In Vivo and *In Silico* Evaluation of *Citrus sinesis* Peel Extracts on Oxidative Stress, Inflammation and Hormonal Parameters in Testosterone Induced Benign Prostate Hyperplasia in Male Wistar Rats

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Certification

This is to certify that Omolola Mary SAMUEL with matriculation number LCU/PG/002341 carried out this research work titled "*in vivo* and *in silico* evaluation of *Citrus sinesis* peel extracts on oxidative stress, inflammation and hormonal parameters in testosterone-induced benign prostate hyperplasia" in the Department of Chemical Sciences (Biochemistry Unit), Faculty of Applied Sciences, Lead City University, Ibadan, Oyo state, for the award of Master Degree (MSc) in Biochemistry and that this has not been previously submitted.

……..... …….....

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Date

Dedication

This work is dedicated to Almighty God who has been my closest companion and my strength, to my parent and my siblings for their care, support, prayers and encouragement.

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Abstract

Benign prostate hyperplasia (BPH) is a noncancerous enlargement of the prostate gland that makes urinating challenging. *Citrus sinesis* peel (CSP) has been reported to have antioxidant, anti-inflammatory, and antitumour properties. This study investigated the *in vivo* and in *silico* effects of CSP extracts on testosterone-induced Benign Prostate Hyperplasia (BPH). Forty-eight rats were randomized into eight groups of six animals each: non-castrated control, castrated control, castrated rats that received testosterone propionate (TP) subcutaneously, castrated rats received TP and 250mg/kg MECSP, castrated rats that received TP and 500mg/kg MECSP, castrated rats that received TP and 250mg/kg HECSP, castrated rats that received TP and 500mg/kg HECSP and castrated rats that received TP and Dutasteride (standard drug). Molecular docking analysis was conducted using Autodock Vina from PyRX. Results indicated that BPH rats had significantly ($p < 0.05$) increased relative organ weight, 5α-reductase activity and MDA levels in serum, prostate and liver in the BPH rats and oxidative stress biomarkers were altered significantly. Liver enzyme activities (AST and ALT) and inflammatory markers (PPARα and NFkB) were significantly increased in the serum of the BPH rats. There was significant reduction in testosterone, progesterone and Luteinizing hormone and histology of the prostate revealed hyperplasia. Treatment with MECSP, HECSP and Dutasteride, respectively attenuated these changes. GC-MS analysis showed 40 compounds in MECSP and 34 compounds in HECSP. Molecular docking shows that interaction of compounds from MESCP with 5α-reductase and Prostrate specific membrane antigen. Cholestan-7-one, cyclic 1, 2-ethanediol acetal, (5.alpha.) and Vitamin E had the highest binding affinities for 5α-reductase and 4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-and Vitamin E had the highest binding affinity for prostate-specific membrane antigen. CSP extracts elicited protective effects on BPH via restoring the relative organ weight, 5α-reductase activity, biomarkers of oxidative stress, inflammatory biomarkers, and hormonal parameters. These extracts contain bioactive compounds that might be chemopreventive against Benign prostate hyperplasia.

Keywords: Benign prostate hyperplasia, *citrus sinesis* peel, 5α-reductase, *in silico*, oxidative stress

Word Count: 300

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List of Acronyms

Abbreviation	Meaning
BPH	Benign Prostate Hyperplasia
DHT	Dihydrotestosterone
CSP	*Citrus sinesis* Peel
MECSP	Methanol Extract of *Citrus sinesis* Peel
HECSP	n-Hexane Extract of *Citrus sinesis* Peel
ADME	Absorption, Distribution, Metabolism, Excretion
Gc-Ms	Gas Chromatography- Mass Spectroscopy
PDB	Protein Data Bank
PK	Peak
RT	Retention Time
CSPE	*Citrus sinesis* Peel Extracts

Chapter One

Introduction

1.1 Background to the Study

Benign Prostate Hyperplasia (BPH) is a condition in which the prostate grows and develops non-cancerously[1]. The prostate gland, characterized by its walnut shape, is an integral part of the male reproductive system. It functions as a supportive organ, playing a crucial role in male reproductive functions. The prostate goes through two main growth periods as a man age. The first occurs early in puberty, when the prostate doubles in size[2,3]. The second phase of growth begins around age 25 and continues during most of a man's life. Benign prostatic hyperplasia often occurs with the second growth phase[1]. The main role of the prostate gland is to generate and release an alkaline solution that envelops the sperm[4,5]. This prostate fluid is essential for maintaining fertility in men. Positioned at the neck of the bladder, the prostate gland surrounds the urethra, serving as a protective and anatomical structure in relation to the urinary and reproductive systems[6].

Globally, BPH typically affects approximately 50% of men in their 50s and 90% of men in their 80s[7,8]. In other words, BPH is typically diagnosed in 9 out of 10 men in their 90s, compared to 5 out of 10 men who are 50 years old[9]. The urethral tissue region of the prostate is affected by this condition when stromal cells and epithelial cells proliferate more frequently[10]. Multiple factors contribute to the development and genesis of BPH[11]. Age, genetics, geographical location, and obesity are factors that contribute to BHP. BPH develops and progresses by other factors, including elevated oxidative stress, protracted inflammation, and hormonal changes[12]. However, hormonal changes are one of the main contributors of BPH growth and metastasis; especially the rise in the synthesis of Dihydrotestosterone (5α-DHT)[13]. 5α-DHT are series of hormones

necessary to keep the prostate's size and functionality stable. As a result, they are suggested to be crucial in the development of BPH[14, 15, 16, 17]. The nuclear membrane contains a lipophilic enzyme called 5-reductases that converts testosterone into dihydrotestosterone. The pharmacological method has been utilized to treat BPH and comprises 5-reductase inhibitors which are basically dutasteride, finasteride and antagonists targeting 1-adreno receptors[18]. 5α-reductase inhibitors help in the management and cure of BPH by reducing the size of the prostate by 20-30% and this contributes to the disruption of the progression[13, 15]. The following conditions has been associated with BPH: age, family history of BPH, medical conditions such as obesity, heart and circulatory disease, and type 2 diabetes, erectile dysfunction, high intake of animal protein and low levels of fruits and vegetables[11]. According to numerous earlier studies, fruits have anticancer properties, which are derived from the phytochemicals they contain. Phytochemicals are biologically active substances found in plants that give them different flavors and colors in addition to protecting them from pests[19].

Citrus fruits are advantageous for commercial purposes because they are nutrient-rich and have a distinctive flavor[20]. According to reports from around the world, 70 million tons of citrus are produced globally between 2020 and 2021, with citrus sinesis accounting for roughly half of all citruses. Forty-five (45) percent of these citruses' peel is produced as a result of the additional processing that turns about 50 percent of them into juice[20]. As a result, enormous quantities of peels are obtainable every year. The citrus peel is considered to be a waste product. Nevertheless, it is composed of varieties of bioactive compounds with a wide range of benefits. Also, these citrus peels composed of bioactive compounds like: limonene, Hesperetin, Nobiletin, Narigin; and phenolic compounds like: polymethoxylated flavones, phenolic acids and few percentages of glycosylated flavanones. The extraction of the biologically active compound derived from the

peel of citrus is necessary to increase the worth and significance of products made from it[21]. Orange peel waste can be harnessed for sustainable value, it is useful in making several value-added bioproducts such as: biochemicals (lactic acid and succinic acid), biopolysaccharides (xanthan and curdlan gum), and bioenergy (biomethane and bioethanol). Therefore, for successful and maximum applications, citrus peel should be investigated extensively. *Citrus sinesis* (orange) peel has been processed locally and valuable portion has been collected which are utilized in manufacturing of food, cosmetics and medications[22]. In addition, biological relevance and effects of citrus peel has been documented. According to reports, *Citrus sinesis* (orange) peel is rich in total antioxidative potentials which are used as antioxidants in the food production and in medicines[14]. However, the efficacy of citrus sinesis peel extracts in the treatment of BPH has yet to be confirmed. This research therefore sought to examine the potential therapeutic effects of extracts from *Citrus sinesis* peel on BPH using male *Wistar* rats.

1.2 Statement of the Problem

Benign Prostate Hyperplasia (BPH) is a prevalent condition that affects a significant number of men, which necessitates appropriate intervention. In Nigeria, BPH affects 50% of men in the 50's and 80% of men in their 90's[19, 20]. If left untreated, BPH can progress to prostate cancer, making it a significant concern[23, 24, 25 ,26]. In Nigeria, benign prostate hyperplasia ranks among the primary causes of cancer-related fatalities in men[27, 28, 29].

1.3 Justification of the Study

Natural products have crucial role in drug discovery[22]. From past eras medicinal plants occurred on earth and with time it become of global and utmost importance. Every plant has its own therapeutic effects due to its unique bioactive compounds, which are usually non-toxic compared

to synthetic compounds. The severe side effects of standard hepatoprotective drugs necessitates the search for alternative treatment options[22]. The popularity of medicinal plants as an alternative to synthetic medications is supported by the fact that they are inexpensive, accessible, and unlikely to experience resistance. Herbal therapies, homoeopathic medications, conventional medications, and functional foods all contain plant products. Traditional communities have employed medicinal plants as a form of treatment for many centuries.

Targeting receptors implicated in prostate hyperplasia has been reported as a possible therapeutic intervention to ameliorate this condition[18]. The citrus peel is considered to be a waste product. Nevertheless, it is composed of varieties of bioactive compounds with a wide range of benefits. Recent studies have reported the antioxidative potential of *Citrus sinesis* (orange) peel; and thus, are used in the food production and in medicines[13, 19]. However, the efficacy of *Citrus sinesis* peel extracts in the treatment of BPH has yet to be confirmed.

1.4 Aim and Objectives of the Study

This study investigated the *in vivo* and in *silico* effects of methanol extract of *Citrus sinesis* peel (MESCP) and n-hexane extract of *Citrus sinesis* peel (HESCP) on testosterone-induced Benign Prostate Hyperplasia (BPH).

The specific objectives are to:

I. determine the effects of MECSP and HECSP on relative organ weight in the prostate and liver of testosterone-induced BPH rats;
II. investigate the effects of MECSP and HECSP on the activity of 5α-reductase in the serum of the animals;

III. determine the effects of MECSP and HECSP on liver function markers in the serum of BPH rats by assaying for alanine aminotransferase (ALT) and aspartate aminotransferase (AST);

IV. determine the effects of MECSP and HECSP on biomarkers of oxidative stress: the activities of enzymatic antioxidants; superoxide dismutase (SOD), catalase (CAT), GSH, GPx and malondialdehyde (MDA) levels in the serum, prostate and liver;

V. determine the effects of MECSP and HECSP on inflammatory biomarkers in the serum of BPH rats by assaying for nuclear factor kappa light chain enhancer of activated B cells (NF-kB) and peroxisome proliferators activator receptors alpha (PPAR-α) in the serum of the rats;

VI. assess the effects of MECSP and HECSP on testosterone, progesterone, follicle stimulating hormone, luteinizing hormone and estradiol in the serum of BPH rats;

VII. determine the effects of MECSP and HECSP on histology of the prostate and liver of rats;

VIII. determine the bioactive compounds present in MECSP and HECSP using GCMS analysis;

IX. identify bioactive compounds in CSPE that has inhibitory effect on proteins (5α-reductases) that facilitate prostate hyperplasia using molecular docking;

X. identify bioactive compounds in CSPE that has inhibitory effect on Prostate specific membrane antigen that facilitate prostate hyperplasia using molecular protein docking.

1.5 Research Questions

i. Can the extracts of *Citrus sinesis* peel be as effective as Dutasteride (standard drug) in the management of BPH?

ii. What are the bioactive compounds in *Citrus sinesis* peel extracts that have anti-hyperplasia efficacy?

iii. Which of the two extracts of *Citrus sinesis* peel has the better *in vivo* effects on BPH rats?

iv. Does BPH affects hormonal levels?

1.6 Significance of the Study

The targeting of receptors associated with benign prostate hyperplasia has been proposed as a potential therapeutic approach to improve this condition[30, 31]. The peel of *Citrus sinesis* is abundant in molecules that possess anti-cancer, anti-inflammatory, anti-oxidant, and immuno-modulatory properties which may possess lead compounds that can be used in the management of this condition[32, 33]. Hence, the need for this study.

1.7 Scope of the Study

This study investigated the *in-vivo* and *in-silico* effects of two extracts of *Citrus sinesis* peel in Testosterone-induced BPH. Testosterone propionate was used to induce BPH in male Wistar rats.

1.8 Limitation of the Study

This study considered the bioactive compounds present in MECSP and HECSP. The study was carried out in male Wistar rats.

1.9 Operational Definition of Terms

Benign: Benign means non-threatening or harmless.

Hormone: Hormones are chemical messengers that are produced by various glands in the body's endocrine system**.**

Hyperplasia: Hyperplasia is an abnormal growth in the number of cells within a tissue or organ resulting in enlargement.

Antioxidant: Antioxidants are compounds that protect the cells from damaging effects of free radicals. Oxidative stress occurs when there is an imbalance between free radicals and antioxidants in the body. Free radicals are highly reactive molecules that can damage cells, proteins, and DNA, potentially leading to various health problems, including cancer, cardiovascular disease, and aging-related conditions.

Orange peel waste (OPW): a discarded part of orange fruit.

Docking: Docking, in the context of molecular biology and bioinformatics, refers to the process of predicting and analyzing the binding interactions between a small molecule (ligand) and a larger macromolecule (target), such as a protein or nucleic acid. It involves computational methods and algorithms that simulate and predict how the ligand binds to the target molecule's binding site.

Histology: Histology is the scientific discipline within biology and medicine that concentrates on the microscopic investigation of cells and tissues. Its primary objective is to analyze and understand the microscopic structure, composition, and arrangement of tissues.

Inflammation: Inflammation is a complex physiological response triggered by the body's immune system to protect and heal tissues in response to injury, infection, or irritation. It is a vital part of the body's defense mechanism and is typically characterized by redness, swelling, heat, pain, and sometimes loss of function in the affected area.

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Chapter Two

Literature Review

2.1 Benign Prostate Hyperplasia (BPH)

Benign Prostate Hyperplasia (BPH) is a characterized by the non-cancerous growth and development of the prostate gland[1]. BPH is a type of histological condition that occurs when the cell count of the stromal and epithelial cells within the prostate gland's transition zone increases[2]. This hyperplasia causes the development of enlarged, distinct prostatic nodules[2]. The prostate undergoes two significant periods of growth as a man increases in age; the first period is the puberty period when the prostate size increases, and the second period begins within the age circle of 25, and this continues all through the man's life. Benign prostatic hyperplasia mostly develops with the second stage of growth[3]. The prostate's enlargement causes the urethra to be compressed and constricted, resulting in pressure on the urethra. This results to a thickened wall of the bladder. Over time, the bladder may deteriorate and lose its ability to completely empty, resulting in urine retention. The majority of symptoms associated with benign prostatic hyperplasia (BPH) arise from the narrowing of the urethra and the inability to fully empty the bladder[3].

An androgen; dihydrotestosterone (DHT), which is synthesized from testosterone through the activity of the enzyme; 5α-reductase in the prostate promotes and stimulate the growth of the prostate cells[4]. One of the risk factors for BPH is hormonal changes, specifically the increased conversion of testosterone to dihydrotestosterone (DHT) in the prostate through the activity of

5α-reductase. These hormonal changes contribute to the enlargement of the prostate and the disease progression. Therefore, inhibiting 5-reductase would result in less DHT being produced and would stop BPH from developing. BPH development is also linked to elevated oxidative stress as well as persistent inflammation[5].

