SPECIMEN FORMAT FOR THESIS OF THE MONTH

School	:	Home Science
Department	:	Food Science and Nutrition
Branch/ Area:	:	Oxidative stress, Environmental Toxicology, Male infertility, In Vivo study
Sub Subject Heading:	:	-
Candidate's Name	:	Amrutha B. Nair
Candidate's Address with email	:	Chirakkara House, Vilang, Elerithattu PO, Nileshwar Via, Kasaragod dt, Kerala-671314 Email: bnairamrutha93@gmail.com
Title of the thesis	:	ANTIOXIDANT POTENTIAL OF *Cucurbita pepo* L. (PUMPKIN) SEED EXTRACT IN THE TREATMENT OF STRESS INDUCED MALE INFERTILITY: AN IN VIVO STUDY
(i) In Roman Script (ii) In roman Script		– –
Nomenclature of Degree:	:	Ph.D. in Food Science and Nutrition
Month & Year of Enrolment:	:	09-02-2017
Month & Year of Registration:	:	09-02-2017
Month &Year of Submission:	:	09-01-2025
Month &Year of Award	:	09-01-2026
Name of Supervisor	:	Dr. P.A. Raajeswari,
Designation of Supervisor	:	Professor & Head
Centre/department/school in which research was conducted	:	Department of Food Science and Nutrition Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043

Abstract (within 300 words)

Infertility is a growing global health concern, with male factors contributing to nearly 50% of reported cases. Environmental toxicants such as lead are known to impair male reproductive function primarily through oxidative stress. Pumpkin (*Cucurbita pepo* L.) seeds, often discarded as biowaste, are rich in bioactive compounds and possess potential antioxidant properties. However, their role in stress-induced male infertility remains inadequately explored. The present study investigated the ameliorative effects of *C. pepo* seed aqueous extract against lead acetate induced reproductive toxicity in male Wistar rats. The study was conducted in five phases involving nutritional and antinutritional profiling, phytochemical and chromatographic analyses, in vitro antioxidant assays, acute oral toxicity evaluation, and an in vivo experimental study. Thirty rats were divided into five groups: control, lead acetate (30 mg/kg bw), seed extract alone (1000 mg/kg bw), lead acetate with low-dose extract (100 mg/kg bw), and lead acetate with high-dose extract (1000 mg/kg bw). Oral administration was carried out intermittently (15^{th}, 30^{th} and 45^{th} days) for a duration of 45 days. The parameters such as body weight and individual organ weight measurements, sperm parameters, hormonal assays, serum and testis antioxidant assays, and histopathology analysis of reproductive organs were carried out using established methodologies. Lead acetate exposure resulted in significant reductions in body and individual organ weights, sperm count, motility, viability, reproductive hormone levels (FSH, LH and testosterone) and antioxidant enzyme levels (SOD, GPx, catalase) along with increased sperm abnormalities, semen pH, lipid peroxidation (MDA), and histopathological alterations in reproductive organ tissues. Co-administration of *C. pepo* seed extract, particularly at the higher dose, significantly ameliorated these alterations in a dose-dependent manner. The key finding of this research was that the *C. pepo* seed extract treatment has mitigated lead induced toxicity and exhibited significant improvement in their reproductive potential owing to the antioxidant property of their phytochemical components.

Keywords: *Male Infertility, Oxidative Stress, Lead Acetate, Cucurbita pepo* L. *Seeds, Antioxidant Potential*

i) Major objectives:

- Assess the nutritional composition of *Cucurbita pepo* L. seeds
- Screen the presence of phytochemical constituents and identify the bioactive compounds in *Cucurbita pepo* L. seed extract
- Assess the in vitro antioxidant activity of *Cucurbita pepo* L. seed extract
- Evaluate *Cucurbita pepo* L. seed extract for its antioxidant potential in treating stress induced infertility in male Wistar rats

ii) Hypothesis:

