

Free radicals are produced in the bodies during naturally occurring biological processes. Such natural processes include breathing, digesting food, metabolizing alcohol and drugs, and converting lipids into energy (Kurutas, 2015). Free radicals consist of hydroxyl ions, superoxide, hydrogen peroxide and peroxy radicals. They can have positive impacts on immune function at low to moderate levels. However, at high concentrations, they cause oxidative stress, a damaging process impairing cell functions and structures (Gulcin, 2020).

Oxidative stress stems from the inability to detoxify (Pizzino *et al.*, 2017) the highly reactive free radicals. It disrupts essential cellular processes, inhibit the function of key enzymes, impede normal cell division, damage deoxyribonucleic acid (DNA), modifies chromosomes, and hinder energy production (Juan *et al.*, 2021). The modern lifestyle characterized by unhealthy dietary habits, insufficient physical activity, and exposure to various chemicals like heavy metals, insect repellents, food preservatives, and ecological toxins, can all contribute to oxidative stress. Chronic diseases and disorders, including AIDS, atherosclerosis, cancer, Central Nervous System (CNS) disorders, diabetes, inflammatory bowel diseases, Parkinson's disease, rheumatoid arthritis, and reproductive failures, have been associated with prolonged oxidative stress (Rudrapal *et al.*, 2022). Over the years, several experimental and human studies have been conducted to explore the association between oxidative stress and various diseases (Fenga *et al.*, 2017; Docea *et al.*, 2018; Fountoucidou *et al.*, 2019; Kostoff *et al.*, 2020).

Rapid industrialization and urbanization over the past 20 years have led to significant heavy metal pollution (Hou *et al.*, 2019). Toxic heavy metals are nonessential xenobiotics that can cause reproductive toxicity and relate with testicular degeneration (Li *et al.*, 2018) and impaired semen quality in men (Wan *et al.*, 2019). Exposure to heavy metals can lead to oxidative stress in the male reproductive system, as evidenced by animal models and in vitro assays (Guvvala *et al.*, 2016; Hassan *et al.*, 2019). Thus, oxidative stress may be a key factor in facilitating the adverse impacts of heavy metal exposure on male reproductive health (Kasperczyk *et al.*, 2015).

Reproductive health is crucial for the social and economic development of families, communities, and nations, and is a key element of a fair and equitable society (Sivakumar *et al.*, 2020). Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse (World Health Organization, 2009). Infertility affects between 50 and 80 million people around the world. In general, one in ten couples experience primary or secondary infertility, but the rates vary from less than five percent to more than thirty percent in different countries (Mohanty and Kastor, 2017). In India, approximately 27.5 million couples actively attempting to conceive face challenges with infertility. Infertility has become an alarming concern in which it is assumed that male infertility is more complex and multifactorial (Skoracka *et al.*, 2020).

It is estimated that male factors contribute to up to half of infertility cases, with oxidative stress responsible for between 25% and 87% of cases of male subfertility (de Ligny *et al.*, 2022). The International Classification of Diseases, 11th Revision (ICD-11), identifies male infertility as being commonly caused by changes in semen ejaculation, a lack of sperm or low sperm count, or alterations in sperm morphology and motility. Male infertility is characterized by oligospermia (low sperm concentration in the sperm); asthenozoospermia (an absolute lack of motility or a reduced motility of spermatozoa); and teratozoospermia (an insufficient number of spermatozoa of normal structure) (Skoracka *et al.*, 2020). Seminal quality is an infertility prognostic factor that can be considered a proxy for male infertility, while fertility often depends on other couple-based covariates (Levine *et al.*, 2017). It has long been believed that male unexplained infertility results from environmental, lifestyle and nutritional factors. Congenital malformations, hormonal, genetic, behavioural factors are also thought to be contributing to male infertility. It negatively affects sperm quality by disrupting spermatogenesis, steroidogenesis, and the functions of Sertoli cells and sperm, declining male fertility (Kumar *et al.*, 2023).

Research over the past decade has consistently emphasized the influence of oxidative stress on male infertility. For instance, Agarwal *et al.*, (2017) demonstrated that raised levels of Reactive Oxygen Species (ROS) in semen are linked to decreased sperm motility and increased DNA fragmentation. Likewise, Sharma *et al.*, (2017) found significantly higher oxidative stress markers in infertile men compared to fertile controls. However, the literature

on this topic remains limited, and clinical studies are even more scarce. Additionally, some similar studies yield conflicting results (Benatta *et al.*, 2020).

