2 Review of Literature

The review of literature pertaining to the study entitled **"ANTIOXIDANT POTENTIAL OF *Cucurbita pepo* L. (PUMPKIN) SEED EXTRACT IN THE TREATMENT OF STRESS INDUCED MALE INFERTILITY: AN IN VIVO STUDY"** has been discussed under the following headings:

2.1. Free Radicals- An Overview

2.2. Concept of Oxidative Stress

2.3. Heavy Metal Exposure and Male Infertility

2.4. Therapeutic Role of Antioxidants

2.5. Antioxidant Potential of *Cucurbita pepo* L. Seeds

2.1. Free Radicals - An Overview

Free radicals are chemical species (atoms, molecules, or ions) containing one or more unpaired electrons in their external orbitals and generally display a remarkable reactivity. The evidence of their existence was obtained only at the beginning of the 20th century. Chemists gradually ascertained the involvement of free radicals in organic reactions and, in the middle of the 20th century, their production in biological systems. For several decades, free radicals were thought to cause exclusively damaging effects. This idea was mainly supported by the finding that oxygen free radicals readily react with all biological macromolecules inducing their oxidative modification and loss of function. Moreover, evidence was obtained that when, in the living organism, free radicals are not neutralized by systems of biochemical defenses, many pathological conditions develop (Di Meo and Venditti, 2020).

In many disease conditions, certain oxygen-containing free radicals are found among which significant ones are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical and peroxy-nitrite radicals. These are highly reactive species which are capable of damaging biological molecules such as nucleic acids viz. dehydroxyribonucleic acid (DNA), proteins, carbohydrates and lipids in the nucleus and

membranes of cells thereby leading to cell rupture and homeostatic disruption (Schieber and Chandel, 2014).

2.1.1. Types of Free Radicals

The damaging free radicals are broadly classified into two types: Reactive Oxygen Species (ROS) and Reactive nitrogen species (RNS). ROS include both oxygen radicals and certain radicals that are oxidizing agents or can easily convert into radicals. RNS is also a collective term including nitric oxide and nitrogen dioxide radicals as well as non-radicals like nitrous acid, N_2O_3, $ONOO^-$

i) Superoxide anion ($O_2^{\cdot -}$): An oxygen molecule with an extra electron that can damage mitochondria, DNA and other molecules. Superoxide generated both in vivo and in foods can undergo several reactions including dismutation to give hydrogen peroxide (H_2O_2).

ii) Hydroxyl radical (OH): A highly reactive molecule formed by the reduction of an oxygen molecule, capable of damaging almost any organic molecule in its vicinity, including carbohydrates, lipids, proteins and DNA. OH cannot be eliminated by an enzymatic reaction.

iii) Singlet oxygen: It is formed by immune system. Singlet oxygen causes oxidation of Low-Density Lipoprotein (LDL).

iv) Hydrogen peroxide (H_2O_2): It is not a free radical itself, but easily converts to free radicals like OH, which then do the damage. Hydrogen peroxide is neutralized by peroxidase (an enzymatic antioxidant).

v) Peroxyl radical (ROO): Formation of peroxyl radicals ($RO_2^{\cdot}$) is the major chain-propagating step in lipid peroxidation and in non-lipid systems such as proteins. Decomposition of both lipid and protein peroxides on heating or by addition of transition metal ions can generate peroxyl and alkoxyl ($RO^{\cdot}$) radicals. Peroxyl radical can easily be generated by allowing O_2 to add to carbon centered radicals. Peroxyl radical is very important in biological systems including lipid peroxidation, DNA cleavage, protein back bone modification and also involved in food spoilage.

vi) Alkoxyl radical ($RO^{\cdot}$): The oxidative deterioration of lipids or lipid peroxidation produces alkoxyl radicals non enzymatically. They are highly oxidizing and can cause DNA mutations and apoptosis (Wagner *et al*., 2018).

2.1.2. Sources of Free Radicals

Free radicals are generated in the body by various endogenous and exogenous systems such as the exposure to various physico-chemical conditions and pathophysiological states (Lobo *et al*., 2010). Immune system cells generate oxy-radicals and ROS in response to pathogens. Free radicals can generate during metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells. Lipid peroxidation, an important source of free radicals can be formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidase and neutrophils. Inflammation releases cytokines and initiates neutrophils and macrophages to produce free radicals. Mental and body's stress can trigger the production of free radicals as a toxic by-product. Additionally, the hormones that mediate the stress reaction in the body like cortisol and catecholamine themselves degenerate into destructive free radicals (Phaniendra *et al*., 2015).

The different types of pollutants like air pollutants (asbestos, benzene, carbon monoxide, chlorine, formaldehyde, ozone, and toluene), chemical solvents (cleaning products, glue, paints, paint thinners, perfumes, and pesticides), and water pollutants (chloroform and other trihalomethanes) are all potent generators of free radicals (Phaniendra *et al*., 2015).

Burning of organic matter during cooking, forest fires, and volcanic activities, UV radiations, medical and dental x-rays, gamma rays, microwave radiation, additives, alcohol, coffee, foods from animal origin, foods that have been barbecued, broiled, fried, grilled or otherwise cooked at high temperatures, foods that have been browned or burned, herbicides, hydrogenated vegetable oils, pesticides, sugar and processed foods containing high levels of lipid peroxides, toxins like carbon tetrachloride, paraquat, benzo pyrene, aniline dyes, toluene and drugs like adriamycin, bleomycin, and mitomycin C increase free radical productions. Automobile exhaust fumes, and smoking of tobacco products also can cause free radical generation (Phaniendra *et al*., 2015).

2.2. Concept of Oxidative stress

It is ironic that oxygen, an element indispensable for life under certain situations has deleterious effects on the human body. Oxidative stress (OS) as a concept in redox biology and medicine has witnessed tremendous development of the past 30-odd years. It is a global concept, defined as an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage (Sies *et al*., 2017). Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins and nucleic acids. They can also serve to shorten the telomere length of chromosome, which many experts believe to be the most accurate biological clock we have (Preiser, 2012).

Short-term-oxidative stress may occur in tissues injured by trauma, heat injury, hypertonia and excessive exercise. These injured tissues produce radical generating enzymes (e.g. xanthine oxidase, lipoxygenase, cyclooxygenase), activate phagocytes, release free iron and copper ions or lead to the disruption of the electron transport chains and oxidative phosphorylation. This in turn produces excess ROS which has been implicated in the induction and complications of diabetes mellitus, age-related eye diseases, neurodegenerative diseases, and cancer (Pickering *et al*., 2013).

Oxidative stress is now thought to make a significant contribution to ischemic diseases (heart diseases, stroke, intestinal ischemia), neurological disorders (Alzheimer's disease, Parkinson's disease, muscular dystrophy), all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematous, adult respiratory disease syndrome), hemochromatosis, AIDS, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, smoking related diseases, infertility, and many others (Akbari *et al*., 2022).

2.2.1. Oxidative Stress and Cardiovascular Diseases

Cardiovascular diseases (CVDs) are one of the leading causes of morbidity and mortality worldwide, and oxidative stress has been identified as a major contributor to their pathogenesis. Cardiovascular disease is of multifactorial etiology linked with multiple risk factors like hypercholesterolemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity. ROS-induced oxidative stress plays a key role in pathogenesis of different

cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure (Sen *et al*., 2010).

Endothelial dysfunction is one of the major causes of cardiovascular diseases, but it is believed that oxidation of low-density-lipoprotein (LDL), loss of nitric oxide and vascular inflammation due to oxidative stress would implicate a potential for antioxidant therapies to ameliorate endothelial dysfunction. Oxidized LDL is atherogenic and is thought to be important in the formation of atherosclerotic plaque. Furthermore, oxidized LDL is cytotoxic and can directly damage endothelial cells (Lobo *et al*., 2010).