- H_0- Male infertility will not be ameliorated by the antioxidant potential of *Cucurbita pepo* L. seeds

iii) Methodology:

The study was carried out in 5 phases. In Phase I, *Cucurbita pepo* L. seeds were collected from the natural habitat, an organic farm in Tudiyalur, Coimbatore district, Tamil Nadu. The collected seeds were identified, authenticated and duly certified by a taxonomist and a voucher specimen number was obtained for the same (BSI/SRC/5/23/2021/Tech/282). The seeds were dehusked and washed thoroughly under running tap water in order to remove any adhering debris. The seeds were shade dried for two weeks in room temperature. The dried seeds were ground and then sieved in 1mm sieve to get a fine powder. The powder was packed in an air tight container for further analyses.

In phase II, qualitative and quantitative phytochemical analyses, as well as the identification of bioactive compounds through GC-MS, were carried out. Qualitative estimation of phytochemicals was conducted using chloroform, ethyl acetate, acetone, methanol, and aqueous extracts of *Cucurbita pepo* L. seeds, following standard procedures. Carbohydrates, proteins, phenols, flavonoids, alkaloids, tannins, steroids, terpenoids, saponins, and quinones were detected qualitatively. The aqueous extract of *Cucurbita pepo* L. seeds, which showed the highest presence of phytochemicals, was selected for administration in stress-induced infertile male Wistar rats. Quantitative analysis was performed on the aqueous extract of *Cucurbita pepo* L. seeds to determine total phenolics, flavonoids, and alkaloids. The identification of secondary metabolites in the *Cucurbita pepo* L. seed extract was conducted using GC-MS.

In Phase III, the aqueous extract of *Cucurbita pepo* L. seeds was examined for its in vitro antioxidant activity using several assays. These included the DPPH radical scavenging assay, Hydrogen Peroxide radical scavenging assay, Superoxide radical scavenging assay (SOD),

and Ferric Reducing Antioxidant Power assay (FRAP), all conducted using standard procedures.

In Phase IV, an acute oral toxicity study was conducted to determine the safe dosage of *Cucurbita pepo* L. seed aqueous extract for administration to experimental rats. Twelve Wistar albino female rats, 12-14 weeks old with a mean body weight (BW) of 120±35 grams, were selected for the acute oral toxicity study. The study was conducted according to OECD 423 guidelines. Animals were assigned into four groups, group I served as control administered with standard diet, EG I administered with low dose 50 mg/kg b.w., EG II with 300 mg/kg b.w. and EG III with 2000 mg/kg b.w. of *Cucurbita pepo* L. seed aqueous extract. The animals were monitored for any kind of toxicity symptoms for duration of 14 days. A single dosage administration was carried out *via* oral gavage.

In Phase V, the animal study was approved by the Institutional Animal Ethical Committee of Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, with the ethical number AIW:IAEC.2020:FSN:02. Thirty adult male Wistar rats, 12-14 weeks old with a mean body weight (BW) of 120±35 grams, were selected for the study. After one week of acclimatization, the male rats (n=30) were divided into five groups: Group I served as the control and was administered with standard diet; Group II (LA) was administered with lead acetate alone (30 mg/kg BW); Group III (PSE) was administered with high dosage of *Cucurbita pepo* L. seed aqueous extract alone (1000 mg/kg BW); Group IV (LA+PSE LD) was co-treated with lead acetate (30 mg/kg BW) and a low dosage (100 mg/kg BW) of *Cucurbita pepo* L. seed aqueous extract and Group V (LA+PSE HD) was co-treated with lead acetate (30 mg/kg BW) and a high dosage (1000 mg/kg BW) of *Cucurbita pepo* L. seed aqueous extract. The dosage administration was carried out by oral gavage on 15th, 30th and 45th day of the experimentation.

