MATERIALS AND METHODS

Over the past few decades, there has been dramatic changes found in the eating pattern, diets and other moves which expose people to a higher risk of developing Non-Communicable diseases rapidly. The increasing prevalence of diabetes among old age population is adversely affecting the quality of life so it needs to be addressed effectively by using non-pharmacological approaches. The rising awareness of consumers for the potential health benefits of food for disease prevention and wellbeing is enhancing functional food and Nutraceuticals market. Therefore, the present study was planned to assess the glycaemic and Lipemic responses of pumpkin seeds among old age population of urban Vadodara.

This chapter summarizes the experimental plan and discusses the materials and methods used to accomplish the objectives of the study in three phases.

PHASE I: Nutrient profiling of Pumpkin seeds (Cucurbita maxima)

PHASE II (A): Sensory evaluation of Pumpkin seed incorporated recipes

PHASE II (B): Assessment of Glycaemic index and Satiety index of pumpkin seed incorporated recipes

PHASE III: Impact evaluation of pumpkin seeds on type 2 diabetic subjects of Urban Vadodara

PHASE I: Nutrient profiling of Pumpkin seeds (Cucurbita maxima)

3.1.1. Identification of pumpkin seed variety for nutrient profiling

3.1.2. Estimation of the Nutritive value of pumpkin seeds

3.1.3. Antioxidant activity of pumpkin seeds

3.1.4. Fatty acid composition of pumpkin seeds

3.1.5. Phytochemical screening of pumpkin seeds

PHASE II (A): Sensory evaluation of Pumpkin seed incorporated recipes

3.2a.1. Development of Pumpkin seeds incorporated recipes

3.2a.2. Selection of 8 eqi carbohydrate recipes

3.2a.3. Procurement of pumpkin seeds

3.2a.4. Procurement of raw ingredients

3.2a.5. Standardization of pumpkin seed incorporated recipes at different dosage of pumpkin seeds

3.2a.6. Selection of panel members for organoleptic evaluation

3.2a.7. Sensory evaluation of pumpkin seeds incorporated recipes

3.2a.8. Organoleptic evaluation

3.2a.9. Composite Scoring test

3.2a.10 Hedonic rating test

3.2a.11.Statistical analysis

PHASE II (B): Assessment of Glycaemic index and Satiety index of pumpkin seed incorporated recipes

3.2b.1. Selection of subjects for the evaluation of Glycaemic index and Satiety index

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PHASE III: Impact evaluation of pumpkin seeds on type 2 diabetic subjects of Urban Vadodara

3.3.1. Study design

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3.3.4. Selection of the subjects for the intervention (Inclusion & Exclusion criteria)

3.3.5. Protocol of the study

3.3.6. Dosage of the pumpkin seeds

3.3.7. Protocol for the consumption of pumpkin seeds

3.3.8. Distribution protocol of the pumpkin seeds

3.3.9. Trial monitoring plan

3.3.10. Personal and lifestyle information

3.3.11. Anthropometric measurements

3.3.12. Biophysical measurements

3.3.13. Life style history

3.3.14. Diet history

3.3.15. Quality of life related data

3.3.16. Biochemical estimation and assay methods

3.3.17. Statistical analysis

Phase I: Nutrient profiling of Pumpkin seeds (Cucurbita maxima).

3.1.1. Identification of pumpkin seed variety for nutrient profiling

Dried, dehusked Cucurbita Maxima variety of pumpkin seed was selected for the analysis and supplementation. They were then identified and authenticated from The Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara.

Table 3.1.1 Taxonomy of selected variety of pumpkin seeds

Kingdom	Plantae- Plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta- land plant
Superdivision	Embryophyta
Division	Tracheophyta- vascular plants, tracheophytes
Subdivision	Spermatophytia- spermatophytes, seed plants, phanérogames
Class	Magnoliopsida
Superorder	Rosanae
Order	Cucurbitales
Family	Cucurbitaceae- gourds, squasches, citrouilles, gourdes
Genus	Vuvurbita L.- gourd
Species	Cucurbita máxima Duschesce- Winter squash

3.1.2. Estimation of the Nutritive value of pumpkin seeds

Analysis of pumpkin seeds was carried out to know its proximate composition, micronutrient content, phytochemicals present, antioxidant activity and fatty acid composition. Indian Institute of Food Processing Technology – IIFPT, Thanjavur [NABL Accredited Laboratory as per ISO/IEC 17025:2017] & recognized by FSSAI, did analysis. Total 750g of pumpkin seeds was sent for analysis purpose.

Table 3.1.2 Analysis Methods used for various parameters

Parameter Analyzed	Method of Analysis
Protein	Standard AOAC methods
Moisture	
Fat	
Ash	
Carbohydrate	
Crude Fiber	
Energy	
Vitamin C	
Vitamin K	HPLC method
Vitamin D	
Riboflavin	HPLC method (NaOH extraction)
Niacin	
Thiamine	
Sodium	Standard AOAC methods
Potassium	
Calcium	
Zinc	
Iron	
Magnesium	
Selenium	

3.1.3. Antioxidant activity of pumpkin seeds

Introduction

The FRAP assay is quick and simple to perform, and the reaction is reproducible and linearly related to the molar concentration of the antioxidant(s) present. It was originally developed as an assay for serum samples only. However, with its various advantages of ease of use etc.; it is very well adapted to botanical samples also.

Principle

This method is based on the reduction of a Ferric Tripyridal Triazine (TPTZ) complex to its ferrous, coloured form in the presence of antioxidant. The FRAP assay directly measures antioxidant with a reduction potential below the reduction potential of the Fe3+ Fe2+ couple (Halvorsen et al, 2002). The FRAP method uses antioxidants as reductants in a redox-linked colorimetric reaction.

Instruments

Spectrophotometer and water bath

Table 3.1.3 Antioxidant Activity of Raw Pumpkin Seeds

Parameter	Reference
ioxidant Activity by FRAP	Benzie, F.F. and strain, J.J. 1999. Ferric reducing/ Antioxidant Power Assay: Direct Measure of Total Antioxidant Activity of Biological Fluids And Modified Version For Simultaneous Measurement Of Total Antioxidant Power And Ascorbic Acid Concentration. Methods in enzymolology, Vol. 299:15-23.

3.1.4. Fatty acid composition of pumpkin seeds

Analysis of Samples

The fat extracted from the given sample was methylated and analyzed through Gas Chromatography – Mass Spectrometry/ Mass Spectrometry for identification of different compounds.

GC Programme

Oven temperature Programme - 110° C hold for 3.50 min

Up to 200° C at the rate of 10 ° C/min-No hold

Up to 250 ° C at the rate of 5° C / min- 12 min hold Injector temperature 250° C

Total GC running time 35.00 min

MS Programme

Library used NIST Version-2011 Inlet line temperature 250° C Source temperature 230 ° C Electron energy 70 eV

Mass scan (m/z) 50-500 amu Solvent Delay 0 - 3.5 min

Total MS running time 35.00 min

3.1.5. Phytochemical screening of pumpkin seeds

Analysis of Samples

The given sample was extracted with Methanol then analyzed through Gas Chromatography – Mass

Spectrometry/ Mass Spectrometry for identification of different compounds.

GC Programme

Oven temperature Programme -

110° C hold for 3.50 min

Up to 200° C at the rate of 10 ° C/min-No hold

Up to 280 ° C at the rate of 5° C / min- 12 min hold

Injector temperature 280° C

Total GC running time 40.50 min

MS Programme

Library used NIST Version-2011

Inlet line temperature 290° C

Source temperature 250 ° C

Electron energy 70 eV

Mass scan (m/z) 50-500 amu

Solvent Delay 0 - 3.5 min

Total MS running time 40.50 min

PHASE II (A): Sensory evaluation of Pumpkin seed incorporated recipes

Development and standardization of pumpkin seed incorporated 8 eqi carbohydrate Indian traditional recipes and studying their various organoleptic attributes and overall acceptability

3.2a.1. Development of Pumpkin seeds incorporated recipes

This section of the study was carried out to develop various pumpkin seeds incorporated eqi carbohydrates recipes viz. Methi Muthiya, Palak Dhokla, Vegetable cutlet, Thalipith, Roasted poha chevda, Poha, Vegetable pulao and Upma at 2g, 5g, 10g dosage of pumpkin seeds and control recipes were also developed without pumpkin seed incorporation.

