CHAPTER-5

MATERIALS AND METHOD

The present investigation entitled “Effect of Sunflower seeds on Hypercholesterolemia, Fatty liver and fasting blood glucose in Diabetes Mellitus type 2 patients” was conducted under the Department of Food Technology and Nutrition, School of Agriculture, Lovely Professional University, Phagwara, Punjab. The material used, detail of the experiments and techniques employed in the investigation have been furnished in this chapter under the following headings.

5.1 Analysis of tocopherol and phenolic content of sunflower seeds

5.1.1 Raw Material

The sunflower seeds’ samples were procured from the local market at Amritsar, Punjab, India. Seed weight varied from 120–200 mg. All seed were sealed up in polythene bags and stored in airtight container until needed.

5.1.2 Sample Preparation

The sunflower seeds were cleaned manually in order to abolish damaged, broken or cracked grains along with the foreign materials if any. The cleaned seeds were then kept in sealed aluminium pouches stored till further analysis and utilisation.

5.1.3 Roasting

The sunflower seeds were positioned on the turntable plate of the oven (Model: Samsung, CE104VD, 230 V-50 Hz, 2450 MHz, 100-900 W-6 Levels) after being placed in a single uniform layer in the 12 cm diameter Pyrex petri dishes (Yoshida et al. 2001). The contents of the dishes were then roasted at 150°C for 5 min. Once the roasting was done the seeds were kept to cool at room temperature (Patricia et al. 2014).
5.2 Analysis of non-roasted and roasted sunflower seeds

5.2.1 Free Radical Antioxidant Scavenging Capacity:

The DPPH assay method works on the principle of the reduction of DPPH. DPPH, the free radical delivers the maximal absorption at 517 nm (purple colour) with one odd electron. When the antioxidant molecule reacts with DPPH, the stable free radical, pairing occurs in the presence of a Hydrogen donor and it’s reduced to DPPHH. Resultant to this the absorbance is decreased DPPH radical to the DPPH-H, which leads to the decolourization (yellow colour). The decolourization is directly proportional to the reducing capacity.

The sunflower seeds have been reported to possess antioxidant properties. So in the present study sunflower seeds, both Roasted and Non Roasted have been evaluated for their possible potential to produce antioxidant action by the DPPH scavenging method. The extract of sunflower seeds was composed by dispersing 0.15 gm of powdered sunflower seeds in 10ml of 70% (v/v) acetone for the conduction of DPPH test. The solution was centrifuged at 20,000 g for 20 minutes after shaking for 30 min continuously at room temperature. Then the extract’s aliquot (50µl) was taken and was made to blend with the acetate buffer (100 mM, pH 5.5, 0.5 ml) and ethanol DPPH solution (0.5 mM, 0.25 ml). After keeping the blend in dark for about thirty minutes, at 517 nm its absorbance was measured with absolute ethanol taken as a blank. Results obtained have been displayed as an IC50 value. It shows the total quantity of sample (in mg) that provided 50% inhibition of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Zilic et al. 2010).
The DPPH scavenging effect percentage was calculated by the given equation:

\[
\text{DPPH scavenging effect (\%) or percent inhibition} = \frac{A_0 - A_1}{A_0 \times 100}
\]

Where \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance of the sample.

### 5.2.2 Total Phenolic Content

Total phenolic content was estimated with Singleton and Rossi method (1965). Similar extract that had been used in the test of DPPH was used. 20\% of 1.25 ml sodium carbonate, Folin reagent 0.25 ml, and 0.4 ml deionized water were taken and then the extract about 0.1 ml was blended with these solutions. At 750 nm the absorbance was measured after the solution was kept at room temperature for about 40 minutes. Gallic acid equivalents (eq.) were put to use in order to analyze the total phenolic content with respect to the gallic acid the calibration curve. The solutions obtained were manifested as milligrams of gallic acid per gram of dry matter (d.m.).