A study by Zhao *et al*., (2023) demonstrated that oxidative stress promotes endothelial cell apoptosis, impairing vascular repair and regeneration, and initiating the early stages of atherosclerosis. The researchers noted that antioxidants like N-acetylcysteine (NAC) were able to reduce ROS-induced endothelial dysfunction and improve vascular health in animal models. Additionally, Bahrami *et al*., (2018) found that oxidative stress could enhance the expression of adhesion molecules such as VCAM-1 and ICAM-1, which further exacerbate the inflammatory response and accelerate the formation of atherosclerotic plaques.

The heart itself is highly susceptible to oxidative damage, particularly in the context of ischemia-reperfusion injury. Sun *et al*., (2023) reviewed the role of ROS in myocardial infarction and found that ROS-induced oxidative damage to cardiac myocytes plays a key role in post-infarction remodelling and heart failure. They also emphasized that antioxidants, including polyphenols, showed promise in mitigating oxidative stress and improving heart function post-infarction.

2.2.2. Oxidative Stress and Neurodegenerative Disorders

Free radical induced oxidative stress contributes to several neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), etc. In these conditions, ROS and RNS contribute to neuronal damage, protein misfolding, and cell death. Neuronal biochemical composition is mainly vulnerable to ROS and causes peroxidation of unsaturated lipids and oxidative modification. The brain consumes an inordinate fraction (20%) of total oxygen for its relatively small weight (2%) and contains high level of fatty acids which are more susceptible to peroxidation. In addition, brain is lower in antioxidant activity and not enriched in

antioxidant defenses when compared to other tissues like liver. ROS are highly reactive to different fundamental molecules in cellular pool and initiate cascade of reactions at same time that leads to neuronal cell death, which is responsible for neurodegenerative diseases (Buccellato *et al*., 2021).

In Alzheimer's disease, Alidoust *et al*., (2023) explored the link between oxidative stress and the formation of amyloid-beta plaques, a hallmark of Alzheimer's disease. The study found that ROS promoted the aggregation of amyloid-beta, which in turn exacerbated oxidative damage in neurons. This cycle of oxidative damage and protein aggregation is a major contributor to the cognitive decline seen in Alzheimer's disease patients. The researchers suggested that therapeutic strategies aimed at reducing ROS levels might offer potential benefits in Alzheimer's disease management.

Similarly, in Parkinson's disease, Choudhury *et al*., (2024) examined the role of oxidative stress in dopaminergic neuron degeneration. Their findings indicated that ROS generated in the substantia nigra contribute to the loss of dopamine-producing neurons, a key feature of Parkinson's disease. The study also discussed the potential therapeutic effect of antioxidant-rich compounds, such as coenzyme Q10 and curcumin, in reducing oxidative damage and preserving neuronal function.

In amyotrophic lateral sclerosis, Liu *et al*., (2022) noted that oxidative stress plays a critical role in the pathophysiology of the disease by damaging motor neurons. The study highlighted the upregulation of oxidative stress markers like 8-hydroxy-2′-deoxyguanosine (8-OHdG) in amyotrophic lateral sclerosis patients and suggested that targeting oxidative stress through antioxidants could slow disease progression.

2.2.3. Oxidative Stress and Cancer

Cancer is another major disease associated with oxidative stress, where ROS contribute to tumour initiation, promotion, and progression. ROS can cause DNA mutations, genomic instability, and activation of oncogenic pathways. However, the relationship between ROS and cancer is complex, as ROS can also induce cell death in cancer cells, leading to the idea of ROS as a double-edged sword in cancer therapy (Wang *et al*., 2021).

Cancer develops through an accumulation of genetic changes. Initiating agents can be tobacco smoking and chewing, ultraviolet rays of sunlight, radiation, viruses, chemical pollutants, etc. Promoting agents also include hormones (androgens for prostate cancer, estrogens for breast cancer and ovarian cancer). Inflammation also induces inducible nitric oxide synthase (INOS) which can initiate carcinogenesis (Kawanishi *et al*., 2017).

Wang *et al*., (2023) reviewed the role of oxidative stress in cancer metastasis, emphasizing that ROS can activate signaling pathways like NF-κB and MAPK, which promote tumour growth and metastasis. Furthermore, Zhu *et al*., (2023) studied the impact of antioxidants in cancer therapy, noting that while antioxidants can protect normal cells from oxidative damage, they may interfere with the effectiveness of certain cancer treatments like chemotherapy and radiation therapy, which rely on ROS to induce cell death in tumour cells.

Lin *et al*., (2023) proposed that modulating oxidative stress could be a therapeutic strategy for cancer. They found that targeting ROS-generating enzymes, such as NADPH oxidase, could reduce cancer cell proliferation and increase sensitivity to chemotherapy agents. They also suggested the potential use of natural antioxidants, like resveratrol, to inhibit cancer progression.

2.2.4. Oxidative Stress and Inflammatory Diseases

Inflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and asthma, have also been linked to oxidative stress. In these conditions, ROS not only contribute to tissue damage but also exacerbate the inflammatory response. Rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by progressive, erosive and chronic polyarthritis. The pathogenesis of this disease is linked with the formation of free radicals at the site of inflammation which leads to lipid peroxidation. Oxidative stress causes modification of low-density lipoprotein, inactivation of α-1-protease inhibitor, DNA damage, lipid peroxidation, NADPH oxidase and endothelial cell xanthine dehydrogenase, which contributes significantly to the inflammatory process. Decreased concentrations of whole blood glutathione and total thiols were found in patients of rheumatoid arthritis. Decreased glutathione concentration has been associated with cell damage, depressed immunity and progression of ageing (Lapenna, 2023).

In RA, Hosseini *et al*., (2023) found that ROS play a central role in joint inflammation and cartilage degradation. The study suggested that antioxidants could reduce ROS levels and alleviate symptoms of RA. Similarly, in IBD, Zhang *et al*., (2023) explored the impact of oxidative stress on the intestinal epithelium and its role in the development of ulcerative colitis and Crohn's disease. The researchers noted that targeting oxidative stress could help to manage these chronic conditions.

In asthma, Lee *et al*., (2021) examined the role of oxidative stress in airway inflammation. The study found that increased ROS production in the lungs contributed to the recruitment of inflammatory cells, leading to airway hyperresponsiveness. Antioxidant therapies, including selenium supplementation, showed promise in reducing oxidative damage and improving lung function in asthmatic patients.

2.2.5. Oxidative Stress and Diabetes

Diabetes mellitus is a global problem associated with increased formation of free radicals and decrease in antioxidant potential, which results in disturbed balance between radical formation and antioxidant protection in normal cell. Both insulin dependent (type 1) and non-insulin-dependent diabetes (type 2) are associated with increased oxidative stress. The chronic hyperglycaemia in diabetic individuals results in the overproduction of ROS, which contributes to insulin resistance, pancreatic β-cell dysfunction, and vascular complications. Hyperglycaemia can also stimulate ROS formation from a variety of sources like oxidative phosphorylation, glucose autooxidation, NADPH oxidase, lipoxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS). Several studies have reported the depletion of antioxidant enzyme levels in patients with diabetes (Panahi *et al*., (2017).

A study by Chen *et al*., (2023) highlighted the role of oxidative stress in the pathogenesis of diabetic nephropathy, one of the major complications of diabetes. The researchers found that high blood glucose levels increased ROS production, leading to the activation of inflammatory pathways and kidney damage. They also explored antioxidant therapies, such as vitamin E and alpha-lipoic acid, that could mitigate oxidative stress and improve renal function in diabetic patients. In another study, Darenskaya *et al*., (2023) focused on oxidative stress in diabetic retinopathy, noting that ROS-induced damage to

retinal cells is a key factor in the progression of the disease. The study suggested that antioxidant supplementation could be beneficial in preventing or slowing the progression of diabetic retinopathy.