3.2a.2. Selection of 8 eqi carbohydrate recipes

Eight eqi-carbohydrates, Indian breakfast recipes were standardized by adding pumpkin seeds with 2gm, 5gm and 10gm. A control recipe was also made to keep it as standard

recipe (without pumpkin seeds incorporation) to evaluate other recipes in comparison to that.

Total eight recipes were developed and standardized and categorized according to the method of preparation.

- Steamed: Methi muthiya and Palak dhokla
- Shallow-fried: Vegetable cutlet and Thalipith
- Roasted: Chevda and Vegetable Poha
- Boiled: Vegetable Upma and Vegetable Pulav

3.2a.3. Procurement of pumpkin seeds

Pumpkin seeds were procured from local market that can supply the required quantity of Vadodara city after confirming from botanist.

3.2a.4. Procurement of raw ingredients

Various ingredients which required to prepare recipes were purchased from local market of the Vadodara city. The list of all raw ingredients along with its purchase source is listed below in the table 3.2a.4.

Table 3.2a.4 List of raw ingredients along with its sources for each recipe

Name of the recipe	Ingredients	Purchase source
Poha	Rice flakes	D-mart
	Onion	Local Market
	Peas	Local Market
	Groundnut Oil	Ankur
	Hing	Ramdev
	Turmeric	Local Market
	Salt	Tata
	Semolina	Uttam Brand
	Onion	Local Market
	Carrot	Local Market

Upma	Green chillies	Local Market
	Udad dal	Local Market
	Groundnut oil	Local Market
	Curry leaves	Local Market
	Hing	Ramdev
	Salt	Tata
Thalipith	Bhajani Mix	Home made
	Spring onion	Local Market
	Carrot	Local Market
	Groundnut Oil	Ankur
	Curd	Amul
	Spices	Local Market
Palak dhokla	Rice	Local Market
	Chana dal	Local Market
	Spinach	Local Market
	Ginger garlic	Local Market
	Spices	Local Market
	Salt	Tata
	Ground nut Oil	Ankur
Methi Muthiya	Wheat flour	Ashirwad
	Besan (gram flour)	Uttam
	Semolina	Uttam
	Methi	Local market
	Green chillies	Local market
	Ginger garlic	Local market
	Salt	Tata
	Ground nut Oil	Ankur
Vegetable Cutlet	Rice flakes	D-mart
	Potato	Local market
	Carrot	Local market
	Beet root	Local market
	Peas	Local market
	Ginger garlic	Local market
	Green chillies	Local market
	Spices	Local market
	Salt	Tata
	Ground nut Oil	Ankur
Roasted Poha Chevda	Rice flakes	Local market
	Peanuts	Local market
	Daliya	Local market
	Curry leaves	Local market
	Spices	Local market
	Salt	Tata
	Ground nut	Ankur

	Oil	
Vegetable pulao	Rice	Daawat Basmati
	Potato	Local market
	Tomatoes	Local market
	Onion	Local market
	French beans	Local market
	Ginger garlic	Local market
	Green chillies	Local market
	Spices	Local market
	Salt	Tata
	Ground nut Oil	Ankur

3.2a.5. Standardization of pumpkin seed incorporated recipes at different dosage of pumpkin seeds

a) Poha: Standard one serving poha was prepared by using 40gm poha, 20gm onion, 10gm peas and 2tsp of oil. Poha was soaked for 10 mins in the water. All the ingredients were sautéed in the oil after adding mustard seeds, cumin seeds, hing, turmeric and salt. It was allowed to cook for 10-15 mins. Addition of pumpkin seed was there at 2gm, 5gm and 10gm dosage.

b) Upma: Standard one serving upma was prepared by using 40gm semolina, 20gm onion, 10gm carrot, 10gm udad dal and 2 tsp of oil. Sauté above all the ingredients in oil after adding mustard seeds, cumin seeds, hing, curry leaves and green chillies and salt. After that hot water was added into sautéed mixture and allowed to cook for 10-15 mins. Addition of pumpkin seed was there at 2gm, 5gm and 10gm dosage.

c) Thalipith: Standard one serving upma was prepared by using 40 Bhajani (Mixure of wheat, rice, Jowar, chana dal and udad dal flour at ratio of 2:1:1:1:1), 20gm spring onion, 10gm carrot and 2 tsp oil for roasting. To prepare dough for Thalipith, above ingredients mixed along with turmeric, chilli powder, 2 tsp of curd and water. After that Thalipith was placed on hot pan by flattening. Roasted from 2 sides by using oil. Addition of pumpkin seed was there at 2gm, 5gm and 10gm dosage.

d) Palak dhokla: for preparing one serving Palak dhokla, 40gm rice and 20g Chana dal were soaked overnight and prepared batter out of it was allowed to ferment for 6-8 hours. Turmeric, ginger-garlic paste, green chillies and 1 tbsp of blanched Palak puree was

added in to that. Dhokla was prepared out of it in the steamer. Addition of pumpkin seed was there at 2gm, 5gm and 10gm dosage.

e) Methi muthiya: for preparing one serving muthiya, 30gm wheat flour, 10gm Besan and 10gm semolina, 2 tbsp chopped methi, ginger garlic paste, green chilli paste, turmeric, sesame seeds were taken and mixed together by adding 100 ml of water. Muthiyas were prepared out of it in the steamer and seasoned in 2 tsp of oil and mustard seeds. Addition of pumpkin seed was there at 2gm, 5gm and 10gm dosage.

f) Vegetable cutlets: for preparing one serving Vegetable cutlet (3 small pcs), 20gm poha (Weight before soaking), 20gm potatoes, 2 tbsp chopped carrot, beet root, peas was used. Turmeric, salt, green chillies, Garam masala, Ginger garlic paste was added to bind cutlets. Crushed powder of pumpkin seed was also added in the cutlets at 2gm, 5gm and 10gm dosage.

g) Roasted Poha Chevda: for preparing one serving of roasted poha chevda, 40 gm nylon poha, 1 tsp peanuts, 1 tsp daliya (roasted chana dal), curry leaves, turmeric, salt and 2 tsp of oil was used. Addition of pumpkin seed was there at 2gm, 5gm and 10gm dosage on the top.

h) Vegetable Pulao: for preparing one serving of vegetable pulao, 30gm rice, 10gm potato, 1 tbsp chopped tomato, onion, peas, French beans were used. 2 tsp oil, turmeric, garam masala, salt, green chillies, ginger garlic paste was also added. Addition of pumpkin seed was there at 2gm, 5gm and 10gm during pulao preparation itself.

Photographs of recipes are given in annexure photo gallery I.

3.2a.6. Sensory evaluation of pumpkin seeds incorporated recipes

The sensory evaluation of pumpkin seeds incorporated recipes were done by adding varying levels of pumpkin seeds in the selected 8 eqi carbohydrate recipes. Overall acceptability was analysed by using appropriate score cards. Selection of the semi trained panel members for the sensory evaluation was done by conducting threshold test.

3.2a.7. Selection of panel members for organoleptic evaluation

In this phase, 20 students from the Department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University were selected as semi trained panel members for sensory evaluation.

3.2a.8. Organoleptic evaluation

Panellist were asked to evaluate and score the products for various sensory attributes like taste, smell, appearance, mouth feel, colour, etc. out of 10. These tests were done to assess the various attributes and acceptability of the pumpkin seed incorporated recipes at varying level.

3.2a.9. Composite scoring test

Composite scoring card was given to semi trained panel members for various attributes of each recipe like colour and appearance, Texture, Taste, after taste and Overall acceptability.

