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3.5. Statistical analysis
SCHEMATIC DIAGRAM FOR THE STUDY

Pumpkin seeds

Processing

Raw          Autoclave          Boiling          Germination          Roasting

Parameters assessed

Physical properties  Proximate composition  Mineral Composition  Amino Acid  Fatty Acid Profile

Efficacy studies using animal model for all processed pumpkin seeds

Anti Depression study  Hypcholesterolemic study

Lipid Profile

Force Swimming Test  Tail Suspension Test

Germinated pumpkin seeds extract shows good result among the five processing techniques

Optimization of germinated pumpkin seeds powder bread

Weight Loss (Y1)  Porosity (Y2)  Specific volume (Y3)  Protein (Y4)  Tryptophan (Y5)

Supplementation of optimized bread (Human model)

Anti Depression study  Hypcholesterolemic study

Underwent Screening for Eligibility-360

Hypercholesterolemic subjects (n=60) Based on lipid profile

Eligible at Screening (n=40) (using Beck Depression Inventory (BDI))

Experimental group-(20) (100 g optimized bread)  Placebo group (20)

Depression Inventory (BDI)

Experimental group-30 (200 g optimized bread)  Placebo group-(30)

Lipid profile (TG, TC, LDL-C, HDL-C, VLDL-C)

Statistical analysis
PHASE I

3.1. Processing and Physiochemical Evaluation of Pumpkin Seeds

Pumpkin seeds also known as pepitas are flat, dark and green, as well as nutty tasting. They belong to the gourd or cucurbitaceae family. Pumpkin seeds are nature’s own little multi-supplement containing a wide variety of nutrients. It has considerable nutritional value for human consumption due to its 37.8-45.4% oil and 25.2-37% protein. Pumpkin seeds are full of iron, zinc, calcium and magnesium, arguably the four most important minerals for human need. Pumpkin seeds are known to be a rich source of the essential fatty acids, phytosterol, amino acid and tryptophan. The different treatment conditions in pumpkin seeds changes its physical properties as well as its nutritional composition.

3.1.1. Morphological and physical characterization of pumpkin seeds

Morphological study of pumpkin seed is based on shape, length, width, thickness, and weight etc.

3.1.1.1. Size of the seeds

To determine the average size of the seeds, 100 seeds were randomly picked and their three linear dimensions namely, length (L), width (W) and thickness (T) were measured using a vernier reading to 0.01 mm (Vilche et al., 2003).

3.1.1.2. Seed shape

The shape was determined in terms of its sphericity. The sphericity (SI) as given by Mohenin (1980) is

\[ SI = \frac{LWT \times 100}{L} \]

3.1.1.3. Thousand Seed weight

Thousand Seed Weight (TSW) was measured by counting 100 seeds and weighing them in an electronic balance to an accuracy of 0.001 g and then multiplied by 10 to give mass of 1000 seeds.

3.1.2. Processing of pumpkin seeds to powder

Food processing is the set of methods and techniques used to transform raw ingredients into food or to transform food into other forms for consumption either in
home or by the food processing industry. The processing techniques help to activate the nutrients present into the seeds. Removal of undesirable components is essential to improve the nutritional quality of seeds. It is widely accepted that simple and inexpensive traditional processing techniques are effective methods of achieving desirable changes in the composition of seeds.

The processing methods such as autoclave, boiling, germination, and roasting were implemented with raw sample to test the effect of processing on physical and nutritional characteristics of pumpkin seeds.

3.1.2.1. Selection of material

Pumpkin (Cucurbita maxima) seeds were obtained from the local market in Salem, Tamil Nadu, India. The seeds were cleaned by hand to remove foreign materials. The seeds were peeled by hand for the process of autoclave, boiling and roasting.

3.1.2.2 Processing techniques

**Raw (Dried)**

The cleaned pumpkin seeds were washed in water. The hulls were removed then the seeds dried in the sun for 6 to 8 hours. The seeds were then kept warm for 3 to 4 hours and powdered (Emenalom and Udedibie, 2005).

**Autoclaving**

The cleaned seeds were autoclaved using vertical autoclave at 15 lb pressure (121 1C) in tap water (1:10, w/v) until 50% of the seeds become soft when felt between the fingers (35 min) (Udensi, 2008).

**Boiling**

The rinsed soaked seeds were cooked in tap water (100ºC) in the ratio of 1:10 (w/v) on a hot plate until they became soft when felt between the fingers (90 min) (Saleh et al., 2006).

**Germination**

The pumpkin seeds were washed in tap water, soaked in the mercuric chloride solution (0.1%) for period (3-4 minutes) and then washed thoroughly with distilled water until free from chloride ions. Further the seeds were soaked in distilled water of depth (3-4 cm) at room temperature for the period (8 hours). The water was changed thrice and then seeds were placed in glass trays covered with wet muslin cloth and
Plate 1

Preliminary preparation of pumpkin seeds for processing

a. Cut the pumpkin
b. Coop all of the pumpkin's stringy insides
c. Separate the seeds from the flesh and strings
d. Place the seeds in a strainer or colander and discard the rest
e. Wash the seed in water
f. Pumpkin seeds for processing
Plate 2
Processing Techniques

1. Processed pumpkin seeds

2. Processed pumpkin seed powder
allowed to germinate for the period (24 hours). The germination of the pumpkin seeds was carried out as per standard method (Sawant, 2007). The germinated pumpkin seeds were dried in oven (50°C) for period (5-6 hours). The dried germinated pumpkin seeds were powdered.

**Roasting**

Roasting involves the application of dry heat to pumpkin seeds using a hot pan or at a temperature of 150 to 200°C for a short time (Nalaini, 2006).

### 3.1.3 Determination of physical properties of processed pumpkin seeds powder

The physical properties of the all processed pumpkin seeds powder were analyzed.

#### 3.1.3.1. Rehydration characteristics

Rehydration quality of dehydrated product was determined by rehydration test (Rangana, 1986). Ten gram of dehydrated samples were placed in glass beakers, 200 ml of water was added and heated at 40-45°C for 60 min. The excess water was drained off through filter paper (Whatman Nr 4). The drained samples were weighed. Rehydration (Rr)

\[
Rr = \frac{C}{D}
\]

C=Drained weight of rehydration sample, D= Weight of hydrated sample

#### 3.1.3.2. Bulk Density (BD)

The BD of the seed powder was determined by the method of Ige et al (1984). A specified quantity of the sample was put into an already weighed 5 ml measuring cylinder (W1); it was gently tapped to eliminate air spaces between the powder in the measuring cylinder and the volume was noted. The new mass of the sample and sample was determined. The BD was computed as

\[
BD = \frac{W2-W1}{Vol.\ of\ sample\ used}
\]

#### 3.1.3.3. Water and Oil absorption

These were determined as described by Beuchat (1977). The powder (1g) was mixed with 10 ml distilled water for water absorption and 10ml of oil for oil absorption in a Kenwood blender for 30 seconds. The samples were then allowed to stand at 25°C
for 30min and centrifuged at 3500ppm for 30min. The supernatant was decanted and discarded. The weight of water or oil absorbed by 1g of flour was calculated and expressed as water or oil absorption capacity (Beuchat 1977; Eke and Akobundu 1993).