### 5.3 Effect of sunflower seeds on the biochemical parameters of sample patients (\(n=300\))

#### 5.3.1 Design of the Study

The present study conducted was a randomised, case controlled and a prospective study. Methodological aspects in the study have been discussed as under:

- Sample selection
- Data collection
- Pre supplementation data collection (Dietary survey, Anthropometric measurement, Biochemical testing)
- Supplementation of Sunflower seeds
- Post supplementation data collection (Biochemical testing, Dietary survey)
- Statistical analysis
Fig 5.2 Flowchart elucidating design of the study
5.3.2 Sample Selection:

The entire sample comprising of 300 patients was selected from Punjab state (from the cities Amritsar, Batala, Jalandhar) amongst people of age ranging from 45-55 years (including both males and females) with raised blood glucose levels, deranged lipid profile or liver function tests. The subjects included in the study were selected from OPD/IPD (Out-Patients Department/ In-Patients Department) of the hospital.

5.3.3 Data Collection:

A schedule for an interview was prepared in order to collect the required information with the help of self-prepared questionnaire so that detailed information could be obtained. The interview schedule (questionnaire) consisted of both closed and open ended questions (Ivan et al. 2000). It was designed in such a manner so as to obtain the information related to:

- General Information
- Dietary Pattern
- History of Diabetes or any other medical problem
- Undergone any major or minor surgery

**Pretesting of Interview Schedule** - Before conducting the actual survey the interview schedule was tested on a few patients having same characteristic to find out the general level of understanding of some basic terms, process and questions and also to find out the need of modification in questions, if any (Richard et al. 1998).

5.3.4 Pre Supplementation Data Collection:

5.3.4.1 Dietary Survey:

Diet survey was carried by 24 hour recall method. The subjects were asked to provide estimates of the amount of meal they had taken during past 1 day or 24 hours. In the given method subject was asked to name the food eaten with approximate amounts during the previous day at each meal and between meals. Quantities were stated in household units such as a glass of milk etc. by providing the subjects the measuring cups or other devices to aid in recalling (Glady et al. 1982).
5.3.4.2 Food Frequency Method:
In this method the subjects were asked about the number of times certain foods or combination of food was consumed per day/per week/per month or any other period of time. The food frequency list is inclusive of a large number of food groups so as to get a clue to the nutritive adequacy (Walter et al. 1984).

5.3.4.3 Anthropometric Measurements:
The Anthropometric measurements taken were:-
(i) Height
(ii) Weight
(iii) BMI (Body Mass Index)

(i) Measurement of Height:
Height is a linear measurement that reflects skeletal growth. It is a measure of chronic malnutrition or under nutrition and should be measured as accurate as possible. It is made up of the sum of component, legs, pelvis, spine and skull (Jatinder et al. 2004). The equipment used for taking height of the subject was a non-stretch tape which was fixed on a flat wall.

(ii) Measurement of Weight:
Recording of weight is the most widely used measurement both for assessing under nutrition as well as for over nutrition. Weight of an individual reflects the more recent nutrition. It is a measurement of body mass. A portable platform weight beam balance was used to assess weight of the subject as it is sturdy, easily transportable and accurate to within limits required (Nisa et al. 2010).
### Table 5.1 Ideal Weight for Height Chart