2.2.6. Oxidative Stress and Renal Disorders

Renal system is also susceptible to ROS induced damage and connection between oxidative stress and renal disease is well established. Vascular (endothelial and smooth muscle cells), glomerular (endothelial and mesangial) and tubular (proximal, distal and collector) cells of renal structure are capable to produce ROS due to stimulating factor like drugs, acute hypertension, radiation exposure and hyperoxia. In addition, large amounts of ROS are also generated by granulocytes, monocyte-macrophages and platelets, which are present in many inflammatory renal processes (vasculitis, glomerulonephritis, pyelonephritis). In chronic renal failure, oxidative stress can be considered as a potentially major cause of patient morbidity and mortality (Jabeen and Noor, 2024).

2.2.7. Oxidative Stress and Pulmonary Disorders

The pathogenesis of chronic pulmonary disorders like asthma and chronic obstructive pulmonary disease (COPD) is complex, which involves both airway inflammations and an oxidant/antioxidant imbalance. An air pollutant such as ozone and cigarette smoking, infections and other allergens increase generation of free radicals and amplify the risk of pulmonary disorders. ROS have been shown to be associated with the pathogenesis of asthma and lung injury by causing oxidative damage to epithelial cells and cell shedding. ROS also evoke bronchial hyper reactivity (Sen and Chakraborty, 2017).

2.2.8. Oxidative Stress and Eye Disorders

Eye is the most susceptible organ to oxidative damage caused by light, toxins (smoke), atmospheric oxygen and abrasion. Oxidative stress has been recognized as an important cause of several eye disorders like cataracts, glaucoma and macular degeneration. Free radical theory of ageing is related with the aetiology of eye diseases, which postulates that ageing and age-related diseases result from the accumulation of cellular damage from ROS. Ultraviolet increases generation of ROS, the conversion of this light into a nerve impulse by the photoreceptors which generates more free radicals such as hydrogen peroxide,

superoxide and hydroxyl radicals. Proteins in the lens are unusually long lived and are subjected to extensive oxidative damage (Sen and Chakraborty, 2017).

2.2.9. Oxidative Stress and Infertility

Reactive Oxygen Species can have beneficial or detrimental effects on reproductive system depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS. Free radicals can act as key signal molecules modulating various reproductive functions and can influence the oocytes, sperm and embryos in their microenvironments, for example follicular fluid and peritoneal fluid. These microenvironments have a direct influence on superiority of oocytes, sperm oocyte interaction, implantation, and early embryo development. Oxidative stress has been implicated in both female and male infertility (Aitken, 2020).

Oxidative stress affects multiple physiological processes, from oocyte to maturation to fertilization, embryo development and pregnancy. Oxidative stress influences both implantation and early embryo development which decides a successful pregnancy. The role of oxidative stress has been demonstrated in female infertility, including fetal dysmorphogenesis, abortions, and intrauterine growth restriction. Oxidative stress has been implicated in many of the causes of infertility, such as endometriosis, polycystic ovarian disease, unexplained infertility, and recurrent pregnancy loss (Wang *et al*., 2021).

ROS are known to be generated from spermatozoa and leucocytes, but elevated production of ROS in semen affects sperm function, especially fusion events associated with fertilization, and direct infertility in male. Increased ROS levels also correlate negatively with sperm concentration and sperm motility. Spermatozoa are highly susceptible to the damage induced by excessive ROS because the plasma membrane of spermatozoa contain large quantities of poly unsaturated fatty acids (PUFA) and cytoplasm contains low concentrations of scavenging enzymes. Peroxidative damage caused by free radicals results in impaired sperm function (Choudhari *et al*., 2010).

2.3. Heavy Metal Exposure and Male Infertility

Human infertility is a global reproductive health issue affecting almost 15% couples worldwide (Fainberg and Kashanian, 2019). Estimates suggest that approximately one in every six people of reproductive age worldwide experience infertility in their lifetime. Half of

the infertility is associated with male factors (Agarwal *et al*., 2015). Infertility can be primary or secondary. Primary infertility is when a pregnancy has never been achieved by a person, and secondary infertility is when at least one prior pregnancy has been achieved. Fertility care encompasses the prevention, diagnosis and treatment of infertility (Zegers-Hochschild *et al*., 2017).

Male infertility could be caused by various factors including psychological disorders (performance anxiety, strained relationship, depression, stress, guilt and fear of sexual failure), androgen deficiencies (testosterone deficiency, hyperprolactinemia), chronic medical conditions like diabetes, hypertension, vascular insufficiency (atherosclerosis and venous leakage), penile disease (Peyronie, priapism, phimosis, smooth muscle dysfunction), pelvic surgery (to correct arterial or inflow disorder), neurological disorders (Parkinson's disease, stroke, cerebral trauma, Alzheimer's disease, spinal cord or nerve injury), drug side effects (anti-hypertensives, central agents, psychiatric medications, antiulcer, antidepressants, and anti-androgens), life style (chronic alcohol abuse, cigarette smoking), aging (decrease in hormonal level with age), systemic diseases (cardiac, hepatic, renal, pulmonary, cancer, metabolic, post-organ transplant) and chronic exposure to environmental toxicants (Kolesnikova *et al.*, 2015; Sharma, 2017).

Reproductive toxicity caused by environmental toxicants has been quite serious concern and increases a serious mental and social problem (Sun *et al*., 2022). Increased human exposure to various environmental toxins are now a significant reason of the progressive decline in the fertility in both male and female (Soave *et al*., 2020). Exposure to environmental pollutants and toxins can be directly toxic to gametes (eggs and sperm), resulting in their decreased numbers and poor quality (Segal and Giudice, 2019). Human semen quality and sperm concentrations has decreased during the last few years (Selvaraju *et al*., 2021) forcing the people towards ART (Assisted Reproductive Technology) resulting only 2% to 4% of child births (Tournaye, 2017).

The exposure to these toxicants is unavoidable as human gets knowingly and unknowingly exposed to deleterious anthropological toxicants/chemicals in their daily life. Humans and animals are exposed to these environmental toxins through several/many routes. After entering in the body, these toxic substances get metabolized and often accumulated in

various organs and tissues that result in a variety of adverse physiological effects and pathological complications, including neural, reproductive, gerontologic and cardiovascular disorders (Zhang *et al*., 2016; Dai *et al*., 2020). Some environmental toxicants such as Bisphenol(s) (BPA, BPS, BPF), fluoride, lead, arsenic, cadmium, and pesticides are found to be associated with decrease in semen quality, sperm count and compromised reproductive health (Venkidasamy *et al*., 2021).

The reproductive effects of lead are complex and appear to involve multiple pathways, not all of which are fully understood. It is still unclear, for example, if male reproductive issues in lead-exposed persons are mostly related to the disruption of reproductive hormones, whether the problems are due to the lead's direct effects on the gonads, or both. This question has been difficult to answer, because lead, especially at high levels, may adversely affect many human organs. Although lead can potentially reduce male fertility by decreasing sperm count and motility, inducing abnormal morphology and affecting functional parameters; not all studies have been able to clearly demonstrate such findings. Future studies should aim to establish more concrete links between lead exposure (especially at low levels) and subsequent male infertility (Vigeh *et al*., 2011).

