3 Methodology

The methodology adopted for the present study entitled **"ANTIOXIDANT POTENTIAL OF *Cucurbita pepo* L. (PUMPKIN) SEED EXTRACT IN THE TREATMENT OF STRESS INDUCED MALE INFERTILITY: AN IN VIVO STUDY"** is discussed under the following phases:

PHASE I

3.1. Nutritional Analysis of *Cucurbita pepo* L. Seeds

3.1.1. Collection and certification of *Cucurbita pepo* L. Seeds

3.1.2. Analysis of nutrient content in *Cucurbita pepo* L. seeds

3.1.3. Antinutrient analysis of *Cucurbita pepo* L. seeds

PHASE II

3.2. Phytochemical Analyses of *Cucurbita pepo* L. Seed Extracts

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3.2.2. Quantitative phytochemical analysis of *Cucurbita pepo* L. seed aqueous extract

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PHASE III

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3.3.3. Superoxide radical scavenging assay

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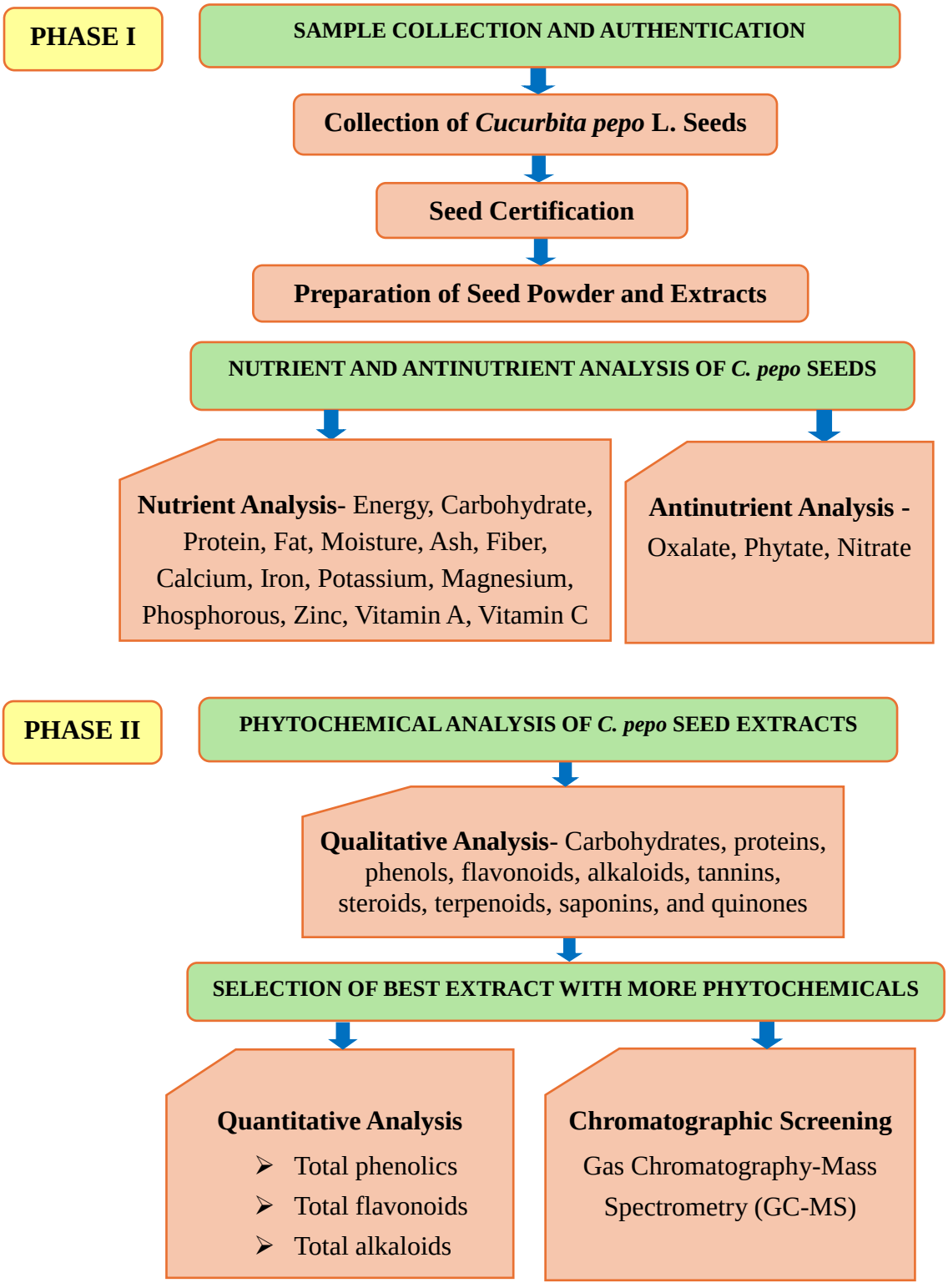
3.5.3. Determination of the effects of *Cucurbita pepo* L. seed extract on stress induced infertile male rats in association with reproductive hormones

3.5.4. Determination of the effects of *Cucurbita pepo* L. seed extract on stress induced infertile male rats in association with antioxidant status

3.5.5. Histopathology of reproductive organs in experimental male rats

3.5.6. Consolidation, statistical analysis and interpretation of the data

The research design of the methodology followed for the study is given in Figure 1.


PHASE I
SAMPLE COLLECTION AND AUTHENTICATION
Collection of *Cucurbita pepo* L. Seeds
Seed Certification
Preparation of Seed Powder and Extracts
NUTRIENT AND ANTINUTRIENT ANALYSIS OF *C. pepo* SEEDS
Nutrient Analysis- Energy, Carbohydrate, Protein, Fat, Moisture, Ash, Fiber, Calcium, Iron, Potassium, Magnesium, Phosphorous, Zinc, Vitamin A, Vitamin C
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PHASE II
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SELECTION OF BEST EXTRACT WITH MORE PHYTOCHEMICALS
Quantitative Analysis
Total phenolics
Total flavonoids
Total alkaloids
Chromatographic Screening
Gas Chromatography-Mass Spectrometry (GC-MS)