<table>
<thead>
<tr>
<th>Height (Meters &amp; Feet)</th>
<th>Males Weight (Kgs)</th>
<th>Females Weight (Kgs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.524 m)5’-0”</td>
<td>50.6 – 54.7</td>
<td>50.7 – 54.3</td>
</tr>
<tr>
<td>(1.549 m)5’-1”</td>
<td>51.8 – 55.5</td>
<td>51.6 – 55.2</td>
</tr>
<tr>
<td>(1.575 m)5’-2”</td>
<td>56.5 – 60.4</td>
<td>53.0 – 56.6</td>
</tr>
<tr>
<td>(1.598 m)5’-3”</td>
<td>57.7 – 61.8</td>
<td>54.3 – 58.0</td>
</tr>
<tr>
<td>(1.625 m)5’-4”</td>
<td>58.8 – 63.6</td>
<td>56.2 – 59.8</td>
</tr>
<tr>
<td>(1.651 m)5’-5”</td>
<td>60.9 – 65.4</td>
<td>57.5 – 61.1</td>
</tr>
<tr>
<td>(1.676 m)5’-6”</td>
<td>62.3 – 66.8</td>
<td>58.8 – 63.4</td>
</tr>
<tr>
<td>(1.701 m)5’-7”</td>
<td>64.1 – 68.6</td>
<td>60.7 – 65.2</td>
</tr>
<tr>
<td>(1.727 m)5’-8”</td>
<td>65.7 – 70.9</td>
<td>62.1 – 66.6</td>
</tr>
<tr>
<td>(1.752 m)5’-9”</td>
<td>67.7 – 72.8</td>
<td>64.1 – 68.4</td>
</tr>
<tr>
<td>(1.778 m)5’-10”</td>
<td>69.3 – 74.5</td>
<td>65.7 – 70.2</td>
</tr>
<tr>
<td>(1.803 m)5’-11”</td>
<td>71.3 – 76.3</td>
<td>67.0 – 71.6</td>
</tr>
<tr>
<td>(1.828 m)6’-0”</td>
<td>73.1 – 78.6</td>
<td>68.4 – 73.8</td>
</tr>
<tr>
<td>(1.854 m)6’-1”</td>
<td>73.4 – 80.8</td>
<td>73.2 – 80.6</td>
</tr>
<tr>
<td>(1.879 m)6’-2”</td>
<td>77.7 – 83.6</td>
<td>77.5 – 83.4</td>
</tr>
<tr>
<td>(1.905 m)6’-3”</td>
<td>79.9 – 85.8</td>
<td>79.7 – 85.8</td>
</tr>
</tbody>
</table>

Source: National Centre for Health Statistics in collaboration with the National Centre for Chronic Disease Prevention and Health Promotion. Ideal weight for height chart for adults (2000).

(iii) BMI:

BMI (Body Mass Index) is the estimation of mass of the human body with respect to an individual’s weight and height. It is the body fat’s measure on the basis of weight and height of adults. It is also called the Quetelet index.

BMI is measured with the help of a simple formula which includes a person’s weight in Kg that is Kilograms to the person’s height taken in meters (m).

\[
\text{BMI} = \frac{\text{Weight in Kg}}{\text{Height in meter square}}
\]
5.3.4.4 Biochemical Testing of the parameters

Biochemical testing helps in determining different parameters, and also identifying the main biological chemical compounds, by using molecular and biochemical tools. It helps to measure the amount of a substance in the body through blood or urine analysis.

(i) Fasting Blood Glucose

A sample of the blood was obtained when the person was in a fasting state and the amount of sugar was assessed. Blood sugar was measured with the help of an apparatus known as glucometer with the help of which fasting as well as random level of blood sugar were determined. First of all, a sharp edge blood lancet was used to the prick over the tip of the finger. A drop of blood from the prick area was taken and put over the specific mark, then the strip was inserted into the glucometer and count-down of time was started. When it reached zero the final value of blood sugar level appeared on screen of glucometer and noted (Kaul et al. 2013).

(ii) Estimation of Glycosylated Haemoglobin (HbA1c)

Hemoglobin A1c (HbA1c) test was done in order to evaluate the control of blood glucose in patients suffering from diabetes (usually type-2). The serum glucose testing on a daily basis gives a view of present control of blood sugar whereas the HbA1c gives a view of blood sugar control of the patient in the past 120 days. Because of the reason that glucose molecule stays attached to hemoglobin molecule for whole life of the red blood cell (about 120 days). This test is done to analyze the blood glucose level on an average in the patient for not just one day but also for previous 2-3 months (Kaul et al. 2013).

The test was performed with the help of the HPLC equipment Bio-Rad D-10, as per the DCCT referral source (i.e. Diabetes Control and Complications) in the latest issues. It is well recorded by the NGSP i.e. the National Glycohemoglobin Standardization Program. Before performing automated analysis, the sample haemosylate was prepared manually. As per the method used for testing the tetra decyltrimethyl ammonium bromide were mixed with the obtained samples consisting of the haemolysing reagent for several mins (1000µl haemolysing reagent + 10µl whole blood). The value of the Glycosylated haemoglobin was estimated and determined with the help of DDS kit (Diasis Diagnostic Systems) as per the
given instructions on its kit. A DDS calibrator was used for its calibration. The total haemoglobin that was required Glycosylated Haemoglobin’s measurement was estimated in a different column of the same equipment in the DDS Kit (Fatih et al. 2010).