A growing number of evidences is indicating that an induction of testicular oxidative stress is the common mode of action of these environmental toxicants (Meli *et al*., 2020). The environmental toxicants somehow, increases oxidative stress in the testes by down-regulating the production of antioxidant enzymes such as SOD, catalase and glutathione peroxidase, consequently excessive amounts of Reactive Oxygen Species (ROS) are produced, that damages the lipids, proteins and DNA in cells (Ritchie and Ko, 2021). Toxicants adversely affect spermatogenesis, subsequently lower sperm count, lead to abnormal sperm morphology and poor semen quality (Li *et al*., 2024). Environmental toxicant causes germ cell loss by disrupting the cell junctions between Sertoli–Sertoli cells and Sertoli–germ cells (Kubincova *et al*., 2019).

Studies observed that toxic substances via various mechanisms including oxidative stress, inflammation, energy impairment and altered cellular communications damages the testicular functions. Sperm damage by ROS is the main cause of infertility in 30–80% of infertile men; and these conditions ultimately damage DNA in the nucleus and mitochondria

(Bui *et al*., 2018). Increased ROS levels also correlate negatively with sperm concentration and sperm motility. Spermatozoa are highly susceptible to the damage induced by excessive ROS because the plasma membrane of spermatozoa contain large quantities of poly unsaturated fatty acids (PUFA) and cytoplasm contains low concentrations of scavenging enzymes. Peroxidative damage caused by free radicals results in impaired sperm function (Choudhari *et al*., 2010).

Sperm cells are highly sensitive to oxidative damages. On the other hand, these cells have cytoplasmic membranes rich in unsaturated fatty acids, and thus they are faced with the lipid peroxidation under the influence of ROS (Wagner *et al*., 2018). Moreover, these cells are incapable of repairing damage by oxidative stress because they suffer from lack of essential cytoplasmic enzymes. Finally, lower motility and death of sperm occur due to the loss of adenosine triphosphate (ATP) caused by lipid peroxidation followed by axonemal injury (Barati *et al*., 2020). OS indirectly affects hypothalamic axes and can disrupt the secretion of sex hormones. ROS reduces levels of male sex hormones and strikes their balance and can disrupt the reproductive system (Darbandi *et al*., 2018).

The most frequent causes of male infertility are associated with spermatogenesis. Because it is relatively easy to conduct, non-invasive and inexpensive to perform, semen analysis (sperm count, semen volume, sperm morphology and assessments of functional parameters) is one of the first laboratory tests commonly performed for infertile couples (Gupta and Rajalakshmi, 2020).

2.3.1. Stress Biomarkers and Male Infertility

Biomarkers of oxidative stress serve as indicators of the extent of cellular damage caused by ROS. These biomarkers can be measured in both in vitro (cell cultures, isolated tissues) and in vivo (whole organisms) models. These markers provide valuable insights into the oxidative stress status of cells and organisms, helping to monitor disease progression and therapeutic efficacy (Sies, 2015).

In vitro models provide the advantage of examining the direct effects of specific stressors on cells, but they also have limitations in replicating the complexity of whole-body systems. These models are useful for initial screening of compounds or treatments that could modulate oxidative stress. In vivo models involve whole organisms, such as rodents or

human clinical trials, and provide a more comprehensive understanding of the systemic impact of oxidative stress. In vivo biomarkers are highly relevant for understanding the physiological implications of oxidative stress in diseases. However, they require careful consideration of factors such as circadian rhythms, diet, and environmental influences, which can affect biomarker levels (Zhao *et al*., 2023).

Over the last decade, research has provided growing support to indicate that excess ROS production leads to abnormal semen parameters and increased sperm damage. Standard semen analysis continues to be the backbone of clinical evaluation of male infertility. Studies have shown that ROS-mediated damage to sperm is a considerable contributing pathology in 30% to 80% of unselected infertile patients. Therefore, it would be reasonable to expect the screening of all infertile men for the presence of increased ROS levels (Liu *et al*., 2021).

Oxidative stress results from an imbalance between ROS production and the intracellular/extracellular antioxidants present in seminal plasma. The hyperviscosity of seminal plasma is also associated with increased levels of seminal plasma MDA and reduced seminal plasma antioxidant status. Direct assays of OS measure the net oxidative result of this imbalance by detecting and measuring the amount of oxidation in the sperm cell membrane. MDA, which is one of the final products of membrane LPO, can be measured via the thiobarbituric acid assay, which is one of the oldest and most widely used direct assays for assessing sperm membrane oxidation. Various authors have reported that increased levels of MDA are associated with decreased sperm motility and sperm-oocyte fusion (Dutta *et al*., 2019).

Sex hormones, such as testosterone, Luteinizing Hormone (LH), and Follicle-Stimulating Hormone (FSH), are crucial for regulating male reproductive health. These hormones not only govern the development and function of the testes but also influence sperm production and quality. The relationship between oxidative stress and sex hormones is complex and bidirectional. Testosterone is an androgen that performs an essential function in the growth, reproduction, and maintenance of a healthy body. Sperm formation (spermatogenesis) is regulated by both endocrine and paracrine systems. FSH and LH are both involved in the endocrine stimulation of spermatogenesis and then work through the intermediate testosterone generated by the Leydig cells of the testis (Bhongade *et al*., 2015).

There are only a few reports which insist on the effect of stress on testosterone synthesis and male infertility. Stress primarily reduces the serum testosterone level, followed by the secondary elicit of serum LH and FSH levels, which may have an impact on the semen quality by reducing the rate of motility, count, and normally functioning spermatozoa (Samplaski *et al*., 2014). Intratesticular testosterone is also an important factor for the spermatogenesis process, virility, and male fertility. Testicular biopsy is the only way to measure intratesticular testosterone concentration, which is invasive and can lead to many complications (Karbel Hadeel *et al*., 2020).

In males, a dimeric glycoprotein gonadotropin hormone called FSH and its receptor play an essential function in regulating the process of spermatogenesis in the testis. It is generated by the anterior portion of the pituitary gland in various isoforms physiologically and targets the cells of gonads. It coordinates its function with LH through G protein-coupled receptors (GPCRs) and alters steroidogenesis and cellular metabolism to regulate reproduction (Retana-Márquez *et al*., 2020). The interrupted secretion of these hormones by the pituitary glands will lead to infertility and testicular functional disruption. Furthermore, this gonadotropin deficiency stands as the major risk factor accounting for about 0.5% of men's infertility. FSH interacts with its receptor on the Sertoli cells, triggering those cells in adults to secrete regulatory nutrients that are essential for germ cell maturation (Goto *et al*., 2020). Furthermore, the circulating concentration of the germ cells directly depends on the volume of Sertoli cells (Ismael *et al*., 2017).

The production of LH is the cornerstone of the effective spermatogenesis process, secondary sexual characteristics, functions, and psychoactive and biosynthetic (anabolic) actions. Abnormalities in the function of LH can affect spermatogenesis, leading to infertility (Ismael *et al*., 2017). In men, in cases of infertility due to hypogonadotropic hypogonadism, sperm can be restored with HCG or human menopausal gonadotropins (hMG). The higher range of body mass index (BMI) will alter the concentrations of seminal plasma and male reproductive hormones, enhance oxidative stress, and affect sperm quality. These consequences may be linked with male infertility (McIntyre *et al*., 2022).

2.3.2. Prevention of Oxidative Stress

In healthy males, sperm DNA is protected from OS by two main mechanisms. Firstly, the DNA is tightly coiled and packaged into chromatin such that the genetic material is minimally exposed to attack by ROS. Secondly, natural antioxidants in the seminal plasma and spermatozoa assist in minimizing the ROS production to normal levels. Some natural antioxidants include enzymes like catalase and SOD as well as non-enzymatic compounds like vitamins C and E and carotenoids. These antioxidants react with and neutralize ROS, assisting in preventing OS onset and preserving the spermatozoa function (Sengupta *et al*., 2024).