3.1.3.4. Foaming Capacity (FC) and Foam Stability (FS)

The FC and FS of both flour samples were determined as described by Narayana et al., (1982). Two grams of pumpkin seeds powder sample was added to 50 ml of distilled water at 30 ± 2°C in a 100 ml graduated cylinder. The suspension was mixed and shaken for 5 min to foam. The volume of foam at 30 seconds after whipping was expressed as FC using the following formula:

\[
FC = \frac{\text{Volume of foam after whipping} - \text{Volume of foam before whipping}}{\text{Volume of foam before whipping}}
\]

The volume of foam was recorded one hour after whipping to determine FS as a percentage of the initial foam volume.

3.1.3.5. Retrogradation

Retrogradation was determined as described by Sosulski et al., (1976). A 5 g powder sample was placed in a 100 ml beaker and mixed with 5 ml distilled water and then gelatinized into a paste with 40 ml boiling water. From the paste, 15 ml measured into a centrifuge tube and allowed to stand overnight. The paste was subsequently centrifuged and the volume of the supernatant was recorded.

3.1.4. Chemical analysis of processed pumpkin seeds powder

Chemical Characteristics like Proximate composition, mineral composition, amino acids analysis and fatty acids profile of the all processed seeds powder was done.

3.1.4.1. Proximate Composition

3.1.4.1.1. Moisture

A known amount of sample was weighed into a previously weighed moisture cup and dried in an oven at 60°C to a constant weight (Anon, 1990) and moisture content calculated as follows:

\[
\text{Moisture} (\%) = \frac{\text{Weight of sample before drying (g)} - \text{Weight of sample after drying (g)}}{\text{Sample weight (g)}} \times 100
\]
3.1.4.1.2. Crude protein

The nitrogen content of the processed pumpkin seeds powder was assessed by Kjeldahl method using Pelican Kelplus equipment. Crude protein was calculated by multiplying with a factor 6.25 (Monteiro et al., 1988).

\[
\text{Protein (\%)} = \frac{\text{Titre value-Blank} \times \text{N. of HCl} \times 14.007 \times 6.25}{\text{Sample weight (g)}} \times 100
\]

3.1.4.1.3. Crude fat

Moisture free powder samples were weighed in moisture free thimbles and crude fat was extracted by refluxing with petroleum ether in a soxhlet apparatus. Per cent crude fat was calculated as follows (Anon, 2002),

\[
\text{Fat (\%)} = \frac{\text{Weight of sample} - \text{Weight of sample before extraction (g)}}{\text{Sample weight (g)}} \times 100
\]

3.1.4.1.4. Crude fibre

Crude fibre was estimated from the moisture and fat free sample. The residue obtained after digestion with acid and alkali was dried in crucible and weighed. The difference in weight of the crucible before and after ashing of the digested residues was taken as weight of the crude fibre (Anon, 2002).

\[
\text{Fibre (\%)} = \frac{\text{Weight of residue before ashing (g)} - \text{Weight of residue after ashing (g)}}{\text{Weight of fat free sample (g)}} \times 100
\]

3.1.4.1.5. Carbohydrate Content

The total carbohydrate content was determined (Egan et al., 1981), by the formula;

\[
\text{Carbohydrate (\%)} = 100-(\% \text{Moisture} + \% \text{Proteins} + \% \text{Lipids} + \% \text{Ash}).
\]

3.1.4.1.6. Energy Value

The energy value was calculated using the Atwater factor method \[(9 \times \text{fat}) + (4 \times \text{carbohydrate}) + (4 \times \text{protein})\] as described by Osborne and Voogt (1978); Eneche (1991); Chinma and Igyor (2007). The proportion of protein, fat and
carbohydrate were multiplied by their physiological fuel values of 4, 9 and 4 kcal, respectively and the sum of the product was taken (Nwabueze, 2007).

3.1.4.1.7. Nitrogen free extracts (NFE)

The NFE was calculated according to the following expression:

\[ \text{NFE} = 100 - (\% \text{ moisture} + \% \text{ crude protein} + \% \text{ crude fat} + \% \text{ crude fiber} + \% \text{ ash}) \]

3.1.4.2. Determination of mineral composition

Using the method described by AOAC (2005) the ash of each sample was digested with 5ml of 2M HNO₃ and heated to dryness on a heating mantle. 5ml of 2M HNO₃ was added to it and heated to boil and filtered through a Whatman No 1 filter paper into a 100 ml volumetric flask. The filtrate was made up with distilled water. Calcium, Potassium and sodium was determined using Jenway Digital Flame Photometer (PFP7 model) while other minerals apart from phosphorus were determined using Buck Scientific Atomic Absorption Spectrophotometer

3.1.4.3. Amino acid composition

All the samples of processed pumpkin seeds were subjected to determination of amino acids composition using an amino acid analyzer according to the method of Schuster (1988). The samples were hydrolyzed with 6N HCl under vacuum at 110°C for 24 h. The hydrolysates were dried in a rotary evaporator at 40°C under vacuum to remove the excess acid (6N HCl). The dry residues were then dissolved in a known quantity of citrate buffer (2.2pH) and filtered to obtain a clean solution of the hydrolysate. An aliquot of it was injected into the column (Shim-pack ISC-07/S1504 Na) of the HPLC. Based amino acid analyzer (RF-10AXL, Shimadzu Corporation, Tokyo, Japan) Equipped with fluorescence detector (FLD-6A). Sodium hypochlorite and ophthalaldehyde solutions were used as reaction solutions.

3.1.4.4. Fatty acids profile analysis by Gas Chromatography-Mass spectrometry (GC-MS)

GC-MS analysis of the fatty acid was carried out after methylation. GC-MS analysis was performed with GC Clarus 500 Perkin Elmer equipment. Compounds were separated on Elite-5MS capillary column (5% diphenyl /95% Dimethyl poly siloxane), 30× 0.25mm×0.25μm df). Oven temperature was programmed as follows: isothermal temperature at 110°C for 2 min., later increased to 200°C at the rate of 10°C/minutes and
then increased up to 280°C at the rate of 5°C/min and held for 9 min. Ionization of the
sample components was performed in the El mode (70eV). The carrier gas flow rate was
1ml/min, and 3 μl of sample was injected. The detector was Mass detector turbo mass
gold-Perkin Elmer. The total running time for GC was 36 min. and software Turbomass
5.2 was used in this GC-MS study.

The determination of the components was done by comparing their retention
time with those of authentic specimens on the capillary column as well as peak
enrichment.

3.1.5. Phytochemical screening of processed pumpkin seeds extract

3.1.5.1. Preparation of aqueous extract

Air dried seeds was homogenized to a fine powder. Hundred grams of powdered
processed pumpkin seeds were infused in 500 ml cold distilled water for 24 h, it was
then brought to boil, after removing it from the heat source and allowed to infuse for 15
min. The extract was filtered, then concentrated over the water bath and brought to
dryness under vacuum (Emamghoreishi et al., 2005).

3.1.5.2. Qualitative analysis on phytochemical constituents

The crude extracts were analyzed for alkaloids, tannins, saponins, flavonoids,
steroids, terpenoids and phenolic acids using standard procedures of analysis (Harborne,
1973).

3.1.5.2.1. Test for flavonoids

A few drops of 1% NH₃ solution was added to the aqueous extract of processed
pumpkin seeds in to a test tube. A yellow coloration was showing the presence of
flavanoids.