2.1.1 Epidemiology of BPH

It has been reported that one third of men diagnosed of BPH whose age are above 50 years say it has a detrimental effect on their living conditions. By 85, histological visibility of BPH is said to occur in up to 90% of men. Up to 14 million men in the US experience BPH symptoms[6].

Age, race, genetics, and lifestyle all contribute to BPH prevalence[7]. As individuals grow older, the incidence of BPH rises considerably. Autopsy study have shown that, the prevalence of histological findings for BPH was 8% in the fourth decade of life, 50% in the sixth decade, and 80% in the ninth decade. Furthermore, experimental studies conducted in the United States, Europe, and Asia have consistently demonstrated that advancing age is a risk factor for the development and progression of clinical BPH[8]. Furthermore, data from the Krimpen and Baltimore Longitudinal Study of Aging show that the prostate gland undergoes an annual growth rate of approximately 2.0 percent to 2.5 percent in older men, supporting the notion that the prostate enlarges with age[9]. Increase in the size of the prostate contributes to the risk of the development of LUTS, and large size of prostates are linked to benign prostatic enlargement (BPE), increased like hood of diagnosis of clinical BPH, urine incontinence, and the requirement for surgery of the prostate[10].

Regarding the relationship between BPH risk and race, no obvious trends have emerged. Observational studies comparing black, Asian, and white men have yielded varying results.

Specifically, research conducted on black males in the United States has shown that their prostate has a larger total volume and transition zone compared to white men. However, extensive studies such as the US Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial and the Health Professionals Follow-Up Study have not found any differences in the risk of clinical BPH between black and white men. According to certain studies, Asian men may have a lower probability of developing clinical BPH than white men[10].

There is significant evidence between BPH and hereditary component. Male cousins and brothers exhibited risks specific to their age group for BPH surgery, with a four-fold and six-fold increase, respectively, according to a case control analysis of men under the age of 64 who received BPH surgery[11]. Furthermore, according to these researchers, heritable BPH affected 50% of males under 60 who underwent surgery for the condition[11]. They found that heritable illness had a greater prostate size and an earlier age compared to sporadic case of BPH in a subsequent investigation. An autosomal dominant mode of inheritance is suggested by these and other data.

More and more evidence suggest that lifestyle variables can significantly affect how BPH develops naturally[12]. Despite the contradictory trends, there are some signs that both macronutrients and micronutrients may have an impact on the risk of BPH[13]. Regarding macronutrients, higher consumption of total energy, energy-adjusted total protein, beef, fatty meals, milk and dairy products, cereals, bread, poultry, and starch may contribute to an increased risk of developing clinical BPH, whereas polyunsaturated fatty acids, fruits, vegetables, vitamin D and linoleic acid have the potential to lower the risk. As regards micronutrients and BPH, elevated circulating levels of vitamin E, lycopene, selenium, and carotene have been inversely associated. Both increased and decreased risk have been linked to zinc[13]. Increased exercise and

physical activity have consistently demonstrated a correlation with reduced likelihood of lower urinary tract symptoms (LUTS), clinical BPH, histological BPH, and BPH surgery. According to a meta-analysis of 11 published studies involving a total of 43,083 participants, moderate-to-vigorous physical activity was associated with a decreased risk of BPH by up to 25% compared to a sedentary lifestyle. Furthermore, the preventive effect appeared to increase with higher levels of physical activity. Similar to exercise, moderate alcohol consumption appears to be preventative for a number of BPH-related outcomes. According to a meta-analysis of nighteen published report involving a total of 120,091 participants, consistent alcohol consumption among males was associated with a reduced risk of developing BPH by up to 35 percent[13].

2.1.2 Aetiology of BPH

While the precise underlying cause of BPH remains uncertain, it is widely accepted that aging, in conjunction with the influence of androgens play significant roles in its onset. The accumulation of the androgen hormone dihydrotestosterone (DHT) is one of the primary contributing factors to the progression of this condition[14].

2.1.3 Symptoms of BPH

BPH typically presents various symptoms, such as difficulty in starting a urine stream, frequent urination, nocturia, pain during urination, and unusual urine color or smell[14].

2.2 Benign and Malignant Tumor

The term "tumor" comes from the Latin word "tumere," which signifies swelling. It refers to an abnormal mass of tissue that serves no beneficial purpose in the body[15]. While all tumors are not desirable, not all of them are cancerous or aggressive. Tumors can be categorized as either

benign or malignant. Determining whether a tumor is benign or malignant requires a pathology examination. Through the examination of biopsy samples under a microscope and conducting molecular tests, pathologists can determine if the tumor is benign or malignant[15].

2.2.1 Malignant Tumours

The term "malignant" originates from the Latin word "malignus" and carries the meaning of being malicious[16]. A malignant growth is characterized by cells that have an aberrant and irregular structure, shape and function in contrast to the surrounding healthy cells. These malignant tumor cells have the ability to divide uncontrollably, giving rise to daughter cells that can essentially live forever. They can invade and destroy nearby tissues and have the metastasize to distant parts of the body via the bloodstream or lymphatic system. This stands as one of the factors why surgical treatment alone is often ineffective for advanced malignancies since it is challenging to completely remove all malignant cells that may have already spread. Leaving behind malignant cells during surgery can lead to the development of new tumors in different locations. To combat malignancy, chemotherapy or radiation therapy is often employed to target a broader area beyond the primary tumor itself. It's important to note that different types of malignant tumors originate from distinct types of cells[17].

Examples of Malignant Tumours

Carcinoma: Carcinomas refer to malignant tumors that arise from parenchyma tissues. These malignant tumors arise from epithelial cells, which are present in the skin and the linings of organs throughout the body. Carcinomas can occur in various organs, including the stomach,

prostate, pancreas, lung, liver, colon, and breast. They represent a common form of malignant tumors.[18]

Sarcoma: Sarcomas denote malignant tumors originating from mesenchymal tissues. These tumors are relatively rare and emerge from connective tissues like cartilage, bones, fat, and nerves[19].

Blastoma: Blastomas originate from embryonic tissue or precursor cells, commonly known as blasts. These tumors are more frequently observed in children. They are named based on their location in the body, such as hepatoblastoma in the liver, nephroblastoma in the kidney, medulloblastoma in the brain, and retinoblastoma in the eye[20].

2.2.2 Benign Tumours

The term "benign" has its roots in the Latin word "benignus," which signifies non-threatening or harmless. A benign tumor, while occasionally causing discomfort, is not cancerous and typically does not pose a threat to life[21]. However, there are instances where they can become problematic, such as when they grow to a significant size (like certain benign ovarian tumors) or when they affect critical organs, blood vessels, or nerves, making surgical removal difficult. For example, benign Schwannomas or meningiomas in the brain can compress vital areas in the brain or spinal cord, and they can also become concerning if they stimulate the excessive production of hormones[22].

Typically, benign growths comprise cellular clusters that closely resemble the normal cells present in the surrounding tissue. They typically do not invade surrounding tissues because they retain surface recognition proteins that enable them to bind together and prevent invasive

behavior or metastasis. Instead, they tend to displace normal tissue. In some cases, benign tumors may progress to a premalignant state and eventually become malignant, although this process usually takes a long time, often spanning decades. Many individuals live with benign tumors throughout their lives, but they are typically removed through surgery, allowing pathologists to confirm their benign nature. Once surgically removed, benign tumors do not usually recur. In most tissues, benign tumors are generally designated by adding the suffix "-oma[21]."

Examples of Benign tumors

Adenomas: Adenomas emerge within the glandular epithelial tissue, which encompasses the thin membrane that covers glands, organs, and several structures in the body. The various examples in this category are stated below:

- Colon polyps, which are usually benign growths on the mucous membrane's surface in the colon, have the potential to remain dormant for several decades;
- fibroadenomas, a prevalent form of benign tumor in the breast; and
- hepatic adenomas, occurring in the liver.
- Adenomas do not initially manifest as cancerous growths, but certain cases may undergo transformations and progress into adenocarcinomas, which are malignant[23].

Fibroids (Leiomyomas): Fibroids, also known as fibromas, are benign tumors that can develop on the fibrous or connective tissue of any organ. They can be classified as "soft" or "hard" based on the proportion of fibers to cells. Some fibromas can cause symptoms and may require surgical intervention. Fibroid tumors are named according to their location in the body, such as uterine fibroids, which are quite common[24]. Despite being benign, uterine fibroids can lead to heavy

vaginal bleeding, pelvic pain or discomfort, and urinary incontinence. In rare cases, fibroids can undergo changes and become fibrosarcomas, which are cancerous. Other types of fibroids include angiofibromas, which may appear as small red bumps on the face, particularly on the nose and cheeks, and dermatofibromas, which typically occur on the skin, often on the lower legs[25,26].

Desmoid tumor: A desmoid tumor is an abnormal growth originating from connective tissues. They typically occur in the abdomen, arms, and legs. Desmoid tumors are often more aggressive than typical benign tumors and may invade nearby tissues and organs, although they do not metastasize. They can exert pressure on blood vessels and nerves, causing pain and impairing the affected body part's functionality. In cases where the leg is affected, it can lead to a limp[27, 28].

2.3 Citruses as Medicine

Natural products have proven to be a valuable resource for discovering new drugs, surpassing synthetic compounds in terms of their structural diversity on a larger scale[29]. They have acted as significant repositories of bioactive substances and will continue to be pivotal in the exploration of innovative pharmaceuticals. The Citrus genus, which falls under the Rutaceae family, holds utmost importance as a fruit tree crop worldwide, with a generation of about 102 million tonnes yearly. Several Citrus species, including lemon (C. limon), citron (C. medica), sour orange (C. aurantium), grapefruit (C. paradisi), mandarin or tangerine (C. reticulata), clementine (C. clementina), and sweet orange (C. sinensis), offer various benefits. These Citrus herbs contain prominent biologically active components including hesperidin, naringin; which are classified as Flavonoid and synephrine; which belongs to the alkaloid family, these active components are beneficial to human's wellbeing. Since citrus species are composed of flavonoids, hence,

contributes to their characteristic properties as an antioxidant, anti-inflammatory, anticancer, anti-lipidemic, and antibacterial[30].

Citrus fruits are abundant sources of essential nutrients and other beneficial phytochemicals[30]. These phytochemicals contribute to cardiovascular health and support proper functioning of the nervous system. Citrus fruits are known for their ability to boost antioxidant activity in the liver, safeguard DNA from lipid-induced damage, and fortify the immune system.

Citrus fruits are valued for more than just their nutritional value; they are also renowned worldwide for their delightful flavors. Citruses are widely cultivated across the globe to satisfy the growing desire of consumers. Consequently, the production of citrus fruits continues to rise each year. Annually, the citrus processing sector produces a notable volume of fruit waste, of which citrus peel waste constitutes over half of the total moisture-laden fruit mass. This waste material from citrus fruits possesses significant economic worth owing to its abundant reserves of essential oils (EOs), ascorbic acid, sugars, carotenoids, flavonoids, dietary fiber, polyphenols, and trace elements. Additionally, the waste harbors a substantial concentration of fermentable sugars that can be effectively harnessed for the production of bioethanol. These constituents hold great value in the creation of pharmaceuticals, cosmetics, and nutritional supplements[30].

2.3.1 Bioactive compounds in Citrus waste

The cultivation of citrus fruits involves extensive processing, which results in the production of diverse wastes that contain high levels of bioactive components and by-products, including pectin, essential oils (EOs), and antioxidants that are both water-insoluble and water-soluble[31].

The citrus by-products and wastes hold significant importance and can be efficiently used for various purposes; including health-related technological. They are composed of biologically active substances that exhibit biological activity, such as carotenoids, polyphenols, and essential oils. The antioxidant properties of carotenoids and polyphenols contribute to a wide range of health benefits[31].

The applications of orange peel waste encompass diverse aspects including: solid biofuel production, bioabsorbents, extraction of essential oils (EOs), extraction of pectin, production of ethanol and methane, industrial enzyme production, and single-cell protein production[32].

2.3.1.1 Dietary Fiber

Citrus fruits are composed of fiber (those which are soluble and those which are not soluble), which can be distinguished. The dietary fiber that are soluble primarily consists of mucus, gum, and pectin, while dietary fiber that are insoluble mainly composed of hemicellulose, cellulose, and lignin. Cellulose and hemicellulose make up around 50-60% of the dry weight of citrus fruits, making them significant sources of these substances[33].

2.3.1.2 Essential Oils (EOs)

Essential oils (EOs) are volatile aromatic compounds produced by plants, these oils are reported to be used as flavors in food, medicine, and cosmetics. Citrus species have gained significant attention as a result of the high concentration of essential oils[34].

Hydrodistillation was utilized to extract citrus essential oils from five different citruses. GC-MS analysis showed distinct phytochemical compositions in each of the five citrus oils. The distinct phytochemical compositions include: alkaloids, tannins, sterols, terpenoids, saponins, and limonene[35]. In a previous study, the essential oil derived from the aerial parts of Citrus

aurantifolia L. was examined for its antioxidant properties, antimicrobial activity, and chemical composition. The oil was extracted using hydrodistillation, and GC-MS analysis was employed to identify and quantify its chemical constituents. Thirty-three compounds were identified, with d-limonene being the major constituent. Minor constituents included 3,7-dimethyl-2,6-octadien-1-ol, geraniol, E-citral, Z-citral, and β-ocimene[35].

2.3.1.3. Carotenoids

Carotenoids, which are hydrocarbons soluble in lipids, and their oxygenated derivatives called xanthophylls, are present in various fruits, vegetables, and green leaves. Carotenes can be found in red and yellow fruits, green leaves, and many root vegetables. Citrus fruits, in particular, are known for their high content of carotenoids. Fruits and vegetables are rich sources of carotenoids, including zeaxanthin, lutein, lycopene, cryptoxanthin, and pro-vitamin A[36].

Carotenoids play a vital role in supporting human health by acting as antioxidants, promoting bone growth and health, positively influencing the immune system, facilitating cell-to-cell communication, enhancing eye health, and reducing the risk of cancer. Quite a number of past studies have demonstrated the numerous health benefits associated with carotenoids. Moreover, carotenoids are the only sole-established substitute to vitamin A in humans due to their ability to generate this essential vitamin. Given their high carotenoid content, citrus fruits serve as a significant dietary source of these beneficial nutrients.

In the extraction of carotenoids from sour oranges, methanol and diethyl ether have been found to be excellent solvents[37].

2.3.2 Health Benefits of Bioactive compounds obtained from *Citrus sinesis*

Citrus fruits contain various secondary metabolites. These secondary metabolites in citrus have a significant impact on human health, offering diverse biological effects including neuroprotection, anti-inflammatory properties, antioxidant activity, anti-cancer potential, and cardiovascular protection[37]. The bioactive compounds present in citrus provide several health benefits, which include the following:

2.3.2.1 Anti-Cancer Properties

Cancer remains a leading cause of untimely death worldwide. In recent years, there has been growing interest among scientists and researchers in developing food products with potent anti-cancer effects. However, many existing food products are limited in the areas of applicability and effectiveness, often associated with significant adverse effects that can negatively impact patients' overall quality of life[37].

Within the realm of functional foods, the use of natural products as a basis for anti-cancer drugs has gained popularity due to their efficacy and low toxicity in managing and treating cancer. Citrus fruit peels and similar extracts have shown promising anti-cancer potential, primarily attributed to their high flavonoid content. These compounds inhibit processes such as inflammation, angiogenesis, proliferation, and apoptosis as part of their therapeutic strategy[37].

2.3.2.2 Mental Health and Metabolism

Citrus fruits are abundant in polymethoxylated flavones and flavanones, which have been found to be beneficial for brain health. Pre-clinical research has demonstrated the neuroprotective properties of these compounds in models of dementia, including Alzheimer's disease. Similarly, clinical and epidemiological studies have revealed that flavonoids present in citrus peels and extracts are associated with improved cognitive performance and a reduced risk of depression

and dementia. These findings provide further evidence of the positive impact of citrus peels and extracts on human cognition and related functions[38].