To remain healthy, a sufficient quantity of antioxidants must be consumed in one's diet to prevent OS from occurring. However, in some patients who suffer from infertility, there may be either an overproduction of ROS or an underproduction of antioxidants, which disrupts the intricate balance and results in OS (Patani *et al*., 2023).

2.4. Therapeutic Role of Antioxidants

Antioxidants are the inhibitory molecules that delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are biological demands for exogenous compounds to counter effects of damaging oxidation to body cells and tissues and ensuring proper health and function of the body. Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes and harmful effects of ROS (Chhikara *et al*., 2018).

In terms of food, antioxidants may be defined as any substances, which are capable of delaying, retarding or preventing the development in food of rancidity or other flavour deterioration due to oxidation (Taslimi and Gulcin, 2018). In food systems, antioxidants have become an indispensable group of food additives mainly because of their unique properties of extending the shelf-life of food products without any adverse effect on their sensory or nutritional qualities (Shahidi and Ambigaipalan, 2015).

From the food preservation perspective, antioxidants can scavenge free radicals, prevent lipid peroxidation (major reason for the deterioration of food) and microbial spoilage of food thereby increase shelf-life working as a natural preservative agent (El Alami El Hassani *et al*., 2024). However, antioxidants for use in food system must be inexpensive, effective and non-toxic at low concentrations; highly stable and capable of surviving processing; have no odour, taste or colour of their own; easy to incorporate and have good solubility in the product (Shahidi and Ambigaipalan, 2015).

Antioxidants can also protect the human body from free radicals and ROS effects. In recent years, there is great interest in identifying alternative natural and safe sources of natural antioxidants, especially of plant origin (Anwar *et al*., 2018). Antioxidants include enzymatic antioxidants (e.g. superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants such as oxidative enzyme (e.g. cycloxygenase) inhibitors, antioxidant enzyme cofactors, ROS/RNS scavengers and transition metal chelators (Pisoschi *et al*., 2021).

Antioxidants act as radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, synergists and metal chelating agents. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Chahal *et al*., 2018). These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Lobo *et al*., 2010). Two principal mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the system. The second mechanism involves removal of ROS/RNS (reactive nitrogen species) initiators by quenching chain-initiating catalyst (Pisoschi *et al*., 2021).

2.4.1. Natural Antioxidants

Experimental as well as epidemiological data indicate that a variety of nutritional factors can act as antioxidants (Martins Gregório *et al*., 2016). Food consumption is a major source of exogenous antioxidants and has been estimated that a typical diet provides more than 25,000 bioactive food constituents and many of this may modify a multitude of processes that are related to different diseases. Antioxidants are abundant in vegetables and

fruits and are also found in grain cereals, teas, legumes, nuts and other food products. A recent study reported that plant-based foods are generally higher in antioxidant content than in animal-based and mixed food products (McClements, 2023).

Consumption of fruits and vegetables has demonstrated an inverse association with mortality from age-related diseases, which may be attributed to their antioxidant activity (Tan *et al*., 2018). Humans take antioxidants directly from fresh and dried fruits and vegetables, which contain enormous quantity of flavonoids and antioxidant supplements that contribute to protection against different types of diseases (Jideani *et al*., 2021).

The major bioactive compounds of these natural sources are especially phenolics and flavonoids, which are responsible for their health benefits (Kaurinovic and Vastag, 2019). Various endogenous radical-scavenging antioxidants viz. vitamin C, uric acid, bilirubin, albumin, and thiols are hydrophilic and others are lipophilic such as vitamin E and Ubiquinol (Lobo *et al*., 2010). Endogenous antioxidant defense systems are incomplete without exogenous antioxidants such as vitamin C, vitamin E, carotenoids and polyphenols since they play an essential role in maintaining redox balance. An interactive and often synergistic action between endogenous and exogenous antioxidants helps to maintain a balance between oxidation and antioxidation. The intake of active antioxidant compounds helps to prevent complex multi-factorial diseases and may slow their development or inhibit their progression. Several clinical studies and basic research have shown that depletion of endogenous enzyme in certain pathological conditions may be mitigated by the use of exogenous antioxidants (Moussa *et al*., 2019).

At present, a variety of synthetic antioxidants are commonly used. However, the use of these compounds has been restricted by legislation due to doubts over their toxic and carcinogenic effects (Lorenzo *et al*., 2017). Therefore, the increased popularity of natural food additives may prompt more food manufacturers to replace synthetic antioxidants with ingredients containing natural antioxidative compounds. Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of plants including vegetables, fruits, seeds, nuts, leaves, roots flours, and barks (Shahidi and Ambigaipalan, 2015).

Plants produce a vast repertoire of secondary metabolites such as flavonoids, essential oils, alkaloids, lignans, terpenes, terpenoids, tocopherols, phenolic acids, phenolics, peptides, polyfunctional organic acids, amongst others in their normal metabolic pathways. These metabolites play a major role in protecting plants from undesired effects. The most prominent representatives of dietary antioxidants are vitamin C, tocopherols, carotenoids, and flavonoids (Shahidi and Ambigaipalan, 2015). In the diet, there may be synergistic effects of these various dietary compounds, which are difficult to assess at present. Indeed, the diet may be considered as an orchestra where interactions between constituents may bring about effects, which are not the necessary properties of the individual constituents (Swallah *et al*., 2021).

2.4.2. Phenolic Compounds

Phenolic compounds known to serve as multipurpose bioactive compounds are widely spread throughout the plant kingdom. Most of the phenolic compounds are an integral part of the human diet and are also consumed as medicinal preparations. Many of the health-protective effects of phenolic compounds have been ascribed to their antioxidant, anticarcinogenic, antimutagenic, antimicrobial, anti-inflammatory and other biological properties. The most widely occurring plant phenolic compounds include phenolic acids, flavonoids, tannins, lignans, and terpenes (Shahidi and Ambigaipalan, 2015).

Phenolic acids are hydroxy derivatives of aromatic carboxylic acids, which arise from either the benzoic acid group or the cinnamic acid group. Phenolic acids, such as *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, and rosmarinic acid, are widely distributed in the plant kingdom (Sarikaya *et al*., 2015).

2.4.3. Flavonoids

Flavonoids are a large group of polyphenolic natural compounds, extensively distributed in plant-based foods. Flavonoids are very effective antioxidants and are among the major antioxidant constituents of diet. Flavonoids are mostly present in human diet, such as fruits, vegetables and plant-derived beverages such as tea. Daily intake of flavonoids is a few hundreds of milligrams per day. Up to now, over 4000 different naturally occurring flavonoids have been identified (Maiti *et al*., 2019).

Flavonoids include flavones, flavonols, isoflavones, flavanones, and chalcones. Flavonoid derivatives vary in their structure around the heterocyclic oxygen ring, but all have the characteristic C_6–C_3–C_6 carbon skeleton (Shahidi and Ambigaipalan, 2015). The ability of flavonoids to inhibit lipid peroxidation is well documented and well known. It was reported that flavonoids may act as antioxidants by scavenging radicals such as lipid peroxyl radicals, superoxide anion radicals, hydroxyl radicals, singlet oxygen quenchers, and metal ion chelators (Gulcin, 2020).

2.4.4. Impact of Antioxidant Supplementation on Male Fertility

Several studies have shown that antioxidant supplementation can improve sperm quality by reducing oxidative stress by improving sperm motility, morphology, and concentration. A meta-analysis by Su *et al*., (2022) demonstrated that antioxidant supplementation led to significant improvements in sperm motility and overall semen quality in infertile men.