3.1.5.2.2. Test for tannins

The 0.5ml of powdered sample of pumpkin seed powder was boiled in 20ml of
distilled water in a test tube and then filtered. The filtration method used here is the
normal method, which includes a conical flask and filter paper. About 0.1% FeCl₃ was
added to the filtered samples and observed for brownish green or blue black colouration,
which shows the presence of tannins.

3.1.5.2.3. Test for carbohydrates

Into 0.5 ml of powdered pumpkin seeds extract, 5ml of Benedict’s reagent was
added and boiled for 5 minutes. Formation of bluish green colour showed the presence of carbohydrate solution when it was boiled for few minutes. In the presence of flavonoids, reddish pink or dirty brown colour was produced.

3.1.5.2.4. Test for protein

In five milliliter of aqueous extract, 5-6 drops of million’s reagent was added. A white precipitate which turns red on heating was formed and it indicates the presence of protein.

3.1.5.2.5. Test for steroids

One milliliter of the extracts was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turned red and sulphuric acid layer turned yellow with green fluorescence. This indicated the presence of steroids.

3.1.5.2.6. Test for terpenoids

Five milliliter of aqueous extract was mixed with 2ml of CHCl₃ in a test tube. Three milliliter of concentrated H₂SO₄ was carefully added to the mixture to form a layer. An interface with a reddish brown colouration was formed showing the presence of terpenoids.

3.1.5.3. Quantitative analysis of phytochemical constituents

3.1.5.3.1. Determination of total phenol

Total phenol was determined from a modified assay described by Chandler and Dodds (1983). The amount of phenolics present in the sample was determined from a standard curve prepared with gallic acid in 95% ethanol. Average values of triplicate estimations were expressed as g/100g sample (dry weight basis).

3.1.5.3.2. Tannin determination

Five hundred mg of the sample was weighted into 100 ml plastic bottle; 50 ml of distilled water was added and shaken for 1 hr in a mechanical shaker. This was filtered into a 50ml volumetric flask and made up to the mark. Then 5 ml of the filtrated was pipette out into a tube and mixed with 3 ml of 0.1m FeCl₃ in 0.1N HCl and 0.008m potassium ferrocyanide. The absorbance was measured in a spectrophotometer at 120nm wavelengths within 10 min. A blank sample was prepared and the colour also developed and read at the same wavelength. A standard was prepared using tannin acid to get 100
ppm and measured (Van and Robinton 1981).

3.1.6. Shelf life evaluation of the processed pumpkin seeds powder

Twenty five grams of processed pumpkin seeds powder was weighed and packed in food grade polyethylene pouches (80 gauges) and heat sealed. The storage quality was assessed in initial, after 3 and 6 months both in refrigerated and ambient conditions. Any change in the visible sensory parameters and moisture content of the stored seed powder was evaluated by expert panels and changes if any were recorded.

PHASE-II

3.2. Study on antidepressant and hypocholesterolemic effect of processed pumpkin seeds aqueous extract on animal model

Pumpkin seeds have many pharmaceutical and health benefits. The healing properties of pumpkin seeds is that reduce cholesterol, boost bladder function, alleviates depression, improve prostate health, protects bones from osteoporosis, improve learning disorders, promote hair growth, reduce arthritis and anthelmintic, diuretic properties. According to World Health Report 2002, cardiovascular diseases (CVDs) will be the largest cause of death and disability by 2020 in India. In 2020 AD, 2.6 million Indians are predicted to die due to coronary heart disease which constitutes 54.1% of all CVD deaths. High serum lipid levels (hypercholesterolemia) are major risk factors of coronary heart diseases that are influenced by lifestyle transition and urbanization. WHO, (2001) estimated that depression will be the 2nd largest global disease for all ages and sexes. Based on WHO reports, the two relevant diseases selected for the study was hypercholesterolemia which leads to CVD and depression. Basically pumpkin seeds are a good source of phytosterol that help to bring down bad cholesterol. Due to the purported L-tryptophan content of pumpkin seeds, they have been suggested to help remedy depression.

All the five processed pumpkin seeds powder extracts were used for efficacy study. The two properties of pumpkin seeds, hypocholesterolemic and antidepressant activities were analyzed in experimental rats.

3.2.1. Anti depression activity of processed pumpkin seeds extract (aqueous)

Depression and anxiety disorders are common public health problems with a 17% lifetime prevalence. The term depression is commonly used to reflect a variety of
experiences ranging from normal, transient unhappiness to pathological states of hopelessness, as defined in the DSM-IV (for Diagnostic and Statistical Manual of Mental Disorders, 4th edition). Depression can be caused by a chemical imbalance. A chemical that the body makes from tryptophan called 5-Hydroxy Tryptophan (5-HTP) regulates behavior. It has a positive effect on sleep, anxiety, mood, appetite and pain (Phillip, 2005). For mild to moderate depression, 5-HTP can be an effective treatment. It increases the serotonin in the brain, which affects mood. Pumpkin seeds are a dietary source of tryptophan, which the body uses to make 5-HTP.

Since research in humans is limited, animal models of depression have been developed. Many symptoms of depression cannot be easily measured in laboratory rodents (e.g. depressed mood, feelings of worthlessness, suicide tendency). However, some behavioral tests have been shown to be very effective to evaluate depressive symptoms and are classically used to predict the antidepressant effect of new medications. The existence of numerous behavioral tests to measure depression in rodents reflects the heterogeneity of depressive-like symptoms. In this way, forced swimming test and tail suspension test are classical paradigms used to evaluate behavioral despair. This immobility, referred as behavioral despair in animals, which is claimed to reproduce a condition similar to human depression (Willner, 1984).

The induction was made twice daily for 30 days. All animals were exposed to a serious of Behavioral paradigms designed to assess the range of depression related behaviors. All behavior testing took place between 10.00am-2.00pm. In this study forced swimming test and tail suspension test was used to evaluate the anti depression activity of processed pumpkin seeds extract on rats.

3.2.1.1. Experimental animals

The research experiments were carried out in 48 male Wister rats weighing 120-140 g when considered for experiment. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Central Animal Research Facility of the Little Flower Medical Research Center (LFMRC), Angamaly, Kerala, which is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Registration no.496/01/9/CPCSEA.
Rats were housed in polypropylene cages (30×22×14 cm). Paddy husk was used as bedding material, which was changed on alternative days. It was kept in a well aerated room with exhaust and ceiling fans. The rats were acclimated to standard environmental conditions of temperature (25±5°C) and humidity (55±5°) and 12 hours dark/light cycle throughout the experimental period.

The rat diet included rat chow obtained from the local market. Each rat was provided approximately 25gm of rat chow and water *ad libitum* per day.

### 3.2.1.2. Induction of depression

In this study, Methyl Isobutyl Ketone (100mg/kg) was used as the depression inducer. Rats were starved for 12 hours before the induction of depression. Each rat in the experimental group (II to VIII) was injected with Methyl Isobutyl Ketone intraperitoneally (100mg/kg body weight, i.p.). The induction was made twice a day for 30 days.

Compared to various pilot experiments the dosage range used in this study was found to be optimum. Umadevi *et al.*, (2010) reported that 30 days induction of Methyl Isobutyl Ketone (100mg/kg) in rats developed depression and standard drug Imipriamine (30 mg/kg) induced in 30 days on depressed rats could decrease the severity of depression.