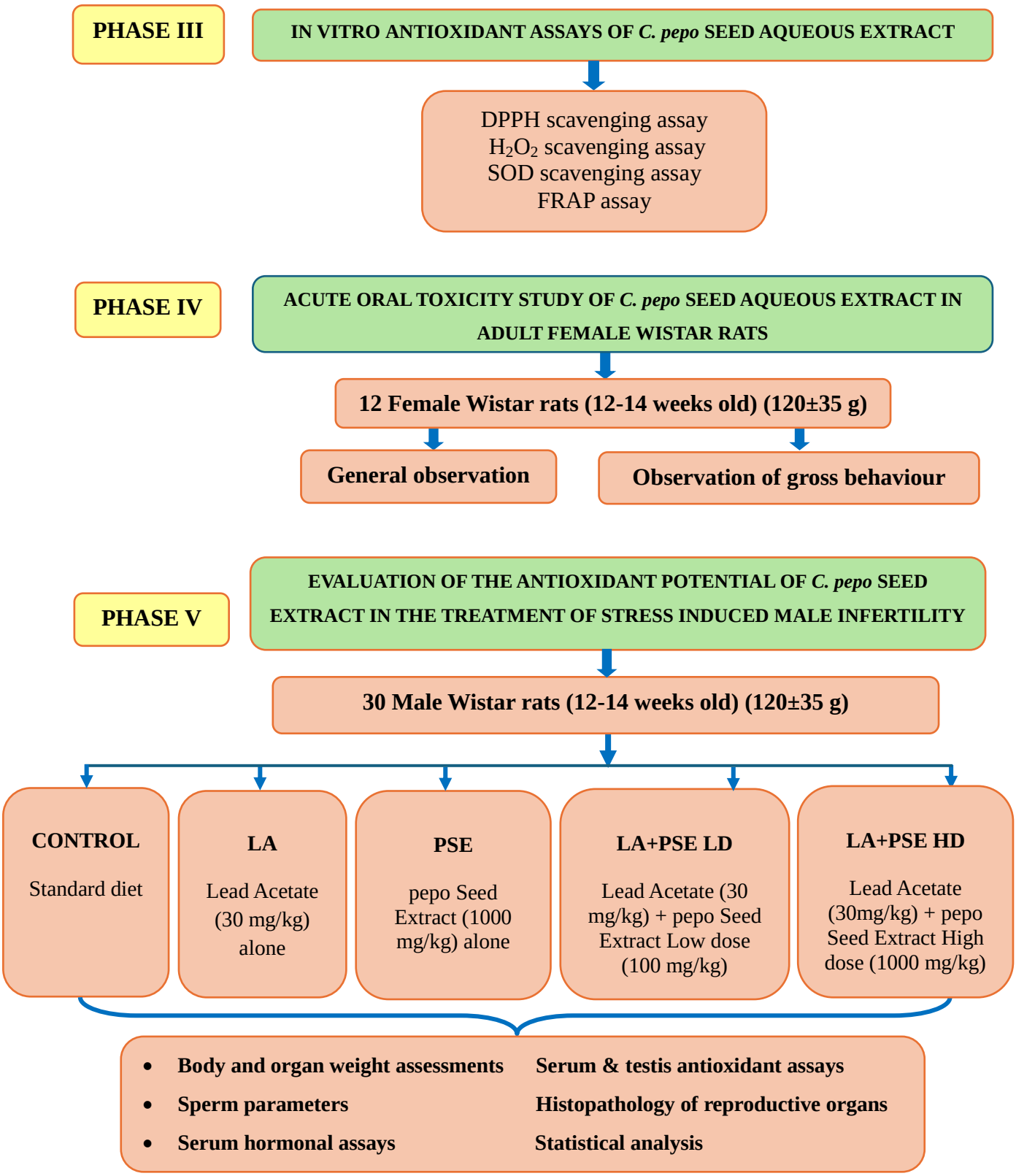

Figure 1: Research Design of the Methodology

PHASE I

3.1. Nutritional Analysis of *Cucurbita pepo* L. Seeds

3.1.1. Collection and certification of *Cucurbita pepo* L. seeds

The *Cucurbita pepo* L. seeds were procured from the Tudiyalur region in Coimbatore district **(Figure 2)**. The seeds were then submitted to a taxonomist at the Botanical Survey of India, Southern regional centre, Tamil Nadu Agricultural University, Coimbatore, for taxonomic certification. The sample seeds were verified and duly certified (voucher specimen number BSI/SRC/5/23/2021/Tech/282) **(Appendix III)**. The collected seeds are depicted in **Plate 1**.

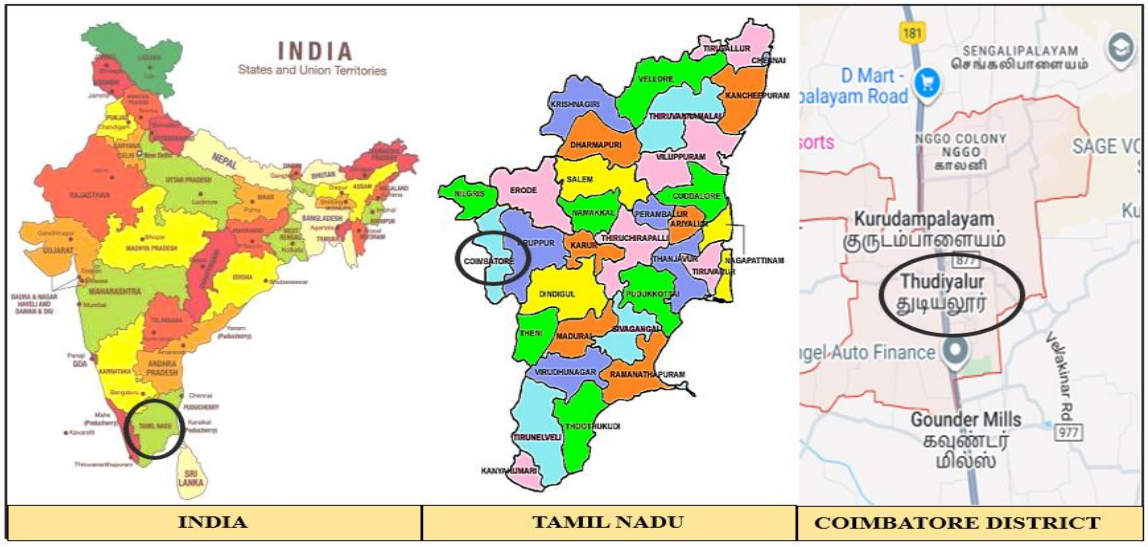

Figure 2: Locale of the Sample Collection

Plate 1: *Cucurbita pepo* L. seeds

3.1.2. Analysis of nutrient content in *Cucurbita pepo* L. seeds

Cucurbita pepo L. seeds are potent alternative sources of oil and protein and could be used in innovative food formulations. Pumpkin seeds are gaining global attention since they possess multiple health benefits. They are rich sources of omega-3 fatty acids (linoleic acid, palmitic acid, oleic acid, and stearic acid) and these unsaturated fatty acids are known to enhance memory, prevent cancer, and lowers inflammation in the body (Batool *et al*., 2022; Abdelnour *et al*., 2023). These seeds are also dense in protein, essential vitamins (thiamine, riboflavin, ascorbic acid, α-tocopherol) along with minerals such as magnesium, potassium, iron, sodium, selenium, phosphorus, zinc, and manganese in addition to phenolic compounds and lutein (Devi *et al*., 2018).