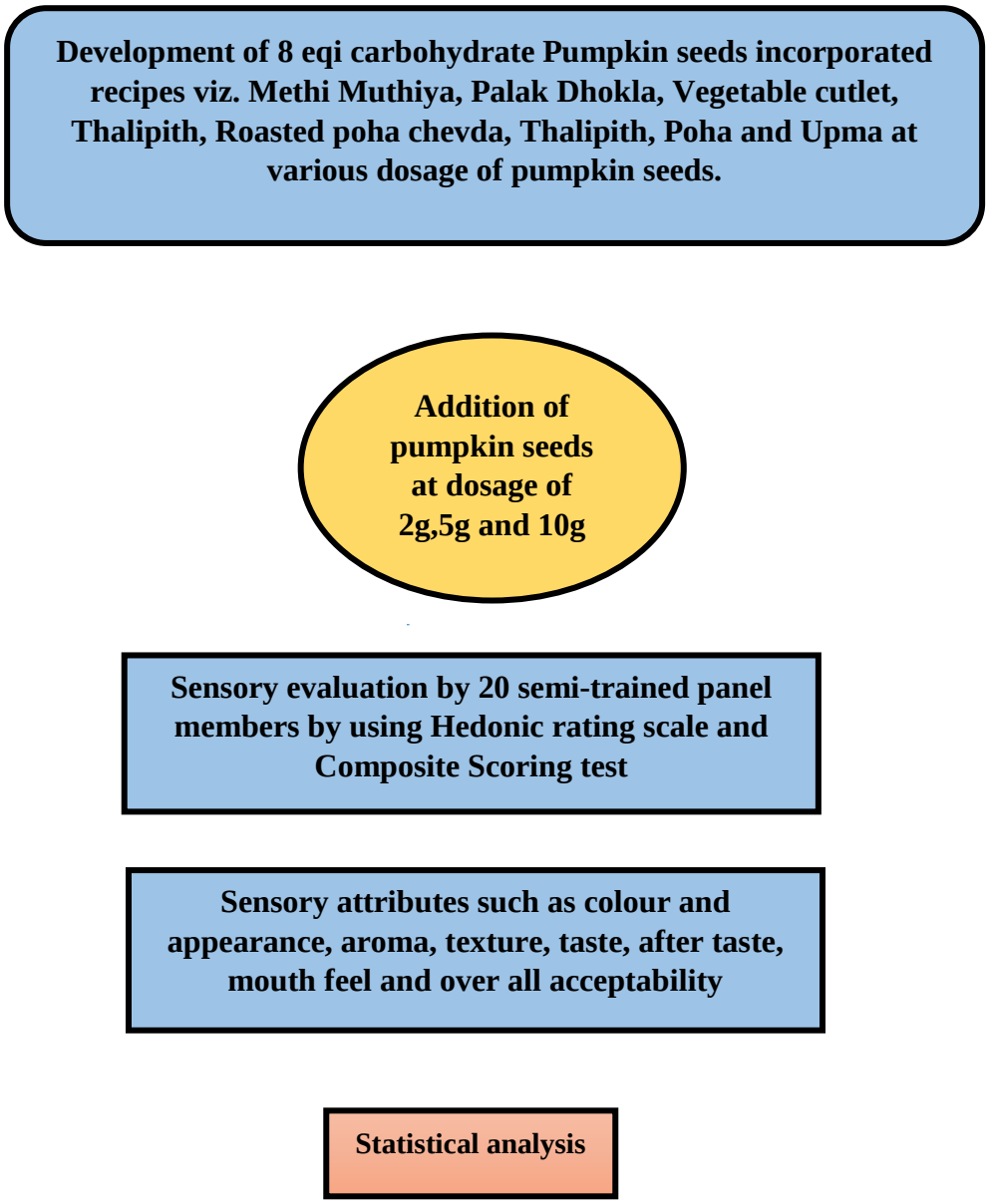
3.2a.10. Hedonic rating scale

For sensory evaluation of pumpkin seed incorporated recipes, products were also rated separately based on 9 point hedonic rating scale ranging from “Like extremely” to “Dislike extremely” with “neither like nor dislike” as a mid score. The resulting scores were compared and analysed further for each sample.

3.2a.11. Statistical analysis

Statistical analysis was performed in the Microsoft excel 2010 by calculating Mean, SD, percentage increase and decrease of all the pumpkin seed incorporated recipes. Mean, F test and Analysis of Variance were also performed to compare the significant differences between standardized recipes.

Figure 3.2a.1 Experimental design for the development of pumpkin seed incorporated recipes



Phase II (B): Assessment of Glycaemic index and Satiety index of pumpkin seed incorporated recipes

3.2b.1. Selection of subjects for the evaluation of Glycaemic index and Satiety index

Subjects were selected from the Department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Vadodara who are healthy and whose age is between 18-30 years. Weight, Height and other anthropometric measurements, initial fasting blood glucose were assessed before the experiment.

The international organization for standardization (ISO) protocol for the estimation of Glycaemic Index of foods (Protocol for GI: ISO 26642:2010) was followed. Eight test recipes (Most acceptable level of incorporation based on sensory evaluation of phase 2a) were selected for the calculation of Glycaemic Index. 10 healthy subjects were enrolled in the study. On first visit subjects were asked to consume 50g glucose and on another visits subjects were asked to consume pumpkin seed incorporated test recipes having approximately 50g of Carbohydrates. Factors such as time of the test, place of the analysis, room temperature were tried to keep constant to prevent variation in the uptake of absorption.

Each study visit included,

- Fasting blood sample taken by lab technician,
- Consumption of specific portion size of test recipes with 10-15 minutes of time and,
- Collection of blood sample by finger prick method at every 15,30,45,60,90 and 120 minutes.

3.2b.2. Assessment of the Glycaemic Index

Figure 3.2b.2 depicts experimental plan for determination of glycaemic index. 8 Pumpkin seed incorporated recipes having 10gm of pumpkin seeds by panellists during sensory

evaluation were tested.

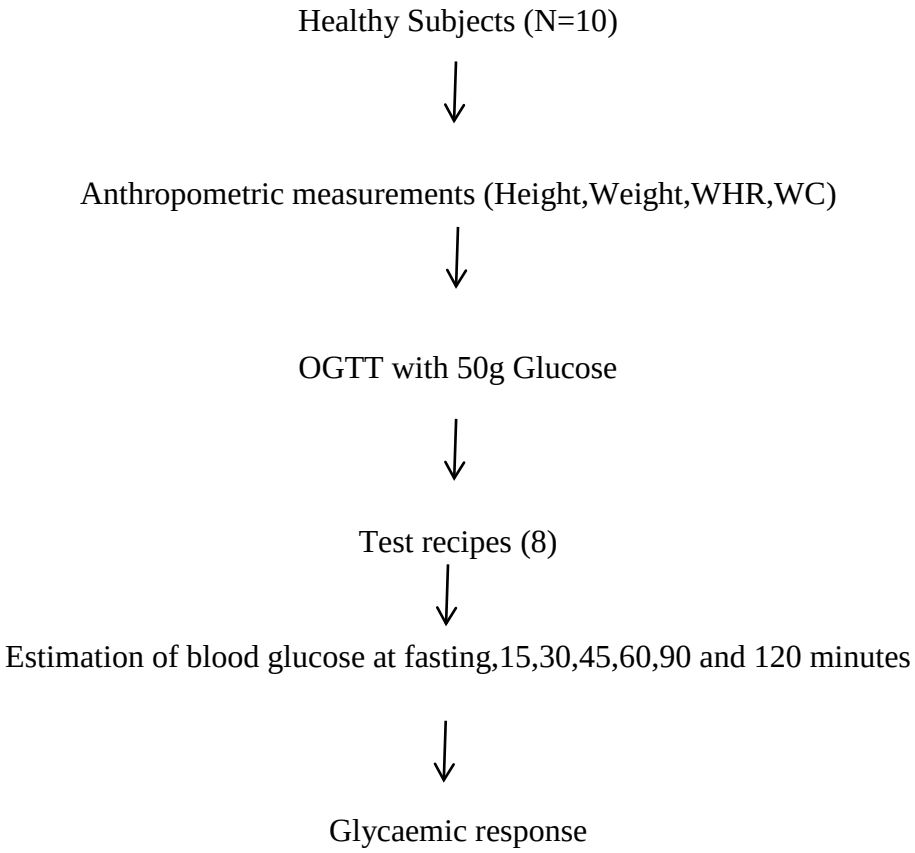
Subjects were studied on separated days in morning after 10-12 hours of overnight fast. After ingestion of food blood glucose estimated at every 15, 30, 45, 60, 90 and 120 minutes initially for test food and then for reference food after 4 days of washout period.

Calculation of GI was done by obtaining glucose values for every point in time over 2 hours with the help of calculation of incremental area under the curve (iAUC) for each recipe. The incremental area under the blood glucose response curve for test and reference food were calculated by using trapezoid rule as per WHO/FAO protocol 1998.

Glycaemic index values were calculated by under curve of test food over reference food by multiplying 100.