### 3.2.1.3. Preparation of the standard drug

Imipramine was used as the reference drug for evaluating the antidepressant activity. Imipramine were dissolved in double distilled water and injected in rats. The chemical is purchased from commercial sources.

### 3.2.1.4. Experimental Design

The experimental rats were grouped into 8 groups based on the type of supplementation. Each group contains 6 animals. Group I was assigned as control (normal) rats, group II was depressive control i.e., the rats were depressed but didn’t take any supplementation, group III rats received the standard drug imipramine which is used as a medicine for the treatment of depression. All the five processed pumpkin seeds extracts (Raw, Autoclave, Boiled, Germinated and Roasted) were supplemented to Group IV to VIII rat’s respectively.
Group I - Control (normal)
Group II - Depressive control
Group III - Standard drug Imipramine
Group IV - Raw pumpkin seeds extract
Group V - Autoclaved pumpkin seeds extract
Group VI - Boiled pumpkin seeds extract
Group VII - Germinated pumpkin seeds extract
Group VIII - Roasted pumpkin seeds extract

From the 31st day onwards, after the confirmation of depression by force swimming test and tail suspension test compared to normal rats, group IV to group VIII received oral administration of aqueous extract (100mg/kg) of processed pumpkin seeds and group III used imipramine (30 mg/kg) respectively (Umadevi et al., 2010).

3.2.1.5. Tools used to assess depression
All animals were exposed to a series of behavioral paradigms designed to assess the range of depression related behaviors. All behavior testing took place between 10 am to 2 pm after 30 days of induced depression and supplementation. In this study two types of tools namely, Forced Swimming Test (FST) and Tail Suspension Test (TST) were used to analyses the depression in rats.

3.2.1.5.1. Forced -Swimming Test

In order to assess the antidepressant activity of processed pumpkin seeds extract, the modified forced swimming test was conducted (Porsolt et al., 2000). Measurement of immobility time was carried out by observing the motoric activity of rats, which were placed in a pool of water. A glass cylinder of 25 cm in diameter of height 23 cm was filled with water to a height of 12 cm. The temperature of the water was 23± 1°C. In the first trial, after the injection of methyl isobutyl keton in 30 days, the rats which were not yet treated (supplemented) were forced to swim in the glass aquarium. In the next exposure, antidepressant activity of processed pumpkin seeds was assessed after 30 days of treatment.
Plate 3
Assessment of Force Swimming Test in Depressed rats

a. Induction of depression

b. Administration of aqueous extracts of processed pumpkin seeds

c. Force Swimming Test in depressed rats
Plate 4
Assessment of Tail Suspension Test in depressed rats
Two sessions were conducted; an initial 15 minute training session (pre-test session) was followed by a 5 min test session after 24 hour. During the test session, the immobility time, swimming and climbing time were observed by a trained observer. The total duration of the immobility was measured during the 5 min test. Upon removal from the water, rats were towel dried and finally returned to the cage.

Shorter immobility time is an indicator of the stronger antidepressant effect of the tested substances.

3.2.1.5.2. Tail Suspension Test (TST)

The tail suspension test is the second method used for assessing the antidepressant effect of the processed pumpkin seeds. This model is based on the principle that suspending rat suspended upside down leads to a characteristic behavior of immobility after initial momentary struggle. This behavior reflects a state of despair which can be reduced by several agents which are therapeutically effective on human depression. (Steru et al., 1985). Rats were suspened on the edge of a table 50cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period (Rodrigues et al., 2002). Animals were considered to be immobile when it did not show any movement of the body and hanged passively. The total duration of immobility was recorded during the next 4 min of total 6 min test (Dhingra, 2005). Duration of immobility was compared with control and within the groups. This test was done to conform depression after methyl isobutyl was injected for 30 days (and after the supplementation).

3.2.2. Hypocholesterolemic effect of processed pumpkin seeds aqueous extract

Hypercholesterolemia is a metabolic disorder, specially characterized by alterations occurring in serum lipid and lipoprotein profile due to increased concentrations of Total Cholesterol (TC), Low Density Lipoprotein Cholesterol (LDL-C), Very Low Density Lipoprotein Cholesterol (VLDL-C), and Triglycerides (TAG) with a concomitant decrease. Phytosterols are compounds found in plants that have a chemical structure very similar to cholesterol, and when present in the diet in sufficient amounts, are believed to reduce blood levels of cholesterol, enhance the immune response and decreases the risk of certain cancers. Pumpkin seeds contain (265 mg/100
g) of phytoserols and 10% omega -3 EFAs which reduces the hypercholesterolemic
levels. Estimation of lipid profile helps to analyses the effect of pumpkin seed on the
rats.

3.2.2.1. Experimental animals

The present research experiments were carried out in 56 male Wister rats
weighing of 120-140 g when considered for experiment. Among the rats, 8 were
selected as control (normal) and rest 48 used for induction of hypercholesterolemia.

The experimental protocol was approved by the Institutional Animal Ethical
Committee (IAEC) of Central Animal Research Facility of the Little Flower Medical
Research Center (LFMRC), Angamaly, Kerala, which is registered with the Committee
for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA),
Government of India, Registration .no.496/01/9/CPCSEA.

Rats were housed in polypropylene cages (30×22×14 cm). Paddy husk was used
as bedding material, which was changed on alternate days. It was kept in a well
aerated room with exhaust and ceiling fans. The rats were acclimatized to standard
environmental conditions of temperature (25±5°C ) and humidity (55±5°) and 12hrs
dark/light cycle throughout the experimental period. The rat diet included rat chow
obtained from the local market. Each rat was provided approximately 25gm of rat chow
and water ad libitum per day.

3.2.2.2. Induction of Hypercholesterolemia

For induction of hypercholesterolemia each rat in the experimental group except
control group (normal) received Metheonine (Nice chemicals pvt.Ltd. Cochin) orally
(1g /kg weight, po) for 30 days.

The dosage range used here is found to be optimum methionine (1g/kg, po) for
30 days from the experiments of Puneet et al., (2008). For confirmation of
hypercholesterolemia in metheonine inducted rats, 8 rats were (3 normal and 5
hypercholesterolemic rats) scarified and lipid profile was analysed. The lipid profile of
methionine inducted rats was compared with normal rat

3.2.2.3. Dose preparation and administration of standard Atorvastatin  Atorvastatin
was used as standard drug. Standard Atorvastatin at a dose of 10 mg per kilogram was
analyzed for the following biochemical parameters like Total cholesterol (TG) by
CHOD Pap, triglycerides (TG) by GPO Pap and high density Lipoprotein-cholesterol prepared by suspending bulk Atorvastatin in aqueous 0.5% methyl cellulose (Henck et al., 1998).

### 3.2.2.4. Experimental Design

The experimental animals were randomly divided into eight groups. Each group contains 6 animals after conformation of hypercholesterolemia.

- **Group I** - Control (normal)
- **Group II** – hypercholesterolemic control
- **Group III** - Standard drug Atorvastatin
- **Group IV** - Autoclave pumpkin seeds extract
- **Group V** - Boiled pumpkin seeds extract
- **Group VI** - Germinated pumpkin seeds extract
- **Group VII** - Raw dried pumpkin seeds extract
- **Group VIII** - Roasted pumpkin seeds extract

Rats were starved for 24 hours. To induce hypercholesterolemia in rats, each rat in the experimental group (II to VIII) received metheonine orally (1g /kg weight, po) for 30 days.