Standard procedures outlined by AOAC, (2005) was employed to determine the nutritional content of *Cucurbita pepo* L. seeds. All components were analyzed in triplicates. Estimation of nutrients **(Appendix I)** was carried out in following methods: ash by incineration using muffle furnace (P-LAB Selec DTC204), moisture content by oven drying (Technico hot air oven), crude fat analysis by Soxhlet method (Socs Plus SCS 6) using petroleum ether (60-80ºC), protein estimation using Kjeldahl (Foss KjelTec^{TM} 2100), crude fiber analysis using digestion (Fibra Plus FES 8), and minerals and vitamin A by colorimetry methods. Vitamin C was estimated by 2,6-Dichloro phenol indophenol dye method. Estimation of carbohydrate was done using the formula given below:

Estimation of Carbohydrate=100- [% protein+% lipid+ fiber+% ash+% moisture]

The calorific value (energy) was calculated by multiplying the percent of crude protein, crude fat, and carbohydrate by 4, 9, and 4 factors, respectively as follows.

Energy (kcal) = 4× (Carbohydrate + protein mass in gram) + 9× (mass of fat in grams)

3.1.3. Antinutrient analysis of *Cucurbita pepo* L. seeds

The nutritional value of foods is influenced not only by their nutrient composition but also by the presence of antinutrients. Antinutrients, either natural or synthetic, interfere with nutrient absorption, reduces nutrient intake, digestion, and utilization, and potentially cause other negative effects. While the antinutrient content in vegetables, whole grains, legumes, and nuts may raise concerns, these effects are primarily an issue when the diet consists solely of raw plant foods. Common antinutrients found in plant-based foods include oxalates,

phytates, and nitrates. For example, oxalates bind to calcium, hindering its absorption in the body (Savage and Klunklin, 2018). Phytates, which are found in grains, nuts, and seeds, can reduce the absorption of essential minerals when consumed in excess (Gupta *et al*., 2015). The methods used to measure these antinutrients are described below.

i) Oxalate

1 gram of seed flour was dissolved in 75 mL of 1.5 N sulphuric acid. The mixture was agitated continuously for 1 hour and then filtered. A 25 mL portion of the filtrate was warmed and titrated with hot potassium permanganate solution (0.1 M) till a pink colour persists for 30 seconds and considered as endpoint (Badu *et al*., 2020).

The oxalate content was estimated as: Oxalate = (titre value x 0.9004) mg/gram

ii) Phytate

The seed flour (4 gram) was added to 100 mL of 2% HCl, agitated for 3 hours, filtered, and 5 mL ammonium thiocyanate (0.3 percent) was added to 25 mL of this filtrate. The solution was titrated against a ferric chloride solution of 0.00195 g/ mL (Badu *et al*., 2020). The phytate content was estimated as:

% phytate = [8.24 (titre value)]/ (1000 x weight of sample) x100

iii) Nitrate

A 5 mL portion of the digest was measured and transferred into a beaker, where it was evaporated to dryness. To this residue, 2 mL of phenol disulphonic acid was added, followed by dilution with 20 mL of distilled water. Ammonia solution (7 mL) was then introduced to the mixture until a yellow permanent colour appeared. The yellow-coloured solution was subsequently transferred to a 50 mL volumetric flask and made up to the mark with distilled water. The absorbance of the solution was measured at 410 nm using a Spectronic 21 spectrophotometer (Milton Roy Co., Rochester, NY, USA), as described by Onyesom and Okoh (2006).

PHASE II

3.2. Phytochemical Analyses of *Cucurbita pepo* L. Seed Extracts

All plants, including grains, legumes, fruits, and vegetables, produce phytochemicals as part of their natural defense system against viral, bacterial, fungal, and parasitic threats. When consumed, these phytochemicals can provide similar protection to humans. Although they are not essential nutrients, their intake helps human cells shield against damage instigated by environmental toxins and the body's natural metabolic processes. The majority of phytochemicals act as antioxidants, substances that protect the body primarily by neutralizing Reactive Oxygen Species (ROS). Foods rich in antioxidants reduce the risk of various chronic degenerative diseases (Sharifi-Rad *et al*., 2020). In addition, when antioxidants are incorporated into foods, they help prevent lipid peroxidation, thereby preserving food quality and potentially extending shelf life (Leyva-Porras *et al*., 2021).

Preparation of *Cucurbita pepo* L. seed extracts

Cucurbita pepo L. seeds were washed under running tap water in order to get rid of any adhering debris or impurities. The cleaned seeds were shade dried for a period of two weeks. The seeds were weighed and coarsely powdered using mortar and pestle and transferred to conical flasks. The solvents were chosen depending upon their increasing order of polarity viz. Chloroform (CF), Ethyl Acetate (EA), Acetone (AC), Methanol (ME) and Aqueous (AQ). Maceration is the extraction technique that involves soaking the dry powder of plant part in a closed container for a definite period of time with frequent agitation (Tiwari *et al*., 2011). 2 grams of *Cucurbita pepo* L. seed powder was measured and dissolved in 20 mL of each solvent and kept in a mechanical shaker overnight at 50ºC for 75-100 rpm. The individual solutions were filtered using Whatman no.1 filter paper and the extracts were collected. The collected extracts were stored in the refrigerator at 4ºC for further analyses. **Plate 2** depicts the process of extraction.