2.3.2.3 Prevention of Oxidative Damage and Cardiovascular Disease

Cardiovascular diseases pose a significant global health challenge, resulting in a high number of annual deaths and an increasing prevalence of treatment resistance. This area of research requires greater attention from scientists and researchers to explore new natural approaches for isolating beneficial compounds from citrus fruits like lemons, sweet oranges, and grapefruits, which have shown potential in hypertension treatment. Citrus extracts have been found to have a notable impact on reducing hemorrhages[39].

Citrus fruits contain flavonoids such as chrysin, luteolin, and 7-hydroxy flavone, which play a crucial role in inducing lipoprotein production in the umbilical vein. Within the human body, endothelial cells oxidize low-density lipoprotein (LDL), resulting in the generation of reactive oxygen species. LDL regulates the bioactive compounds in our system, and these compounds have demonstrated effectiveness in controlling hypercholesterolemia and atherosclerosis. Citrus flavonoids possess various qualities, including scavenging free radicals, altering lipid metabolism, improving glucose tolerance and insulin resistance, promoting adipocyte differentiation, alleviating endothelial dysfunction, and reducing inflammation and apoptosis. Studies have indicated that flavonoids found in citrus fruits can contribute to improved cardiovascular health outcomes[39].

Effective self-care for heart failure has been associated with depression, although recent research suggests a more complex relationship between the two. Insufficient daily intake of citrus fruits has been linked to an increased risk of developing depression in patients with chronic heart

failure. These findings support the idea that incorporating citrus fruits into daily dietary habits may help prevent and manage depression in individuals with chronic heart failure[39].

2.3.2.4 Prevention of Obesity

Obesity is a widespread and life-threatening condition that affects individuals globally. Conventional anti-obesity medications often come with significant side effects, and their long-term effectiveness is uncertain. consequently, there has been increasing interest in complementary and alternative medicines for their potential health benefits[40]. Citrus phytochemicals have emerged as promising candidates in the treatment of obesity-related disorders. Citrus fruits, known for their abundance of flavonoids and p-synephrine, are recognized as the primary active biological ingredients. Numerous studies have demonstrated the anti-obesity properties of citrus fruits through various mechanisms, including the regulation of the consumption of energy and the expenditure of energy, modulation of lipid metabolism, and control of adipogenesis.

Research has highlighted the anti-obesity potential of citrus phytochemicals, and recent studies have provided an update on their active components and mechanisms of action in combating obesity. These phytochemicals have shown promising effects in reducing the risk of obesity development. In vitro and in vivo investigations have focused on hydroxyl polymethoxyflavones (PMFs) and polymethoxyflavones present in citrus peels, highlighting their potential in combating obesity[40].

2.3.2.5 Regulation of Lipoprotein Metabolism

Citrus fruits are widely acknowledged as a plentiful source of beneficial compounds, including flavonoids, vitamins, carotenoids, and polyphenolic compounds, known for their remarkable

biological properties. Recent studies have primarily centered on exploring the potential of citrus flavonoids to impact lipid metabolism and various metabolic factors associated with conditions such as metabolic syndrome, heart disorders, type 2 diabetes, and atherosclerosis. Notably, major citroflavonoids, namely nobiletin, naringenin, tangeretin, and hesperidin, have emerged as promising agents with nutraceutical and therapeutic potential in addressing metabolic dysregulation[37].

2.3.2.6 Phytochemicals as Antiaging Agents

Citrus fruits are widely acknowledged as a plentiful source of beneficial compounds, including flavonoids, vitamins, carotenoids, and polyphenolic compounds, known for their remarkable biological properties[41]. Recent studies have primarily centered on exploring the potential of citrus flavonoids to impact lipid metabolism and various metabolic factors associated with conditions such as metabolic syndrome, heart disorders, type 2 diabetes, and atherosclerosis. Notably, major citroflavonoids, namely nobiletin, naringenin, tangeretin, and hesperidin, have emerged as promising agents with nutraceutical and therapeutic potential in addressing metabolic dysregulation[41].

Epidemiological studies have consistently revealed that the consumption of citrus flavonoid-rich foods is linked to a decreased risk of cardiovascular disorders, inflammation, and metabolic disorders[42].

Recent investigations have also confirmed the potential of citrus flavonoids in addressing insulin resistance, dyslipidemia, obesity, hepatic steatosis, and atherosclerosis[42].

2.3.3 Citrus sinesis (sweet orange)

Citrus sinensis, belonging to the Rutaceae family, is a widely cultivated and commercially important citrus species[43]. Oranges, which are classified as hesperidia, consist of distinct layers. The outer layer, known as the peel, comprises the flavedo (epicarp or exocarp) and the albedo (mesocarp). Within the orange, there is an inner material known as the endocarp or pulp, which contains vesicles filled with juice. It is worth noting that the orange peel contains citral, an aldehyde that counteracts the action of vitamin A. Therefore, individuals who consume significant quantities of orange peel should ensure that their dietary intake of vitamin A is adequate[43].

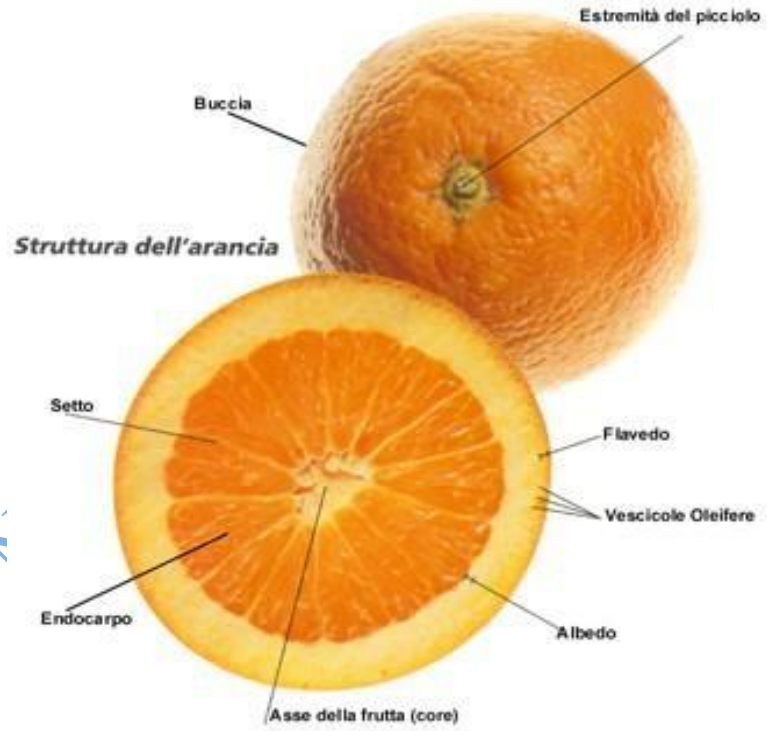

Plate 2.1: Structure of *Citrus sinesis*

Source: [43, 44, 45]

C. sinensis is the predominant citrus cultivar group cultivated worldwide, contributing approximately 70% of the total annual production of Citrus species[44]. Originally native to Asia, C. sinensis has now spread to Pacific regions and warm areas across the globe. This evergreen flowering tree typically reaches a height of 9-10 meters and is characterized by large spines on its branches. The leaves are arranged alternately, with narrowly winged petioles measuring 3-5 mm in width and 6.5-15 cm in length. They have varying shapes, ranging from elliptical and oblong to oval, and are often bluntly toothed. The leaves emit a distinct citrus odor due to the abundant presence of oil. The flowers, borne either singly or in whorls of 6, have a diameter of 5 cm and consist of five white petals and 20-25 yellow stamens[44].

The fruit of *C. sinensis* can be globose or oval-shaped, measuring 6.5 to 9.5 cm in width, and it ripens to an orange or yellow color. Anatomically, the fruit is composed of two distinct regions: the pericarp, also known as the peel, skin, or rind, and the endocarp or pulp containing juice sac glands. The skin is made up of an epidermis coated with epicuticular wax and numerous small aromatic oil glands that give it its characteristic smell. The pericarp consists of the outer flavedo or epicarp, primarily composed of parenchymatous cells and a cuticle layer. Beneath the flavedo lies the albedo or mesocarp, which consists of tubular-like cells compressed into the intercellular space. The fruit typically contains a sweet pulp and several to numerous seeds. The pulp is divided into eleven segments, each filled with juice that ranges in flavor from sour to sweet.

While sensitive to frost in orchards, the *C. sinensis* fruit is perennial and has adapted to various climatic conditions[44].

2.3.3.1 *Citrus sinesis* Peel

The outer layer or skin of the orange fruit, known as *Citrus sinensis* (Orange) peel, encloses the fruit and the white spongy pith material within. Orange peel contains a variety of beneficial phytochemicals, flavonoids, antioxidants, as well as essential vitamins A, B, C, copper, calcium, and magnesium[45].

The peel of *Citrus sinensis* (Orange) has demonstrated potential health benefits for humans and has been utilized in food, pharmaceutical, and cosmetic products. However, the demand for orange peels remains relatively insignificant as their applications have not been widely explored and recognized. The main constituents of orange peels include flavonoids such as polymethoxy flavonoids and terpenoids like limonene and linalool. These flavonoids exhibit antioxidant, antimutagenic, antiallergic, anti-inflammatory, and antimicrobial activities[45].

In addition to their widespread consumption as fresh fruits or processed into juice, citrus fruits generate considerable waste in the form of peels, seeds, and pulps, accounting for approximately 50% of the raw processed fruit. These by-products present a potential source of valuable resources. Citrus fruits hold high economic value due to their versatility in various industries, including food, cosmetics, and traditional medicine[45].

Plate 2.2: *Citrus sinesis* peel

Source: [45, 46]

2.3.3.1.1 The Health Benefits of *Citrus sinesis* Peel

2.3.3.1.1.1 Reduces Cancer Risk

Research findings indicate that the flavonoids present in orange peels have the ability to inhibit RLIP76, a protein associated with cancer. Moreover, orange peels contain limonene, a substance known to decrease the risk of developing cancer. Several studies highlight the remarkable potential of citrus peels to impede carcinogenic activities[46].

The consumption of orange peels has shown to effectively slow down the growth of cancer cells. Additionally, regular intake of orange peels has been associated with a reduced incidence of squamous cell carcinoma, a type of skin cancer known for its life-threatening nature. Individuals who incorporate orange peels into their diet on a consistent basis demonstrate a lower likelihood of being diagnosed with lung cancer and skin cancer[46].

2.3.3.1.1.2 Regulates blood sugar levels

Orange peel is rich in pectin, a dietary fiber known for its ability to regulate blood sugar levels, making it beneficial for individuals with diabetes[47]. Furthermore, research studies have indicated that incorporating orange peel extract into therapy can help prevent diabetic nephropathy. By removing RLIP76, a protein associated with diabetes, orange peel exhibits a preventive effect against the development of diabetes. It's worth noting that the glycemic load of an orange is low, with a value of only 5, which means that consuming the peel has a minimal impact on blood sugar levels[47].

2.3.3.1.1.3 Better Digestion

The anti-inflammatory properties of orange peels make them beneficial in the treatment of various digestive and gastrointestinal problems, including diarrhea, heartburn, and acidity[48]. With a high content of dietary fiber, orange peels aid in digestion by regulating bowel movements and preventing constipation. They serve as an effective remedy for digestive issues such as heartburn, indigestion, gas, and irritable bowel syndrome. Additionally, the presence of pectin in orange peels promotes the growth of beneficial bacteria in the stomach, further supporting digestive health[48].

2.3.3.1.1.4 Cardiovascular Health

Researchers at the UF Institute of Food and Agricultural Sciences have discovered that orange peels can have a positive impact on gut flora, potentially contributing to the prevention of atherosclerosis, a condition characterized by the accumulation of fatty substances on artery walls[49]. The breakdown of choline and carnitine by gut bacteria produces trimethylamine (TMA), which can be converted into trimethylamine N-oxide by enzymes. However, components found in orange peels hinder the proper functioning of these TMA enzymes, thereby aiding in the prevention of atherosclerosis. To further investigate these findings and determine if consuming orange peel extract can reduce the risk of cardiovascular diseases, researchers are conducting animal studies and enzyme tests. By incorporating orange peels into your diet, you can benefit from their fiber content and diverse range of nutrients, which can significantly improve cardiovascular health, including lowering blood pressure and preventing cholesterol buildup[49].

2.3.3.1.1.5 Orange peels are rich in vitamin C and A.

Orange peels possess natural antioxidant properties that can enhance the immune system and aid in the defense against germs and viruses[49].

2.4 Organs of the Male Reproductive System

The male reproductive system comprises a collection of organs that constitute the reproductive and urinary system of a man[50].

2.4.1 External Organs of the Male Reproductive System

The majority of the male reproductive system is situated outside of the abdominal cavity or pelvis. External components of the male reproductive system encompass the penis, scrotum, and testicles[51, 52].

2.4.1.1 Penis

The penis serves as the male organ for sexual intercourse and can be divided into three parts:

- The root: This section of the penis attaches to the abdominal wall[53, 54, 55].
- The body or shaft: Resembling a tube or cylinder, the body of the penis consists of three internal chambers. Within these chambers, there is a specialized sponge-like erectile tissue that contains numerous large spaces. When sexually aroused, these spaces fill with blood, causing the penis to become rigid and erect. The skin of the penis is elastic and loose, allowing for changes in size during an erection[55].

- The glans: This is the cone-shaped tip of the penis, also known as the head. The glans is covered by a loose layer of skin called the foreskin, which may be removed in a procedure called circumcision.

At the tip of the glans penis, the urethra, the tube responsible for carrying both semen and urine out of the body, has its opening. The penis is also rich in sensitive nerve endings.

During sexual climax or orgasm, semen, which contains sperm, is expelled (ejaculated) through the end of the penis. When the penis is erect, the flow of urine is blocked from the urethra, allowing only semen to be ejaculated[54, 54, 55].

2.4.1.2 Scrotum

The scrotum is a relaxed, pouch-like sac of skin located behind the penis. It houses the testicles, also known as testes, along with numerous nerves and blood vessels. The scrotum plays a crucial role in safeguarding the testes while also serving as a climate control system. To facilitate optimal sperm development, the testes require a temperature slightly lower than the body temperature. The scrotum possesses specialized muscles in its wall that enable it to contract, tightening and relaxing as needed. This movement brings the testicles closer to the body for warmth and protection or moves them farther away to lower the temperature[56, 57].

2.4.1.3 Testicles (testes)

The testes, which resemble large olives in size and shape, are oval organs located within the scrotum. They are anchored at each end by a structure known as the spermatic cord. Typically, men have a pair of testes[58, 59]. The testes play dual roles in the male reproductive system. Firstly, they are responsible for producing testosterone, the primary male sex hormone. Secondly, they

are involved in the production of sperm. Inside the testes, there are coiled masses of tubes called seminiferous tubules. These tubules carry out spermatogenesis, the process by which sperm cells are produced[59].

2.4.1.4 Epididymis

The epididymis is a lengthy and convoluted tube situated behind each testicle. Its primary functions are to transport and store sperm cells that are produced in the testes[60]. Additionally, the epididymis plays a vital role in facilitating the maturation process of sperm. When sperm emerges from the testes, they are initially immature and unable to fertilize an egg. However, within the epididymis, the sperm undergo maturation processes that equip them with the ability to fertilize an egg. When sexual arousal occurs, contractions within the epididymis propel the mature sperm into the vas deferens[60].