From the 31st day onwards, after the confirmation of hypercholesterolemia by analysis the lipid profile in metheonine induced rats (8 rats), group III to group VIII was supplemented with oral administration of aqueous extract (100mg/kg) of processed pumpkin seeds for 30 days. Group II was treated with standard drug atorvastatin (30mg/kg) for 30 days. The dosage range used in the processed pumpkin seeds extract was based on the study of Puneet et al., (2008).

### 3.2.2.5. Analysis of lipid profile

To find out the impact of processed pumpkin seeds extract on hypercholesterolemic rats, the rats were scarified then serum collected from all the groups of rats for analyze the lipid profile.

Twenty four hour after the last dose was administrated, animals were scarified using ether anesthesia and blood samples were collected from hepatic portal vein in centrifuge tubes to determine the effect of processed pumpkin seeds extracts on hypercholesterolemic rats. Blood sample was left for 15m at 25°C temperature and then centrifuged at 4000rpm for 20 minutes in order to separate the serum. Serum was
Plate 5
Hypercholesterolemia in rats

a. Hypercholesterolemic rats

b. Induction of hypercholesterolemia
(HDL-C) by direct method using commercial enzymatic kits (Randox, UK) and Photometer model BTR-830 (Biotech, Spain). Low density lipoprotein-cholesterol (LDL-C) and very low density lipoprotein-cholesterol (VLDL-C) were calculated using Friedewald’s formula.

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

\[
\text{VLDL-Cholesterol} = \frac{\text{Triglyceride}}{5}
\]

**PHASE – III**

3.3. **Optimization of germinated pumpkin seeds powder incorporated bread using Response Surface Methodology (RSM)**

3.3.1. **Product development**

Based on efficacy (animal) study, germinated pumpkin seeds extract showed more positive result in reducing depression as well as hypercholesterolemia. Therefore germinated pumpkin seeds powder was used for further study like formulation of bread using Response Surface Methodology (RSM) and also for the assessment of supplementary impact on selected human subjects.

3.3.2. **Preparation of bread dough**

The composition of the dough for standard was; 100% flour, 8% sugar, 2% salt, yeast, 8% butter, 55% water. For optimum amount of ingredients to the germinated pumpkin seeds bread the composition of ingredients was changed.

Dough was prepared by using straight dough method. First, all the dry ingredients (flour, sugar, salt, and milk powder) were mixed for 1 min in a mixer at 58 rev/min. Then, yeast dissolved in 30°C water and melted butter along with pumpkin seed powder. Then, yeast dissolved in 30°C water and melted butter along with pumpkin seed powder was added to the dry ingredients. All the ingredients were again mixed for 2.5 min by the help of the same mixer at 85 rev/minutes and during mixing, water was added to the mixture. After mixing, the dough was fermented in an incubator at 30°C with 85% relative humidity.

The total fermentation time was 105 min. After the first 70 min, the dough was punched to remove the carbon dioxide and again placed into the incubator. The dough was punched for a second time after 35 min. Then, the dough was divided into 50 g
pieces and shaped. The shaped samples were placed in greased baking pan and again placed into the incubator for 20 min in order to maintain the proofing step, which is defined as the last fermentation. The samples were the ready for baking.

3.3.3. Experimental Design

Response Surface Methodology (RSM) was used to optimize the ingredients of the bread. RSM is a statistical technique that uses quantitative data to determine and simultaneously solve multivariate equations, which specify the optimum product for a specified set of factors through mathematical models. These models consider interactions among the test factors and can be used to determine how the product changes with changes in the factor levels (Giovanni, 1983). In this study, central composite design was used. There were three independent variables, such as wheat flour \( (X_1) \), germinated pumpkin seeds powder \( (X_2) \) and butter \( (X_3) \). For convenience actual values were converted to coded values. Table 2 shows coded and uncoded levels of variables used in the experiment. Experimental design is shown in Table 1.

**Table 1.**

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Symbol</th>
<th>Coded levels</th>
<th>Uncoded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1.68</td>
<td>-1</td>
</tr>
<tr>
<td>Wheat Flour</td>
<td>( X_1 )</td>
<td>56.59</td>
<td>60</td>
</tr>
<tr>
<td>Germinated Pumpkin seed powder</td>
<td>( X_2 )</td>
<td>21.59</td>
<td>25</td>
</tr>
<tr>
<td>Butter</td>
<td>( X_3 )</td>
<td>4.32</td>
<td>5</td>
</tr>
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</table>
Plate 6
Formulation of germinated pumpkin seeds bread

a. Ingredients

b. Mixing of ingredients

c. Dough for baking

d. Germinated pumpkin seeds bread
Table 2
Independent variables and their coded and actual values used in RSM optimisation

<table>
<thead>
<tr>
<th>Variation no.</th>
<th>Coded</th>
<th>Uncoded</th>
<th>Wheat flour (g) (X₁)</th>
<th>Germinated pumpkin seed flour (g) (X₂)</th>
<th>Butter (g) (X₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x₁</td>
<td>x₂</td>
<td>x₃</td>
<td></td>
<td></td>
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<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>65</td>
<td>30</td>
</tr>
</tbody>
</table>

\[
Y = \alpha_0 + \sum_{i=1}^{n=3} \alpha X_i + \sum_{j=i+1}^{n=1} \sum_{i=1}^{n} \alpha X_i X_j + \sum_{i=1}^{n} \alpha X_i^2
\]

Where, Y is response variable,

\( \alpha_0 \) - Constant and coefficient

\( \alpha_i \) - Linear coefficient

\( \alpha_{ii} \) - quadratic coefficient

\( \alpha_{ij} \) - cross product coefficient
\(X_i, X_j\) – levels of the independent variables

\(k\) – Number of the factors tested \((k = 3)\)

\(X_i\) and \(X_j\) are coded independent variables, i.e., \(x_1, x_2\) and \(x_3\) are coded levels of wheat flour \((X_1)\), germinated pumpkin seeds powder \((X_2)\) and butter \((X_3)\). The dependent variables for calculation of optimum product are Quality Measurements of bread like weight loss \((Y_1)\), porosity \((Y_2)\), specific volume \((Y_3)\), protein \((Y_4)\) and tryptophan \((Y_5)\).

The regression analysis was done using Design Expert Software. The adequacy of the model was tested using F-ratio and co-efficient of determination \((R^2)\). The model was generally considered adequate when (a) the calculated F ratio was more than table F-value and (b) the \(R^2\) value was more than 80%. The effect of variables at linear, quadratic and interactive levels on the response was described using significant at 1, 5 and 10% level. The counter plot was used to select the range of different ingredients required to get the desired level of response. All the responses under investigation for optimized using Design Expert Software to determined the individual optimum of above responses and level of different ingredients. This process gave 5 estimated formulations corresponding to the five optimized levels of responses. Quadratic model was used to describe the response variables.

3.3.4. Determination of dependent variables (Y variable)

The quality measurements were performed after baking the breads, inorder to determine the optimum point. The quality parameters were weight loss, specific volume, and porosity of the breads.