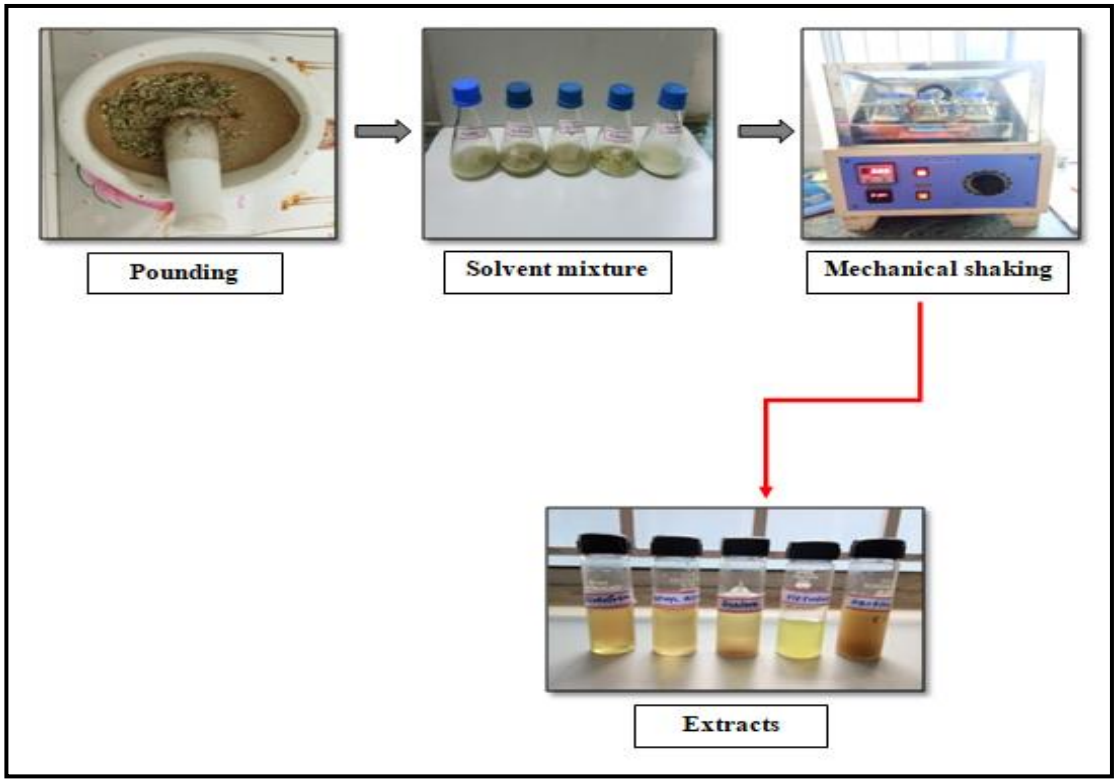

Plate 2: The Process of Extraction

3.2.1. Qualitative phytochemical analysis of *Cucurbita pepo* L. seed extracts

i) Alkaloids (Mayer's test)

To 1 mL of extract, 1 mL of Mayer's reagent and a few drops of Iodine solution were added. Formation of yellow colour indicated the presence of alkaloids (Tiwari *et al*., 2011).

ii) Terpenoids (Salkowski test)

To 1 mL of crude extract, 1 mL of concentrated Sulphuric acid was added and kept in hot water bath for 2-4 minutes. Formation of greyish colour indicated the presence of terpenoids (Sheel and Nisha, 2014).

iii) Phenolics and Tannins (Ferric chloride test)

To 1 mL of crude extract, 1 mL of Ferric chloride was added. Formation of blue green or black colour indicated the presence of tannins (Iyengar, 1995).

iv) Carbohydrates (Molisch test)

To 2 mL of extract, 2-3 drops of Molisch reagent (10% solution of α-naphthol in ethanol) was added. Subsequently, 2 mL of concentrated Sulphuric acid was added down the

side of the test tube so that it forms a layer beneath the aqueous solution without mixing. A purple ring at the interface of the two layers indicated the presence of carbohydrates (Schreck and Loffredo, 2017).

v) Saponins (Foam test)

To 1 mL of extract, 2 mL of distilled water was added. The solution was shaken well and the formation of one centimeter layer of foam indicated the presence of saponins (Ndam *et al*., 2014).

vi) Flavonoids (Shinoda's test)

To 1 mL of extract, a few fragments of Magnesium ribbon and a few drops of concentrated Hydrochloric acid were added. Appearance of scarlet pink colour confirmed the presence of flavonoids (Siddiqui and Ali, 1997).

vii) Quinones

To 1 mL of extract, 1 mL of 1% Sodium hydroxide was added. Appearance of blue green/red colour indicated the presence of quinones (Archana *et al*., 2012).

viii) Proteins (Xanthoproteic test)

To 1 mL of extract, a few drops of Mercuric chloric acid or Nitric acid were added. Formation of yellow colour indicated the presence of protein (Tiwari *et al*., 2011).

ix) Steroids (Salkowski's test)

To 1 mL of extract, 1 mL of Chloroform and 1 mL of concentrated Sulphuric acid were added along the sides of the test tube. Formation of red colour at the lower chloroform layer indicated the presence of steroids (Siddiqui and Ali, 1997).

Justification for selecting aqueous extract for further study

Based on the high polar property, detection of more phytochemicals and water being the universal cooking medium in addition to the safety of administration to experimental animals, aqueous extract was used for further analyses.

3.2.2. Quantitative analyses of phytochemicals in *Cucurbita pepo* L. seed aqueous extract

For the purpose of quantifying these phytochemicals, *Cucurbita pepo* L. seed aqueous extract was analyzed for quantification of total phenolics, total flavonoid and total alkaloids using standard assays **(Appendix II).**

i) Total phenolics

The total phenolic content of *Cucurbita pepo* L. seed aqueous extract was done by using Folin-Ciocalteu's method of Bhalodia *et al*., (2011). The standard curve of gallic acid (GAE) was plotted between absorbance versus concentrations, and the unknown sample concentration was calculated in terms of mg GAE/100g. All the estimations were carried out in three replicates.

ii) Total flavonoids

The total flavonoid content of *Cucurbita pepo* L. seed aqueous extract was determined by following the method of Pallab *et al*., (2013). A linear calibration curve of quercetin (QE) was used to calculate the concentration of flavonoid content and expressed as milligrams of quercetin equivalent mg QE/100 g. All the determinations were carried out in triplicates.

iii) Total alkaloids

Accurately measured aliquots (0.4, 0.6, 0.8, 1, and 1.2 mL) of atropine standard solution were transferred into different separatory funnels. To each funnel, 5 mL of pH 4.7 phosphate buffer and 5 mL of Bromocresol Green (BCG) solution were added. The mixture was then shaken with extracts using 1, 2, 3, and 4 mL of chloroform. The resulting extracts were collected in 10 mL volumetric flasks and diluted with chloroform to the mark. The absorbance of the resulting complex in chloroform was measured at 470 nm using a UV-Spectrophotometer (SHIMADZU UV-1800), with the blank prepared in the same way but without the addition of atropine (Ajanal *et al*., 2012).

3.2.3. Chromatographic screening of bioactive compounds in *Cucurbita pepo* L. seed extract

GC-MS screening of secondary metabolites in *Cucurbita pepo* L. seed extract

According to Yeshi *et al*., (2022), secondary metabolites produced by plants are biologically active. In general, secondary metabolites are important sources because of their properties and structural diversity. Thus, the active compounds present in *Cucurbita pepo* L. seed extract were identified using Gas Chromatography Mass Spectrometry (GC-MS). Molecular structure and molecular mass of the phytochemical compounds were screened by GC-MS analysis as per the method of Srinivasan *et al*., (2014).

To 10 grams of *Cucurbita pepo* L. seed powder, 80 mL of ethanol was added for soaking and left overnight. The mixture was then filtered through Whatman filter paper No. 41, with the addition of 2 g of sodium sulfate to remove any sediments and trace amounts of water in the filtrate. Prior to filtration, the filter paper was wetted with ethanol. The resulting filtrate was concentrated to 1 mL by passing nitrogen gas through the solution. Both polar and non-polar phytocomponents were present in the extract. The chemical composition of 1mL of the extract was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The mass spectrum data were interpreted using the National Institute of Standards and Technology (NIST) database, which contains over 162,000 reference patterns. Peak area normalization was employed to quantify the compounds.