2.4.2 Internal Organs of the Male Reproductive System

2.4.2.1 Vas deferens

The vas deferens is an extended and muscular tube that extends from the epididymis and passes through the pelvic cavity, reaching a position just behind the bladder[61, 62]. Its primary function is to carry mature sperm from the epididymis to the urethra in readiness for ejaculation[62].

2.4.2.2 Ejaculatory ducts

The fusion of the vas deferens and the seminal vesicles gives rise to these ducts. The resulting ejaculatory ducts ultimately discharge into the urethra[63].

2.4.2.3 Urethra

The urethra serves as a tube responsible for conveying urine from the bladder to the outside of the body. In males, it has an additional role of expelling (ejaculating) semen during orgasm. When engaged in sexual activity and the penis is erect, the urethra functions to block the flow of urine, enabling the expulsion of semen exclusively during orgasm[64].

2.4.2.4 Seminal vesicles

The seminal vesicles are pouch-like structures that connect to the vas deferens near the lower part of the bladder. Their main function is to produce a fluid rich in sugar (fructose), which serves as an energy source for sperm and aids in their movement (motility). The fluid produced by the seminal vesicles constitutes a significant portion of the ejaculatory fluid or ejaculate volume[65].

2.4.2.5 Bulbourethral glands

The bulbourethral glands, also known as Cowper's glands, are small structures about the size of peas situated alongside the urethra, just below the prostate gland. Their main function is to generate a transparent and lubricating fluid that directly enters the urethra. This fluid serves the purpose of lubricating the urethra and counteracting any acidity that might be present due to leftover urine drops in the urethra[66].

2.4.2.6 The Prostate Gland

The prostate gland, approximately the size of a walnut, is positioned between the bladder and the penis, specifically in front of the rectum[67, 68, 69]. The urethra passes through the center of the prostate, facilitating the passage of urine out of the body. One of the key functions of the prostate is to secrete a fluid that provides nourishment and protection to sperm. When ejaculation occurs, the prostate contracts, releasing this fluid into the urethra, where it is expelled along with sperm as semen[68, 69].

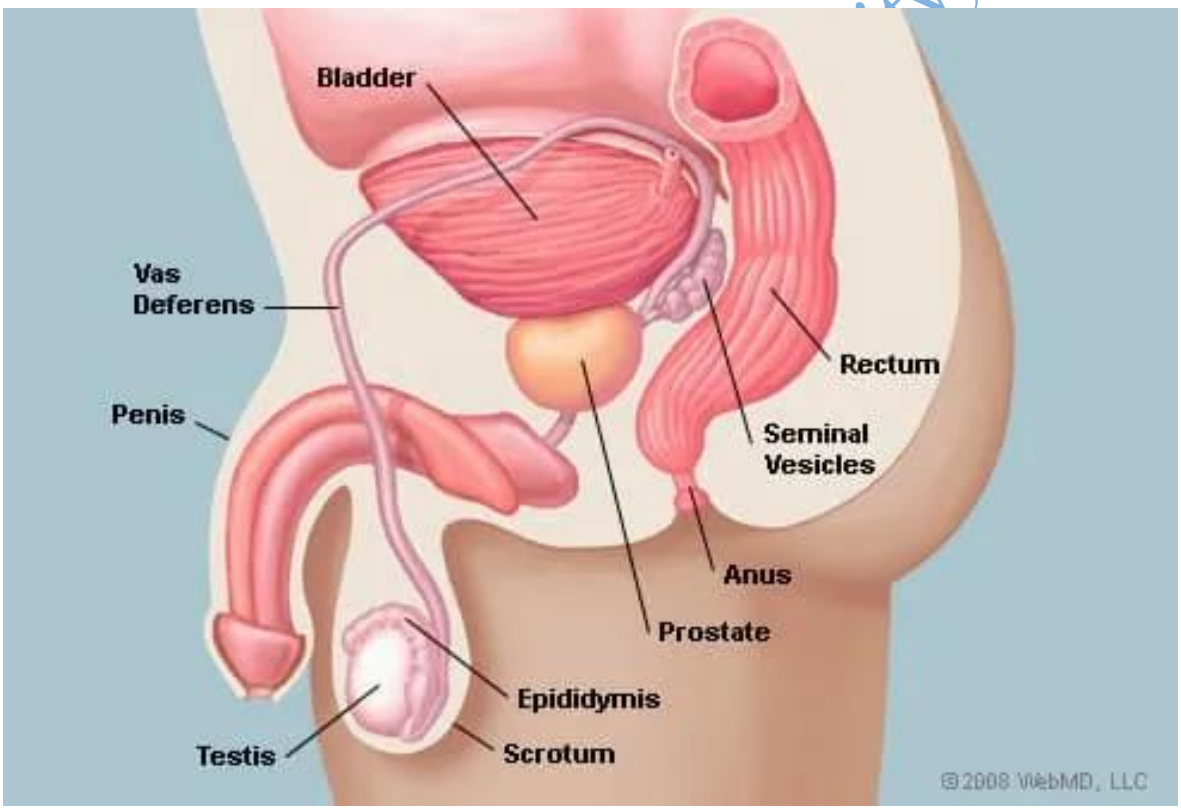

Plate 2.3: Overview of the male reproductive organ

Source: [50, 51, 52]

2.4.2.6.1 The Structure of the Prostate Gland

Traditionally, the prostate has been categorized into anatomical lobes, including the inferoposterior, inferolateral, superomedial, and anteromedial lobes[70,71]. These divisions are based on the pathways of the urethra and ejaculatory ducts as they pass through the organ. However, from a clinical standpoint, a more significant classification is the histological division of the prostate into three zones, which are as follows:

- Central zone: This zone surrounds the ejaculatory ducts and constitutes approximately 25% of the normal prostate volume. The ducts of the glands in the central zone empty obliquely into the prostatic urethra, making them less susceptible to urine reflux.[71]
- Transitional zone: Positioned centrally, the transitional zone encompasses approximately 5-10% of the normal prostate volume. It surrounds the urethra, and the glands in this zone are prone to undergoing benign hyperplasia (BPH), a noncancerous enlargement.
- Peripheral zone: The peripheral zone constitutes the major portion (approximately 65%) of the prostate gland and is located posteriorly. The ducts of the glands in the peripheral zone empty vertically into the prostatic urethra. This arrangement may explain the tendency of these glands to permit urine reflux[72].

These histological divisions provide valuable insights into the different characteristics and susceptibilities of the prostate zones.

2.4.2.6.2 Functions of the Prostate Gland

2.4.2.6.2.1 Production of Semen

The primary role of the prostate gland is to contribute prostatic fluid to semen[73]. Approximately 20-30% of the total semen volume is derived from the prostate. The remaining portions are provided by the seminal vesicles (50-65%) and the testicles (5%). Prostatic fluid contains various components that create an optimal environment for sperm cells. These include enzymes, zinc,

and citric acid. One crucial enzyme is prostate-specific antigen (PSA), which helps to liquefy and make the semen more fluid[74].

The fluid present in semen plays a vital role in assisting the movement of sperm through the urethra and supporting their survival during their journey towards an egg, which is essential for reproduction. Prostatic fluid is slightly acidic, but the overall alkaline nature of semen is maintained through other components. This alkalinity helps to counteract the natural acidity of the vagina and provides protection to the sperm, preventing potential damage along the way[75].

2.4.2.6.2.1.2 Closing the urethra during ejaculation

During the process of ejaculation, the prostate gland undergoes contractions that propel prostatic fluid into the urethra[76,77]. In the urethra, this fluid combines with sperm cells and fluid from the seminal vesicles, forming semen. The body then expels the semen outward. When the prostate contracts during ejaculation, it effectively closes off the opening connecting the bladder and urethra. This action enables the forceful expulsion of semen. As a result, under normal anatomical conditions, it is not possible to urinate and ejaculate simultaneously[78].

2.4.2.6.2.1.3 Hormone metabolism

For proper functioning, the prostate relies on androgens, which are male sex hormones, including testosterone[79]. Within the prostate, there exists an enzyme known as 5-alpha reductase, responsible for converting testosterone into a biologically active form called dihydrotestosterone (DHT). DHT plays a vital role in the normal development and functioning of the prostate.

Additionally, during male development, DHT is essential for the emergence of secondary sexual characteristics, such as the growth of facial hair[79,80].

2.5 Sex Hormones

Hormones are chemical messengers that are produced by various glands in the body's endocrine system[81, 82]. They play a crucial role in regulating and coordinating many physiological processes and functions. Hormones are released into the bloodstream and travel to target cells or organs, where they bind to specific receptors and initiate a response. They help control processes such as growth and development, metabolism, reproduction, mood, and many others. Some examples of hormones include insulin, estrogen, testosterone, cortisol, and thyroid hormones. Hormones play a fundamental role in maintaining the overall balance and homeostasis within the body[82].

Sex hormones are a subset of hormones that primarily regulate sexual development and reproduction. They are responsible for the development of primary and secondary sexual characteristics, as well as the functioning of the reproductive system. In males, the primary sex hormone is testosterone, which is produced by the testes. Testosterone is involved in the development of male reproductive organs, sperm production, and the development of secondary sexual characteristics such as facial and body hair, deepening of the voice, and muscle growth.

These sex hormones also have broader effects on other aspects of the body, such as bone density, mood regulation, and metabolism. They work in a delicate balance, and any disruptions or imbalances in sex hormone levels can lead to various health issues and reproductive disorders[83, 84]

2.5.1 Testosterone

Testosterone, the most abundant and clinically relevant androgen, is primarily produced by the interstitial cells of Leydig in the testes and also by the adrenal glands in both males and females[85]. Its secretion is regulated by luteinizing hormone (LH) from the anterior pituitary, which is stimulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus. When in circulation, testosterone binds to albumin or sex hormone-binding globulin. In healthy males, serum testosterone concentrations range from 12 to 31 nmol/L. After a few hours, testosterone is either transported to target tissues or degraded[85, 86].

At a cellular level, testosterone or its intracellular metabolite, dihydrotestosterone, binds to a nuclear receptor protein complex. This complex translocates to the cell nucleus, initiating DNA transcription. Testosterone has well-documented anabolic properties, such as inducing hypertrophy of both type I and II muscle fibers, increasing the number of skeletal muscle satellite cells, and promoting the differentiation of multipotent mesenchymal cells into myocytes while inhibiting their differentiation into adipocytes[86, 87].

During acute illness, testosterone levels tend to decrease, while LH levels increase. In patients with prolonged critical illness, serum concentrations of testosterone, LH, and GnRH are low. Synthetic androgen administration has shown benefits in some conditions, such as chronic

obstructive pulmonary disease and human immunodeficiency virus-associated wasting syndromes, where it has induced gains in muscle mass and strength, as well as improvements in respiratory function. In men with severe burn injuries, testosterone has been found to reduce protein catabolism[85].

2.5.1.1 Physiology of Testosterone Production

Testosterone, the primary male sex hormone, plays a crucial role in the development of male characteristics and virilization. It belongs to the class of steroid hormones and is derived from cholesterol[87]. The majority of testosterone production (about 95%) occurs in the testes, while a smaller amount is produced in the adrenal glands. The synthesis of testicular testosterone begins with the release of gonadotropin-releasing hormone (GnRH) from the mediobasal hypothalamus in a periodic pattern every 60 to 90 minutes[88].

GnRH stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in a pulsatile manner. LH acts on the Leydig cells within the testes, triggering the production of testosterone, while FSH stimulates the Sertoli cells, supporting spermatogenesis. Testosterone and its metabolites, estradiol and dihydrotestosterone (DHT), exert negative feedback on the hypothalamus and pituitary, inhibiting further testosterone release. Aromatase converts testosterone to estradiol, and 5α-reductase (predominantly found in the skin and prostate) converts it to DHT[88, 89].

Testosterone levels exhibit a diurnal pattern, with highest concentrations in the morning and lowest in the evening. However, this diurnal variation is less pronounced in older men. In the body, the majority of testosterone is bound to proteins, while a small portion exists in its free form. Approximately 60% of testosterone binds tightly to sex hormone-binding globulin (SHBG),

and about 38% binds weakly to albumin[91]. Only around 2% of testosterone circulates freely. Bioavailable testosterone, which is the active form, consists of both free testosterone and albumin-bound testosterone. Changes in SHBG levels can affect the amount of bioavailable testosterone. Elevated SHBG levels can occur in conditions such as chronic liver disease, HIV-AIDS, and with the use of certain antiepileptic drugs. On the other hand, decreased SHBG levels can be associated with obesity, diabetes mellitus (DM), or hypothyroidism[88, 89].

2.5.1.2 Functions of Testosterone

Testosterone plays a crucial role in primary sexual development, which encompasses various processes such as testicular descent, spermatogenesis, enlargement of the penis and testes, and the development of libido[93]. Normally, the testes begin descending into the scrotum around the seventh month of gestation when they start producing significant amounts of testosterone. If a male child has undescended but otherwise normal testes that fail to descend by the age of 4 to 6 months, administration of testosterone can facilitate their descent through the inguinal canals.

Additionally, testosterone is involved in the regulation of secondary male characteristics, which are responsible for the masculine traits[92, 93]. These characteristics include patterns of male hair growth, changes in vocal cords resulting in a deeper voice, and anabolic effects such as growth spurts during puberty. During puberty, testosterone stimulates tissue growth at the epiphyseal plate, contributing to growth, and later plays a role in the closure of the plate. Testosterone also promotes skeletal muscle growth by stimulating protein synthesis.

Furthermore, testosterone stimulates erythropoiesis, the process of red blood cell production, leading to a higher hematocrit in males compared to females. However, testosterone levels tend to decrease with age[93]. Consequently, men often experience a decrease in testicular size, a

decline in libido, lower bone density, a decrease in muscle mass, an increase in fat production, and a reduction in erythropoiesis, which can potentially result in anemia.

2.5.2 Progesterone

Progesterone, both a steroid and a hormone, plays significant roles, particularly in the reproductive process[94]. It is produced by the adrenal glands and testes in males and is involved in sperm development. The absence of progesterone in men can have various implications that affect their well-being, sleep patterns, and overall hormonal balance. Progesterone serves as a foundational component for numerous hormones, including testosterone, which is crucial for defining male characteristics. It contributes to building bone mass, participates in regulating blood sugar levels and brain activity, and functions throughout the body to maintain a healthy balance. Progesterone also influences the conversion of fat into energy and the production of thyroid hormones[94, 95, 96].

As progesterone serves as a precursor to testosterone, low levels can often lead to a decrease in sex drive, as it acts as an antagonist to estrogen. Insufficient balance of progesterone in men can result in a condition known as "estrogen dominance," commonly associated with women. This condition may manifest as nervousness, erectile dysfunction, fatigue, prostate enlargement, an increased risk of prostate cancer, and a decrease in libido.

Furthermore, alcohol consumption has been shown to elevate blood estrogen levels in men[97]. Even moderate drinking has been linked to lower levels of progesterone in men, and it has also been associated with reduced zinc absorption, which negatively affects progesterone production.

2.5.3 Follicle Stimulating Hormone

Follicle stimulating hormone (FSH) is one of the gonadotrophic hormones, along with luteinising hormone (LH). Both hormones are released into the bloodstream by the pituitary gland[98]. FSH plays a crucial role in pubertal development and the proper functioning of the ovaries in women and testes in men. In women, FSH stimulates the growth of ovarian follicles in the ovary, leading to the release of an egg from one follicle during ovulation. It also promotes the production of oestradiol by the ovaries. In men, FSH acts on the Sertoli cells in the testes to stimulate the production of sperm (spermatogenesis)[99].

In males, FSH is essential for testicular growth and plays a crucial role in male fertility. It aids in the creation of normal sperm cells and helps maintain them until they are mature and ready for release. Abnormal FSH levels can affect sperm production and may lead to infertility.