3.3.4.1. Weight Loss \((Y_1)\)

The weight loss of the breads was calculated by measuring the weight of the dough before and of the bread after the baking process. The following equation was used to express the weight loss:

\[
\text{Weight loss (\%)} = \frac{W_i - W_f \times 100}{W_i}
\]

Where,

\(W_i\): weight of the dough before baking,
3.3.4.2. Specific Volume *(Measurement Loaf bread volume)* *(Y₂)*

Loaf volume expressed in cubic centimeters was determined by the seed displacement method according to Pyler (1973). The loaf was placed in a container of known volume into which millet seeds were run until the container is full. The volume of a seed displaced by the loaf was considered as the loaf volume. The specific volume of the loaf was calculated according to the AACC (1986) by dividing volume of the loaf (cm³) by its weight (g). Triplicate measurements were taken.

\[
W_{seeds} = W_{total} - W_{bread} - W_{container}
\]

\[
V_{seeds} = W_{seeds} / \rho_{seeds}
\]

\[
V_{bread} = V_{container} - V_{seeds}
\]

Where, \( W \) represents ‘weight (g)’, \( V \) is ‘volume (cm³)’, and \( \rho \) is ‘density (g/cm³)’.

The specific volume was calculated by dividing the volume of the bread by its weight;

\[
SV_{bread} = V_{bread} / W_{bread}
\]

Where \( SV \) is the specific volume (cm³/g)

3.3.4.3. Porosity *(Y₃)*

Porosity was measured by using the method of Zanoni *et al.*, (1995). Porosity can be defined as the ratio of the volume of the pores to the total volume of the product:

\[
\varepsilon = (V_T - V_{np}) / V_T
\]

Where, 

\[
V_T = \text{total volume of the sample},
\]

\[
V_{np} = \text{volume of the non-porous material in the sample}.
\]

An apparatus having a constant basement area was designed, which allowed pores to be removed from the bread samples, to measure porosity. The prepared samples were put inside this apparatus and constant force was applied for 1 min. Since the basement area was constant, porosity can be defined as:

\[
\varepsilon = (H_0 - H_f) / H_0
\]

Where,
H0 = initial height of the sample (mm),
Hf = Height of the sample (mm) after compression

3.3.4.4. Protein (Y4) and tryptophan (Y5)

Protein and Tryptophan, content in germinated pumpkin seeds bread was calculated based on NIN food nutritive value and nutritive value analysis of germinated pumpkin seed powder by AOCS, (1998) method.

3.3.5. Organoleptic evaluation of germinated pumpkin seeds bread

Ten panellists who had completed a graduate course and were familiar with bread were chosen. Instructions were given in full to panellists beforehand. Bread samples were evaluated on a scale of 1 - 5 for five quality parameters: crust colour, crumb colour, external appearance and shape, taste and aroma, and mouth feel and texture. A ballot sheet was prepared to evaluate sensory attributes of breads after modifying parameters and scores of various flat breads to Lavash (Qarooni et al., 1987; Saxena and Rao, 1996). Consistency of the panel was checked by subjecting data for the indicated attributes from three replicate rating of bread samples to principal component analysis (Kwan and Kowalski, 1980; Powers, 1984). Samples, selected at random from the different treatments, were removed from polyethylene bags before evaluation. The breads were rated in comparison to regular wheat bread.

**Crust Colour**

(5) Creamish yellow colour with light brown patches
(4) Cream colour with light brown patches
(3) Light cream colour with light brown patches
(2) Lighter gray colour with pale or dark brown patches
(1) Gray colour with pale or dark brown patches

**Crumb Colour**

(5) Whitish cream colour
(4) Light yellowish cream colour
(3) Yellowish cream colour
(2) Light grayish cream colour
(1) Gray colour
External Appearance and Shape
(5) Crust is smooth with few blisters and few cracks on the edges
(4) 75% of crust is smooth with small amount of blisters and cracks on the edges
(3) 50% of crust is smooth with moderate amount of blisters and cracks on the edges
(2) 25% of crust is smooth with high levels of blisters and high levels of cracks on the edges
(1) Crust is not smooth, very high levels of blisters and cracks

Taste and Aroma
(5) Characteristic aroma and taste
(4) Light smell and taste from additive
(3) Perceptible smell and taste from additive
(2) Definite undesirable taste and smell from additive
(1) Very definite unacceptable smell and taste

Mouth feel and Texture
(5) Very pleasant and easy to chew
(4) Pleasant and easy to chew
(3) Slightly sticky when chewing
(2) Sticky when chewing
(1) Very sticky and doughy when chewing (waxy texture)

Ballot sheet for bread samples (Qarooni et al., 1987; Saxena and Rao, 1996) with some modifications.

3.3.6. Nutrient analysis of optimized bread and standard bread
The nutrients like protein, carbohydrate, energy, fat, calcium, zinc, magnesium, manganese and iron are calculated based on NIN nutritive value (Gopalan et al., 2011) and nutrients screening using AOAC method. Analysed optimized germinated pumpkin seed compared with standard bread.

3.3.7. Phytosterol analysis of optimized and standard bread
The phytosterol of the optimized germinated pumpkin seeds bread were compared with normal standard bread. The phytosterol was analysed using HPTLC Chromatogram.
Chromatographic conditions

Mobile phase: Chloroform: Acetone (15:1) or (20:1)
Tank saturation: 20 minutes
Solvent front: 90mm
Drying: 5 minutes
Detection/visualization: At 366nm, 254nm & after derivatization
Derivatization: Anisaldehyde sulphuric acid

3.3.8. Shelf life of evaluation of the optimized germinated pumpkin seeds bread

The optimized germinated pumpkin seeds bread was stored in room temperature. Sensory evaluation was done to the sample in 3 periods (1st day, 2nd and 3rd day of the storage).

PHASE –IV

3.4. Therapeutic trials of germinated pumpkin seeds bread on depressed and Hypercholesterolemic human models

3.4.1. Assessment of supplementary impact of optimized germinated pumpkin seeds Bread on depressed human models

Pumpkin seeds are nutritional power house. It is an excellent nutritional source of vitamins, minerals, healthy oils and fiber. 100 g of pumpkin seeds contributed 24.3 g of protein, 4.2g of fat, 584 kcal of energy and 830mg of phosphorus (Gopalan et al., 2011). Apart from the nutritional contribution of pumpkin seed, it also possesses various medicinal properties. Curcurbitin is a constituent in pumpkin seeds. The other principal active ingredients in pumpkin seeds are essential fatty acids, amino acids, and phytosterols. The processing especially germination, changes the amino acid and nutrients in pumpkin seeds and increased tryptophan which helps to reduce depression.

Pumpkin seeds are a good dietary source of tryptophan, which the body uses to make 5-Hydroxytryptophan (5HTP), increase the serotonin in the brain, which affect mood and regulate behavior. In this study the subjects consumed the supplement orally in bread form. The optimized bread used for supplementation contains 35 g germinated pumpkin seed, 70 g wheat flour and 5g butter. Typical dosage of tryptophan for sleep disorders and depression is 1-3 g daily (Frank,1999). In this study 100 gram of
germinated pumpkin seeds bread or standard bread used for supplementation in one month.

Based on the above studies the present work was carried out by the investigator to find out the effect of germinated pumpkin seeds bread on the reduction of depression among the selected subjects evaluated by Beck Depression inventory (BDI) compared by placebo group.

3.4. 1.1. Selection of the Area

Subjects were recruited from Stella Maris Hospital and Nazareth Institutions in Paduapuram at Ernakulam district, Kerala, India.