PHASE III

3.3. In vitro Antioxidant Activity of *Cucurbita pepo* L. Seed Aqueous Extract

3.3.1. DPPH radical scavenging assay

To determine the antioxidant activity of purified phenolics and organic plant extracts, the DPPH (1,1-diphenyl -2-picrylhydrazyl) assay is widely used (Geng *et al*., 2015). According to Celik *et al*., (2010), spectrophotometric methods for the estimation of antioxidant capacity in plant extracts are commonly used due to the simple, speedy, sensitive, and repeatable procedures involved in assays based on the use of 1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) radical.

Different concentrations (10–100 µg/mL) of the extract were prepared by diluting 1 mL of the extract in ethanol and then mixed with 1 mL of a 0.2 mM DPPH solution in ethanol as the free radical source. The mixture was incubated for 30 minutes at room temperature, and the absorbance was measured at 517 nm. L-Ascorbic acid was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100,$$

where A_0 represents the absorbance of the control (without the sample), and A_1 represents the absorbance of the sample.

3.3.2. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging capacity of *Cucurbita pepo* L. seed extract was determined using the method of Ruch *et al*., (1989). A 40 mM hydrogen peroxide solution was prepared in phosphate buffer (pH 7.4). To this solution, 100 µg/mL of the extract in distilled water was added. The absorbance of hydrogen peroxide at 230 nm was measured using a hydrogen peroxide-free phosphate buffer as the blank. The percentage of hydrogen peroxide scavenged by the *Cucurbita pepo* L. seed extract was calculated using the following formula:

$$\text{\% recovered } [H_2O_2] = [(Ac\text{-}As)/Ac] \times 100,$$

where Ac is the absorbance of the control (hydrogen peroxide solution without extract), and As is the absorbance of the sample (hydrogen peroxide solution with extract).

3.3.3. Superoxide radical scavenging assay

In this experiment, superoxide radicals were generated in 3 mL of sodium phosphate buffer (100 mM, pH 7.4), which contained 1 mL of Nitroblue tetrazolium (NBT) solution (150 µM), 1 mL of Nicotinamide Adenine Dinucleotide (NADH) solution (468 µM), and varying concentrations of the seed extract (20–100 µg/mL) dissolved in water. The reaction was initiated by adding 1 mL of PMS solution (60 µM) to the mixture. After incubating the reaction mixture at 25°C for 5 minutes, absorbance was measured against the corresponding blank. L-Ascorbic acid was used as a positive control. The reduction in NBT, indicated by the

change in absorbance, was inversely related to the superoxide radical scavenging activity of the seed extract (Kostyuk *et al*., 2007). The superoxide radical scavenging activity was calculated using the formula:

$$\text{Superoxide radical scavenging activity (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

3.3.4. Ferric Reducing Antioxidant Power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) assay of the extract was conducted following the method outlined by Oyaizu (1986). Substances with a reduction potential react with potassium ferricyanide to form potassium ferrocyanide, which then interacts with ferric chloride to produce a ferric-ferrous complex that absorbs at 700 nm. As the ferric ion is reduced to ferrous ion, the absorbance increases, indicating the extract's reducing ability.

Various concentrations of aqueous seed extract of *Cucurbita pepo* L. (20–100 µg/mL in double-distilled water) were mixed with 2.5 mL of phosphate buffer and 2.5 mL of 1% potassium ferricyanide solution. The mixture was incubated at 50°C for 20 minutes. After incubation, 1.5 mL of trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. From each tube, 0.5 mL of the supernatant was mixed with 1 mL of distilled water and 0.5 mL of ferric chloride solution. The absorbance was then measured at 700 nm using a spectrophotometer. An increase in absorbance indicated a higher reducing power of the extract. Water served as the blank.

PHASE IV

3.4. Acute Toxicity Study of *Cucurbita pepo* L. Seed Aqueous Extract in Adult Female Wistar Rats

Toxicity testing of new compounds is a crucial step in the drug development process. Preclinical toxicity testing, conducted across various biological systems, helps identify species-, organ-, and dose-specific toxic effects of an investigational product (Parasuraman, 2011). Acute toxicity studies are designed to determine the lethal dose or concentration (LD_{50}) of a substance, which is the amount that causes death in 50% of the test population during short-term exposure. These studies are the only standardized toxicity tests in which

death is the intended endpoint. A fundamental principle of toxicology, known as dose-response, involves evaluating clinical effects based on the amount of exposure. The dose refers to the total amount of a chemical absorbed during exposure. It depends on both the concentration of the chemical and the duration (contact time) of the exposure.

Acute oral toxicity study

An acute oral toxicity study was performed prior to the extract supplementation study to arrive at the maximum safety dosage of aqueous extract of *Cucurbita pepo* L. seeds for administration in experimental animals. Acute oral toxicity defined as toxic effects which occur within 24 hours after supplementation of a single or multiple doses of any substance by oral route (OECD, 2001). The study was carried out as per OECD 423 guidelines.

Selection of animals

Twelve healthy female (nulliparous and non-pregnant) rats of Wistar strain (12-14 weeks old with mean body weight 120 gram) were selected to conduct acute toxicity study. According to OECD (2000), the preferred rodent species for acute toxicity study is female rats. Lipnick *et al.,* (1995) pointed out that studies of conventional LD_{50} tests demonstrated that there was a slight variation detected in sensitivity amongst male and female and female animals were found to be extra sensitive than male.

Housing and feeding of animals

Twelve female Wistar rats were selected to conduct acute toxicity study. Animals were housed in polypropylene cages. Temperature was maintained at 22±3ºC and in a sequence of 12 hours of light/dark cycles all throughout the study period. Humidity in the laboratory was 40-60 percent. The animals were fed with commercially available normal rat chow (dehulled soybean meal, ground corn, wheat middling's, ground wheat, soybean oil, cane molasses, ground oats, calcium carbonate, dried beet pulp, dehydrated alfalfa meal, wheat germ, brewer' dried yeast, salt, calcium propionate, di-calcium phosphate) with water *ad libitum*. Prior to dosing, rats were kept in cages for one week for acclimatization in the laboratory conditions. While the extract was fed to the rats, utmost care was taken to avoid the entry of the drug from gavage to the respiratory system of the rats. After the administration of the stipulated quantity of the extract, further uptake of feed except water by the test animals was ceased for

3-4 hours and the animals were supervised for 24 hours. Test animals were continuously examined for treatment related behavioural changes and observations were recorded.

Grouping of animals

The animals were randomly categorized into 4 groups containing 3 animals in each group. Grouping of animals for the acute toxicity study was as follows.