The levels of FSH can be measured through a simple blood test. Normal FSH levels in adult males typically range from 1.5 to 12.4 mIU/mL, although the specific normal values may vary slightly among different laboratories due to variations in testing standards[100, 101].

High FSH levels in males may indicate improper testicular function. Several factors can contribute to high FSH levels, including testicular damage from radiation, trauma, or alcohol abuse, genetic issues, aging, hormonal disorders, certain medications (such as pain medications or steroids), diseases like HIV/AIDS or Type 2 diabetes, or, in rare cases, pituitary gland tumors[98, 99].

On the other hand, low FSH levels in males may indicate dysfunction of the pituitary gland in the brain, although this is less common than high FSH levels[99].

2.5.3.1 Regulation of FSH

The production and release of follicle stimulating hormone (FSH) are regulated by various hormones released by the ovaries and testes, operating within the hypothalamic-pituitary-gonadal (HPG) axis[100]. The hypothalamus releases gonadotropin-releasing hormone (GnRH), which binds to receptors in the anterior pituitary gland, stimulating the synthesis and release of both FSH and luteinising hormone (LH). Once released, FSH travels through the bloodstream and binds to receptors in the testes and ovaries[101].

In men, the production of FSH is influenced by the levels of inhibin circulating in the body. When spermatogenesis is impaired, less inhibin is produced by the testes. Normally, inhibin acts to inhibit the release of FSH from the pituitary gland. Therefore, impaired spermatogenesis leads to decreased inhibin levels and consequently an increase in FSH release from the pituitary gland[100, 101].

Spermatogenesis also relies on testosterone levels within the testes. The production of testosterone in the testes is stimulated by luteinising hormone. This system of "negative feedback" control involves testosterone regulating LH secretion and spermatogenesis influencing inhibin secretion. This feedback mechanism helps maintain steady production of spermatogenesis and sex steroids. However, excessive testosterone in the body, such as from the use of testosterone-like drugs to enhance muscle growth, can lead to low levels of FSH and LH, ultimately impairing spermatogenesis[102].

2.5.4 Luteinizing hormone (LH)

Luteinizing hormone (LH) is a glycoprotein hormone produced by the gonadotrophin cells in the anterior pituitary gland[103]. It is an integral part of the neurological pathway that involves the hypothalamus, pituitary gland, and gonads. The release of LH is stimulated by gonadotropin-

releasing hormone (GnRH) and inhibited by estrogen in females and testosterone in males. The functions of LH differ between men and women[104].

In both sexes, LH plays a role in the maturation of primordial germ cells[106]. In men, LH stimulates the Leydig cells in the testes to produce testosterone. In women, LH triggers the production of steroid hormones from the ovaries[107]. LH also plays a crucial role in regulating the menstrual cycle in women by influencing ovulation and the implantation of the egg in the uterus. Testosterone, influenced by LH, brings about various male characteristics such as increased muscle mass, deepening of the voice due to larynx enlargement, and the growth of facial and body hair.

Abnormally high levels of luteinizing hormone may indicate infertility. As LH secretion is tightly controlled by the hypothalamic-pituitary-gonadal axis, elevated levels of LH in the bloodstream can suggest reduced sex steroid production from the testes or ovaries, as seen in conditions like premature ovarian failure[104].

On the other hand, low levels of luteinizing hormone can be observed in conditions such as Kallmann's syndrome in men. This syndrome is associated with a deficiency in the secretion of gonadotropin-releasing hormone from the hypothalamus[103].

2.5.4.1 Luteinizing Hormone at the Cellular level

Luteinizing hormone (LH) is produced by gonadotroph cells located in the anterior pituitary gland. These cells have distinctive features, including large round cell bodies with well-developed Golgi apparatus and endoplasmic reticulum[104]. They are dispersed throughout the anterior pituitary and make up approximately 10 to 15% of its functional cell mass. When

observed under a microscope, gonadotroph cells do not exhibit strong affinity for acid or basic stains, appearing either basophilic or chromophobic[104].

LH and follicle-stimulating hormone (FSH) share similarities as they are derived from similar genes and possess comparable characteristics. Both hormones are glycoproteins composed of an alpha and beta subunit. The alpha subunit is identical between LH and FSH, while the beta subunit of each hormone differs, giving them their specific biological functions. The alpha subunit of LH consists of 92 amino acids, and the beta subunit comprises 120 amino acids. Together, these two subunits have a combined mass of 28 kDa[104].

2.5.5 Estrogen

Estradiol, which is the primary form of estrogen, plays a crucial role in male sexual function. It is involved in regulating libido, erectile function, and spermatogenesis in men[108]. Organs important for sexual function, such as the brain, penis, and testis, contain abundant estrogen receptors and the enzyme aromatase, responsible for converting testosterone into estrogen[109].

In the brain, there is an increase in estradiol synthesis in areas associated with sexual arousal. Moreover, the penis contains estrogen receptors distributed throughout the corpus cavernosum, with a higher concentration around neurovascular bundles[108].

Both low testosterone levels and elevated estrogen levels independently contribute to an increased incidence of erectile dysfunction. In the testes, estrogen modulates spermatogenesis at various stages, starting from the hypothalamus-pituitary-gonadal axis, followed by the involvement of Leydig cells, Sertoli cells, germ cells, ductal epithelium, epididymis, and mature sperm. The regulation of testicular cells by estradiol exhibits both inhibitory and stimulatory

influences, reflecting a complex interplay of dose-dependent and temporally sensitive modulation[110].

2.6 Oxidative Stress

Oxidative stress is characterized by an imbalance between oxidants and antioxidants in the body, where oxidation surpasses the capacity of antioxidant systems[111]. This imbalance can lead to potential damage. Oxidative stress is not limited to harmful events like lipid peroxidation and oxidative DNA damage; it also influences physiological adaptation and the regulation of intracellular signal transduction. Interestingly, oxidative stress can be beneficial in certain situations. For instance, it induces apoptosis to prepare the birth canal for delivery[112]. Furthermore, oxidative stress enhances biological defense mechanisms during appropriate physical exercise and ischemia[111].

Additionally, oxidative stress plays a vital role in modulating messengers that regulate essential functions of cell membranes crucial for survival[112]. It impacts the intracellular redox status, resulting in the activation of protein kinases, which include receptor and non-receptor tyrosine kinases, protein kinase C, and the MAP kinase cascade. Consequently, this activation leads to various cellular responses. Protein kinases play a significant role in cellular activation, proliferation, differentiation, and other important functions. As a result, protein kinases have garnered considerable attention in exploring the relationship between oxidative stress and disease[113].

2.6.1. Free Radicals

Free radicals are compounds characterized by the presence of unpaired electrons in their outer shells[117]. These unstable molecules or atoms can cause damage to cells and tissues by engaging

in chemical reactions with other molecules in the body. Free radicals are highly reactive and can initiate chain reactions, leading to oxidative stress and potential harm to biological systems. While excessive free radicals are associated with various diseases and aging processes, they also play essential roles in physiological processes such as immune function, cell signaling, and cellular defense mechanisms[114, 117]. The body has built-in antioxidant systems to help neutralize and regulate free radicals, maintaining a balance to ensure optimal health.

Free radicals are substances that have been implicated in various diseases and aging processes[114]. However, they also play a role in important physiological conditions within the body. Free radicals are compounds that possess an unpaired electron in their outer shell. Reactive oxygen species (ROS) is a collective term used to describe oxygen-containing free radicals based on their reactivity and oxidizing potential[115]. ROS engage in numerous chemical reactions with biomolecules, leading to a pathological state known as oxidative stress. The redox degenerative reactions occurring in the biological system generate reactive oxygen species (ROS) and their derivatives, commonly referred to as free radicals. Under physiological conditions, the irreversible production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) represents a significant event in living organisms[115].

2.6.1.1 Various categories of Free Radicals

There are highly reactive species known as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) that play significant roles in various biological processes[117].

• Reactive Oxygen Species (ROS) encompass both radicals and non-radicals. Radicals include Superoxide, Hydroxyl, Peroxyl, Alkoxyl, and Hydroperoxyl. Non-radicals in this category

include Hydrogen Peroxide, Hypochlorous Acid, Hypobromous Acid, Ozone, and Singlet Oxygen[117, 118, 119].

• Reactive Nitrogen Species (RNS) also consist of radicals and non-radicals. Radicals in this group include Nitric Oxide and Nitrous Acid. Non-radicals comprise Nitrogen Dioxide, Nitrosyl Cation, Nitrosyl Anion, Dinitrogen Tetroxide, Dinitrogen Trioxide, Peroxynitrite, Peroxinitrous Acid, and Alkylperoxynitrites[117, 118, 119].

2.6.1.2 Sources of Reactive Species

Oxidants are generated as a result of normal cellular metabolism occurring in mitochondria and peroxisomes, as well as various cytosolic enzyme systems[120]. Additionally, several external factors can trigger the production of Reactive Oxygen Species (ROS)[120]. To maintain physiological balance, enzymatic and non-enzymatic antioxidant defense systems, such as catalase (CAT), superoxide dismutase (SOD), and reduced glutathione (GSH), work in concert to counteract and regulate ROS levels[120]. The primary ROS generated during oxygen metabolism is superoxide, which is highly reactive and cytotoxic. Superoxide is enzymatically converted to a less reactive compound, hydrogen peroxide (H2O2), by metalloenzymes called superoxide dismutase (SOD). These enzymes play a critical role in facilitating the breakdown of superoxide into molecular oxygen and peroxide, thereby protecting cells against the toxic byproducts of aerobic respiration[121].

The sources of different ROS are as follows:

• Hydrogen Peroxide (H2O2): Produced through the dismutation of O2- by SOD.

• Organic Hydroperoxide (ROOH): Generated through radical reactions involving cellular components.

• Hydroxyl Radical (OH): Formed via the Fenton reaction.

• Superoxide Ion (O2-): Generated through autooxidation reactions and the electron transport system (ETS)[122].

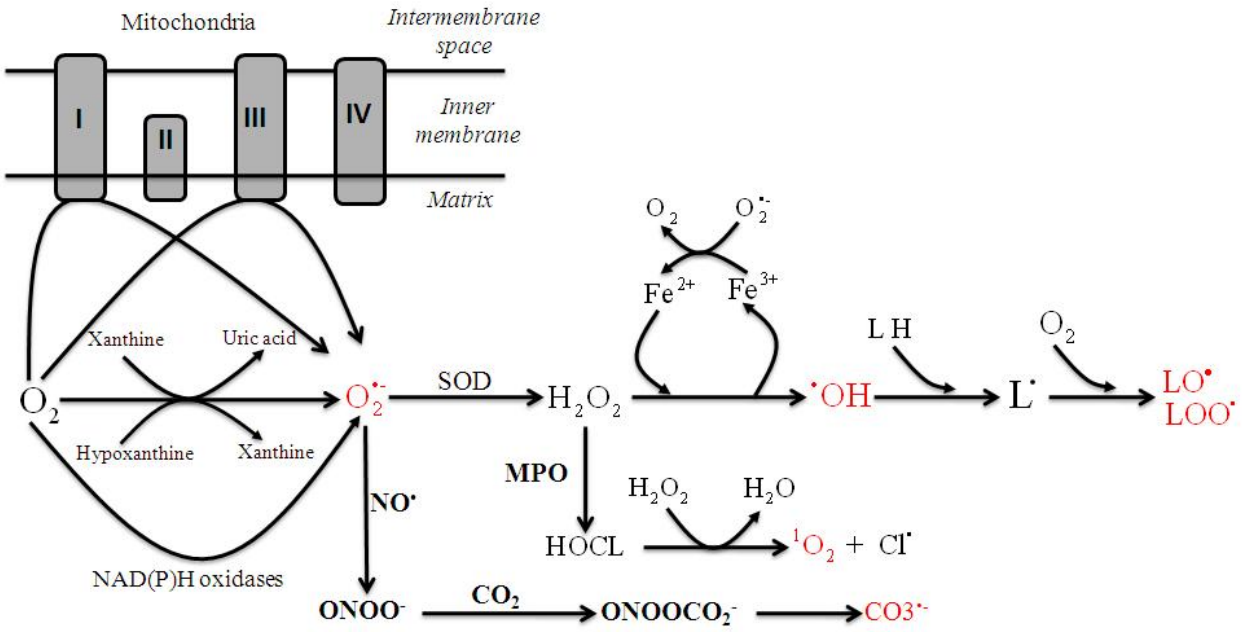

Plate 2.4: Production of free radicals through different routes

Source: [117, 118, 120, 121, 122]

2.6.1.3 Benefits of Free Radicals

Optimal levels of free radicals play a crucial role in normal physiological functions, including defense against infections, gene expression, and cellular growth[123, 124]. These reactive oxygen species (ROS) serve as stimulants for various biochemical processes within cells. The effects of

reactive oxygen species (ROS) are exerted through the reversible oxidation of active sites in transcription factors such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1), resulting in gene expression and cellular growth. Additionally, free radicals can indirectly stimulate transcription factors by activating signal transduction pathways. One instance of a signal transduction molecule activated by ROS is the mitogen-activated protein kinases (MAPKs)[122].

Furthermore, free radicals serve as secondary messengers during different developmental stages. For instance, in sea urchins, ROS levels rise during fertilization. Similarly, in mammals, free radicals play a regulatory role in prenatal and embryonic development[123]. They also participate in the biosynthesis of molecules such as thyroxine and prostaglandins, which facilitate developmental processes. Within the immune system, free radicals are utilized to trigger the proliferation of T cells through NF-κB activation. Macrophages and neutrophils generate free radicals to eliminate engulfed bacteria via phagocytosis. Tumor necrosis factor (TNF-α) mediates the cytotoxicity of tumor and virus-infected cells by generating ROS and inducing apoptosis[124].

2.6.1.4 Damaged caused by Free Radicals

Reactive free radicals can initiate lipid peroxidation by reacting with membrane lipids, leading to the formation of lipid hydroperoxides (LOOH)[125]. The hydroperoxides can undergo additional decomposition, leading to the production of aldehydes like malonaldehyde, 4-hydroxy nonenal (4-HNE), cyclic endoperoxides, isoprostanes, and hydrocarbons. The outcomes of lipid peroxidation encompass the cross-linking of membrane proteins, changes in membrane fluidity,

and the formation of lipid-protein and lipid-DNA adducts. These effects can have detrimental effects on cellular function[126].

Proteins can undergo direct and indirect damage as a result of interaction with reactive oxygen species (ROS), leading to peroxidation. This, in turn, can cause changes in their tertiary structure, proteolytic degradation, protein-protein cross-linkages, and fragmentation. The side chains of various amino acid residues in proteins, particularly tryptophan, cysteine, and methionine, are susceptible to oxidation by free radicals. Protein oxidation products include carbonyls such as aldehydes and ketones[125].

Despite the stability and well-protected structure of DNA, free radicals can interact with it and cause various types of damage[127]. These include the modification of DNA bases, single and double strand DNA breaks, loss of purines, damage to the deoxyribose sugar, DNA-protein cross-linkages, and impairment of the DNA repair system.

It is important to note that not all free radicals have the potential to cause DNA damage[128]. The hydroxyl radical (OH·) is one of the main inducers of DNA damage. When OH· reacts with DNA, a variety of adducts are formed. The OH· radical can attack purine and pyrimidine bases, resulting in the formation of oxidizing and reducing OH· radical adducts. Free radicals can also target the sugar moiety, leading to the production of sugar peroxyl radicals and subsequent strand breakage. The outcome of DNA damage is the alteration of genetic material, which can contribute to cell death, mutagenesis, carcinogenesis, and aging.