(iii) Estimation of Lipid Profile

A set of blood tests that help in identifying the range of lipid content in the blood as like cholesterol, triglycerides etc. is known as the Lipid profile. The results of this test help to identify approximate risks for disease if any as like coronary artery disease, arthrosclerosis etc. also it helps to determine certain genetic diseases. The lipid profile includes complete cholesterol, triglycerides, LDL, HDL. Here, LDL is commonly known as the bad cholesterol whereas HDL is known as the good cholesterol (Sidhu et al. 2012).

The lipid profile and its relative tests were estimated by enzymatic calorimetric method especially the total cholesterol (Allain et al. 1974). The triglycerides were estimated by Van Denmark and Jacobs enzymatic method. The High Density and Low Density Lipoprotein were analysed with the help of Gordon and Gordon method 1977 and Friedewald formula 1972. All the above mentioned tests were estimated in the blood serum. All parameters were determined in the blood of the patients and controls bringing into use special kits of the reagent which are commercially available.

(iv) Estimation of Liver Function Tests (LFT)

The LFTs are groups of laboratory assays of blood in biochemistry which have been designed to analyse the status of an individual’s liver. The parameters measured in this include albumin, bilirubin (indirect, direct), globulin, SGOT (AST) and SGPT (ALT) the Liver transaminases. The LFTs prove to be a helpful screening tool in detecting hepatic dysfunction in a patient if any (Sultana et al. 2004).

For enzymes- SGOT/AST (Serum Glutamate Oxaloacetic Transaminase/ Aspartate Aminotranferase), SGPT/ALT (Serum Glutamic Pyruvate Transaminase/ Alanine Aminotranferase the procedure approved worldwide is used with p- nitrophenol phosphate taking part as a substrate, in an environment of basic pH. Un-haemolysed fresh blood was used as the sample for the evaluation (Thapa et al. 2007).
5.3.5 Grouping and Supplementation

The roasted sunflower seeds were advised to be added to hot or cold beverages or cereals (2gm).

(i) Control Group: It comprised of patients with high serum lipid levels, high blood glucose levels or high LFT levels than normal with minor medications for the same like lovastatin, glycomet, avas etc. along with the specific diet modifications. The groupings were as follows:

**Group 1** - Deranged lipid levels on specific medications and diet modifications.

**Group 2** - Raised blood glucose levels on medications and diet modifications

**Group 3** - Increased LFT levels on medications and diet modifications

**Group 4** - Deranged lipid levels + increased blood glucose + deranged LFT levels with the medications and the diet modifications

**Group 5** - Patients with high serum lipid levels or high blood glucose levels or high LFTs and only on diet modifications but not on any medications.

(ii) Case Group: - comprised of as follows:

**Group 1 (a)** - Deranged lipid levels receiving 2 g of sunflower seeds in addition to the medications and the dietary modifications

**Group 2 (a)** - Raised blood glucose levels receiving 2 g of sunflower seeds in addition to the medications and the dietary modifications

**Group 3 (a)** - Increased LFT levels receiving 2 g of sunflower seeds in addition to the medications and the dietary modifications

**Group 4 (a)** - Deranged lipid levels + increased blood glucose + deranged LFT levels receiving 2 gram of sunflower seeds in addition to the medications and the dietary modifications

**Group 5 (a)** - Patients receiving 2gram of sunflower seeds including patient with high serum lipid levels or high blood glucose levels or high LFTs but not on any medications.
Fig 5.3 Flowchart showing the classification of the Case and the Control Group
5.4 To prepare and evaluate the sunflower seeds’ enriched food product

Product formulation process is a systematic set of activities targeted at development of an acceptable product (Earle et al. 2007). Keeping in mind the nutritional attributes of sunflower seeds, different flour blends were used to design and develop healthy sunflower based cookies made from these blends. The result obtained was put to use for the nutritional therapeutic purpose.