Physical parameters, such as body weight and the weights of the individual organs such as liver, brain, kidney, testes, caudal epididymis, prostate, and seminal vesicle were assessed using electronic weighing balance. Sperm parameters such as count, motility, viability, and abnormality, were analyzed using staining techniques and microscopical examination. Semen pH was tested using pH paper. Serum reproductive hormonal analysis (luteinizing hormone, follicle-stimulating hormone, and testosterone) and antioxidant assays (serum superoxide dismutase, glutathione peroxidase, catalase, lipid peroxidation (malondialdehyde) and testicular tissue superoxide dismutase, catalase, glutathione peroxidase, and lipid peroxidation concentration were carried out. Histopathology analysis was conducted using staining

techniques and microscopical examination at varying magnifications for reproductive organs such as testis, prostate, caudal epididymis and seminal vesicle.

Statistical analysis

All data were analyzed using software IBM SPSS V. 21.0. One way ANOVA with Dunnett's comparison test was carried out for statistical analysis and data interpretation. P values <0.001, <0.01 and <0.05 were accepted as statistically significant values.

iv) Salient Findings:

- *Cucurbita pepo* L. seed powder contained ash (4%), moisture (4.5%), energy (559.4 kcal), carbohydrates (11.5±0.98g), protein (29.8±0.72g), total fat (43.8±1.02g), dietary fibre (6.4±0.65g), calcium (48±0.25mg), iron (7.5±0.06mg), phosphorous (900±1.10 mg), potassium (450±0.98 mg), magnesium (520±0.43mg), zinc (8.2±0.12 mg), vitamin A (13.3±0.13 µg) and vitamin C (2.6±0.18mg) per 100 g.

- *Cucurbita pepo* L. seed powder contained only negligible amount of antinutrients such as oxalates (0.16±0.05 mg), phytates (8.24±0.18 mg) and nitrates (2.11±0.04 mg) per 100 g.

- Among chloroform, acetone, ethyl acetate, methanol and aqueous extracts of *Cucurbita pepo* L. seeds, aqueous and methanol extracts contained most of the phytochemicals and a few were in appreciable levels. However, only aqueous extract showed the presence of all the phytochemicals under investigation in it. Based on the presence of phytochemicals, the aqueous extract may exhibit more therapeutic properties as compared to other extracts. Hence aqueous extract was chosen to carry out the further study.

- Total phenolics using Folin–Ciocalteu method (F-C) method analyzed in the *Cucurbita pepo* L. seed aqueous extract revealed a higher phenolic content (32.7±0.89 mg GAE/100g), total flavonoids analyzed using Aluminium chloride method revealed 16.8±0.63 mg QE/100g and total alkaloid revealed 11.3±1.12 mg AE/100g thus confirming the quantity of these major bioactive compounds.

- GC-MS screening on secondary metabolites in *Cucurbita pepo* L. seed extract identified seventeen prominent peaks with compounds such as 1-Tetradecanol, 1-Octadecene, n-Hexadecanoic acid (Palmitic acid), Hexadecanoic acid, ethyl ester, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, Stigmasterol, .gamma.-Sitosterol, Squalene, Undec-10-ynoic acid, tetradecyl ester, 1,6,10,14,18,22-

Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-, .gamma.Tocopherol, .gamma.-Sitostenone that are considered to possess antioxidant, anti-inflammatory, anti-tumour, cancer and chemo preventive, diuretic, immunostimulant, anti-ageing, gastro and hepatoprotective, detoxifier, antimicrobial, anti-arthritic, anti-obesity, cardio and neuroprotective, anti-degenerative, prostate protective, vasodilator, antispasmodic, hypolipidemic, analgesic, anti-androgenic activities.

- DPPH radical scavenging assay revealed that *Cucurbita pepo* L. seed aqueous extract showed the percentage of inhibition (IC_{50}) at 90.35 µg/mL whereas for standard ascorbic acid, it was found to be 46.94 µg/mL.