2.6.2 Antioxidants

Antioxidants are compounds that have the ability to inhibit or slow down the oxidation process of other chemicals[129, 130]. They are often referred to as oxidation inhibitors. Oxidation is a chemical reaction in which atoms lose electrons, resulting in the production of free radicals. These free radicals can initiate chain reactions that may lead to cellular damage. Therefore, antioxidants are molecules that can counteract the harmful effects of free radicals and help protect cells from damage[131].

2.6.2.1 Antioxidant Classification

Antioxidants can be classified based on their activity into two categories: enzymatic antioxidants and non-enzymatic antioxidants.

Enzymatic Antioxidants: Examples in this category are: Glutathione peroxidases (GPx), Superoxide dismutases (SOD), and Catalase (CAT), among others[134].

Non-enzymatic Antioxidants can be further divided into two groups:

- Endogenous non-enzymatic antioxidants, such as glutathione (GSH), which are naturally produced within the body[134].
- Exogenous non-enzymatic antioxidants, which are obtained from external sources. Examples of exogenous non-enzymatic antioxidants include Carotenoids, Vitamin E, and Vitamin C[132, 135].

Furthermore, antioxidants can also be categorized based on their solubility in water:

Water-Soluble Antioxidants are found in the cellular fluid, like the cytosol. Vitamin C is a notable example of a water-soluble antioxidant[133].

Lipid-Soluble Antioxidants, on the other hand, are located in the cell membrane. Examples of lipid-soluble antioxidants include lipoic acid, carotenoids, and vitamin E[133].

2.6.3 Markers of Oxidative Stress

Biomarkers play a crucial role in evaluating oxidative stress and assessing the antioxidant capacity within the body[135]. Some important biomarkers in this context include:

- Catalase
- Superoxide dismutases (SODs)
- Glutathione-S-Transferases (GSTs)
- Malondialdehyde (MDA)

2.6.3.1 Malondialdehyde

Malondialdehyde (MDA) is an organic compound with the chemical formula $CH_2(CHO)_2$, although its actual structure is more complex than this simple formula suggests. It serves as one of the biomarkers for assessing oxidative stress and occurs naturally within the body. Malondialdehyde predominantly exists in the enol form[137].

As a highly reactive compound, pure malondialdehyde is not commonly observed. However, it can be generated in the laboratory by hydrolyzing 1,1,3,3-tetramethoxypropane, which is commercially available. In aqueous environments, the trans-isomer of MDA is more prevalent, while the cis-isomer is present in organic solvents.

Polyunsaturated lipids are degraded by reactive oxygen species, leading to the formation of malondialdehyde. This compound, being a reactive aldehyde, contributes to cellular toxic stress and forms covalent protein adducts known as Advanced Lipoxidation End-products (ALE),

similar to advanced glycation end-products (AGE). Measurement of malondialdehyde production serves as a biomarker for assessing the level of oxidative stress in an organism[137].

In DNA, malondialdehyde reacts with deoxyadenosine and deoxyguanosine, forming DNA adducts, with the primary one being M1G, which has mutagenic properties. Additionally, the guanidine group of arginine residues can condense with malondialdehyde, resulting in the formation of 2-aminopyrimidines[137].

2.6.3.2 Super Oxide Dismutase

Superoxide dismutase (SOD) is an enzyme that facilitates the conversion of the superoxide (O2−) radical into either molecular oxygen (O2) or hydrogen peroxide (H2O2)[138]. Its role involves the dismutation of the superoxide anion, breaking it down into oxygen and hydrogen peroxide. Superoxide is generated as a by-product of oxygen metabolism and, if left unregulated, can cause various types of cellular damage[138]. Hydrogen peroxide, while also harmful, is broken down by other enzymes like catalase. Hence, SOD plays a crucial role as an antioxidant defense mechanism in almost all oxygen-exposed living cells.

SOD enzymes are found in aerobic cells and extracellular fluids. They can be categorized based on their metal cofactors, resulting in three groups:

• Cu/Zn Family: This type of SOD binds copper and zinc metals.

• Fe and Mn Type: This group of SOD binds iron and manganese.

• Ni Type: This SOD variant binds to nickel.

Different isozymes of SOD have been identified in various cellular compartments of higher plants[141]. For example, Mn-SOD is present in mitochondria and peroxisomes, while Fe-SOD is

found in chloroplasts, peroxisomes, and also chloroplasts. CuZn-SOD, on the other hand, has been detected in cytosol, chloroplasts, peroxisomes, and apoplast[140, 141].

In humans, all three forms of SOD exist but are located in different parts of the body. SOD1 is found in the cytoplasm, SOD2 is present in mitochondria, and SOD3 is located in extracellular organelles. SOD1 is a dimer composed of two subunits, while SOD2 and SOD3 are tetramers with four subunits. SOD1 and SOD3 contain copper and zinc as cofactors, while SOD2 utilizes manganese in its reactive center.

SOD acts as a superior competitor against harmful superoxide reactions, thereby protecting cells from superoxide toxicity. The reaction between superoxide and non-radicals is spin-forbidden. In biological systems, this means that superoxide primarily undergoes dismutation or reacts with another biological radical like nitric oxide (NO) or a transition-series metal. The spontaneous dismutation of the superoxide anion radical (O2−) leads to the rapid formation of O2 and H2O2 (~105 M−1s−1 at pH 7). SOD is necessary because superoxide can react with critical and sensitive cellular targets. For instance, it can react with the NO radical, forming the toxic peroxynitrite.

The spontaneous dismutation reaction of superoxide requires the interaction of two superoxide molecules, resulting in a second-order dismutation rate relative to the initial concentration of superoxide. As a result, the half-life of superoxide is extremely short at high concentrations (e.g., 0.05 seconds at 0.1mM), but significantly longer at low concentrations (e.g., 14 hours at 0.1 nM). Conversely, the reaction between superoxide and SOD follows a first-order dependence on the concentration of superoxide. Additionally, superoxide dismutase displays the highest kcat/KM (a measure of catalytic efficiency) among all known enzymes (~7 x 109 M−1s−1), with the reaction

rate solely limited by the frequency of collisions between SOD and superoxide. In other words, the reaction rate is "diffusion-limited"[142].

The exceptional efficiency of superoxide dismutase is crucial because even at subnanomolar concentrations achieved by the abundant levels of SOD[140].

2.6.3.3 Catalase

Catalase, an enzyme found in organisms exposed to oxygen, including plants, animals, and sometimes bacteria, plays a vital role in the breakdown of hydrogen peroxide into water and oxygen[145]. One of its crucial functions is to protect organism cells from damage caused by reactive oxygen species (ROS) through its catalytic action. Catalase is composed of four polypeptide chains, forming a tetramer with a length of over 500 amino acids. It contains four porphyrin heme groups, each containing iron, which enables the enzyme to interact with hydrogen peroxide[143].

The optimal pH for catalase in humans is approximately 7, and it exhibits a relatively broad range of pH values within which its activity remains unchanged, typically between 6.8 and 7.5. The optimum temperature for catalase varies depending on the organism's species, as different organisms have different temperature preferences[143]. The pH optimum for catalases in other species may range from 4 to 11, depending on the specific organism[144].

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Decomposition reaction catalyzed by catalase

2.6.3.4 Glutathione-S-Transferases (GSTs)

GSTs, previously known as ligandins, serve as important biomarkers of oxidative stress. They belong to the GST superfamily, which comprises three major superfamilies: cytosolic, mitochondrial, and microsomal (also known as MAPEG proteins). The GST superfamily members exhibit significant variations in amino acid sequences, and many sequences in public databases have unknown functions. The Enzyme Function Initiative (EFI) is employing GSTs as a model superfamily to discover new functions associated with GSTs[146].

In certain mammalian organs, GSTs can account for up to 10% of cytosolic proteins[148]. They play a crucial role in facilitating the conjugation of glutathione (GSH) to electrophilic centers on diverse substrates, enhancing their water solubility. This enzymatic activity enables the detoxification of endogenous compounds such as peroxidized lipids and aids in the metabolism of xenobiotics.

Additionally, GSTs can bind toxins and act as transport proteins, which initially led to their designation as ligandins. The activity of GSTs depends on a continuous supply of GSH, which is synthesized by gamma-glutamylcysteine synthetase and glutathione synthetase. Specific transporters are also involved in eliminating GSH conjugates from the cell.

The main function of GSTs is to facilitate the detoxification of xenobiotics through the catalytic reaction of GSH's nucleophilic attack on electrophilic carbon, sulfur, or nitrogen atoms present in non-polar xenobiotic substrates. This mechanism effectively prevents the interaction of these substrates with essential cellular proteins and nucleic acids. The function of GSTs in this role involves two main aspects:

Binding both the substrate and GSH at distinct sites within the enzyme's structure, forming the active site.

Activating the thiol group of GSH, which enables the nucleophilic attack on the substrate.

The glutathione molecule binds in a crevice between the N-terminal and C-terminal domains of GSTs, with catalytically important residues residing in the N-terminal domain. Each subunit of the GST dimer, whether it is a hetero- or homo-dimer, contains a single non-substrate binding site and a GSH-binding site. In hetero-dimeric GST complexes like those formed by the cytosolic mu and alpha classes, an additional high-affinity non-substrate xenobiotic binding site is present in the crevice between the two subunits, potentially contributing to the formation of hetero-dimers[146].

2.7. Inflammation

Inflammation plays a critical role in the immune system's response to injuries and infections[149]. It is a complex process through which the body communicates with the immune system to initiate healing, repair, and replacement of damaged tissues. In the absence of inflammation as a natural physiological response, wounds would struggle to heal, and infections could become life-threatening. However, prolonged inflammation has been linked to various conditions such as cardiovascular disease, stroke, and autoimmune disorders like rheumatoid arthritis and lupus[150]. Adopting a healthy diet and lifestyle can help regulate and manage inflammation. It is widely acknowledged that inflammation can be assessed by various markers, such as the levels of pro-inflammatory cytokines[150].

2.7.1 Markers of Inflammation

Markers of inflammation are measurable substances or indicators that reflect the presence or extent of an inflammatory response in the body[151]. These markers can be assessed through

laboratory tests or clinical evaluations and are useful for diagnosing and monitoring inflammatory conditions[152]. Some common markers of inflammation include:

C-reactive Protein (CRP): CRP is a hepatically synthesized protein that is generated in response to inflammation. Elevated levels of CRP in the blood are indicative of inflammation, and the test is commonly used to assess the presence and severity of systemic inflammation[153].

Erythrocyte Sedimentation Rate (ESR): ESR is a test that measures the rate at which red blood cells settle in a tube over a given period. Increased ESR is a nonspecific marker of inflammation, as it is influenced by factors like increased blood protein levels and red blood cell aggregation[154].

Pro-inflammatory Cytokines: Cytokines are compact proteins secreted by immune cells to regulate immune responses and inflammation. Inflammatory conditions are often accompanied by increased levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β)[155].

White Blood Cell Count (WBC): An increase in the total number of white blood cells, specifically neutrophils (a type of immune cell), can indicate an inflammatory response[156].

Imaging Tests: Imaging techniques like MRI, CT scan, or ultrasound can provide visual evidence of inflammation in specific organs or tissues. Examples include joint inflammation observed in rheumatoid arthritis or lung inflammation seen in pneumonia[157].

Clinical Signs and Symptoms: The presence of classic signs of inflammation, such as redness, swelling, heat, and pain in a specific area, can also serve as indicators of inflammation[150].

It's important to note that these markers are not specific to any particular condition and may also be influenced by other factors. A thorough assessment, which involves reviewing medical history,

conducting a physical examination, and performing supplementary diagnostic tests, is usually necessary for accurate diagnosis and assessment of inflammation.

2.7.1.1: NF-κB (Nuclear Factor-kappa B)

NF-κB (Nuclear Factor-kappa B) is a transcription factor that assumes a vital role in the regulation of immune response, inflammation, cell survival, and diverse cellular processes. It constitutes a protein complex that governs the expression of numerous genes associated with immune and inflammatory responses. This transcription factor is found in almost all cell types and is typically inactive in the cytoplasm, bound to inhibitory proteins called IκBs (inhibitors of κB)[158].

Upon activation by various stimuli, such as pro-inflammatory cytokines, microbial products, or stress signals, NF-κB is released from its inhibitory proteins and translocates into the nucleus. In the nucleus, NF-κB binds to specific DNA sequences called κB sites and promotes the transcription of target genes involved in immune and inflammatory responses[160]. These target genes include cytokines, chemokines, adhesion molecules, and other proteins that regulate inflammation, immune cell activation, and cell survival[159].

The activation of NF-κB is a tightly regulated process and serves as a critical mediator of the immune response. It is involved in both acute and chronic inflammation and has been implicated in various diseases, including autoimmune disorders, chronic inflammatory conditions, and cancer. Modulating NF-κB activity is a target for therapeutic interventions aimed at controlling inflammation and related diseases.

Nuclear factor-kappa B (NF-kB) is not considered a marker of inflammation itself, but rather a key transcription factor that regulates the expression of genes involved in the inflammatory

response. NF-kB plays a central role in the initiation and regulation of immune and inflammatory processes[160].

While in an inactive state, NF-kB resides in the cytoplasm where it is bound to inhibitory proteins known as IκBs. Upon activation by different stimuli, such as pro-inflammatory cytokines or pathogen-associated molecular patterns, IκBs undergo phosphorylation and degradation. This process enables NF-kB to translocate into the nucleus. Once inside the nucleus, NF-kB binds to specific DNA sequences and facilitates the transcription of pro-inflammatory genes, including cytokines, chemokines, adhesion molecules, and enzymes involved in the inflammatory cascade[163].

The activation of NF-kB is considered a key step in the inflammatory response and is often associated with increased production of inflammatory mediators. Therefore, the measurement of NF-kB activity or levels can serve as an indirect marker of inflammation. Researchers often assess NF-kB activation through techniques such as electrophoretic mobility shift assay (EMSA), immunohistochemistry, or reporter gene assays to detect NF-kB-responsive gene expression.

It's important to note that NF-kB is involved in a wide range of cellular processes beyond inflammation, including cell survival, proliferation, and immune responses. Its activity can be influenced by various factors, and its dysregulation has been implicated in numerous inflammatory diseases. Therefore, while NF-kB itself is not a direct marker of inflammation, monitoring its activation and function can provide valuable insights into the inflammatory status of cells and tissues[161].

2.7.1.2: PPAR Alpha

Peroxisome proliferator-activated receptor alpha (PPAR-alpha) is not typically considered a direct marker of inflammation. Instead, PPAR-alpha is a nuclear receptor that plays a crucial role in regulating lipid metabolism and inflammation. It is involved in the control of genes related to fatty acid oxidation and the reduction of pro-inflammatory mediators[162].

PPAR-alpha activation can have anti-inflammatory effects by inhibiting the expression of inflammatory cytokines and chemokines, reducing the adhesion of immune cells to blood vessels, and suppressing the production of reactive oxygen species. It also promotes the expression of anti-inflammatory factors and enhances tissue repair mechanisms.

While PPAR-alpha is not a direct marker of inflammation, its activity and expression levels can be altered in various inflammatory conditions[162]. Changes in PPAR-alpha expression or activity can serve as indirect indicators of the presence or severity of inflammation. However, the assessment of PPAR-alpha is typically performed through laboratory techniques that measure its expression or activity rather than considering it a direct marker of inflammation.

It's worth noting that the role of PPAR-alpha in inflammation is complex and can vary depending on the specific context and disease. Further research is ongoing to better understand the interplay between PPAR-alpha and inflammation in different pathological conditions.

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Chapter Three

Methodology

3.1 Reagents

Testosterone Propionate and Dutasteride were of pharmaceutical grade, and were products of Laborate Pharmaceuticals India LTD; and GlaxoSmithKline United Kingdom Pharmaceuticals respectively. The Bioassay kits used for this study were purchased from Randox (UK) and Biobase (Jinan, China). Analytical grade chemicals and reagents were utilized, and all essential precautions were meticulously adhered to[1].

