe to generate colour at 570nm. A large portion of the cholesterol in blood is in the form of cholesteryl esters, cholesterol esterase hydrolizes cholesteryl esters into cholesterol. Cholesterol is then oxidised by cholesterol oxidase to yield H₂O₂ which interacts with a sensitive cholesterol probe to produce resorufin which can be detected by spectrophotometrically. The desirable serum cholesterol level is less than 200mg/dl.

Since HDL–cholesterol level is inversely related to the tendency of heart disease, its estimation is useful. Serum levels lower than 45mg/dl in males and 55mg/dl in females indicate increased risk of heart disease. The HDL level may be increased to up to 60mg/dl for males and up to 75mg/dl for females, which indicate the safe margin. The LDL cholesterol level may be maintained to the value less than 150mg/dl and treatment is
required if it exceeds this value. Increase in LDL level indicates increased risk of heart disease. The safe limit of LDL fraction is 60-90mg/dl and increased risk if the range is between 150-200mg/dl.

### 3.9.4. Estimation of Hemoglobin

Estimation of Hemoglobin was conducted by Cyanmethemoglobin Method. In solution ferrous ions (Fe$^{2+}$) of the hemoglobin are oxidized to the ferric state (Fe$^{3+}$) by potassium ferric cyanide to form methemoglobin. In turn, methemoglobin reacts with cyanide ions provided by potassium cyanide to form cyanmethemoglobin, which has an absorbance at 540 nm (Raghuramulu et al., 2003). According to WHO (2005), diagnosis of anemia is confirmed if the hemoglobin value is less than 12g/dl for adult men and women. The normal blood hemoglobin level is between 14-16g/dl for males and 13-15g/dl for females.

### 3.9.5. Estimation of Vitamin A and Beta Carotene

Vitamin A and carotene are extracted into petroleum ether after protein precipitation with ethanol. The intensity of the yellow colour of the carotenes is measured directly. The petroleum ether is evaporated off and the residue is taken up in chloroform. Carr-Price reagent (SbCl$_3$) is added and the amount of blue colour produced is read spectrophotometrically at 460nm and correction applied for the colour due to carotenes to obtain the level of vitamin A present at 520nm (Winsten and Dalal, 1972).

One International Unit (IU) of Vitamin A is defined as having the activity of 0.344 µg of vitamin A acetate. The blood level is greatly influenced by dietary carotenes. The normal range for vitamin A has been reported as 30 to 60 µg/100 ml in the U.S. and 20 to 50 µg/100 ml in England. It may be assumed that there is no deficiency when the serum vitamin A level is above 20 µg/100 ml. Impaired dark-light adaptation is noticed if the serum vitamin A concentration is Below 10µg/100 ml (Winsten and Dalal, 1972).
3.9.6. Estimation of Tocopherol (Vitamin E)

Vitamin E estimation is based on the reduction of ferric ions to ferrous ions by tocopherols after xylene extraction of blood samples. The ferrous ions react with α-α dipyridyl to give a red colour which is measured at 520 nm (Winsten and Dalal, 1972). Normal values for Vitamin E in serum are considered to be 0.8 to 1.2mg%.

3.9.7. Estimation of Ascorbic Acid (Vitamin C)

Ascorbic acid is oxidized to dehydroascorbate by the action of 2,6-dichlorophenolindophenol. The dehydroascorbate is hydrolyzed to diketogulonic acid in the strong acid medium. This forms an osazone with 2-4 dinitrophenyl hydrazine. The osazone rearranges to a stable reddish-brown product which can be measured photometrically. The estimation is based on the methods stated by Winsten and Dalal, (1972). Normal values for human plasma or serum is 0.4 to 1.5 mg/100 ml.

3.9.8. Estimation of Super oxide dismutase

Super oxide dismutase (SOD) activity was determined based on the method prescribed by Stefan Marklund and Gudrun Marklund (1974), where the ability of the enzyme to inhibit the autooxidation of pyrogallol is measured. One unit of SOD is the amount of enzyme that inhibits 50% of the autooxidation of pyrogallol. The activity was measured using a spectrophotometer at 420 nm for 3 minutes.