Cookies are very common and well acceptable in all the countries. The percentile as discussed might differ but the final product is always expected to be same sweet, crunchy and nutty. Cookies are named variedly in different areas in accordance to the place of origin. From a very long time cookies have been served as or with a dessert and even nowadays they are a very common snack consumed at various times of the day with tea/coffee and even used as a gift item.

5.4.1 Procurement of raw material

To prepare the cookies, the materials needed were procured from the local market: sunflower seeds, wheat flour, sodium bicarbonate and white butter.

5.4.2 Flour Preparation

The seeds were first graded, then sorted and finally cleaned. The seeds then were soaked for 24 hrs in water. Post to soaking the seeds were washed thoroughly and then oven dried for 24 hrs at 60°C or till the moisture content came to around 11.4%. Seeds once dried were ground with the help of a grinding machine, the ground seeds were then sieved through a 1-mm sieve. The ground and sieved seeds were then stored in airtight containers or sealed packets until further analysis at room temperature. (Morton, 1987)

5.4.3 Experimental Plan

The experimental plan is given in Table 5.2 and Table 5.3 shows the different composition of flour. In Table 5.4 the different ingredients used in making the cookies were given in gm and Fig 5.4 shows flowchart of the preparation of cookies.
Table 5.2 Experimental plan for product formulation

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>Level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Product</td>
<td>1</td>
<td>Cookies</td>
</tr>
<tr>
<td>2.</td>
<td>Ingredient</td>
<td>5</td>
<td>sunflower seeds, white butter, wheat flour, salt and sodium bicarbonate</td>
</tr>
<tr>
<td>3.</td>
<td>Samples</td>
<td>4</td>
<td>A₁, A₂, A₃ and A₄</td>
</tr>
<tr>
<td>4.</td>
<td>Analysis</td>
<td>4</td>
<td>Physical analysis, Sensory analysis, Functional Analysis, Physicochemical Analysis</td>
</tr>
</tbody>
</table>

Table 5.3 Composition of various flour blends

<table>
<thead>
<tr>
<th>S.No</th>
<th>Flour Blend</th>
<th>Wheat Flour (WF), %</th>
<th>Sunflower Seeds Flour (SSF), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>A₁</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>A₂</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>3.</td>
<td>A₃</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>4.</td>
<td>A₄</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 5.4 Ingredients for cookies’ preparation

<table>
<thead>
<tr>
<th>S.No</th>
<th>Ingredients</th>
<th>A₁</th>
<th>A₂</th>
<th>A₃</th>
<th>A₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>WWF (gm)</td>
<td>250</td>
<td>200</td>
<td>175</td>
<td>150</td>
</tr>
<tr>
<td>2.</td>
<td>SSF (gm)</td>
<td>0</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>3.</td>
<td>White Butter (gm)</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>4.</td>
<td>Sodium Bicarbonate [Baking powder] (gm)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Fig 5.4 Flowchart of cookies’ preparation
5.4.4 Evaluation of functional properties of the sunflower enriched flour

5.4.4.1 Bulk Density

A known amount of sample was weighed into 50 ml graduated measuring cylinder. The sample was packed by gently tapping the cylinder on the bench top 10 times from a height of 5 cm. The volume of the sample was recorded.

Bulk Density (g/ml or g/cm\(^3\)) = Weight of sample/Volume of sample after tapping.

5.4.4.2 Water Absorption Capacity

15 ml of distilled water was added in a centrifuge tube of 25 ml with 1 gm of sample in it. The centrifuge tube was agitated for two minutes on a vortex mixer. It was then centrifuged for 20 minutes at 4000 rpm. The supernatant was decanted and discarded. The adhering water drops were removed and the tube was weighed again.

WAC % = (Weight Tube + Sediment – Weight of empty tube)/ Weight of Sample × 100

5.4.4.3 Oil Absorption Capacity

10 ml of oil was added in a centrifuge tube of 25 ml with 1 gm sample in it. The centrifuge tube was agitated for 2 minutes on a vortex mixer. It was then allowed to stand for 30 minutes at room temperature.