3.2 Plant Collection and Extraction

The fresh peel of *Citrus sinesis* were collected from a local vendor and authenticated at Botany Department, University of Ibadan. The peels were washed, rinsed and air dried in a shady area for four weeks. The well dried peels were blended into fine powder using a blender. The resultant powder was weighed and recorded (990 g).

The fine powder was extracted using three different solvents based on the solvents 'increasing order of polarity. The *Citrus sinesis* powder was first extracted with 2970 mL of n-hexane (ratio 1:3). The solution was stirred using a stirrer intermittently for 72 hours. After 72 hours, the mixture was filtered using a muslin cloth, and thereafter with Whatman filter paper No 1 to obtain the filtrate. The solvent was then evaporated using a water bath at 40^{o}C to obtain the oil[2]. The solute obtained after the filtration was air dried, and the procedure described above was repeated for the two other solvents (methanol and acetone) [2].

3.3 Experimental Animals

Forty-eight sexually matured male *Wistar* rats were purchased from a private animal farm. The animals were transported to the animal house, Department of Biochemistry, Lead City University, Ibadan. The rats were treated with care, housed in suspended cages made of plastic, and kept in a rat facility that had adequate ventilation and hygiene standards. The temperature and humidity conditions were suitable for their well-being. The rats were fed with rat pellets (Top feeds), given access to water, and maintained under a natural light-dark cycle of 12 hours each. Prior to the start of the study, the animals were given a two-week acclimatization period[6].

Before the commencement of study, forty-two animals were castrated and allowed to recover for four weeks. At the end of the study, the rats were sacrificed by cervical dislocation and the prostrate was rapidly excised and weighed[6].

3.3.1 Castration of rats

To eliminate the impact of endogenous testosterone during the study, the rats underwent castration using an anesthetic agent called ketamine at a dose of 25 mg/kg[7]. The castration procedure involved the removal of both testis and the epididymal fat, following the method Coppenolle et al. as described by Obisike et al[8]. The blood vessels and the spermatic cord were tied off using suture materials (3.0 mm) and then resected. Subsequently, the animals were given a recovery period of 4 weeks before the study commenced.

3.4 Experimental design

The rats were allocated into eight groups (with six rats per group) using a random assignment method. These groups were labeled as groups A to H.

The groupings were as follows:

Group A: Non-castrated control

Group B: Castrated control

Group C: Castrated control + Testosterone propionate only (BPH)

Group D: BPH + 250 mg/kg bw methanol extract of *Citrus sinesis* peel (MECSP)

Group E: BPH + 500 mg/kg bw methanol extract of *Citrus sinesis* peel (MECSP)

Group F: BPH + 250 mg/kg bw n-Hexane extract of *Citrus sinesis* peel (HECSP)

Group G: BPH + 500 mg/kg bw n-Hexane extract of *Citrus sinesis* peel (HECSP)

Group H: BPH + 0.5mg/kg bw Dutasteride (standard drug)

3.4.1 Body Weight determination

The body weight of the rats was determined after acclimatization using a weighing balance and recorded as the initial weight. This was employed to calculate the dosage of testosterone

propionate administered for inducing toxicity. The body weight was then measured on a weekly basis to appropriately adjust the treatment doses[9].

3.4.2 Induction of testosterone propionate

To induce Benign Prostate Hyperplasia (BPH), a daily subcutaneous administration of testosterone propionate was performed at a dosage of 2.5 mg/kg body weight for a duration of thirty days[9,10,11].

3.4.3 Standard drug

Dutasteride was purchased from a local drug store and administered at a dose of 0.5 mg/kg per day[12, 13].

3.4.4 Blood collection

The blood sample was collected from each rat into plain tubes for biochemical assays. Serum was separated from the whole blood by centrifuging at 4000 rpm for 10 minutes, and was kept under ice for biochemical assays.

3.4.5 Organ Collection and Determination of relative Organ Weight of the rats

Prostate, kidney and liver were excised and rinsed in a rinsing buffer (1.15% KCL)[14]. The tissues were weighed and their values were recorded as relative organ weight per 100 g rat weight.

$$\text{Relative Organ Weight} = \frac{\text{Tissue weight}}{\text{Animal weight}} \times 100$$

3.4.6 Preparation of post-mitochondrial fraction

The rats were then sacrificed, the prostate and the liver were excised and washed in 1.15% KCL, blotted and weighed. The tissue was then suspended in homogenizing buffer (1gram tissue: 4mL buffer). The tissue was then homogenized with Telfon Elvehjem glass homogenizer and centrifuged at 10000 rpm for 20 minutes at 4^0C to obtain the post-mitochondrial fraction[15,16]. The supernatant was collected for oxidative stress markers bioassays.

3.5 Histology

A portion of the prostate gland and liver was preserved in a 10% formalin solution, dehydrated using 95% ethanol, and subsequently fixed in paraffin and cleared with xylene. Micro sections of approximately 4 µm thickness were prepared and stained with haematoxylin and eosin. The slides were examined by a histopathologist for evaluation.

3.6 Determination of Benign Prostate Hyperplasia Biomarkers

3.6.1 Determination of 5 alpha reductase

Biobase ELISA kit for the determination of 5-α Reductase concentration in Rat serum, plasma, and other biological fluids was used[38].

Standards

S0, S1, S2, S3, S4 and S5 were 0, 20, 40, 80, 160, and 320 ng/mL respectively

Preparation of wash solution:

Wash solution was diluted with distilled water in ratio 1:20.

Assay procedure:

All Standards and Samples were added in duplicate to the Microelisa Stripplate.

1. All reagents needed for the assays were before starting assay procedure.

2. Standards (50µl) were added in duplicate into the standard well.

3. Sample (10 µL) was added in to sample well and sample diluent (40 µL) was added to each testing sample in the well. The blank well was left empty.

4. HRP-conjugate reagent (100 µL) was added to each well and cover with an adhesive strip and incubate for 60 minutes at 37°C.

5. The liquid content of each well was aspirated and washed with wash Solution (400 µL); this process was repeated four times for a total of five washes. For good performance, the plate was inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well.

6. chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, gently mix and incubated for 15 minutes at 37°C, the plate was protected from light.

7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow.

8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

The standard curve was generated by plotting the average O.D. (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding level of testosterone

concentration on the horizontal (X) axis. The standard curve was used to determine the unknown testosterone concentration in the sample.

3.7 Determination of Liver function Tests in Testosterone-induced Benign prostate Hyperplasia male rats

3.7.1 Determination of Aspartate aminotransferase (AST) activity

This assay was carried out using RANDOX AST assay kit[20,21].

Assay principle

The measurement of AST was performed by monitoring the formation of oxaloacetate hydrazone with 2,4-dinitrophenylhydrazine.

Reagent composition for AST activity estimation

R1: The solution used consisted of a phosphate buffer with a concentration of 100 mmol/L at pH 7.4, along with L-aspartate at a concentration of 100 mmol/L and α-oxoglutarate at a concentration of 2.0 mmol/L.

R2: 2,4-dinitrophenylhydrazine 2.0 mmol/L

Procedure

For the reagent blank preparation, 0.5 mL of reagent 1 and 0.1 mL of distilled water were carefully transferred into a test tube. Each serum sample was then pipetted into separate test tubes, with 0.1 mL of each sample added. Following this, 0.5 mL of reagent 1 was added to all

test tubes. The resulting mixtures were thoroughly mixed and incubated at a temperature of 37ºC for a duration of 30 minutes.

After the incubation period, 0.5 mL of reagent was added to both the reaction blank and the sample tubes. Once again, the mixtures were mixed and allowed to incubate at room temperature for 20 minutes. Subsequently, 5.0 mL of sodium hydroxide was added to all of the reaction mixtures. The mixtures were once again mixed and after 5 minutes, the absorbance of each sample was measured at 546 nm against the reagent blank[21, 22].

Calculation

The activity of AST was obtained from the standard curve generated from the values provided in the kit's manual.

3.7.2 Estimation of alanine amino transferase (ALT) activity in serum

This assay was carried out using the RANDOX ALT assay kit[20].

Assay principle

The measurement of Alanine Aminotransferase (ALT) was conducted by monitoring the formation of pyruvate hydrazone with 2,4-dinitrophenylhydrazine[22].

Reagent composition for ALT activity estimation

Reagent 1: The solution used in the assay comprised a phosphate buffer with a concentration of 100 mmol/L at pH 7.4, along with L-alanine at a concentration of 200 mmol/L and α-ketoglutarate at a concentration of 2.0 mmol/L.

Reagent 2: 2,4 dinitrophenylhydrazine 2.0 mmol/L

Procedure

To prepare the reagent blank, 0.5 mL of solution R1 and 0.1 mL of water were carefully pipetted into a test tube. Each serum sample (0.1 mL) was pipetted into separate test tubes, followed by the addition of 0.5 mL of solution R1 to all tubes. The resulting reaction mixtures were thoroughly mixed and incubated at a temperature of 37ºC for precisely 30 minutes.

After the incubation period, 0.5 mL of solution R2 was added to both the reagent blank and all the sample tubes. The mixtures were thoroughly mixed again and incubated at room temperature for 20 minutes. Following this, 5.0 mL of sodium hydroxide was added to both the reagent blank and the sample tubes. The mixtures were mixed once more, and their absorbances were measured against the reagent blank at a wavelength of 546 nm after 5 minutes[22].

Calculation

The activity of ALT for each sample was obtained from the standard curve generated from the values provided in the kit's manual.

3.8 Determination of Oxidative Stress/Antioxidant Markers in Testosterone-induced Benign Prostate Hyperplasia male rats

Total protein concentration, MDA concentration, GSH concentration, SOD activity, CAT activity and GPx activity were determined in the serum and tissue homogenates (Liver and prostate).

3.8.1 Determination of Total Protein

The protein concentration of the various homogenates was determined by means of the biuret method as described by Gornall *et al*[23]. with some modifications: the addition of potassium iodide to prevent precipitation of Cu^{2+} ions as cuprous oxide.

Principle

When proteins are exposed to an alkaline solution, their amino groups react by reducing cuprous ions to the cupric form. The resulting cupric ions form a complex with the proteins, imparting a blue color to the solution. This complex exhibits maximum absorption at a wavelength of 540 nm. Sodium potassium tartrate is included in the solution to stabilize the complex and maintain its integrity.

Reagents

1. 0.2M Sodium Hydroxide (NaOH)

   1.6 g of NaOH was dissolved in distilled water and the solution made up to 200 ml with the same.

2. Biuret reagent

   0.6 g of copper sulphate ($CuSO_4$. $5H_2O$) and 1.8 g of sodium potassium tartrate were dissolved in 150 ml of 0.2 M NaOH. Potassium iodide (1 g) was added and the solution made up to 200 ml with 0.2M NaOH.

3. Stock bovine serum albumin (BSA) solution

20 mg of BSA was dissolved in 4 ml of distilled water to give a stock solution of 5 mg/ml.

Standard protein (BSA) curve

Several dilutions of the stock solution containing 2-10 mg protein/ml were made. Into 1 ml of each protein standard solution in a test tube was added 3 ml of biuret reagent. The mixture was allowed to stand at room temperature for 30 min and the optical densities of the resulting solutions were read in a spectrophotometer at 540 nm against a blank of 1 ml of distilled water and 3 ml of biuret reagent. A curve of absorbance against protein concentration was plotted.

Table 3.1 Protocol for protein standard curve preparation

TEST TUBE NO	1	2	3	4	5
Stock BSA (ml)	0.2	0.4	0.6	0.8	1.0
Distilled water (ml)	0.8	0.6	0.4	0.2	0
Biuret reagent (ml)	3	3	3	3	3
BSA concentration (mg/ml)	0.25	0.50	0.75	1.00	1.25

Procedure for samples

To obtain a 1 in 5 dilution of the sample, 0.2 ml of the sample was combined with 0.8 ml of distilled water. Following this, 3 ml of biuret reagent was added to the mixture. The resulting solution was then incubated at room temperature for 30 minutes. After incubation, the absorbance was measured at 540 nm using the reagent blank. The protein content of the samples was determined by extrapolating from the standard curve, and the obtained value was multiplied by 5 to obtain the actual protein concentration in the sample.

3.8.2 Determination of malondialdehyde level

The method developed by Varshney and Kale was employed to assess lipid peroxidation in the test sample by quantifying the presence of thiobarbituric acid reactive substances (TBARS)[24].

Principle

Under acidic conditions, the chromogenic reagent 2-thiobarbituric acid reacts with malondialdehyde (MDA) generated from the peroxidation of fatty acids. This chemical reaction gives rise to a pink-colored complex, which demonstrates its highest absorbance at a wavelength of 532 nm.[24].

Reagents

1. 30% Trichloroacetic acid (TCA)

   4.5 g of TCA (CCl_3COOH) was dissolved in distilled water and made up to 15 mL with the same.

2. 0.1M Hydrochloric acid (HCl)

   13 µl of concentrated HCl (36.5-38%) was added to distilled water and the volume made up to 15 mL with the same.

3. 0.75% Thiobarbituric acid (TBA)

   0.1125 g of TBA was dissolved in 0.1 M HCl and made up to 15 mL with the same. Dissolution was aided by stirring in a hot water bath (50˚C).

4. 0.15 M Tris-KCl buffer (pH 7.4)

0.559 g of KCl and 0.909 g of Tris base were dissolved in 45 mL of distilled water, the pH was then adjusted to 7.4 with HCl and the volume made up to 50 mL with the same.

Procedure

An aliquot of 0.4 mL of the test sample was mixed with 1.6 mL of Tris-KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 mL of 0.75% TBA was added and placed in a water bath for 45 minutes at 80°C. This was then cooled in ice to room temperature and centrifuged at 3000 rpm for 10 min. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm.

Calculation

The MDA concentration was determined utilizing an extinction coefficient of 0.156 µM-1cm-1 for the calculation.

$$\text{Lipid peroxidation (nmol MDA/mg protein)} = \frac{Absorbance\ x\ volume\ of\ mixture}{\text{E532nm x volume of sample x } total\ protein\ (mg/mL)}$$

Where E_{532nm} = Extintion coefficient of MDA-TBA adduct (0.156 $\mu M^{-1}cm^{-1}$)

3.8.3 Determination of reduced glutathione (GSH) level

The estimation of reduced glutathione (GSH) level was conducted following the method established by Beutler.

Principle

This method depends on producing a stable yellow compound when sulfhydryl compounds, mainly glutathione found in tissues, react with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), also known as Ellman's reagent. The resulting-colored product shows its maximum absorption at a wavelength of 412 nm[25].

Reagents

1. GSH stock solution

   40 mg of GSH was dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 100ml with the same.

2. Phosphate buffer (0.1 M, pH 7.4)

   Dipotassium hydrogen phosphate trihydrate (2.098 g) and potassium dihydrogen phosphate (0.791 g) were dissolved in 120 mL of distilled water, the pH adjusted to 7.4 and the volume made up to 150 mL with distilled water.

3. Ellman's Reagent

   60 mg of Ellman's reagent was in dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 150 mL with the same.

4. Sulphosalicylic Acid (4% solution)

   0.8 g of sulphosalicylic acid was dissolved in 20 mL of distilled water.

GSH Standard Curve Preparation

Serial dilutions of the GSH stock solution were prepared as shown in the table below. The absorbance of the yellow colour formed upon the addition of Ellman's reagent was read within

30 min at 412 nm against a blank of 1.5 mL of Ellman's reagent and 0.5 mL phosphate buffer. A plot of absorbance against concentration of reduced GSH was then plotted.