SOD units were obtained from standard curve using percentage inhibition of the sample. SOD units/ml of whole blood is absolute activity and was converted to SOD units/gram of hemoglobin which is the specific activity. The reference value established by Bogdanska et al., (2003) for superoxide dismutase-absolute value 122.4-333.9±2SD U/ml and for specific value 884.2-2119±2SD U/gHb
3.9.9. *Estimation of Catalase*

Catalase assay was done as explained in the method of Aebi, (1974) by decomposing hydrogen peroxide into water and oxygen and the rate of decomposition of H₂O₂ by catalase was measured spectrophotometrically at 240 nm for 3 minutes. It can be expressed as:

\[
2\text{H}_2\text{O}_2 \overset{\text{Catalase}}{\longrightarrow} 2\text{H}_2\text{O} + \text{O}_2.
\]

Absolute activity was expressed as k-rate constant of first order reaction defined by Aebi, (1974) and k/g hemoglobin is the specific gravity. The reference value established by Bogdanska *et al.*, (2003) for Catalase-absolute value 17.9-41.1 ±2SD k and for specific value 118.8-222.0 ±2SD k/g Hb.

3.10. **STATISTICAL ANALYSIS OF THE DATA**

The data was statistically analysed using Windows based SPSS (Chicago IL Version 17). 95% confidence intervals for proportion were calculated using square root method (Radhakrishna *et al.*, 1992). The following statistical analysis were done to test the reliability and significance of the data, and to derive at meaning full conclusion. Details of the statistical analysis are given in Appendix 7.

- **Mean test** –
  - To Find The Percentage Distribution Of Subjects With Diabetes Mellitus By Their Socio-Economic And Demographic Characteristics And Type Of Diabetes
  - Type Of Diabetics And Sex-Wise Classification of Anthropometric Measurements
- **Standard Deviation** –
  - The Mean Age Of Onset Of Diabetics, The Current Age And The Duration Of Disease By Type Of Diabetes
• **Chi-Square($\chi^2$) test** –
  - Percentage Distribution of Subjects with Diabetes Mellitus by Family History and Test of Significance
  - Percentage Distribution of Subjects with Diabetes Mellitus by Measures Taken to Control Diabetics and Test of Significance
• **Pearson Chi-Square** –
  - Percentage Distribution of Subjects with Diabetes Mellitus by Source of Vigorous Physical Activity with Test of Significance
• **Z test** –
  - Percentage Distribution of Subjects with Diabetes Mellitus by Source of Moderate Physical Activity with Test of Significance
  - Percentage Distribution of Subjects with Diabetes Mellitus by Nature of Mental Strain
• **Weighted Index Numbers** –
  - Group Wise Analysis Of The Most Frequently Consumed Food Stuffs In Basic 5 Food Groups
• **Multiple Regression Analysis** –
  - Mean scores of the patients with diabetics mellitus in total awareness and awareness in the four major areas of management of diabetics and antioxidants were subjected to multiple regression analysis
• **Multiple Regression Analysis Using Backward Selection Method** –
  - First and final regression coefficients of the multiple regression analysis using backward selection method of awareness regarding symptoms and complications of diabetics
• **t-Test** –
  - Awareness Score Of Selected Diabetic Subjects Before And Three Months After Counseling
  - Comparison of Dietary Antioxidant and Mean Nutrient Intake of Diabetic Subjects Before and After Counseling using Weighment Method.
• **Standard Deviation, test of significance, F test and Scheffe** –
• Mean Antioxidant Status and other parameters in the blood of Type I, Type II and Non-diabetic subjects with corresponding standard/reference value and with Standard Deviation, test of significance, F test and Scheffe

• Antioxidant Status and other parameters in the blood of Diabetic subjects Before and After Spirulina Supplementation with corresponding standard/reference value and with Standard Deviation and test of significance.