To 1 g of the sample, 10 ml of oil was added in a 25 ml centrifuge tube and agitated on a vortex mixer for 2 minutes. It was allowed to stand at room temperature for 30 minutes. The mixture was then centrifuged for 30 minutes at 500 µg in a high speed micro centrifuge. The supernatant was then decanted and discarded. The adhering oil drops were removed and the tube was reweighed again.

OAC % = (Weight tube + sediment – weight of empty tube)/ weight × 100
5.4.4.4 Swelling Power

The swelling power (SP) was measured at 70ºC and 80ºC independently for each flour sample. 0.1 g of sample was taken and heated for 15 minutes at 70ºC and 80ºC in a water bath with intermittent shaking. A high speed micro centrifuge was used to centrifuge the sample. The supernatant was decanted into a test tube and the sediment was weighed. The decanted supernatant was also collected, dried and weighed.

\[ \text{SP} \% = \frac{\text{dry matter weight}}{\text{sediment weight}} \times 100 \]

5.4.5 Physical evaluation of sunflower seeds enriched cookies

5.4.5.1 Weight

A digital top loading balance was used to check the weight of cookies which consists of different units of weight as like gram, milligram etc.

5.4.5.2 Diameter and Height

A Vernier calliper was used to measure the cookie diameter and height.

5.4.5.3 Spread ratio

Spread ratio is calculated as diameter/height

5.4.6 Physicochemical composition of sunflower seeds enriched cookies

5.4.6.1 Protein Content

The protein content had been determined by Lowry’s method. Different dilutions of BSA solutions were prepared by mixing stock BSA solution (50 mg/ 50 ml) and water in standard flask. Extraction of sample was carried out with buffers used for enzyme assay. 0.5 gm of sample was weighed and ground in a pestle mortar with 5ml of buffer. The mixture was centrifuged and supernatant was collected. 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard was taken into series of test tubes. 0.1 ml of sample was taken in other test tube. 5 ml of alkaline copper solution was added in each test tube including blank (1ml distilled water). Mixture was mixed properly and was allowed to stand for 10 minute. 0.5 ml of Folin-Ciocalteau reagent was added to each test tube and kept in dark for 30 minutes. Readings
were taken at 660 nm. Standard graph was plotted and amount of protein was calculated as mg/g or 100gm of sample (Lowry et al. 1951).

5.4.6.2 Fat Content

Soxhlet extraction method was used to determine the fat content using petroleum ether (40-600°C) as the reagent. Dried samples (2gm) were extracted with petroleum ether in Soxhlet extraction apparatus for 6-8 hours in pre weighed round bottom flask. The extract containing fat and petroleum ether was evaporated over boiling water bath and dried in an oven at low temperature and weighed. The difference in the weight of the round bottom flask represented the ether extract (fat content) present in the sample (AOAC, 2000)

Weight of sample = W (g)

Weight of empty round bottom flask = W₁ (g)

Weight of empty round bottom flask + Fat content = W₂ (g)

Fat Content % = \( \frac{\text{Amount of Ether extract}}{\text{Weight of Sample (g)}} \times 100 \)

Fat content % = \( \frac{W_2-W_1}{W} \times 100 \)

5.4.6.3 Total Carbohydrate Content

Total carbohydrates were calculated by the given formula (Rangana, 1986)

Total CHO% = 100- (Moisture + Crude Ash + Crude Protein + Crude Fat + Crude Fiber)

5.4.6.4 Crude Fiber Content (AOAC, 2000)

In order to analyse the crude fiber content moisture and fat free sample (2g) were mixed with 200 ml of 1.25 percent H₂SO₄ by gentle boiling for half an hour. The contents were filtered and the residue was washed many times with distilled hot water till it all the acid washes off. Acid free residue was then transferred to the same flask to which 200ml of 1.25 per cent of NaOH was added. The contents were mixed again for half an hour, filtered it and residue was again washed with hot distilled water till it became alkali free. The residue was dried overnight at 100°C and weighed and then placed in muffle furnace at 600°C (±50°C) for
4 hours. The loss in weight after ignition of the sample represented the fiber in the sample (AOAC, 2000). The per cent crude fiber was calculated as follows:

\[
\text{Fiber content \%} = \frac{(W_2 - W_1) - (W_3 - W_1)}{W} \times 100
\]