GI= (iAUC test food/iAUC reference food) × 100

Figure 3.2b.2 Experimental plan for determining glycaemic index



3.2b.3. Assessment of the Satiety Index

Subjects were asked to evaluate their satiety using a visual analogue scale. Ten healthy subjects were enrolled in the study.

During each 2 hour test session, the subjects were asked to consume the entire portion of food at a comfortable pace, within 10 minutes of time. Subjects were free to drink water during entire test time.

The time taken for eating that specific portion by subjects was recorded. Immediately after consumption, subjects were asked to report their desire for prospective consumption using a Visual Analogue Scale (VAS) developed by Holt (1995). A measurement scale that tries to measure a characteristic; or attitude is believed to range across a continuum

of values.

Prospective consumption was assessed with questions like ‘how much more would you like to eat?’(Nothing at all to a large amount); ‘do you like eating something else?’ (Nothing at all to large amount), etc.

3.2b.4. Statistical analysis

The data obtained was entered and analysed in Microsoft excel 2010 after sorting and cleaning. Mean, standard deviations, percentage and t-tests were used to find out statistical significance

Phase III: Impact evaluation of pumpkin seeds on type 2 diabetic subjects of Urban Vadodara

3.3.1. Study design

A randomized control trial was performed to assess the effect of pumpkin seed supplementation on Glycaemic and Lipemic responses of type 2 diabetic old age (more than 60 years) population of urban Vadodara. In this total subject were divided into 3 groups where group 1 were received 15gm of pumpkin seeds, group 2 were received 10gm of pumpkin seeds on a daily basis for 90 days. Group 3 was the control group. Biochemical and anthropometric data were collected before and after intervention.

3.3.2. Sample size calculation

For the sample size calculation Glycated haemoglobin was taken as the primary outcome. About 1% reduction (15mg/dl) in HbA1C level is anticipated. The standard deviation for HbA1c is taken of old age population of urban Vadodara from past departmental study (Agarwal 2017). Power was set as 80% at a two-sided alpha of 0.05. The sample size of 21 in each phase was computed by using above details. Further on adding 20% attrition, sample size has finally arrived to 23 (Hulley,2007).

ES= 0.91

Thus, 23 subjects were enrolled for each group for the intervention but to perform proper statistical analysis 30 subjects were enrolled in each group.

3.3.3. Ethical clearance and considerations

This study was approved by the Institutional Ethics Committee for Human Research of Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Vadodara (IECHR/FCSc/2020/63). Consent was taken from the participants in the local language.

3.3.4. Selection of the subjects for the intervention (Inclusion & Exclusion criteria)

This study was conducted at the urban Vadodara city of the Gujarat. The Vadodara city was divided into 4 zones. Samples were collected from each zone from clinics, senior citizen associations and societies. To enroll the subjects for the intervention, old age subjects above 60 years were contacted, Willing and eligible 90 subjects (as per the inclusion and exclusion criteria) were enrolled in the study for the supplementation of the pumpkin seeds. Further all the subjects were distributed equally into three groups for intervention. Group 1 received 10g pumpkin seed supplementation, Group 2 received 15g pumpkin seed supplementation and Group 3 (No supplementation) was control group without supplementation.

Data on baseline information, anthropometry, blood pressure, morbidity profile, blood glucose level, physical activity, food intake, quality of life, mental health, diabetes distress, kidney function test, liver function test, lipid profile were collected before intervention and post data was collected on same parameters after supplementation of 90 days.

Before the collection of baseline data and final enrolment of diabetic subjects, they were asked to be in the fasting state overnight and at morning their fasting blood glucose levels were monitored by using glucometer.

Then enrolled subjects were further screened by following inclusion and exclusion

criteria for the supplementation.

Inclusion criteria

- Subjects ≥ 60 years of age of urban Vadodara
- Subjects who were willing to participate
- Subjects whose FBS levels were between 125-250mg/dl.
- Subjects who were on hypoglycaemic drugs or medications.

Exclusion criteria

- Subjects suffering from any chronic illness.
- Subjects above 80 years of age.
- Subjects whose FBS levels were more than 250mg/dl. (further referred to doctors) and whose HbA1c was more than 9%.
- Subjects consuming other functional foods to control diabetes.

3.3.5. Protocol of the study

After enrolment of the subjects according to above criteria, they were explained about the protocol of the study, study design and possible benefits of the pumpkin seeds consumption. After taking the consent, subjects were randomly distributed into three groups, as experimental group 1 and 2 (2 different dosage) and control group for intervention of 90 days.

Subjects were also informed that data (baseline information, anthropometry, blood pressure, morbidity profile, latest blood glucose level, physical activity, food intake, quality of life, diabetes distress, MMSE score , kidney function test, liver function test, lipid profile) would be collected twice, at the beginning and at the end of the study.

Details were provided regarding the financial standings, biochemical tests to be included in the study, safety standards to be followed while withdrawing the blood, provision of availability of results, confidentiality and right to withdraw from the study at any point of time.

For the baseline data collection, anthropometry data and biochemical data, convenient time was set for the old age population who has given consent for the study. Subjects were asked to be in the 10-12 hours of fasting state for the fasting blood sample. Blood sample was taken by the trained lab technician by using disposable blood syringes. Samples were labelled carefully and sent to the accredited lab for the analysis.

All other data was collected by the researcher by using appropriate tools. Data regarding diet was also collected by using 24-hour diet recall and food frequency checklist

3.3.6. Dosage of the pumpkin seeds

Subjects enrolled in Experimental group 1 were asked to consume 10gm of pumpkin seeds on a daily basis for 90 days whereas in experimental group 2 subjects were asked to consume 15gm of pumpkin seeds on a daily basis for 90 days. Control group was not given any type of treatment.

3.3.7. Protocol for the consumption of pumpkin seeds

Both experimental groups were asked to consume pumpkin seeds at any time of the day in the raw form. If any subject was found to be difficult to consume full dosage of seeds to consume at a time, were allowed to divide in 2-3 dosage in a day. Subjects were also instructed to chew the seeds properly.

3.3.8. Distribution protocol of the pumpkin seeds

Sachet of 10gm and 15gm were prepared manually, seeds were packed in air tight small zip lock bag. Dosage was distributed to the subjects at the start of every month. Empty sachets were collected back before distributing new samples.

3.3.9. Trial monitoring plan

Sachets of pumpkin seeds were provided on a monthly basis and total 30/31 sachets were distributed to the subjects per week either 10gm or 15gm according to the respective group. Empty sachets were collected back before distributing new samples. WhatsApp/SMS reminders were sent to ensure pumpkin seed consumption. A compliance card was prepared for each subject in which subject was asked to mark "√" in the card after consuming daily dose. Investigator also checked the compliance at every week before giving new samples. After 3 months, all ticks (√) were counted and compliance was calculated.

Figure 3.3.1 Compliance card sample in English and Gujarati

Compliance Card						
Name: _____						
Month: _____						
Subject Code: _____						
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				
*** Please make √ on each day when you consume the given sachet of Pumpkin Seeds.**						
*** Please return the empty / unconsumed sachet.**						