Antioxidants are our primary protection against harm caused by oxidative stress and are critical to preserving optimal health and well-being (Yadav *et al.*, 2016). These compounds prevent or slow down oxidation when present in low concentrations relative to the oxidizable substrate (Shahidi and Zhong, 2010). The exogenous intake of antioxidants from plants, animals and minerals has been exposed to be advantageous for human health and effective in reducing the occurrence of diseases caused by oxidative stress. As of late, there has been mounting interest in the therapeutic potential of antioxidants for treating conditions associated with oxidative stress (Tian *et al.*, 2017). While antioxidants are widely accessible and affordable compared to other fertility treatments, many remain unregulated, and their effectiveness is still uncertain (de Ligny *et al.*, 2022).

Despite synthetic chemistry becoming the dominant approach in drug development and processing, bioactive components of the plants still hold tremendous potential for providing novel and effective drugs for disease treatment and prevention (Dehelean *et al.*, 2021). Fruits, seeds, pulp, and/or peel/pericarp from dietary plants are rich in bioactive compounds such as proteins, amino acids, phenolic compounds, carotenoids, and vitamins, which contribute to their vital role in human nutrition and health (Huang *et al.*, 2010). However, a key challenge in validating the health benefits of dietary antioxidants lies in the absence of clinical evidence and reliable molecular markers to assess their effects not just on oxidative stress, but also on overall health status (Huang, 2018).

Since ancient times, the plants with medicinal properties have been used and it is also labelled as the origin of modern medicine (David *et al.*, 2015). Researchers have always been interested in plants and other natural resources, primarily for the treatment of numerous medical conditions as well as the development of new medications (Wangchuk, 2018). Plant secondary metabolites have several health promoting qualities in living beings according to several reviews (Clemensen *et al.*, 2020). Therapeutic potential of many plants is owing to its phytochemical properties. Secondary metabolites are used to treat many disease conditions in both traditional and folk medicine. In modern medicine, they act as a leading compound in the production of various drugs for treating migraine to cancer (Hussein and El-Ansary, 2019). Many plants with hidden medicinal properties are yet to be identified.

Pumpkin belongs to the family of Cucurbitaceae. It is typically classified into five species such as *Cucurbita pepo*, *Cucurbita moschata*, *Cucurbita mixta*, *Cucurbita maxima*, and *Cucurbita stilbo* (Dotto and Chacha, 2020). *Cucurbita pepo* Linn (pepita) is most widely used species among the 15 species of genus *Cucurbita* in Cucurbitaceae family. Pumpkin is also known as squash due to their identical nature of gourd like fruits; despite the dissimilarity in their species (Paris and Lust, 2019). It is believed that this annual crop was first domesticated in South America and Mexico before spreading to Europe, Asia, and North America (Paris, 2018). The plant is widely grown in both temperate and tropical regions, primarily for its edible fruit, as well as its leaves, seeds, oil, and medicinal properties. Both its fruits and its aerial components are typically eaten as vegetables (Pooja *et al.*, 2024).

Seeds are the ideal plant components used for the treatment of diseases such as prostate cancer, nephritis, bronchitis, haemorrhoids, hyperplasia and anaemia (Omotayo and Borokini, 2012). Pumpkin seeds are smooth, flat and greenish and contain many phytocomponents (Ramachandran *et al.*, 2017). Apart from using as anthelmintic, the seeds are also used as antitumor, antihypertensive, anti-inflammatory, diuretic and nervine tonic. They are also beneficial in treating taeniasis, strangury and nerve weakness (Ethiraj and Balasundaram, 2016). Pumpkin seeds are rich in minerals like potassium, magnesium, selenium, zinc, copper, molybdenum, and chromium, as well as bioactive compounds such as tocopherols (especially γ -tocopherols) and carotenoids (primarily lutein). They also have a high content of fats, proteins, and essential amino acids (Özbek and Ergönül, 2020).

The high content of magnesium and antioxidants in pumpkin seeds helps in maintaining heart health by reducing blood pressure and cholesterol levels. Studies have shown that pumpkin seeds can lower lipid levels and improve overall cardiovascular health (Patel *et al.*, 2023). Some studies suggest that pumpkin seeds regulate blood glucose levels and improve insulin regulation (Zhang *et al.*, 2022). Pumpkin seeds have been shown to support prostate health and ease symptoms of Benign Prostatic Hyperplasia (BPH) (Dordević *et al.*, 2016). The antioxidants in pumpkin seeds help in reducing inflammation and protecting cells from oxidative damage (Bardaa *et al.*, 2020). Research has indicated that pumpkin seeds possess antimicrobial properties against various pathogens (Singh and Kumar, 2022).