Antioxidants such as vitamin C, vitamin E, and CoQ10 are particularly effective in reducing sperm DNA fragmentation. DNA damage in sperm is a critical factor in male infertility, as it can impair fertilization and embryo development. Studies have found that antioxidants can reduce sperm DNA fragmentation, improving the chances of successful conception. Oxidative stress can disrupt the balance of sex hormones, such as testosterone, estrogen, and follicle-stimulating hormone (FSH). Antioxidant supplementation may help restore normal hormonal function, which is crucial for maintaining sperm production and quality. In particular, antioxidants can help reduce the oxidative stress-induced decline in testosterone levels, which is associated with reduced fertility. Agarwal *et al*., (2021) reviewed several clinical trials and concluded that antioxidant therapy, including the use of vitamin E, vitamin C, and CoQ10, significantly improved semen parameters in infertile men, leading to better fertility outcomes.

2.4.5. Antioxidant Assays

In order to evaluate the efficacy of phytocompounds as therapeutic agents as well as to elucidate their mode of action, researchers today are using a wide range of experimental models, simple chemical antioxidant assays ranging from biologically relevant cellular based assays to the most accurate animal models, and ultimately clinical studies in humans. The latest scientific knowledge offers a more detailed understanding of the biological effects of

phytocompounds and their role in human health promotion and disease prevention (Vladimir-Knežević *et al*., 2012). As therapeutic activity cannot be concluded based on single method or model system, in practice, several in vitro test procedures are carried out for evaluating the samples of interest for therapeutic activities. Another aspect is that the test models vary in different respects. Therefore, it is difficult to compare fully one method to other one and hence, different in vitro methods are needed to validate the therapeutic effects of a particular plant extract (Alam *et al*., 2013).

Different antioxidant methods have been introduced to measure and investigate the antioxidant property and capacity of commercial antioxidants, foods, medicinal, pharmaceuticals and biological samples (Amorati and Valgimigli, 2015). The concept of antioxidant capacity first originated from chemistry and was later adapted to biology, medicine, epidemiology and nutrition (Floegel *et al.*, 2011). In recent years, a wide range of spectrophotometric assays has been adopted for measurement of antioxidant capacity of foods and pharmaceuticals. Therefore, it is desirable to establish and standardize methods that can measure the antioxidant level directly from plant-based materials, food extracts and biological samples (Apak *et al*., 2019).

However, up to now, the most commonly used methods for in vitro determination of antioxidant capacity of food constituents are inhibition of lipid peroxidation, ferric ion (Fe^{3+}) reducing antioxidant power assay (FRAP), $DPPH^{\cdot}$ radical scavenging, superoxide anion radical scavenging, and hydrogen peroxide scavenging activities. Most of the methods employ the same principle: a synthetic coloured radical or redox-active compound is generated; and the ability of a biological sample to scavenge the radical or to reduce the redox-active compound is monitored by spectrophotometer, applying an appropriate standard to quantify antioxidant capacity (Floegel *et al.*, 2011).

2.5. Antioxidant Potential of *Cucurbita pepo* L. Seeds

Cucurbita plants were first explained by Linnaeus in the middle of the eighteenth century and are among the earliest known plants that have been grown by mankind (Neelamma *et al*., 2021). The Cucurbitaceae family frames a huge gathering with roughly 130 genera and 800 species. Cucurbitaceae plants are commonly known as cucurbits, extensively cultivated in tropical and subtropical countries. *Cucurbita* species also show

diverse habitat use, growing in terrestrial and wetland environments, as monoecious climbers or annuals; they may be found in meadows, fields, and on the shores of rivers or lakes. *Cucurbita* species include pumpkins, squashes, gourds and melons (Wehner *et al*., 2020).

Cucurbita pepo L. is considered as one of the most seasoned and developed varieties, by Mexican archaeological evidence of 7000 BC. Along these lines, it was extensively developed through indigenous people of Central, North America and Mexico, earlier the landing of Europeans. *Cucurbita pepo* L. is local to Northern Mexico and Southwestern and eastern USA (Paris, 2016). *Cucurbita pepo* L. is a medium sized *cucurbit* grown for its fruits and edible seeds and used as food and in herbal formulations. All anatomical parts of the plant are edible, but seeds and pulp are particularly important for food processing and nutrition (Elinge *et al*., 2012).

Cucurbita pepo L. is a widely used for human consumption and in traditional medicine. Seeds are used in folk medicine for the treatment of irritable bladder, spleen and lungs diseases, gastritis, headache, anaemia, stomach ulcer, and so on. Pumpkin seeds have aphrodisiac properties (Martha and Gutierrez, 2016). The seeds can be ground into a powder and mixed with cereals for making bread or roasted and consumed as snacks. The presence of a multitude of biologically active compounds in high concentrations such as α- and β-carotene, β-cryptoxanthin, lutein and zeaxanthin, polysaccharides, phytosterols, unsaturated fatty acids, proteins, and peptides make Cucurbita species very interesting for the natural ingredients industry (Durante *et al*., 2014).

Extracts (from different parts of the plant) contain biologically active components with antidiabetic, antibacterial, hypocholesterolemic, antioxidant, anticancer, antimutagenic, immunomodulatory, anthelmintic, anti–bladder stone and other miscellaneous activities. In last few decades, interest for polyphenols in oilseeds is growing because they represent health-promoting substances because of their antioxidant properties. However, until recently, the literature about the presence of phenolic compounds in *Cucurbita pepo* L. was scarce. The most abundant phenolic acid present in pumpkin is p-hydroxybenzoic acid, but the presence of other phenolics such as caffeic, p-coumaric, ferulic, sinapic, protocatechuic, syringic acid, and p-hydroxybenzaldehyde is also confirmed (Krimer-Malešević, 2020).

2.5.1. Botanical Description

Cucurbita pepo L. has creeping plants which are compact or semi-shrubby, annual, broadly ovate-cordate to triangular-cordate leaves, with or without white spots, often with three to five deep lobules. Tendrils have two to six branchlets or are simple and little developed. It has pentamerous flowers. The fruit is variable in size and shape with a rigid skin varying in color from light to dark green, plain to speckled with cream or green contrasting with yellow, orange, or two coloured. The flesh is cream to yellowish or pale orange; it ranges from soft and not bitter to fibrous and bitter, and has numerous seeds which are narrowly or broadly elliptical or rarely orbicular, slightly flattened. Pumpkin seeds are dark green (because of the presence of protochlorophyll). Some are encased in a yellow-white husk (hulled or husked seeds), although some pumpkins produce seeds without shells (hull-less or naked seeds), which have only very thin dark green skin (Krimer-Malešević, 2020).

2.5.2. Nutritional Composition

Pumpkin has major amounts of pro-vitamin A carotenoids, which imparts different colors to it because of the presence of components such as lutein (bright yellow color) and β-carotene (orange colour). However, α-carotene, lutein, lycopene, cryptoxanthin and cis-β-carotene are present in trace amounts (de Carvalho *et al*., 2017). β-carotene is converted into vitamin A in the body which is responsible for vision, growth, embryonic development and its deficiency may lead to blindness and infant mortality. Carotenoid act as antioxidants and also reduces the risk of certain diseases such as cardiovascular diseases and cancer by scavenging the free radicals (Takahashi *et al*., 2011).

The pumpkin seed contains crude protein (39.25 g/100 g), crude oil (27.83 g/100 g), ash (4.59 g/100 g), crude fiber (16.84 g/100 g), energy (2,401 kcal/100 g), potassium (5,790 mg/100 g), chromium (3 mg/100 g), sodium (6.9 mg/100 g), magnesium (5,690 mg/100 g), aluminum (9.21 mg/100 g), barium (1.16 mg/100 g), cobalt (0.29 mg/100 g), zinc (113 mg/100 g), iron (106 mg/100 g) and calcium (346 mg/100 g) on dry weight basis (Kim *et al*., 2012). The pulp contains low amount of fat (2.3 g/100 g), carbohydrates (66 g/100 g), protein (3 g/100 g) and the carotenoid content (171.9 mg/g) on dry weight basis. The vitamin C and E contents of pumpkin pulp are 2-10 mg/100 g and |

9-10 mg/100 g respectively (Martha and Gutierrez, 2016).