- Hydrogen peroxide scavenging assay indicated an inhibition of the radicals with IC_{50} values 153.33 (µg/mL) and 245.81 (µg/mL) exhibited by the standard ascorbic acid and the *Cucurbita pepo* L. seed aqueous extract respectively.

- Superoxide dismutase scavenging assay indicated an excellent scavenging activity with IC_{50} values 63.18 µg/mL and 39.15 µg/mL for the *Cucurbita pepo* L. seed aqueous extract and ascorbic acid respectively.
- Ferric Reducing Antioxidant Power (FRAP) assay revealed an IC_{50} of 50 µg/mL and 25.57µg/mL for *Cucurbita pepo* L. seed aqueous extract and ascorbic acid respectively. The above findings demonstrated the in vitro antioxidant activity of *Cucurbita pepo* L. seed aqueous extract, which might be due to the presence of phenolic and flavonoid compounds.

- A single dosage of *Cucurbita pepo* L. seed aqueous extract ranging from 50 mg/kg body weight (low dose) to 2000 mg/kg body weight (high dose) was orally administered to female rats and were observed for 14 days. None of the rats in the experimental study showed toxicity signs in their general behaviour. Central nervous system stimulant activities such as irritability and hyperactivity exhibited immediately after supplementation of high dosage of 2000 mg/kg body weight which was subsided after 3-4 hours.

- Central Nervous System (CNS) depression activities namely hypoactivity, passivity, relaxation, and ataxia were absent in the experimental groups after supplementation of *Cucurbita pepo* L. seed aqueous extract. The Autonomous Nervous System (ANS) activities such as salivation and frequent urination after supplementation of high dosage (2000 mg/kg body weight) got diminished after 3-4 hours.

- At the end of the study (after 45 days), body weight assessment revealed a reduction in body weight of LA (lead acetate alone) group when compared to control and other treated groups. On the other hand, PSE (*Cucurbita pepo* L. seed aqueous extract high dosage alone) group exhibited a weight gain similar to that of control group. A dose dependent restoring effect on the body weight was observed in LA+PSE LD and LA+PSE HD groups.
- Organ weight assessment indicated that detoxifying and excretory organs such as liver and kidney were susceptible to lead toxicity as evidenced by their weight increments in LA group. There was also a remarkable reduction in the weights of reproductive organs such as testes, seminal vesicle and prostate observed in LA group. However, there was no significant alteration to cauda epididymis and brain weights in all the treated groups. *Cucurbita pepo* L. seed extract when administered alone showed no significant difference in any of the organs when compared to control group whereas co-administration with lead acetate exhibited a dose dependent reversal effect on the altered organ weights.
- Sperm count results are indicative of severe lead toxicity induced oxidative stress as evidenced by a significant reduction of total number of sperms in LA group when compared to the control. On the other hand, *Cucurbita pepo* L. seed extract when treated alone showed a significant increment in the sperm count when compared to control. The co-treatment of seed extract with lead acetate dose dependently mitigated the lead toxicity by improving the sperm count as evidenced by LA+ PSE LD & LA+ PSE HD groups.
- Sperm motility results indicated that there was a remarkable decline of motile sperms in the lead acetate treated alone LA group which was dose dependently improved upon the administration of *Cucurbita pepo* L. seed extract as evidenced by LA+PSE LD & LA+ PSE HD groups. Interestingly, *Cucurbita pepo* L. seed extract alone treated group PSE revealed a significantly higher percentage of sperms motility when compared to the control group.
- Sperm viability results indicated that there was a notable reduction in the percentage of viable sperms in the LA group which was dose dependently improved upon the administration of *Cucurbita pepo* L. seed extract as evidenced by LA+PSE LD & LA+ PSE HD groups. *Cucurbita pepo* L. seed extract alone treated group PSE revealed a higher percentage of viable sperms compared to control group.
- Sperm abnormality results indicated a remarkable increment in the percentage of total abnormal sperms in the lead acetate alone treated group. There was dose dependent

decrement observed upon the co-administration of *Cucurbita pepo* L. seed extract as evidenced in LA+PSE LD & LA+ PSE HD groups. *Cucurbita pepo* L. seed extract alone treated group PSE revealed a lower percentage of abnormal sperms when compared to control group.