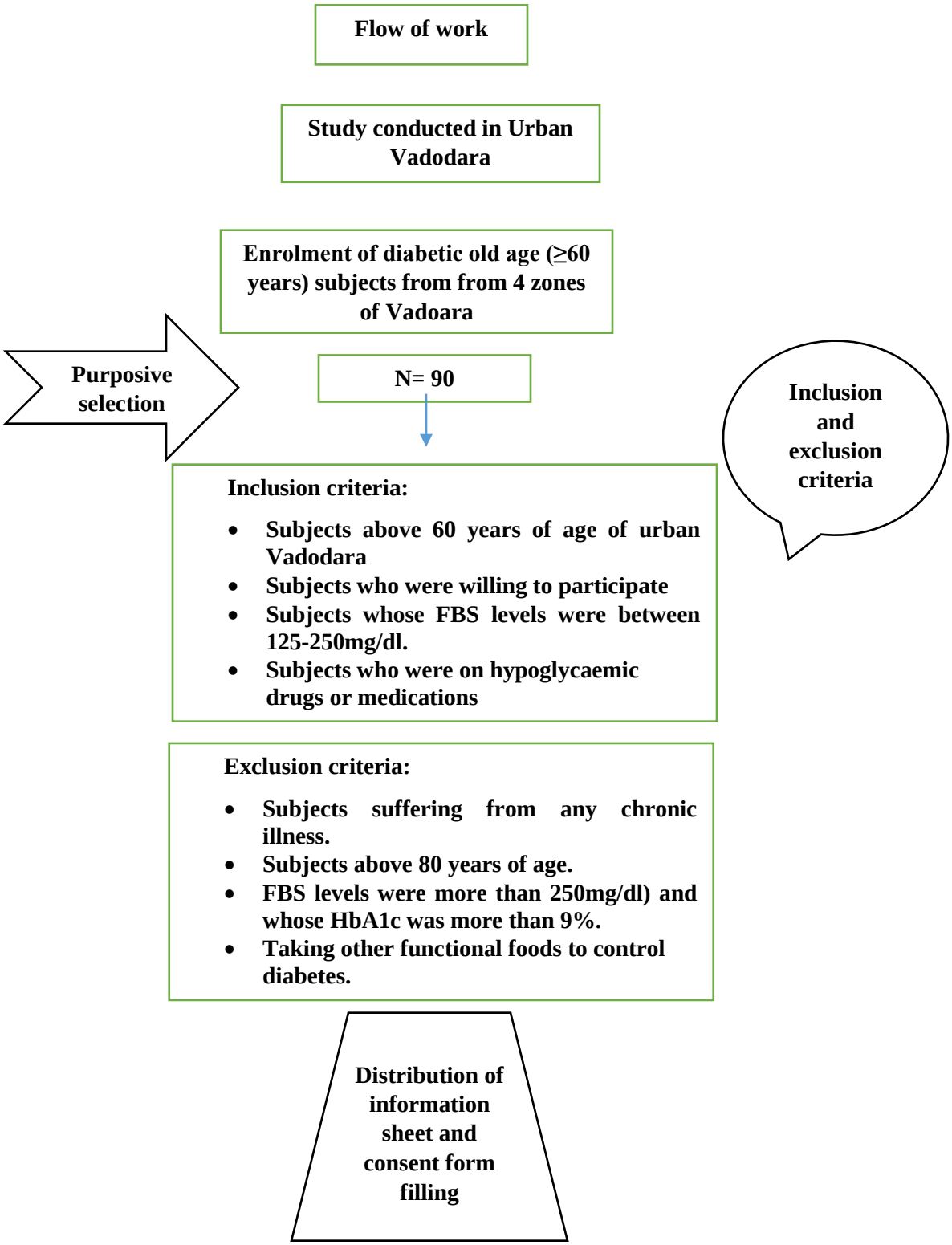
અનુપાલન કાર્ડ						
નામ : _____						
મહિનો : _____						
સહભાગી કોડ : _____						
૧	૨	૩	૪	૫	૬	૭
૮	૯	૧૦	૧૧	૧૨	૧૩	૧૪
૧૫	૧૬	૧૭	૧૮	૧૯	૨૦	૨૧
૨૨	૨૩	૨૪	૨૫	૨૬	૨૭	૨૮
૨૯	૩૦	૩૧				
* કૃપા કરીને દરરોજ કોળા નાં બીજ નું સેવન કરી √ કરવું .						
* મહેરબાની કરી ખાલી / બીનઉપયોગી કોથળી પરત કરવી						

3.3.10. Statistical analysis

The data was entered in the Microsoft excel Spreadsheet 2010. The data was sorted and cleaned before analysis. Data analysis was done by using Microsoft excel 2010 and SPSS (Version). Mean, Standard deviation, percentage difference, correlation was calculated according to data. Student pair t test was calculated to check the significant difference between before and after intervention, among various groups and among experimental and control group.

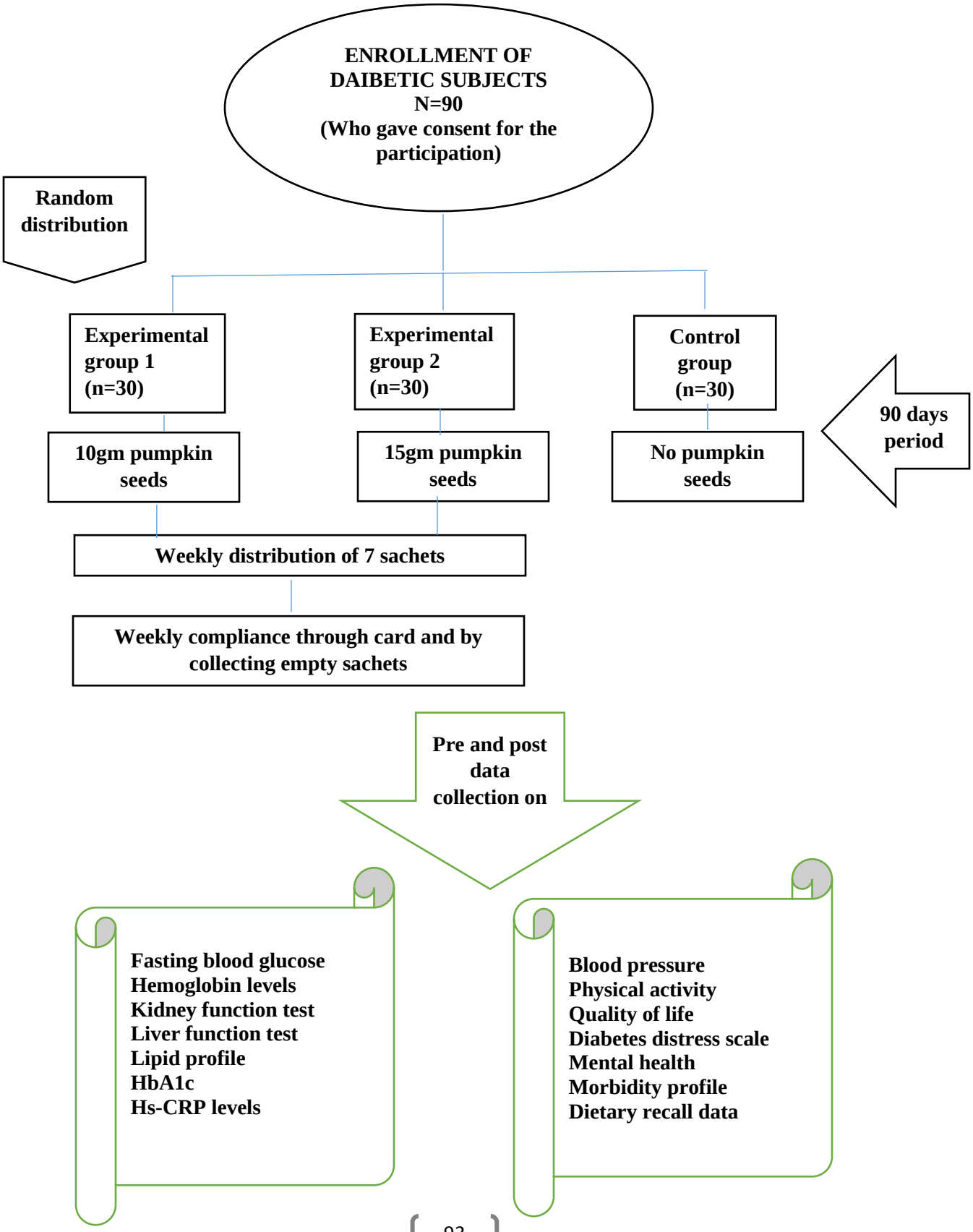
Figure 3.3.2 Experimental design:

Phase III (a)



Experimental design:

Phase III (b)



Methods and tools used for data collection

Parameters	Techniques
Personal and lifestyle information	
Baseline information, Medical history, family history, life style variables, health practices	Pre tested questionnaire
Diet history	
24 hours dietary recall	Semi structured questionnaire
Food frequency method	Semi structured questionnaire
Life style history	
Physical Activity	International Physical activity Questionnaire (Short), 2005
Quality of life	WHOQOL, Diabetes distress scale
Mental health	MMSE Score
Morbidity profile	Semi structured questionnaire
Bio physical measurements	
Blood Pressure	Mercury sphygmomanometer
Anthropometric measurements	
Weight	Digital weighing balance
Height	Fibre glass tap
BMI	BMI calculation, Asia Pacific Classification
Blood glucose levels	
Fasting Blood Sugar	Enzymatic kit method
HbA1C (Glycated hemoglobin)	High Pressure Liquid Chromatography
CBC	Differential Analyzer
Lipid profile	
TC, HDL-C, TG	Enzymatic kit method
LDL-C	Derived from TC and TG
Renal function test	
Creatinine, BUN and GFR	Photometry
Liver function test	
SGPT,SGOT	Photometry
Inflammatory Markers	
CRP (C Reactive Protein)	Nephelometry

3.3.11. Personal and lifestyle information

Baseline information and family history

Information regarding name, age, address, gender, religion, education, occupation, marital status, medical history, family history for non-communicable diseases was collected.