Studies have elucidated the mechanisms through which pumpkin seeds exert their antioxidant effects. For example, Peiretti *et al.*, (2017) identified various phenolic compounds in pumpkin seeds that contribute to their high antioxidant activity. Additionally, Shaban and Sahu, (2017) reported that pumpkin seed oil contains tocopherols and sterols, which play a vital role in counteracting free radicals. Several animal studies have explored the impact of pumpkin seeds on reproductive health. For example, Al-Masri, (2015) inspected and found significant protective effect of combined action of pumpkin seed oil and vitamin E on the testicular impairment induced by lead.

Another study by Aghaei *et al.*, (2014) reported that pumpkin seed extract recovered the altered epididymal histology and improved sperm quality in rats exposed to Cyclophosphamide induced oxidative stress. Al-Salhie *et al.*, (2017) conducted a comparison of the antioxidant activity between pumpkin seed extract and vitamin E in mitigating testicular oxidative stress and found that both treatments were effective, with pumpkin seed extract showing comparable results to vitamin E. This highlights the potential of pumpkin seeds as a natural alternative to synthetic antioxidants.

In order to produce dietary antioxidants having adequate potency for use in supplements or functional food additives, it is essential to successfully extract and isolate the antioxidant compounds from raw plant tissues. The chemical diversity of antioxidants and the complexity of plant tissue composition require various extraction methods to efficiently isolate specific plant antioxidants. These methods can be chemical, physical, or based on phytochemicals, and they are used to extract bioactive compounds for use in dietary supplements, nutraceuticals, food additives, pharmaceuticals, or cosmetic products. Phenolic compounds can be extracted from plant samples that are fresh, frozen, or dried. Typically, the plant materials are first milled, ground, or homogenized before the extraction process (Fan *et al.*, 2022).

Due to their simplicity, effectiveness, and wide applicability, extractions are the most extensively used processes for collecting bioactive molecules from plant sources. The efficiency of chemical extraction is affected by factors such as the type of solvent (which varies in polarity), extraction time and temperature, sample-to-solvent ratio, and the chemical and physical properties of the plant material. The solubility of phenolics is primarily governed by the chemical composition of the plant sample and the polarity of the solvents

used. Depending on the solvent method, a mixture of phenolics can be extracted from plant materials. Common solvents, including methanol, ethanol, acetone, ethyl acetate, and their combinations (often with varying amounts of water), are frequently used to extract phenolics (Alara *et al.*, 2021). Given the growing demand for herbal products with health benefits, the most efficient methods for extracting the active ingredients have to be established (Mohammad *et al.*, 2016). Aqueous extraction offers several advantages over traditional solvent extraction, such as lower initial capital investment, safer operation, the ability to perform discontinuous operations, and greater flexibility in product manufacturing (Sorita *et al.*, 2023).

Animal studies are conducted for a variety of scientific, ethical, and practical reasons. They play a critical role in understanding disease mechanisms, which can lead to the development of new treatments and therapies. By studying animals, researchers can observe disease progression and the effects of potential treatments in a controlled setting (Perlman, 2016). Before new drugs are tested in humans, they must undergo extensive testing in animal models to ensure their safety and efficacy, a crucial step in the drug development process as required by regulatory agencies globally (Van Norman, 2019). Many animals possess considerable genetic and physiological similarities to humans, making them important models for investigating human biology and diseases. For instance, mice and humans share approximately 85% of their genes (Domínguez-Oliva *et al.*, 2023). Animal studies allow researchers to control variables such as diet, environment, and genetics, factors that are often difficult to manage in human studies (Pound and Ritskes-Hoitinga, 2018).

Scope of the Study

The present study explores the intricate relationship between heavy metal toxicity, oxidative stress, male infertility, and the potential therapeutic role of antioxidants, with a specific focus on the promising effects of *Cucurbita pepo* L. seeds. Understanding these relationships is crucial for developing effective strategies to mitigate the adverse effects of toxicity induced oxidative stress on male reproductive health and enhances fertility outcomes.

Significance of the Study

The findings of this study may offer valuable insights into the antioxidant potential of *Cucurbita pepo* L. seeds in addressing male infertility associated with oxidative stress. These findings may pave the way for natural, plant-based interventions in the management of male infertility, offering a safer and potentially more effective alternative to conventional therapies thereby contributing to the broader field of reproductive health research.

With this in consideration, the present study is an effort to evaluate the antioxidant potential of *Cucurbita pepo* L. seed extract in the treatment of stress induced male infertility using in vivo models with the following objectives.

Aim of the Study

- Evaluate the antioxidant potential of *Cucurbita pepo* L. seed extract in the treatment of stress induced infertility in male Wistar rats.

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