- Semen pH results revealed that lead acetate alone treated group exhibited a highly significant increment in the pH making it highly alkaline in nature. On the other hand, *Cucurbita pepo* L. seed extract maintained a neutral pH (ideally 7.2-8) which is desirable for an effective sperm health.
- Serum antioxidant assays disclosed a highly significant reduction in the Superoxide dismutase, Glutathione peroxidase, and Catalase levels in the lead acetate alone treated LA group. LA group also markedly increased the lipid peroxidation as evidenced by high MDA level indicating the high oxidative stress induced by lead. Co-administration of *Cucurbita pepo* L. seed extract improved the antioxidant levels in a dose dependent manner and decreased the lipid peroxidation to a great extent. *Cucurbita pepo* L. seed extract when administered alone showed a highly significant improvement in these antioxidant levels when compared to control.
- Testicular tissue antioxidant assays indicated a highly significant decline in the Superoxide dismutase, Glutathione peroxidase, and catalase levels of LA group. LA group also markedly increased the lipid peroxidation as evidenced by high Malondialdehyde MDA level. Co-administration of *Cucurbita pepo* L. seed extract in both low and high dosages improved these antioxidant levels besides decreasing the lipid peroxidation in a concentration dependent manner. *Cucurbita pepo* L. seed extract when administered alone showed a highly significant increment in these antioxidants when compared to control.
- Serum reproductive hormonal assays indicated that LA group exhibited a highly significant reduction in Follicle Stimulating Hormone, Luteinizing Hormone and testosterone levels when compared to control group. *Cucurbita pepo* L. seed extract treated alone PSE group retained the LH level intact similar to control group indicating no significant alteration in the hormone levels. However, co-administration of seed extract in low and high dosages concentration dependently improved the hormonal levels.
- In testis histopathology, LA group showed a decrement in the number of Leydig cells and spermatozoa with mild interstitial thickening indicating impairment in normal spermatogenesis. PSE group showed a recovery in the tissue indicating an increased spermatozoa and normal spermatogenesis with varying stages of maturation. Co-

treatment with seed extract in both low and high dosages reduced the interstitial thickening and Leydig cells were found in normal number.

- Histopathology of prostate indicated that LA group showed edematous surrounding stroma and scattered mononuclear inflammatory infiltrates whereas PSE showed normal architecture characterized by acini lined by simple columnar epithelium and basal cells luminal secretions. Surrounding stroma was found edematous and scattered mononuclear inflammatory infiltrates. LA+PSE LD showed edematous stroma but there was a reduction in the mononuclear inflammatory infiltrates. However, PSE HD exhibited stroma with normal luminal secretions.
- Histopathology of caudal epididymis indicated that all groups showed normal architecture characterized by hyperplastic acini lined by simple columnar epithelium and basal cells with luminal villi projections filled with secretions.
- Histopathological examination of seminal vesicle showed degenerative changes such as glandular alterations and inflammatory infiltrates in LA group whereas PSE showed normal glandular epithelium, tubule-alveolar glands, smooth muscle and supportive stroma. Co-treatment with *Cucurbita pepo* L. seed aqueous extract showed signs of dose dependent restoration in the histoarchitecture as evident from normal glandular epithelium, tubule-alveolar glands, smooth muscle, supportive stroma in LA+PSE LD and LA+PSE HD groups.

Examiners

Internal Examiner: Dr. Uma Dutta
Professor, Department of Zoology,
Cotton University, Assam-781001

External Examiner: Dr. W.A.J.P. Wijesinghe
Professor, Department of Food Science and Technology,
Uva Wellassa University, Sri Lanka-90000