Diabetes history

Information regarding duration, causes and symptoms of diabetes was collected. Information about Present treatment for diabetes, other medicines, nutrient and diet supplement and its frequency was also taken care of.

Life style information

Information regarding habit of tea, coffee, gutkha, pan, cigarette, tobacco and alcohol was also collected along with its frequency.

3.3.12. Anthropometric measurements

Anthropometric measurements are a series of quantitative measurements of the bone, muscles, adipose tissues used to assess mainly the composition of the body. It is an essential feature for the evaluation of nutritional status among geriatric population for the determination of malnutrition, muscular mass loss, fat mass gain and adipose tissue redistribution. The anthropometric measurements were conducted on the basis of the standard reference guidelines by Lohman et al 1988.

Height

It is a linear measurement made up of the sum of four components, i.e. Legs, Pelvis, Spine and Skull (Jellife 1966). A Non-stretchable glass tap was used to measure the height of the old age subjects. A convenient flat wall was identified at the selected

diabetes clinics. Subjects were asked to stand straight after removing foot wears. The scapula and the buttock were ensured to be in contact with the measuring wall. The head was placed in the Frakfort plane (with the tragus of the ear and the lateral angle of the eye in a horizontal line) for the accurate measurement. In this position, a mark was made on the wall and height was recorded to the nearest 0.1cm. two consecutive measurement was taken to ensure accuracy.

Weight

It is the most widely used and simplest reproducible anthropometric measurement. Weight indicates the body mass and is a composite of all body constituents like water, minerals, fat, protein, bone, etc. (Robinson et al 1998). A digital weighing scale was used to measure the weight of the old age subjects. The subjects were asked to wear simple indoor clothing, no jewellery and any stuff in the pockets with bare feet. Scale was set at "Zero" before taking weight of the subjects. It was calibrated after weight of every third subject by using standard weights to maintain accuracy.

Computation of anthropometric indices

Body Mass Index (BMI)

It is a convenient and valid measure of adiposity. The formula to calculate BMI is

BMI (Kg/m^2) =Weight(kg)/Height (m^2)

Table 3.3.12 Cut-offs for Body Mass Index (BMI)

Category	BMI
Underweight	<18.5
Normal	18.5-22.9
Over weight	23-24.9
Grade I obesity	25-29.9
Grade II obesity	30-34.9
Grade III obesity	≥35

Asia Pacific Classification 2004

3.3.13. Biophysical measurements

Blood pressure

It is the pressure of blood in circulatory system which is related to force and rate of heartbeats and the diameter of the arterial walls. The blood pressure of the subjects was measured in the relaxed sitting position by using mercury sphygmomanometer and stethoscope on the left arm.

The blood pressure was taken when subjects were sited quietly for at least 5 minutes. Blood pressure cuff was proper in size which was equal to 80% of the circumference of the upper arm. Then cuff was wrapped around the upper arm surface area with the cuff's lower edge one inch above the antecubital fossa. Stethoscopes 'bell was placed over the brachial artery just below the cuff's edge.

Cuff was rapidly inflated to 180mmHg. Air was released from cuffof at a moderate rate (3mm/sec). The first knocking sound (Korotkoff) was the subject's systolic pressure. When the knocking sound disappears, that was the diastolic pressure. We have taken measurements twice with the interval of few minutes if blood pressure was recorded abnormally high or low. The recommendations of the Joint National Committee VIII (JNC8) were adopted for screening of the blood pressure.

Table 3.3.13 Classification of blood pressure for adults

Classification of blood pressure	SBP (mmHg)	DBP (mmHg)
Normal	<120	<80
Pre hypertension	120-139	80-89
Stage 1 hypertension	140-159	90-99
Stage 2 hypertension	≥160	≥100

Joint National Committee VIII (JNC8), 2014

Pulse rate

Pulse rate is also known as heart rate. It is the number of times heart beats in each minute. Pulse rate is an important indicator of overall health. A normal resting heart rate

for adults ranges from 60 to 100 beats per minute. Generally, a lower heart rate at rest implies more efficient heart function and better cardiovascular fitness. For example, a well-trained athlete might have a normal resting heart rate closer to 40 beats per minute.

To measure heart rate, pulse oximeter was used. It was an easy, painless measure of how well oxygen was being sent to parts of your body furthest from your heart, such as the arms and legs and to know pulse rate. It was a clip-like device called a probe placed on finger of the subject. After 5 minutes of placing, measurement was noted. The reading takes time to steady, so we need to Keep the oximeter in place for at least a minute or longer if the reading is not stable.

3.3.14. Life style history

Physical activity

Global Physical Activity Questionnaire (GPAQ) developed by World Health Organization in 2002 as part of the WHO STEP wise approach to chronic disease risk factor surveillance for PA observation. We used this tool to collect information on physical activity pattern as well as sedentary behaviour among old age population of urban Vadodara. GPAQ covers various aspects such as from vigorous to moderate activity at work and during leisure time, transport activity and sitting. It is a checklist contains questions on various activities along with time spent on it to assess the activity pattern.

Metabolic equivalents (METs) are commonly used to express intensity of physical activity. In GPAQ existing guidelines are there for the analysis of physical activity. METs are different according to the level of physical activity from sedentary to vigorous-high intensity activity.

MET values used for the analysis of GPAQ data were:

Walking = 3.3 METs

Moderate PA = 4.0 METs

Vigorous PA = 8.0 METs

Total MET values for a week were calculated using the below mentioned formulas

Walking MET-minutes/week = 3.3 × walking minutes × walking days

Moderate MET-minutes/week = 4.0 × moderate-intensity activity minutes × moderate days

Vigorous MET-minutes/week = 8.0 × vigorous-intensity activity minutes ×vigorous-intensity days

So therefore,

Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous MET minutes/week scores.

3.3.15. Diet history

Information regarding type of diet, number of meal consumption per day, water intake, type of oil, amount of consumption for oil, sugar and salt per month, type of reuse of deep fried oil, frequency of eating out, frequency of consumption of various types of foods was obtained.

24 hours dietary recall method

The 24-hour dietary recall method is a quantitative method for the assessment of total dietary intake per day. In this method, subjects were asked to keep the record of food items consumed by him/her of entire day for consecutive 3 days (avoid Sundays and festival holidays). Standard cups and spoons were used to record the exact quantity of food. Reported food intake was converted into raw ingredients with its amounts. After that nutritive value was calculated by using Diet Cal software (Developed by dietician Gurdeep Kaur, AIMS) to know the nutrient intake consumed by specific person per day.

Food frequency method

A semi structured food frequency checklist was prepared to know the consumption pattern of Fruits, Vegetables, milk and milk products, egg, Meat, Fish, poultry, fried foods, sweet items and foods which are high in carbohydrates among old age population. The frequency of consumption was obtained as per daily, weekly, monthly or occasionally basis. Amount was also reported along with frequency to get idea about the actual amount of consumption. Further data was separated according to its frequency of consumption.

3.3.16. Quality of life related data

WHO Quality of Life

WHOQOL-BREF (WHO Quality of Life) was developed by World Health Organization in 2004 which includes the measures of the impact of disease and its impairment on daily life, activities and behaviour. WHOQOL focuses upon respondents' "perceived" quality of life, it is not expected to provide a means of measuring in any detailed fashion symptoms, diseases or conditions, nor disability as objectively judged, but rather the perceived effects of disease and health interventions on the individual's quality of life. The WHOQOL is, therefore, an assessment of a multi-dimensional concept incorporating the individual's perception of health status, psycho-social status and other aspects of life (WHO,2012).

Quality of life (QoL) in diabetic patients is the primary goal of care. Today, there is an increasing awareness suggesting that patient's QoL and treatment satisfaction were improved after good glycemic control. Today, attention towards patient's QoL is increasing rather than patient's longevity. Thus, quality of life of diabetic patients should be maintained because it can aggravate metabolic disorders. There is an increasing awareness suggesting that patient's QoLand treatment satisfaction were improved following good glycemic control (Amelia et al, 2018).