3.4.1.2. Selection of Subjects

A total of 360 adults from both sexes belonging to the age of 20-50 years were screened for supplementation. The Beck Depression Inventory (BDI) scale was used for screening. The Participants were categorized into normal, mild, moderate and severe depression based on BDI. Forty moderate depressed (Score -20-28) subjects, 16 male and 24 female were selected for the study.

The study was approved by the institutional ethics committee. All patients gave written informed consent and were recruited for the study.

3.4.1.3. Study Design

This study design was double-blind, randomized, placebo-controlled. The subjects randomized to supplement with germinated pumpkin seeds bread (Experiment group) or standard bread (placebo group) for one month. One person was taking optimized germinated pumpkin seeds bread and the next person was randomized to standard bread (placebo) and vice-versa. Effects of supplementation was determined based on BDI score compared with baseline (day 0) and end (day 30) of a one-month of supplementation period in all group of subjects.

3.4.1.4. Tools selected for data collection

Internationally accepted tools based on earlier studies were selected for data collection.

3.4.1.4.1. Interview schedule

A detailed interview schedule was developed by the investigator in order to elicit
information pertaining to the socio economic status, dietary pattern, health status and personal habits of the selected subjects. All the 40 subjects underwent an interview.

Interview schedule is a method of collecting data where the questions are asked and filled by the investigator with face to face contact with the subjects from whom the information is elicited (Gupta, 2006).

**3.4.1.4.2. Assessment of nutritional status**

The following techniques were employed to carry out the assessment of nutritional status

a) Anthropometric measurement

b) Dietary survey

**3.4.1.4.2.1. Assessment of anthropometric measurements**

Anthropometrics is the gold standard for assessment of nutritional status (Elizabeth, 2000). To add, anthropometry is the single point portable invasive method of assessing body composition reflecting health and nutrition, predicting performance, health and survival. While height is used to assess the past nutritional status, weight helps to assess the present. Body Mass Index (BMI) is frequently used as a popular and rapid clinical measure of relative obesity and malnutrition (Priyatomako et al., 2001).

While height is used to assess the past nutritional status, weight helps to assess the present. Body Mass Index (BMI) is frequently used as a popular and rapid clinical measure of relative obesity and malnutrition (Priyatomako et al., 2001).

Accordingly, the anthropometric indicators namely weight (kg), height (cm), BMI, waist and hip circumferences were measured for all the 40 depressive subjects.

**3.4.1.4.2.2. Weight**

Measurement of weight serves as the indicator to profess the presence and progress of ailment. The weight of all the selected subjects were determined by making them stand barefooted and erect on a portable weighing scale to the accuracy of 0.1kg before supplementation (Brahmam et al., 2005).

**3.4.1.4.2.3. Height**

Height is a constituent factor in the calculation of BMI and hence the height of all subjects was measured using a vertical measuring height chart. The subjects were made
to stand erect, barefooted on a leveled surface, with heels together and toes apart. A mark was made with a scale rested flat on the head (Brahmam et al., 2005).

3.4.1.4.2.4. Body Mass Index (BMI)

BMI determines if weight is appropriate for the height and thus has good correlation with fitness (Bamji et al., 2004). WHO (2001) has explained BMI as a simple index of weight for height that is commonly used to classify adults as
Plate 7
Anthropometric Measurements of the subjects

a. Height
b. Weight
c. Hip circumstance
d. Waist circumstance
underweight or overweight. BMI was calculated for all the selected subjects using the following formula, BMI = \frac{\text{Weight (kg)}}{\text{Height}^2 (\text{m}^2)}.

3.4.1.4.2.5. Waist circumference

Waist circumference was measured for all the selected subjects. The subjects were asked to stand erect with weight evenly balanced on both feet, which were placed about 25 to 30 cm apart. A mark was made at the level of the lowest rib margin. The iliac crest in the mid auxiliary line was felt and a mark was made. The measuring tape was passed around the waist horizontally midway between the lowest rib margin and iliac crest and the circumference in centimeter was measured up to the nearest millimeter. The observer sat on a stool in front of the subjects while taking the measurement (Brahmam et al., 2005).

3.4.1.4.2.6. Hip circumference

Hip circumference was measured for all the subjects. For measuring the hip circumference, the measuring tape was placed horizontally over the buttocks and the circumference was measured at the point yielding the maximum circumference in centimeter up to the nearest millimeter (Brahmam et al., 2005).

According to Boyle et al., (2001) the waist circumference should be taken at the narrowest circumference between ribs and hips. For all the selected subjects Waist Hip Ratio (WHR) was computed by dividing subject’s waist circumference in centimeter by hip circumference in centimeters.

3.4.1.4.2. Dietary survey

According to Bamji et al., (2004), diet is a vital determinant of health and nutritional status of people. Precise information of food consumption pattern of people through application of appropriate methodology is often needed not only for assessing the nutritional status of people, but also for elucidating the relationship of nutrient intake with deficiency as well as degenerative diseases. Precise information on food consumption pattern was collected through 24 hour recall method from the selected subjects. The raw food equivalent of the cooked food was determined and the intake of macro and micro nutrients was computed using the values given in the ‘Nutritive Value of the Indian Foods’ (ICMR, 2004).
underweight or overweight. BMI was calculated for all the selected subjects using the following formula, \( \text{BMI} = \frac{\text{Weight (kg)}}{\text{(Height) (m}^2\text{)}} \).

### 3.4.1.4.2.5. Waist circumference

Waist circumference was measured for all the selected subjects. The subjects were asked to stand erect with weight evenly balanced on both feet, which were placed about 25 to 30 cm apart. A mark was made at the level of the lowest rib margin. The iliac crest in the mid auxiliary line was felt and a mark was made. The measuring tape was passed around the waist horizontally midway between the lowest rib margin and iliac crest and the circumference in centimeter was measured up to the nearest millimeter. The observer sat on a stool in front of the subjects while taking the measurement (Brahmam et al., 2005).

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3.4.1.5. Assessment of depression

Beck Depression inventory scale was used for screening the moderate depressed subjects and analyses the effect of supplementation on the selected 40 subjects.

The selection of self-rating symptom scales has been mainly based on their concordance with the DSM-IV or ICD-10, with a couple of exceptions. Beck Depression Inventory (BDI) has been included due to its widespread use and as it represents the gold standard for self-rating depression scales.

The BDI-II (Beck et al., 1996) is a 21-item self-report depression screening measure. Each item is rated on a 4-point Likert-type scale ranging from 0 to 3, with higher scores indicating higher levels of depression. The measure asks respondents to endorse statements characterizing how they have been feeling throughout the past 2 weeks. The maximum total score for all 21 items is 63. According to Beck et al., 1996, scores of 0 to 13 denote minimal depression; scores of 14 to 19 denote mild depression, scores of 20 to 28 denote moderate depression, and scores of 29 to 63 denote severe depression.