2.5.3. Phytochemistry

The phytochemicals present in pumpkin are responsible for distinct physiological action on the body of human because of their vast medicinal characteristics. The major bioactive constituents of plants are phytosterols, tannins, alkaloids, flavonoids, phenolics, tocopherol and cucurbitacin. The pumpkin seeds also possess numerous health benefits and it has many biologically active components such as polysaccharides, para-aminobenzoic acid, fixed oils, sterols and proteins. Pumpkin seed kernel was found to be richest in phytosterols (265-289 mg/100 g) and these phytochemicals have a significant hypocholesterolemic effect (Kaur *et al*., 2020).

Cucurbita pepo L. seed and seed oil have macro- and micro compounds. The protein level is high in seeds (25–51%) and in seed oil (40% to 60%). The seed oil contains fatty acids oleic (up to 46.9%), linolenic (up to 40.5%), palmitic and stearic up to 17.4%. There are 1% phytosterols presented in free and bound forms; squalene; chlorophyll pigments, 4–5% minerals including selenium, calcium, copper, iron, manganese, phosphorous, potassium, and zinc, which is necessary in male reproductive system (Abd El-Ghany *et al*., 2010).

Anti-allergic, anti-inflammatory activities were reported with in vitro studies (Rajasree *et al*., 2016) by phenolic compounds (0.3 mg/100 g) present in pumpkin. Phenolics have reactive oxygen scavenging ability because of their electron-donating capability, which play a vital role in disease prevention in plants and animals such as Alzheimer, Parkinson and cancer (Kumar *et al*., 2021). Tannins have astringent properties such as healing of wounds and inflamed mucous membrane (Rajasree *et al*., 2016).

2.5.4. Health Benefits

Pharmacologically, the *Cucurbita pepo* L. has various uses, including anti-tumour, antioxidant, anti-inflammatory, anti-diabetic, anti-carcinogenic, anti-bacterial, anti-hypercholesterolemic, anti-hypertensive, and intestinal benefits. The plant contains several phyto-constituents such as linoleic acid, oleic acid, alkaloids, flavonoids, and palmitic acid, which may contribute to its medicinal properties. *Cucurbita pepo* L. is rich in protein, carbohydrates, fats, and minerals, making it beneficial for both humans and

animals (Yadav *et al*., 2017).

2.5.5. Antioxidant activity

The determination of antioxidant activity in Cucurbitaceae seed protein usually comprises different in-vitro measures, such as radical scavenging activity, reducing power and metal chelating activity. On hydrolysis, the antioxidant activity has been reported to increase depending on the degree of hydrolysis (DH) and molecular weight distribution of peptides in hydrolysates. In general, the higher the DH and the smaller the size of the peptides, the better the antioxidant activity of Cucurbitaceae seed protein hydrolysates (Ozuna and Leon Galván, 2017). The pumpkin seed oil contains the isomeric mixture of beta and delta tocopherol. Tocopherols are non-glycoside compound present in the vegetable oil and acting as natural antioxidants. Pumpkin polysaccharide extracted with hot water extraction method shows good antioxidative properties in both natural and sulfated forms (Chen and Huang, 2019).

2.5.6. Hypotensive activity

Hypotension mainly occurs due to low blood pressure, especially in the arteries of the systemic circulation. Positive hypotensive effects of pumpkin seed were reported in relaxing vessels on chemical-induced hypertension in rats (El-Mosallamy *et al*., 2012) by decreasing the elevated levels of malondialdehyde with increase of NO and metabolites to normalcy. The results showed the protective effect against pathological alterations in the heart and aorta along with the reduced risks of heart attacks by reason of high magnesium content. In another animal model of hypertensive rats, 2 g/day administration of pumpkin seed oil for 12 weeks, showed symbolic increase in high-density lipoprotein cholesterol and a significant decrease in diastolic blood pressure in postmenopausal women in recent clinical trial (Yoshinari *et al*., 2015).

2.5.7. Antidiabetic activity

Diabetes mellitus is a chronic metabolic disorder represented by insulin shock due to its non-secretion or intolerance. Hypoglycemic results were obtained for alloxan-induced diabetic rats with flax and pumpkin seed mixture though diabetic patients are fasten from consuming pumpkin considering abundant carbohydrates (Makni *et al*., 2011). The study examined partly preserved pancreatic function, improved peripheral glucose and contracted

the increased plasma enzyme levels, produced by induction of diabetes. Also, increment in hepatic glycogen content was noticed that proposed the maintenance of hepatic glycogen and the gluconeogenesis rate was impaired. Tocopherol isomers are present in the pumpkin seed and are effective in alleviation of diabetes because it contains some antioxidant activity (Bharti *et al*., 2013; Gutierrez, 2016).

2.5.8. Hepatoprotective activity

The hepatoprotective effect of pumpkin seed oil against fatty liver progression was attributed to the presence of biologically-active compounds as it contains phenolic compounds, tocopherol, β -carotene, unsaturated fatty acids and sterols. Unsaturated fatty acids such as oleic acid and linoleic acid turn down the blood cholesterol in humans and rats, which, perhaps, affiliated to depletion of cholesterol synthesis and elevated cholesterol catabolism in the liver (Al-Okbi *et al*., 2014).

A study was performed by Makni *et al*., (2008) on 30 male rats by dividing into three groups: a control group (CD), CD-chol group fed diet with 1% cholesterol and MS-chol group fed diet enriched with flax and pumpkin seed mixture. Liver histological sections showed lipid storage in hepatocytes of CD-chol group and an improvement was noted in MS-chol group and study showed that flax and pumpkin seed mixture had anti-atherogenic and hepatoprotective effects, which were probably mediated by unsaturated fatty acids present in seed mixture.

2.5.9. Anticancer activity

The antioxidants and polyphenolic compounds in food are described as able to control aberrant inflammatory signals, and signaling routes, associated with cancer stem cells (Sardana *et al*., 2018). *Cucurbita pepo* L. extract has potential to be developed as a new chemotherapeutic agent to prevent or to inhibit the growth of tumors and cancer (Gutierrez, 2016). Apart from lowering the risks of gastric, breast, lung and colorectal cancer, carotenoids from pumpkin seed diet have also been correlated to prostate cancer. A study disclosed inhibition of the growth of leukemia K-562 cells from some basic proteins named microtubule-associated proteins (MAP)2 and MAP4 present in the seeds. Although, other proteins inhibit melanoma proliferation are also reported. Pumpkin polysaccharides exhibit good anticancer effects by removing various free radicals generated in the body during

metabolism, including superoxide anions, hydroxyl radicals and other Reactive Oxygen Species (Chen and Huang, 2019).

Research reports have proven that the extracts of rind, flesh and seed oil of *Cucurbita pepo* L. inhibited cancer cell breast carcinoma (MCF7) and liver carcinoma (HEPG2). The seed oil showed cytotoxicity against breast carcinoma (MCF7) with IC_{50} in the range of 0.40–1.01 mg (Adnan *et al*., 2017). Linolenic acid present in *Cucurbita pepo* L. seeds is useful in the prevention of many diseases such as heart diseases, diabetes and different types of cancer, particularly breast cancer (Bialek *et al*., 2016). The moderate dose of fatty oil in pumpkin seed was reported non-toxic to animals and human beings, and also has protective effects against genotoxicity induce by azathioprine diseases (Elfiky *et al*., 2012).