### 5.4.6.5 Moisture Content (AOAC, 2000)

Moisture Content in the edible immature seeds of pulses was determined by following the oven drying method. 5g of sample was taken in a previously weighed, dried aluminium cups. These cups are kept in a hot air oven at 60°C (±5°C) for 8 hours. The aluminium cups were taken out from the oven and kept in the desiccator for cooling for 30 minutes in order to attain a constant weight. After cooling, the samples were weighed with aluminium cups. The loss in the weight represented the moisture content of the sample.

\[
\text{Moisture content \%} = \frac{\text{Loss in weight (g)}}{\text{Weight of sample (g)}} \times 100 = \frac{X - Y (g)}{X (g)} \times 100
\]

### 5.4.6.5 Ash Content

The weighed amount of sample (1g) was taken and put in previously dried and weighed silica crucibles. Samples were first incinerated over an electric hot plate followed by ashing in muffle furnace at a temperature of 550°C (±25°C) for 6 hours (until pale white residue was
obtained). These ashed samples were taken out from the muffle furnace and kept in desiccator for 2 hours for cooling (AOAC, 2000). After cooling samples were weighed again and per cent ash content was calculated as follows:

- Weight of empty crucible = W (g)
- Weight of crucible + sample before ashing = W₁ (g)
- Weight of crucible + sample after ashing = W₂ (g)

\[
\text{Ash content } \% = \frac{W₂ - W₁}{W₁ - W} \times 100
\]

### 5.4.7 Total Phenolic Content

Similar to section 5.2.2; 0.2 g of finely ground sample was weighed and taken in a beaker and 10 ml of 70 per cent acetone was added. The beaker was placed in a water bath (adjusted at 37°C for 2 hours). Frequent shaking was given for better extraction. After expiry of this period, extract was centrifuged for 20 minutes at 3000 rpm. The supernatant was collected in a test tube and was further used for the estimation of total and simple phenols. 0.1 ml of aliquot extract as obtained above was taken and volume was made 1ml with distilled water. 2.5 ml of 20 per cent sodium carbonate solution was added followed by 0.5 ml Folin-Ciocalteau reagent. Contents were left for 40 minutes for colour development (purplish blue). Absorbance was read at 725 nm after 40 minutes against a suitable bank and calculations were done for total phenols using standard curve which was prepared using gallic acid (0.1 mg/ml) (Makkar et al. 1997).

### 5.4.8 Antioxidant activity by DPPH Assay (Brand-William et al. 1997)

Similar to section 5.2.1; the antioxidant properties were evaluated using the DPPH radical scavenging method. Ascorbic acid was used as the natural antioxidant for the antioxidant activity comparison. Each sample’s antioxidant activity was expressed as IC 50, and was calculated in accordance to the standard protocol from the graph after plotting inhibition percentage against extract concentration DPPH assay. 1.5 ml of 0.1 mm DPPH
solution was mixed with 1.5 ml of various concentrations (10-500 µg/ml) of extract. The experiment was replicated in three independent assays. Ascorbic acid was used as positive controls.

The DPPH scavenging effect percentage was calculated by the given equation:

$$DPPH\,scavenging\,effect(\%)\,or\,percent\,inhibition = \frac{A_0-A_1}{A_0} \times 100$$

Where $A_0$ was the absorbance of the control and $A_1$ was the absorbance of the sample.

All tests were run in triplicates ($n=3$) and average values were calculated.

5.4.9 Organoleptic evaluation:

Nine point Hedonic scale method as given by Amerine et al. (1965) was followed for conducting the sensory evaluation of sunflower seeds incorporated cookies. The panel of 10 judges comprising of faculty members and post-graduate students of the Department of Food Technology and Nutrition, Lovely Professional University were selected with care to evaluate the cookies for sensory parameters such as colour, crispiness, taste, mouthfeel and overall acceptability. Efforts were made to keep the same panel for sensory evaluation throughout the entire period of study. The samples were presented to judges and plain water was given to them to rinse their mouth in between the evaluation of samples. No discussion during evaluation was allowed.