Each of the inventory items corresponds to a specific category of depressive symptom and/or attitude. Each category purports to describe a specific behavioural manifestation of depression and consists of a graded series of four self-evaluative statements. The statements are rank ordered and weighted to reflect the range of severity of the symptom from neutral to maximum severity. Numerical values of 0, 1, 2, or 3 are assigned to each statement in order to indicate degree of severity. The 21 categories identified in the BDI are:

1. Sadness or Mood
2. Hopelessness or Pessimism
3. Sense of Failure
4. Anhedonia or Lack of satisfaction
5. Guilty Feelings
6. Sense of Punishment
7. Self-dislikes
8. Self-blame or Self-accusations
9. Suicidal Ideation
10. Crying
11. Agitation or Irritability
12. Loss of Interest in Activities or Social Withdrawal
13. Indecisiveness
14. Distortion of Body Image
15. Work Inhibition or Loss of energy
16. Insomnia or Sleep Disturbance
17. Fatigability or Irritability
18. Loss of Appetite
19. Weight Loss
20. Somatic Preoccupation or Fatigue
21. Loss of Libido

(Beck, 1961)

3.4.1.6. Conduct of the Supplementation Study for Depression

Based on Beck Depression inventory scale moderate depressive (Score between 20-28), 40 subjects were selected for supplementation. The selected subjects were divided into 2 groups, experimental group (20) and placebo group (20). Hundred grams of optimized germinated pumpkin seed bread for experimental group (contains 1 gm tryptophan) or the same amount of standard bread for placebo group supplemented daily for the period of one month. The optimized bread included, 35g germinated pumpkin seeds, 70 g wheat flour and 5 g butter.

3.4.1.7. Supplementation study for hypercholesterolemic human models

Pumpkin seeds have a high nutritional value, provides good quality oil, and excellent source of protein. The main monounsaturated fatty acids (MUFA) present in non-irradiated pumpkin seeds was oleic acid (17.2), with minute levels of erucic (0.71%) and palmitoleic acid (0.15). The major polyunsaturated fatty acid (PUFA) present was linoleic acid (52.64) with small amounts of linolenic acid (0.40). The major saturated fatty acids were palmitic acid (19.01) and stearic acid. Processing of pumpkin seeds significantly changes the fatty acid composition. Germination increased the unsaturated fatty acid and decreased the saturated fatty acids in pumpkin seeds. The
most dynamic component in pumpkin seeds is phytosterol is (β– sitosterol), it lowers hypercholesterolemia.

According to Hendrinks et al., (1999), supplementation of 0.8 g/day of plant sterols derived from three vegetable oils, for a period of 3.5 weeks, LDL-C concentration was significantly reduced by 8%. Results suggest that phytosterol esters can be used safely to provide “additional” cholesterol lowering effect conjunction with cholesterol-lowering drugs.

The subjects consumed the germinated pumpkin seeds bread or standard bread as supplementation. The optimized bread developed with the proportion of 35g germinated pumpkin seeds, 70g wheat flour and 5g butter. In this study 200 gram of optimized germinated pumpkin seeds bread or standard bread was used for supplementation in one month.

Based on the above study the present work was carried out by the investigator to find out the effect of germinated pumpkin seeds supplementation on the reduction of hypercholesterolemia in human model.

3.4. 2.1. Selection of the Area

Subjects were selected from Stella Maris Hospital, Paduapuram at Ernakulam district, Kerala, India.

3.4.2.2. Selection of Subjects

For assessing the effect of pumpkin seeds on hypercholesterolemia, 60 hypercholesterolemic subjects of both sexes (female-42, male-18) belonging to the age of 20-50 years were selected. The subjects were already on medication but they were still hypercholesterolemic. Subjects were selected based on screening values for total cholesterol (TC) 225mg/dl to 300mg/dl, triacylglycerol (TG) concentrations above 150mg/dl, LDL cholesterol 125mg/dl to 232 mg/ dl (between 3.25 and 6.0 mmol/l) and HDL cholesterol below 45mg/dl and subjects were excluded from this study if they are diabetic patient.

The study was approved by the institutional ethics committee. All patients gave written informed consent and were recruited for the study.

3.4.2.3. Study Design

The study design was double-blind, randomized, placebo-controlled. The subjects
randomized to supplement with germinated pumpkin seeds bread (Experiment group) or a placebo (placebo group) for one month. One subject was taking optimized germinated pumpkin seed bread and the next person was randomized to standard bread (placebo) and vice-versa. Effects of treatment were compared at the beginning (day 0) and the end (day 30) of a one-month supplementation period in two groups of subjects.

3.4.2.4. Tools selected for data collection

Internationally accepted tools based on earlier studies were selected for data collection.

3.4.2.4.1. Interview schedule

A detailed interview schedule was developed by the investigator in order to elicit information pertaining to the socio economic status, dietary pattern, health status and personal habits of the selected subjects. All the 60 subjects were undergone for interview. Interview schedule is a method of collecting data where the questions are asked and filled by the investigator with the face to face contact with the subjects from whom the information is elicited (Gupta, 2006)

3.4.2.4.2. Assessment of nutritional status

The following techniques were employed to carry out the assessment of nutritional status;

a) Anthropometric measurement
b) Biochemical study
c) Dietary survey

The analysis of anthropometry and dietary survey were done the same methods used in the study of supplementary impact of optimized germinated pumpkin seeds bread on depressed human models.

3.4.2.4.3. Biochemical analysis for determination of hypercholesterolemia

The lipid profile like total serum Cholesterol (TC), triglyceride (TG) and low density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and very low density lipoprotein (VLDL) and were analyzed in initial (baseline) and final (after 30 day) day of supplementation.

Blood was collected in morning (fasting condition) and separated the serum. Serum was used for estimation of total cholesterol (TG), triglycerides (TG) by GPO Pap
and high density Lipoprotein-cholesterol (HDL-C) by direct method using commercial enzymatic kits (Randox, UK) and Photometer model BTR-830 (Biotech, Spain). Low density lipoprotein-cholesterol (LDL-C) and very low density lipoprotein-cholesterol (VLDL-C) were calculated using Friedewald's formula.

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

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

In the development of any deficiency diseases, biochemical changes can be expected to occur prior to clinical manifestations. Therefore biochemical tests which can be conducted on easily accessible, body fluids such as blood and urine, can help to diagnose disease at the sub clinical stage, and confirm clinical diagnosis at the disease stage. An ideal biochemical test should be sensitive, specific, easy to carry out, noninvasive, preferably inexpensive and should reveal information on the extent of tissue instaurations rather than short term fluctuations in the diet (Bamji, et al., 2004).

### 3.4.2.5. Conduct of the Supplementation Study for Hypercholesterolemic subjects

Sixty people suffering from hypercholesterolemia, who were already on medication but still hypercholesterolemic were selected for supplementation.

The samples divided into experimental (30 subjects) and placebo (30 subjects) group. Two hundred gram (per day) of optimized germinated pumpkin seed bread (provide 804 mg phytosterol daily) supplemented to the experimental group and same quantity standard bread, supplemented for placebo group as twice in a day. Lipid profile was used to assess the effect of optimized germinated pumpkin seed bread or placebo into the subjects. It was done before and after the supplementation.

### 3.5. Statistical analysis

The data was compiled and analyzed by using statistical methods. The results are represented as Descriptive statistics mean, standard error mean, standard deviation, one way ANOVA, followed by Duncan’s multiple comparison tests. A p-values <0.05 were considered significant. Differences in baseline characters were analyzed by t-test. Paired comparison test are computed using statistical software IBM SPPS Statistics (Version 19, 2010). Duncan’s multiple range tests were applied to determine the significant differences between samples. Design Expert version 8.0 was used to analyze the optimization techniques.