Control: Rats treated with oral dose of distilled water

EG I: Rats treated with 50 mg/kg body weight of *Cucurbita pepo* L. seed aqueous extract

EG II: Rats treated with 300 mg/kg body weight of *Cucurbita pepo* L. seed aqueous extract

EG III: Rats treated with 2000 mg/kg body weight of *Cucurbita pepo* L. seed aqueous extract

Preparation of extract

Aqueous extract of *Cucurbita pepo* L. seeds was administered to the animals. Aqueous extract was prepared by maceration extraction technique which involves immersing of dried and powdered seed sample in water in an air tight container for a variable time. After which the extract was intermittently stirred for a period of one week and filtered. The filtrate was used for subsequent experiments.

Administration of extract

Doses varying from 50 mg, 300 mg and 2000 mg//kg body weight (b.w.) of aqueous extract of *Cucurbita pepo* L. seeds were administered to the animals by oral gavage tube. Prior to administration of dosage, animals were fasted overnight and weighed for recording body weight. Food was withheld for 3-4 hours soon after administration of extracts.

3.4.1. General observation of acute toxicity of *Cucurbita pepo* L. seed extract in adult female rats

Measurement of body weight

Before beginning drug administration, on the 7th day, and on the 14th day, the body weight of the animals in all the groups was noted by weighing them in a weighing balance (ISHTAA-ITA/ITB SW series).

3.4.2. Observation of gross behaviour of acute toxicity of *Cucurbita pepo* L. seed extract in adult female rats

Following dosage administration, the animals were carefully observed for the first 30 minutes and occasionally for the first 24 hours, with special attention given to gross behaviour such as Autonomous Nervous System (ANS) and Central Nervous System (CNS) activity during the first four hours. When compared to the control group, the animals were observed for clinical signs and symptoms of acute toxicity and/or mortality, abnormalities, food and water intake, and changes in body weight for 14 days.

PHASE V

3.5. Evaluation of the Antioxidant Potential of *Cucurbita pepo* L. Seed Aqueous Extract in the Treatment of Stress Induced Male Infertility

Selection of experimental animals

Wistar rats (*Rattus norvegicus*) are among the most commonly used rat strains in research, valued for their calm temperament, consistent size, and ease of handling, as well as their high survival rate. These rats play a crucial role in biomedical research, serving as valuable models for studying human and animal health. Research involving Wistar rats contributes to a better understanding of disease mechanisms, the effects of drugs, responses to treatments, and gene expression, among other areas. They are also instrumental in the development and safety evaluation of new medications.

This study involved thirty (30) healthy adult male Wistar rats (12-14 weeks old), with a mean weight of 120±35 gram. The rats were obtained from Biogen Animal Facility in Bangalore (971/PO/RcBiBt/S/06/CPCSEA) and had not been previously used in any other experimental studies. The animals were housed in clean aluminium wire cages with sterile

paddy husk bedding, which was changed weekly along with regular cage cleaning. They were kept at a temperature of 28±2ºC, with 75–80% relative humidity, under a 12-hour light/dark cycle. The rats had unrestricted access to a standard rat pellet diet and water (Offor *et al*., 2017). The animals were given one week to acclimatize before the study began. All procedures followed ethical guidelines and were approved by the Institutional Animal Ethical Committee of the Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore (Ethical Approval No: AIW:IAEC.2020:FSN:02) **(Appendix IV)**.

Experimental design

After one week of acclimatization period, the animals were randomly categorized into 5 groups containing 6 animals in each group. The duration for the study was 45 days. The lead acetate and *Cucurbita pepo* L. seed aqueous extract administered via oral gavage on 15th, 30th and 45th day of study. The grouping was done as follows.

Control: Rats were treated with oral dose of distilled water

LA: Rats were treated with lead acetate solution (30 mg/kg body weight) alone

PSE: Rats were treated with pepo seed extract (1000 mg/kg body weight) alone

LA+PSE LD: Rats were treated with lead acetate solution (30 mg/kg body weight) and pepo seed low dosage extract (100 mg/ kg body weight)

LA+PSE HD: Rats were treated with lead acetate solution (30 mg/kg body weight) and pepo seed high dosage extract (1000 mg/ kg body weight)

Induction of stress

Induction of oxidative stress in experimental animals was achieved by administration of lead acetate. The lead acetate doses were provided to the animals based on earlier researches. Lead acetate trihydrate was sourced from Oxford Lab. Co., India. It was dissolved in distilled water to prepare a 1% solution, with a concentration of 30 mg/kg body weight. The lead acetate solution was administered intermittently to 18 rats excluding control group and *Cucurbita pepo* L. seed aqueous extract high dosage alone treated group (PSE) on 15th, 30th and 45th day of the study.

3.5.1. Determination of the effects of *Cucurbita pepo* L. seed extract on stress induced infertile male rats in association with physical parameters

i) Body weight assessment

The body weights of the animals were assessed during the initial day (1st day) of the experimental study and on the day of sacrifice (45th day) by using an electronic weighing balance (ISHTAA-ITA/ITB SW series)

ii) Individual organ weight assessment

Within 24 hours after the final dose, the animals were sacrificed via decapitation. The following organs—brain, kidney, liver, caudal epididymis, prostate, seminal vesicles, and testes—were carefully excised according to the standard operating procedure **(Plate 3)**. They were then thoroughly cleaned in phosphate buffer to remove any adhering fat. After washing, the weights of each organ were recorded **(Plate 4)**.

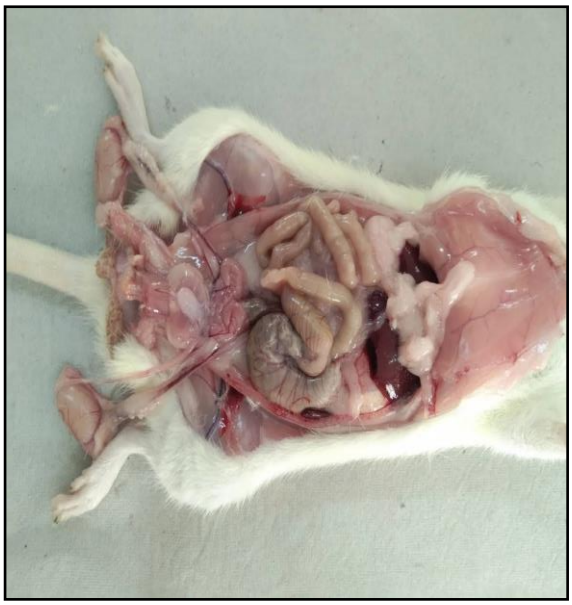

Plate 3: Organ harvesting

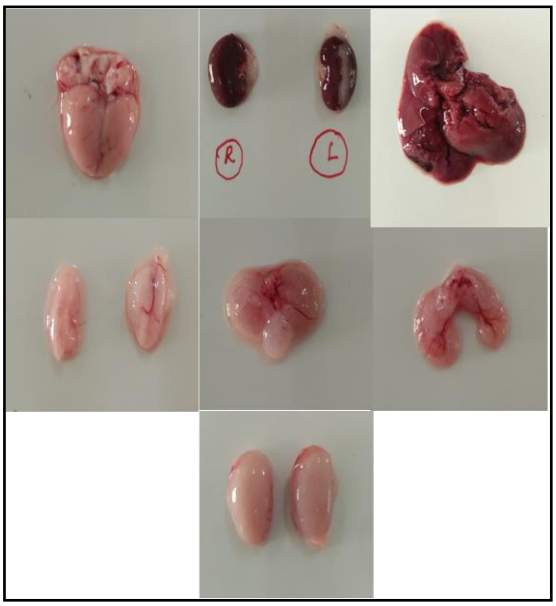

Plate 4: Organ weight assessment

3.5.2. Determination of the effects of *Cucurbita pepo* L. seed extract on stress induced infertile male rats in association with sperm parameters