The concentration of squalene was found at range 583.2-747 mg/100 g in pumpkin seed. Squalene is a triterpene and precursor of steroid hormone, cholesterol and vitamin D biosynthesis in the human body and has been found beneficial for the curing of many types of cancer (Martha and Gutierrez, 2016). Several studies (in vitro and in vivo) with crude pumpkin extracts and its various purified fraction including proteins and polysaccharide shows anticancer activity against malignant tumor associated with skin cancer and leukemia. Besides these, boiled pumpkin juice comparably suppressed the rate of the division of tumor cells (Ahmad and Khan, 2019).

2.5.10. Antimicrobial activity

Many researchers reported that the extracted oil and proteins from pumpkin have importance to clinical microbiology and possess therapeutic applications. A peptide from the pumpkin seed was proved to inhibit *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola* at dose of 375 mg (Wang and Ng, 2003). Pumpkin oil-soluble components have antimicrobial activity against *Pseudomonas aeruginosa*, *Candida albicans*, *Acinetobacter baumanii*, *Enterococcus faecalis*, *Klebsiella pneumonia*, *Escherichia coli* and *Staphylococcus aureus* (Ahmad and Khan, 2019).

Alkaloids (180 mg/100 g) present in pumpkin are in great demand for pharmaceutical formulations as these are the therapeutic molecules that scavenges the free radicals or binds with catalysts of some metal ions (oxidative reactions) to retard the growth

of microorganisms (bacteria, fungi, protozoans, etc.) and so prevent the onset of different diseases.

Three different proteins extracted from the pumpkin rinds, seeds and pulp showed antimicrobial activity against the tested microbial isolates (El-Aziz and El-Kalek, 2011). Aqueous extract against aflatoxin B1 (AFB1) toxicity and *Aspergillus flavus* fungus infection-induced lung histomorphological damage in rats was investigated by Saddiq *et al.*, (2019). Oral co-administration of aqueous extract of pumpkin fruits (1.0 mg/kg of body weight) to either rat groups intoxicated with AFB1 or infected with *Aspergillus flavus* for 20 consecutive days showed more or less normal histological structure of rat lungs.

2.5.11. Antirheumatic activity

Pumpkin seeds oil is an extraordinarily rich source of diverse bioactive compounds having functional properties and with positive effects on small urinary disorders, prostate gland and urinary bladder (Rezig *et al*., 2018). In a study in South Africa, it was revealed that arthritis and blood booster can be treated with orally taken *Cucurbita pepo* L. leaves (Asowata-Ayodele *et al*., 2016). Pumpkin-based foodstuffs are well-recognized as a source of anti-inflammatory remedies, which can be useful in arthritis treatment (Tawheed *et al*., 2013). Pumpkin seed oil prevented adjuvant-induced arthritis in rats and the clinical applicability as an antioxidant was also assessed on rheumatoid arthritis by Van Vugt *et al*., (2008).

2.5.12. Antiulcerative activity

Pumpkin fruits, pulp, and seeds are reported to have antiulcer activity (Gutierrez, 2016). Gill and Bali (2011) have shown the antiulcer and antioxidant activities of tetracyclic triterpenoids *(Cucurbitacins*) extracted from *Cucurbita pepo* L. seeds. Herbal preparations from pumpkin fruits were used to treat duodenal ulcers; recent attempts were made to treat ulcer and development of new anti-ulcer drugs by using natural resources like plants (Jain *et al*., 2017). Albino rats treated with fruit pulp extract of pumpkin were reported with increased alkaline phosphate activity and decreased mucosal thickness and ulcer index (Sarkar and Guha, 2008) indicating that pumpkin pulp has gastroduodenal protective and antiulcer properties.

2.5.13. Other Health Benefits

The pumpkin supplemented food is considered a better way for anti-inflammatory substances (Caili *et al*., 2006). The higher iron content in the pumpkin seed is also helpful in reducing the anemia caused by the iron deficiency (Białek *et al*., 2015). The oil of pumpkin seed reduces many diseases such as lower the bladder pressure and also the urethral pressure (Rezig *et al*., 2018). Consumption of raw or processed pumpkin seeds could be helpful in curing depression. The protein isolates from the pumpkin seed could inhibit the trypsin and activated the Hageman factors (Ratnam *et al*., 2017). The fruit of the *Cucurbita pepo* L. plant is used as a cooling agent and is effective in relieving constipation. It also supports dental health, aids in treating throat and eye infections. The leaves are edible, serve as an analgesic, help eliminate biliousness, and are applied externally to treat burns. The seeds are diuretic, stimulant, and beneficial for conditions such as painful chest, bronchitis, fever, excessive thirst, and kidney issues, while also supporting brain health. The extract from *Cucurbita pepo* L. fruit and seed is known to improve urinary dysfunction as well as prostatic hyperplasia (Yadav *et al*., 2017).

2.5.14. Reproductive Health

Pumpkin seeds improve libido and semen quality by elevating the serum testosterone level. It has been demonstrated that pumpkin seeds and daily rich diet of zinc can decrease the undesirable side effect of lead contaminants and improve the sexual health status (Abd El-Ghany *et al*., 2010). Also, pumpkin seed can inhibit citral induced hyperplasia of the ventral prostate lobe by reducing protein binding prostate levels (PBP), weight of ventral prostate lobe and improve histology of testis. Therefore, it may be helpful in the management of benign prostatic hyperplasia (Henkel *et al*., 2005).

Pumpkin seeds improve sexual stimulation and intromission and ejaculatory latency (Gundidza *et al*., 2009). Pumpkin causes a significant reduction in sperm count with primary and secondary abnormalities by producing further zinc and protein. Therefore, pumpkin is proposed for both the prevention and treatment of infertility in male animals (Oyeyem *et al*., 2008). Fluted pumpkin seed oil (FPSO) has been reported to have some essential components (vitamin A, tannins, linoleic acid, oleic acid and alkaloids) which suppress lipid peroxidation, improve testicular function, enhance fertility, and improve sperm count and testicular

histology. FPSO improves semen parameters and has little or no effect on testicular histology when administered at a low dose of 400 mg/kg body weight (Hwang *et al*., 2004). According to Nworgu *et al*., (2007), tannins are classified as antioxidants at a high dose and could become pro-oxidant by increasing lipid peroxidation. This explains for the drop in sperm count and motility including hormonal levels at a high dose of 800 mg/kg body weight FPSO (Hwang *et al*., 2004). pumpkin seed extract ameliorated Cyclophosphamide toxicity as evidenced in the epididymis histology and sperm parameters by preventing oxidative stress (Aghaei *et al*., 2014).

It has been suggested that extracts derived from *Cucurbita pepo* L. may help protect human sperm from damages caused by chemotherapy and autoimmune diseases (Elfiky *et al*., 2012). Gossell-Williams *et al.,* (2006) revealed that the oil from the pumpkin seed is useful in the management of benign prostatic hyperplasia. Hyperplasia was induced by subcutaneous administration of testosterone (0.3 mg/100 g of body weight) for 20 days. Simultaneous oral administration of either pumpkin seed oil (2.0 and 4.0 mg/100 g of body weight) or corn oil (vehicle) was carried out for 20 days. Testosterone significantly increased prostate size ratio, and this induced increase was inhibited in rats fed with pumpkin seed oil at 2.0 mg/100 g of body weight. The protective effect of pumpkin seed oil was significant at the higher pumpkin seed oil dose.

Continued research is needed to identify therapeutically important constituents and dietary components that have antioxidant action, quantify these components and assess their potential for in vivo antioxidant activity and their interactions with target tissue (Sen and Chakraborty, 2017). There is no sufficient information available about the antioxidant potential of *Cucurbita pepo* L. seed extract in mediating oxidative stress induced male infertility that needs a lot of attention.