The information regarding quality of life of the diabetic subjects was collected using the

WHO QoL BREF questionnaire which was developed in an attempt to know the perception of people regarding their quality of life. The WHO-QoL BREF was developed from WHO-QoL 100. The data was analyzed with the calculations as guided by the WHO-QoL 100 scoring guide.

Due to lack of a universally agreed upon definition of quality of life, the first step in the development of the WHO-QoL was to define the concept. According to WHO, quality of life is defined as "Individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns". The quality of life was assessed based on WHOQOL-BREF questionnaires consists of 26 items. Two items are related to the overall QOL and general health, and the remaining 24 items are related to four domains of physical health (seven items), psychological health (six items), social relationship (three items) and environmental health (eight items). Each item is scored on a four-point Likert scale ranging from one (strongly agree) to five (strongly disagree) with the highest scores representing better QOL.

The data was collected using interview technique and all the diabetic subjects, were asked to answer keeping in mind about their last years from onset of diabetes

Diabetes Distress

Diabetes distress (DD) refers to the unique, often hidden emotional burdens and worries that are part of the spectrum of patient experience when managing a severe, demanding chronic disease like diabetes . High levels of DD are common (prevalence, 18–35%; 18-month incidence, 38–48%) and persistent over time, and they are distinct from clinical depression in their linkages with glycemic control and disease management. High levels of DD have been significantly associated with poor glycemic control, poor self-care, low diabetes self-efficacy, and poor quality-of-life, even after controlling for clinical depression.

The depression status of the type 2 diabetes mellitus subjects was assessed using Diabetes distress scale. It's a scale of 17 questions and yields a total diabetes distress scale score

plus 4 sub scores, i.e Emotional burden, Physician related distress, Regimen related stress, Interpersonal distress.

Mean item score less than 2 is considered as little or no distress, score between 2-2.9 is considered as moderate distress and scores more than 3 is considered as high distress

Mini mental state exam (MMSE)

The mini mental state exam (MMSE) was used to test the cognitive function among elderly which includes tests of orientation, attention, memory, language and visual spatial skills. It was developed by Folstein et al (1975) was one of the tests used for cognitive assessment of the MCI condition. The maximum score obtained is 30. Categorization of respondents was subjected to the following cut-offs:

Table 3.3.16 Cut-offs for MMSE Score

Criteria	MMSE Score
Normal cognitive function	27-30
Mild cognitive impairment	21-26
Moderate cognitive impairment	11-20
Severe cognitive impairment	0-10

3.3.17. Biochemical estimation and assay methods

The enrolled subjects were asked for overnight fasting before the collection of blood sample. Samples were collected by using disposable syringes with the help of experienced lab technician. Samples were kept in vacutainers having the respective EDTA, clot activator or gels for the serum formation. Such tubes may contain some additional substances that helps to preserve blood before its processing. Samples in the vacutainers were stored at -80◦C after the separation of serum until analysed. The blood

was then analysed for various biochemical parameters such as fasting blood sugar, Glycated haemoglobin, lipid profile, kidney function test, liver function tests for two times (Before and after intervention), CRP test by using standardised kits.

Blood glucose levels

Fasting blood sugar

FBS was estimated using the accepted enzymatic reference method with hexokinase (Kunst et al 1984). Hexokinase (HK) catalyses the phosphorylation of glucose in the presence of adenosine-5'-triphosphate (ATP) and magnesium to form glucose-6phosphate (G-6-P) and adenosine diphosphate (ADP). G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of nicotinamide adenine dinucleotide (NAD) to produce 6-phosphogluconate and NADH. One mole of NAD is reduced to one mole of NADH for each mole of glucose present. The absorbance due to NADH (and thus the glucose concentration) is determined using a biochromatic (340 and 383 nm) endpoint technique.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \xrightarrow[\text{MG}^{++}]{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADH} + \text{H}^+$$

Glycated haemoglobin (HbA1c)

HbA1c was quantified and assayed using IFFC and FDA approved automated dedicated high-performance liquid chromatography (HPLC) technique (Geistanger 2008). The principle involved ion exchange of HPLC. The samples are automatically diluted on the d-10, injected into the analytical flow path, and applied to the analytical cartridge. The d-10 delivered a programmed buffer gradient of increasing ionic strength to the cartridge,

where the haemoglobin is separated based on their ionic interactions, then pass through the flow cell of the filter photometer, where the change in the absorbance at 415 nm are measured. The d-10 software performs reduction of raw data collected from each analysis; two level calibrations are used for quantization of HbA1c values. A sample report and a chromatograph are generated for each sample.

The A1c area is calculated using an Exponentially Modified Gaussian (EMG) algorithm that excludes the labile A1c and carbamylated peak areas from the A1c peak area. HbA1c covers all fractions; this includes labile HbA1a, HbA1b and HbA1c. The former two fractions are liable and hence do not represent the stable or long term change. HbA1c represents the true long term glycaemic control.

Table 3.3.17.1 Cut-offs for HbA1C

Classification	HbA_{1C}
Good	≤6
Borderline	7-8
Poor	>8

American Diabetes Association 2007 Standards

Lipid Profile test

Total Cholesterol (TC)

Total cholesterol was estimated using end point enzymatic colorimetric technique. Cholesterol esterase (CE) catalyses the hydrolysis of cholesterol esters to produce free cholesterol which, along with preexisting free cholesterol, is oxidized in a reaction catalysed by cholesterol oxidase (CO) to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidise (HPO), the hydrogen peroxide thus formed is used to oxidize N, N diethylaniline-HCl/4-aminoantipyrine (DEAHCl/AAP) to produce a chromophore that absorbs at 540 nm. The absorbance due to oxidized DEA-HCl/AAP is directly proportional to the total cholesterol concentration and is measured using a polychromatic (452,540, 700nm) endpoint technique.

$$\text{Cholesterol esters} \xrightarrow{\text{CE}} \text{Cholesterol + Fatty acids}$$

CO

Cholesterol + O2 ⟶ cholest-4-ene-3-one + H2O2

HPO

2H2O2+DEA.HCI/AAP ⟶ 4H2O+Oxidised DEA.HCI/AAP

Table 3.3.17.2 Cut-offs for Serum Cholesterol

Classification	Cholesterol Value (mg/dl)
Desirable	<200
Borderline high	200-239
High	≥240

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2013

Triglycerides (TG)

Enzymatic colorimetric method (GPO/PAP) with glycerol phosphate oxidase and 4aminophenazone was used to assess triglycerides. Triglycerides are hydrolysed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol- 3- phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H2O2). In the presence of peroxidise (POD), hydrogen peroxide affects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form red coloured quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample (Fossati and Prencipe, 1982).

LPL

Triglycerides ⟶ glycerol + fatty acids

GK

Glycerol +ATP⟶ Glycerol-3-phosphate+ ADP

GPO

Glycerol-3-phosphate+ O2 ⟶ dihydroxyacetone phosphate + H2O2

POD

2 H2O2 + 4- aminophenazone + 4-chlorophenol ⟶ quinoneimine + 4 H2O

Table 3.3.17.3 Cut-offs for Serum triglycerides

Classification	Triglycerides Value (mg/dl)
Desirable	<150
Borderline high	150-199
High	200-499
Very high	≥500

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2013

HDL Cholesterol (HDL-c)

HDL fraction of cholesterol was determined using enzymatic, colorimetric method (CHOD/PAP) without sample pre-treatment. The principle of HDL Cholesterol direct is based on the absorption of synthetic polyanions to the surface of lipoproteins. LDL, VLDL, chylomicrons are thereby transformed into a detergent resistant form, whereas HDL is not. Combined action of polyanions and detergent solubilises cholesterol from HDL, but not from LDL, VLDL, chylomicrons. Solubilized cholesterol is oxidized by the sequential enzymatic action of cholesterol esterase (CE) and cholesterol oxidase (CHOD). The hydrogen peroxide formed reacts with N, N-bis (4-sulfonyl)-m-toluidine (DSBmT) and 4-aminoantipyrine (AAP) in the presence of peroxidise (POD) and forms a red quinoneimine dye. The colour intensity of the red quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 552nm (Sugiuchi et al 1995).