Sperm collection

The caudal epididymis of each rat was carefully separated from the testes as described by Raji *et al*., (2005). It was then minced in 1 mL of phosphate-buffered saline (PBS) at pH 7.2 and incised to facilitate the release of sperm from the epididymis. To obtain the epididymal fluid, the minced tissue was gently swirled in the buffer solution to allow proper diffusion of the sperm cells. Approximately 50 µL of the sperm concentrate was then pipetted, diluted 20 times with phosphate-buffered saline, and used for the assessment of sperm parameters.

i) Sperm count

Sperm counting was performed using the improved two-chambered Neubauer haemocytometer. The original sperm suspension was thoroughly shaken to ensure even dispersion of the sperm. Approximately 10 µL of the diluted sperm suspension was added to each chamber and allowed to stand for 5 minutes. Counting was carried out following the method described by Raji *et al*., (2005). The sperm concentration was expressed as sperm number $\times 10^6$/mL.

ii) Sperm motility

A sufficient quantity of the prepared sperm suspension was introduced into the counting chamber. After charging the haemocytometer, the chamber was allowed to stand for two minutes to allow immotile sperm to settle. Sperm cells within five blocks of the 16 small squares, which constitute 1/5th of the entire field (80 squares in total), were counted as described by WHO (2010). Sperm cells that lay on the left and top lines of the squares were included in the count, while those on the bottom and right lines were excluded.

$$\text{Sperm motility (\%)} = \frac{\text{No. of motile sperms}}{\text{Total number of sperms}} \times 100$$

iii) Sperm viability

Sperm viability was assessed as described by Sudjarwo *et al.*, (2017). Two drops of eosin stain solution (1% dissolved in distilled water) were added to a test tube consisting one drop of the freshly collected sperm suspension. The eosin stain stained the heads of dead sperm (pink), while the heads of viable sperm remained colourless. Sperm viability was determined by counting the viable and non-viable sperm in each chamber. Viable sperm do not absorb the eosin stain, while non-viable sperm absorb it. The dye exclusion was evaluated by examining 100 sperm cells.

$$\text{Sperm viability (\%)} = \frac{\text{Total viable sperms observed}}{\text{Total number of sperms observed}} \times 100$$

iv) Semen pH

The semen sample was thoroughly mixed, and a drop of semen was evenly spread onto the pH paper. After allowing less than 30 seconds for the colour in the impregnated zone to become uniform, the resulting colour was compared with the calibration strip to determine the pH.

v) Sperm morphology

Sperm morphology was done by eosin-negrosin staining method. For the same, about 10 µL of eosin and negrosin stain mixture was mixed with 40µL volume of sperm cell suspension. After incubating in the room temperature for staining, the cells were resuspended using a Pasteur pipette to microscopic slides. At 40-100X magnifications in the light microscope, the number of spermatozoa with abnormal morphology were counted and recorded in percentage.

3.5.3. Determination of the effects of *Cucurbita pepo* L. seed extract on stress induced infertile male rats in association with reproductive hormones

Blood collection

After 45 days of treatment period, blood was collected from retro-orbital plexus of rats using capillary tubes under mild anaesthesia with ether. Blood samples from each rat were collected in EDTA (anti-coagulant) containing fresh vials and separated serum in

cooling electric centrifuge (REMI Cooling Centrifuge–VCBF-1322) at 3000 rpm for 10 minutes for further estimation of biomarkers. The serum (supernatant) was collected and stored at −20ºC for biochemical analyses (Ochei and Kolhatkar 2000). Serum level of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) were estimated using a Rat/Mouse ELISA kit (Cosmo Bio Co. Ltd. Japan) as per the manufacturer's instructions.

i) Assay of Testosterone

The serum testosterone concentration was estimated quantitatively using the human serum testosterone enzyme immunoassay kit, following the manufacturer's protocol. The measurement was based on a direct (competitive) assay principle, utilizing the general antibody-antigen reaction in an ELISA format, as outlined by Tietz (1995) and Span Diagnostics, Surat, India.

ii) Assay of Luteinizing Hormone (LH)

The LH concentration was determined using a solid-phase Enzyme-Linked Immunosorbent Assay (ELISA). In this assay, mouse monoclonal anti-α-LH antibodies were immobilized on the solid phase (microtiter wells), while mouse monoclonal anti-β-LH antibodies were used in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample reacted with both antibodies, resulting in LH molecules being sandwiched between the solid-phase and enzyme-linked antibodies. After a 45-minute incubation at room temperature, the wells were washed with water to remove any unbound labelled antibodies. TMB reagent was added, and after a 20-minute incubation, a blue colour developed. The colour development was stopped by adding the stop solution, changing the colour to yellow, which was then measured spectrophotometrically at 450 nm. The LH concentration was directly proportional to the colour intensity of the test sample.

iii) Assay of Follicle Stimulating Hormone (FSH)

The FSH concentration was determined using ELISA kit. In this assay, anti-Bovine FSH antibody was used for the immobilization on the solid phase (microtiter wells), while a goat anti-FSH antibody conjugated to horseradish peroxidase enzyme was used in the

conjugate solution. The test sample was allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 3-hour incubation period, the wells were washed with buffer to remove any unbound labelled antibodies. TMB solution was added, and the mixture was incubated for 20 minutes, resulting in the development of a blue colour. The colour development was stopped by adding 2N HCl, and the absorbance was measured spectrophotometrically at 450 nm. The intensity of the colour formed was proportional to the amount of enzyme present and inversely related to the amount of unlabelled FSH in the sample. By comparing the results to a series of FSH standards assayed in the same way, the concentration of FSH in the unknown sample was quantified.

3.5.4. Determination of the effects of *Cucurbita pepo* L. seed extract on stress induced infertile male rats in association with antioxidant status

Disruption of the balance between Reactive Oxygen Species (ROS) and the antioxidant system can lead to oxidative stress, which plays a significant role in the pathophysiology of various diseases (Liguori *et al*., 2018). Herbal antioxidants have been widely used as rejuvenators for centuries in Indian systems of alternative medicine (Yadav *et al*., 2020). Therefore, assessing the tissue antioxidant status is crucial to understanding the effects of *Cucurbita pepo* L. seed extracts on infertile male rats.