LDL, VLDL, chylomicron ns +polyanions ⟶ lipoprotein-polyanion

HDL + detergent ⟶ micelle complexes

CE/CHOD

Micelle complexes ⟶ oxidised cholesterol + H2O2

POD

H2O2 + 4-aminoantipyrine + DSBmT ⟶ quinoneimine dye

Table 3.3.17.4 Cut-offs for HDL Cholesterol

Classification	HDL Cholesterol Value for male (mg/dl)	HDL Cholesterol Value for female (mg/dl)
Low	<45	<35
Optimal	45-55	35-45
High	>55	>45

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2013

LDL Cholesterol (LDL-c)

Enzymatic colorimetric method (CHOD/POD) was used for the direct estimation of LDL, HDL, VLDL and chylomicrons are specifically hydrolysed by a detergent. The released cholesterol content in these lipoproteins reacts immediately in the enzymatic action of cholesterol esterase (CE) and cholesterol oxidase (CHOD) generating hydrogen peroxide. The latter is consumed by a peroxidase (POD) in the presence of 4-aminoantipyrine to generate a colourless product. During this first step, LDL particles remain intact. The reaction of LDL cholesterol is initiated by the addition of another detergent together with a coupler, N, N-bis (4-sulfonyl)-m-toluidine (DSBmT). The second detergent releases cholesterol in the LDL particles which are subjected to the enzymatic reaction in the

presence of a coupler to produce a coloured product. The colour intensity of the red quinoneimine dye formed is directly proportional to the LDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 520nm (Sugiuchi et al 1995).

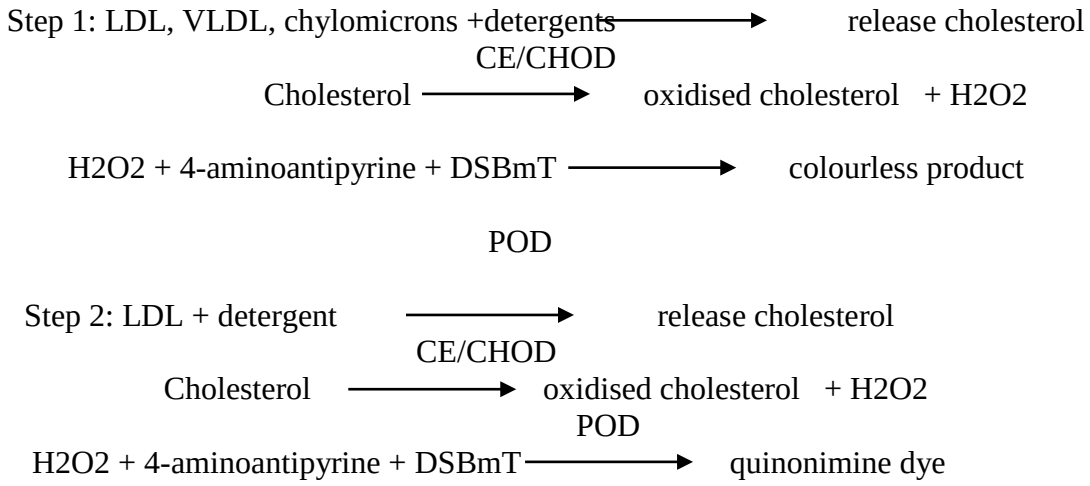


Table 3.3.17.5 Cut-offs for LDL-Cholesterol

Classification	LDL Cholesterol Value (mg/dl)
Low	<100
Optimal	100-129
Borderline High	130-159
High	160-189

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2013

Kidney and liver function tests

Blood urea

Blood urea in the test serum was detected by standard enzymatic method (UV KINETIC/GLDH). The ready-to-use urea agent is added in test tubes kept in a series forming ammonium and carbonate. α-Ketoglutarate and ammonium together with Nicotinamide adenine dinucleotide (NADH) gave glutamate and coenzyme NAD by catalysing with glutamate dehydrogenase (GLDH). The results were expressed in mg/dL

(Fawcett and Scott 1960).

$$\text{Urea} + 2H2O \xrightarrow{\text{Urease}} 2NH_4^{-} + CO_3^{-2}$$

$$\alpha\text{-Ketoglutarate} + NH_4^{--} + \text{NADH} \longrightarrow \text{L- Glutamate} + NAD^{+} + H2O$$

Table 3.3.17.6 Cut-offs for Blood Urea (60-90 years)

Classification	Blood urea value (mg/dl)
Normal	15-40
High	>40

Rose 1933

Serum creatinine

The creatinine in test serum was estimated by standard enzymatic (Picric acid) method. The creatinine in alkaline solution reacts with picrate to form a red-orange compound. Under the specific conditions of the assay, the rate of development of the color is proportional to the concentration of creatinine in the sample when measured at 500nm (Henry et al 1974).

$$\text{Creatinine} + \text{Picrate} \xrightarrow{\text{Alkaline medium}} \text{Orange colour}$$

Table 3.3.17.7 Cut-offs for serum creatinine (60-90 years)

Classification	Creatinine value (mg/dl)
Normal	0.8-1.2
High	>1.2

Liver function tests

Serum glutamic-oxaloacetic transaminase (SGOT) or Aspartate amino (AST)

The Aspartate amino transferase in the test serum was tested by standard enzymatic method (Siemens kit). The L-aspartate was added to α-ketoglutarate resulting in oxaloacetate and glutamate in presence of Aspartate Aminotransferase (AST). The

reaction of oxaloacetate, NADH and hydrogen ion with catalyst Malate Dehydrogenase (MDH) yielded malate and coenzyme NAD. The result values were expressed in IU/L (IFFC, 1986a).

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H} \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}$$

Table 3.3.17.8 Cut offs for SGOT (AST) (60-90years)

Classification	SGOT values for Males (U/L)	SGOT values for Females (U/L)
Normal	19-48	9-36
High	>48	>36

Serum Glutamic-Pyruvic Transaminase (SGPT) or Alanine transaminase (ALT)

The SGPT estimation was determined by standard enzymatic method (Siemens kit). The L-aspartate was added to α-ketoglutarate resulting in oxaloacetate and glutamate in presence of Alanine Aminotransferase (ALT). The reaction of oxaloacetate, Nicotinamide adenine dinucleotide (NADH) and hydrogen ion with catalyst Lactate Dehydrogenase (LDH) yielded malate and coenzyme NAD. Test values were expressed in IU/L (IFCC, 1986 b).

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^{+}$$

Table 3.3.17.9 Cut offs for SGPT (ALT) (60-90years)

Classification	SGPT Value (U/L)
Normal	30-65
High	>465

Inflammatory Markers

C Reactive Protein

In this method a soluble analyte and corresponding antibodies that are bound to polysterene particles are made to react. The test specimen is mixed with latex particles coated with monoclonal antibodies (anti-CRP antibodies), so the CRP present in the specimen will bind with the latex bound antibodies. This method quantifies C-reactive protein (CRP) by latex-enhanced nephelometry. Particleenhanced assays are based on the reaction between a soluble analyte and the corresponding antigen or antibody bound to polystyrene particles. For the quantification of CRP, particles consisting of a polystyrene core and a hydrophilic shell are used in order to link anti-CRP antibodies covalently. A dilute solution of test sample is mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP present in the test sample will form an antigen-antibody complex with the latex particles.

Light scattering, measured by a nephelometric procedure after 6 min, is proportional to the concentration of the analyte present in the sample. An automatic blank subtraction is performed. CRP concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve (NHANES 2007–2008). Reference range for HsCRP provided by AHA/CDC (Pearson et al, 2003) was used for categorization of risk associated with HsCRP levels

Table 3.3.17.10 Cut offs for CRP levels

Classification	CRP level (mg/L)
Normal	≤3
High	>3

3.3.18. Data Monitoring and Management

To ensure the quality of the research, data collection, monitoring and handling was done

using checkpoints as described in table 3.3.18.

Table 3.3.18. Checkpoints for Data Monitoring and Management

Data collection	Through pretested questionnaires and appropriate tools
Blood parameters	By Trained Technician of accredited laboratory/ secondary data if available
Supplementation	Delivered at Every month
Compliance	Reminder through WhatsApp/SMS and by collecting Compliance card
Data storage	MS excel, 2016
Data analysis	Using Microsoft excel by applying appropriate techniques