Serum preparation

Blood was collected in a dry test tube and allowed to clot at ambient temperature for 30 minutes. Serum was then separated by centrifugation at 2000 rpm for 10 minutes. The right testis was immediately fixed in formalin solution for subsequent histopathological analysis, while the left testis was used for homogenization and subsequent biochemical assay studies.

Preparation of the tissue homogenate

Rats were sacrificed and dissected. A vertical midline incision was made in the thoracic and abdominal regions to expose their viscera. Specimens from the testes were homogenized in a 1.17% Potassium chloride (KCl) solution, and ice-cold phosphate buffer with a molarity of 0.1 and pH 7.4 was used to prepare a 10% homogenate of each tissue

sample. For the estimation of lipid peroxidation levels, a portion of the homogenate was used, and the remaining solution was centrifuged at 10,000 rpm for 10 minutes at 4ºC (in a cold centrifuge). The supernatant was collected and stored at -20°C for lipid peroxidation and antioxidant analyses (Ochei and Kolhatkar, 2000).

i) Determination of Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is a metal-containing protein that catalyses the dismutation of the highly reactive superoxide anion (O_2^-) into oxygen (O_2) and less reactive hydrogen peroxide (H_2O_2). The activity of SOD in testis homogenate was measured using the method described by Kakkar *et al*., (1984).

To assay SOD activity, 0.5 mL of testis homogenate was diluted with an equal volume of deionized water. To this, 0.25 mL of ethanol and 0.15 mL of chilled chloroform were added, and the mixture was shaken for 1 minute before centrifuging at 2000 rpm. 1.5 mL of buffer was then added to 0.5 mL of the supernatant. The reaction was initiated by adding 0.4 mL of epinephrine, and the change in optical density per minute was measured at 480 nm using a double beam UV-VIS spectrophotometer (UV 1700, Shimadzu). SOD activity was expressed as units/min/mg of tissue protein. One unit of enzyme activity was defined as the change in optical density per minute at 50% inhibition of the adrenochrome transition.

ii) Determination of Glutathione peroxidase (GPx)

Glutathione peroxidase is a cytosolic enzyme that catalyses the reduction of hydrogen peroxide to water and oxygen, as well as the reduction of peroxide radicals to alcohol and oxygen, thus limiting their harmful effects. The activity of glutathione peroxidase was measured using the method described by Rotruck *et al*., (1973).

To determine glutathione peroxidase activity, the following reagents were mixed: 0.2 mL of EDTA, sodium azide, reduced glutathione, hydrogen peroxide (H_2O_2), 0.4 mL of buffer, and 0.1 mL of enzyme (testis homogenate). The mixture was incubated at 37°C for 10 minutes. The reaction was stopped by adding 0.5 mL of trichloroacetic acid (TCA), and the tubes were then centrifuged. To 0.5 mL of the supernatant, 3 mL of sodium hydrogen phosphate and 1 mL of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) were added. The colour

developed was read immediately at 412 nm using a double-beam UV-VIS spectrophotometer. Peroxidase activity in serum was expressed as μg/mg of tissue.

iii) Determination of Catalase (CAT)

Catalase is an important enzyme that catalyses the decomposition of hydrogen peroxide into water and oxygen. It is present in all major body organs, with particularly high concentrations in the liver and erythrocytes. During β-oxidation of fatty acids by flavoprotein dehydrogenase, hydrogen peroxide is produced, which is then broken down by catalase in peroxisomes. The reaction catalysed by catalase is as follows:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

To assess catalase activity, 0.1 mL of testis homogenate was mixed with 1 mL of phosphate buffer and hydrogen peroxide. The reaction was stopped by adding 0.2 mL of dichromate acetic acid reagent. Standard hydrogen peroxide solutions, ranging from 4 to 20 μL, were treated in the same manner. The tubes were placed in a boiling water bath for 10 minutes. The green colour developed in the reaction was measured at 570 nm using a double-beam UV-VIS spectrophotometer. Catalase activity was expressed as μmoles of H_2O_2 consumed per minute per milligram of protein, following the method described by Sinha (1972). The high levels of hydrogen peroxide (H_2O_2) can inhibit catalase activity. This method is based on the reduction of dichromate to chromic acetate in acetic acid when heated in the presence of water, forming an unstable perchromic acid.

iv) Determination of Lipid Peroxidation (MDA)

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acid peroxidation in cells. An increase in free radicals leads to the overproduction of MDA, making it a common marker of oxidative stress.

To measure MDA levels, 1 mL of testis homogenate was mixed with 0.2 mL of 4% (w/v) sodium dodecyl sulfate, 1.5 mL of 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5), and 15 mL of 0.8% thiobarbituric acid (TBA, pH 7.4). The mixture was incubated in a hot water bath at 85°C for 1 hour. Afterward, the developed pink colour was read at 532 nm against a reagent blank following centrifugation at 1200 g for 10 minutes. The concentration

of MDA was expressed as n moles of MDA per mg of protein, using 1,1,3,3-tetra-ethoxypropane as the standard (Ohkawa *et al*., 1979).

3.5.5. Histopathology of reproductive organs in experimental male rats

Histopathology is the microscopic examination of tissues to detect pathological changes. This process involves the collection of affected tissues through biopsy or necropsy, followed by fixation, section preparation, staining, and microscopic analysis.

After the final dose, the animals were sacrificed within 24 hours by decapitation. The reproductive organs, including the testes, caudal epididymis, seminal vesicles, and prostate, were excised, weighed, rinsed in saline, and preserved in 10% neutral buffered formalin for histopathological examination. The tissues were then dehydrated in a series of increasing concentrations of alcohol and cleared with xylene. After clearing, the tissues were embedded in paraffin wax, and thin sections (about 5 µm) were cut using a microtome. Each section was mounted on a clean glass slide, stained with Haematoxylin and Eosin, and a mounting medium (Canada balsam) was applied. A cover slip was placed on each slide and allowed to dry. The tissue sections were then examined under a light microscope, and photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic China Group Ltd.) attached to the microscope. Each tissue section was evaluated for histological changes, including epithelial atrophy, interstitial edema, and vacuolation of epithelial cells.

3.5.6. Consolidation, statistical analysis and interpretation of the data

The data obtained from the experimental study were analyzed statistically using IBM SPSS (Statistical Package for Social Sciences) version 21. Descriptive statistics were used to represent the values, including the mean and standard deviation. Intra-group variations were assessed using One-Way ANOVA, followed by Dunnett’s post-hoc comparison test. The results from the animal model experiments were compared between the control and treatment groups to evaluate statistical significance. A p-value of <0.001, <0.01, and <0.05 was considered statistically significant.