Procedure for samples

0.4 mL of sample was added to 0.4 mL of precipitating solution which was vortexed and centrifuged at 4000 rpm for 5 minutes. Thereafter, 0.5 mL of the supernatant was added to 1.5 mL of Ellman's reagent. The absorbance of the reaction mixture was read at 412 nm against a reagent blank.

Table 3.2: GSH Standard Curve Protocol

GSH Stock (ml)	Phosphate Buffer (mL)	Ellman's Reagent (mL)	GSH Conc. (µg/mL)
0.01	0.49	1.5	2
0.03	0.47	1.5	6
0.05	0.45	1.5	10
0.10	0.40	1.5	20
0.15	0.35	1.5	30
0.20	0.30	1.5	40

3.8.4 Determination of superoxide dismutase activity

The method developed by Misra and Fridovich was used to measure the activity of SOD[26].

Principle

The capacity of SOD to prevent the autoxidation of epinephrine at pH 10.2 provides the foundation for a straightforward assay to measure this dismutase. The oxidation of epinephrine to adrenochrome is induced by the superoxide radical, and the amount of adrenochrome produced per superoxide radical introduced rises with higher pH and greater concentration of epinephrine[26].

Reagents

Preparation of 0.05M Carbonate buffer (pH 10.2): 1.573 g of $Na_2CO_3.10H_2O$ and 0.588 g of $NaHCO_3$ were dissolved in 200 mL of distilled water. The pH of the solution was then adjusted to 10.2, and distilled water was added to bring the total volume up to 250 mL.

Preparation of 0.3M Epinephrine in HCl: 0.05 g of epinephrine was dissolved in 200 mL of distilled water containing 0.5 mL of concentrated HCl (37%).

Procedure:

In a cuvette, 50 µL of sample was added to 2.5 mL of 0.05M carbonate buffer (pH 10.2) and 0.3 mL of epinephrine. The mixture was then inverted and the change in absorbance was monitored every 30 seconds for a total of 2.5 minutes at 480 nm. For the reference cuvette, water was used instead of the samples.

Calculation

$$\% \text{ inhibition} = 100 - \left(100 \times \frac{\text{Increase in absorbance per min for sample}}{\text{Increase in absorbance per min for blank}}\right)$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of epinephrine.

3.8.5 Determination of Catalase Activity

The method developed by Claiborne was employed to determine the catalase activity[27].

Principle

The technique relies on measuring the decrease in absorbance at 240 nm when catalase breaks down hydrogen peroxide. Although hydrogen peroxide does not have a specific absorbance peak at this wavelength, its absorbance is closely related to its concentration, making it suitable for a quantitative analysis. To quantify the hydrogen peroxide concentration, an extinction coefficient of 0.0436 mM-1cm-1 (Noble and Gibson[28]) was employed.

Reagents

1. Phosphate buffer (0.05 M, pH 7.4)

   Dipotassium hydrogen phosphate trihydrate (0.696 g) and potassium dihydrogen phosphate (0.265 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

2. Hydrogen peroxide (19 mM)

   194 µl of 30% H_2O_2 was added to 50 mL of 0.05 M phosphate buffer, pH 7.4 and the volume made up to 100 ml with the same.

Procedure

A volume of 2.95 mL from a 19 mM solution of hydrogen peroxide was carefully transferred into a quartz cuvette with a 1 cm path length. Then, 50 µl of the sample was added to the cuvette. The mixture was quickly inverted to ensure thorough mixing and promptly inserted into a spectrophotometer. The absorbance change was measured at 240 nm every minute for a duration of 5 minutes.

Calculation

$$\text{Catalase activity} = \frac{\Delta A_{240}/\text{min} \times \text{reaction volume} \times \text{dilution factor}}{0.0436 \times \text{sample volume} \times \text{mg protein/ml}}$$

= µmole H_2O_2/min/mg protein

3.8.6. Assay for Glutathione Peroxidase Activity

Glutathione peroxidase (GPX) activity was measured according to the procedure of Rotruck*et al*[28]. with some modifications.

Principle

After a specific duration of time, glutathione peroxidase is permitted to catalyze the conjugation of hydrogen peroxide with glutathione. Once the allotted time has passed, the reaction is terminated. The remaining glutathione is then subjected to a reaction with Ellman's reagent, and the extent of GSH consumption is utilized as an indicator of enzyme activity.

Reagents

1. Sodium azide (10 mM)

   3.25 mg of sodium azide was dissolved in 50 ml of distilled water.

2. Reduced glutathione (4 mM)

   12.3 mg of GSH was dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4.

3. Hydrogen peroxide (2.5 mM)

   14 µl of 30% hydrogen peroxide was added to distilled water and the volume made up to 50 ml with the same.

4. Trichloroacetic acid (10%)

   2 g of TCA was dissolved in distilled water and the volume made up to 20 ml with the same.

5. Dipotassium hydrogen orthophosphate (0.3 M)

   4.11 g of $K_2HPO_4.3H_2O$ was dissolved in distilled water and the volume made up to 60 ml with the same.

6. Ellman's reagent (DTNB)

   19.8 mg of DTNB was dissolved in 50 mL of 0.1 M phosphate buffer, pH 7.4.

7. Phosphate buffer (0.1 M, pH 7.4)

In 90 mL of distilled water, 1.399 g of dipotassium hydrogen phosphate trihydrate and 0.527 g of potassium dihydrogen phosphate were dissolved. The pH was then adjusted to 7.4, and distilled water was added to bring the total volume to 100 mL.

Procedure

In a test tube, 0.5 mL of phosphate buffer was combined with 0.1 mL of NaN3, 0.2 mL of GSH, 0.1 mL of H_2O_2, and 0.5 ml of sample (added last). The resulting mixture was incubated for 3 minutes at 37˚C. After incubation, 0.5 mL of TCA was added, and the final mixture was

centrifuged at 3000 rpm for 5 minutes. To 1 mL of the resulting supernatant, 2 mL of K_2HPO_4 and 1 mL of DTNB were added. The absorbance was then measured at 412 nm against a reagent blank composed of 1 mL of distilled water, 2 mL of K_2HPO_4, and 1 mL of DTNB.

Calculation

GSH consumed = initial GSH amount (129.39 µg) – GSH remaining (µg/mL × 4 mL)

GPX activity = GSH consumed/mg protein

= µg GSH/mg protein

3.9. Determination of Inflammatory Biomarkers

Nuclear factor kappa light chain enhancer of activated B cells (NF-kB) and Peroxisome proliferator-activated receptor (PPAR)-alpha were assayed in the serum of the rats. The methods used for each of this assay were explained in the subsection.

3.9.1 Determination of level of NF-κb using ELISA

The examination was conducted utilizing a rat-specific Enzyme Linked Immunoassay (ELISA) kit (manufactured by Biobase, China) designed for the quantitative measurement of NF-κb in various biological fluids such as rat serum, blood plasma, and urine[29]. This kit is characterized by high sensitivity and exceptional specificity in detecting NF-κb, with a minimum detectable concentration typically lower than 15 ng/L. The assay range of the kit spans from 50 ng/L to 1000 ng/L, providing a reliable and accurate measurement of NF-κb levels.

Principle

The kit utilizes a solid-phase enzyme immunoassay based on the "sandwich" principle to measure NF-κb levels in the sample. The Microelisa strip plate included in the kit is coated with purified NF-κb antibody, creating a solid-phase antibody. NF-κb is added to the wells, where it binds to the NF-κb antibody labeled with HRP, forming an antibody-antigen-enzyme complex. After thorough washing to remove unbound enzyme, Chromogen Solution A and Chromogen Solution B are added, resulting in a color change to blue. The addition of acid subsequently changes the color to yellow. The extent of color change is measured spectrophotometrically at a wavelength of 450 nm. By comparing the absorbance values to a standard curve, the concentration of NF-κb in the samples is determined through interpolation[29].

Standards

S0, S1, S2, S3, S4 and S5) were 0, 1, 2, 4, 8, and 16 ng/mL respectively

Preparation of wash solution:

Wash solution was diluted with distilled water in ratio 1:20.

Assay procedure:

All standards and samples were added in duplicate to the Microelisa Stripplate.

1. All reagents needed for the assays were before starting assay procedure.

2. Standards (50µl) were added in duplicate into the standard well.

3. Sample (10µL) was added in to sample well and sample diluent (40µL) was added to each testing sample in the well. The blank well was left empty.

4. Each well was supplemented with 100 µL of HRP-conjugate reagent, covered with an adhesive strip, and incubated for 60 minutes at a temperature of 37°C.

5. The liquid in each well was removed by aspiration, followed by washing with 400 µL of wash solution. This washing process was repeated four times, resulting in a total of five washes. To ensure thorough removal of liquid from the wells, the plate was inverted and gently blotted against clean paper towels. This step was carried out to optimize the performance of the assay.

6. In each well, 50 µL of chromogen solution A and 50 µL of chromogen solution B were added. The contents were gently mixed and incubated for 15 minutes at a temperature of 37°C, while ensuring that the plate was protected from light.

7. In each well, 50 µL of stop solution was added. The addition of the stop solution caused a noticeable color change in the wells, transitioning from blue to yellow.

8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

The standard curve was created by plotting the average optical density (OD) values obtained at 450 nm for each of the six standard concentrations on the vertical (Y) axis, against the corresponding NF-κb concentration levels on the horizontal (X) axis. This standard curve was then utilized to determine the unknown NF-κb expression in the sample.

3.9.2 Determination of level of PPAR-alpha in serum using ELISA

This was done using Biobase ELISA kit for the determination of PPAR-α concentrations in Rat serum, plasma, tissue homogenates and other biological fluids[30]. The kit has assay range 37.5 pg/mL - 1200 pg/mL with minimum detectable level to less than 1.0 pg/mL

Principle

The kit is designed to assess Rat PPAR-α levels in the sample. It utilizes purified Rat PPAR-α antibody to coat microtiter plate wells, creating a solid-phase antibody. PPAR-α is then added to the wells, and an antibody labeled with HRP is combined, resulting in the formation of an antibody-antigen-enzyme-antibody complex. After thorough washing, TMB substrate solution is added, causing TMB substrate to turn blue due to the HRP enzyme-catalyzed reaction. The reaction is eventually halted with the addition of a sulfuric acid solution, and the resulting color change is measured spectrophotometrically at a wavelength of 450 nm. By comparing the absorbance values to a standard curve, the concentration of PPAR-α in the samples is determined through interpolation.

Standards

S0, S1, S2, S3, S4 and S5 were 0, 75, 150, 300, 600, and 1200 pg/mL respectively

Preparation of wash solution:

Dilute wash solution with distilled or deionized water 1:20.

Assay procedure:

All Standards and Samples be added in duplicate to the Microelisa Stripplate.

1. All reagents needed for the assays were before starting assay procedure.

2. Standards (50 µL) were added in duplicate into the standard well.

3. Sample (10 µL) was added in to sample well and sample diluent (40 µL) was added to each testing sample in the well. The blank well was left empty.

4. HRP-conjugate reagent (100 µL) was added to each well and cover with an adhesive strip and incubated for 60 minutes at 37°C.

5. The liquid content of each well was aspirated and washed with wash Solution (400 µL); this process was repeated four times for a total of five washes. For good performance, the plate was inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well.

6. To each well, 50 µL of chromogen solution A and 50 µL of chromogen solution B were introduced. The contents were gently mixed and allowed to incubate for 15 minutes at 37°C while ensuring that the plate was shielded from light.

7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow.

8. The Optical Density (O.D.) of the well content was measured at a wavelength of 450 nm using a microtiter plate reader, and this measurement was taken within a time frame of 15 minutes.

Calculation of results

By plotting the average O.D. (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis against the corresponding levels of PPAR-α concentration on the horizontal (X) axis, the standard curve was established. This standard curve served as a tool to determine the unknown expression of PPAR-α in the sample.

3.10. Determination of Reproductive Biomarkers

3.10.1 Determination of Follicle stimulating hormone (FSH) concentration

Enzyme Immunoassay for the Quantitative Determination of FSH in Rat serum, blood plasma, and other biological fluids was used for the study (Biobase, China)[32].

Test principle:

The kit employs an enzyme-linked immunosorbent assay (ELISA) based on the double antibody sandwich principle to measure the level of FSH in the sample. The Microelisa strip plate included in the kit is coated with FSH antibody to create a solid-phase antibody. FSH is added to the wells and combines with FSH antibody labeled with HRP, forming an antibody-antigen-enzyme-antibody complex. After thorough washing to remove any unbound enzyme, Chromogen Solution A and Chromogen Solution B are added, resulting in a color change to blue. Subsequently, the addition of acid causes the color to change to yellow. The extent of the color change is measured spectrophotometrically at a wavelength of 450 nm. By comparing the optical density (O.D.) of the samples to a standard curve, the activity of FSH in the samples can be determined[32].

Standards

S0, S1, S2, S3, S4 and S5 were 0, 7.5, 1.5, 3.0, 6.0, and 12.0 IU/L respectively

Preparation of wash solution:

Wash solution was diluted with distilled water in ratio 1:20.

Assay procedure:

All Standards and Samples be added in duplicate to the Microelisa Stripplate.

1. All reagents needed for the assays were before starting assay procedure.

2. Standards (50 µL) were added in duplicate into the standard well.

3. Sample (10 µL) was added in to sample well and sample diluent (40 µL) was added to each testing sample in the well. The blank well was left empty.

4. HRP-conjugate reagent (100 µL) was added to each well and cover with an adhesive strip and incubate for 60 minutes at 37°C.

5. The liquid content of each well was aspirated and washed with wash Solution (400µL); this process was repeated four times for a total of five washes. For good performance, the plate was inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well.

6. chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, gently mix and incubated for 15 minutes at 37°C, the plate was protected from light.

7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow.

8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

The standard curve was generated by plotting the average O.D. (450 nm) obtained for each of the six standards on the vertical (Y) axis versus the activity of FSH concentration on the horizontal (X) axis. The standard curve was used to determine the unknown FSH activity in the sample.

3.10.2 Testosterone determination in the serum sample

Biobase ELISA kit for the determination of testosterone concentrations in Rat serum, plasma, and other biological fluids was used[33].

Standards

S0, S1, S2, S3, S4 and S5 were 0, 20, 40, 80, 160, and 320 pg/mL respectively

Preparation of wash solution:

Wash solution was diluted with distilled water in ratio 1:20.

Assay procedure:

All Standards and Samples be added in duplicate to the Microelisa Stripplate.

1. All reagents needed for the assays were before starting assay procedure.

2. Standards (50 µL) were added in duplicate into the standard well.

3. Sample (10 µL) was added in to sample well and sample diluent (40 µL) was added to each testing sample in the well. The blank well was left empty.

4. HRP-conjugate reagent (100 µL) was added to each well and cover with an adhesive strip and incubate for 60 minutes at 37°C.

5. The liquid content of each well was aspirated and washed with wash Solution (400 µL); this process was repeated four times for a total of five washes. For good performance, the plate was

inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well.

6. chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, gently mix and incubated for 15 minutes at 37°C, the plate was protected from light.

7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow.

8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

To create the standard curve, the average optical density (O.D.) values at a wavelength of 450 nm were plotted on the vertical (Y) axis, while the corresponding testosterone concentrations were plotted on the horizontal (X) axis. This standard curve served as a reference to estimate the unknown testosterone concentration in the sample[33].

3.10.3 Progesterone determination in the serum sample

ELISA kit for the determination of Progesterone concentration (Monobind, USA) in serum, plasma, and other biological fluids was used[34].

Standards

S0, S1, S2, S3, S4 and S5 were 0, 0.3, 2.0, 5.0, 15, and 30 ng/mL progesterone concentration respectively

Preparation of wash solution:

Dilute wash solution with distilled or deionized water 1:20.

Assay procedure:

All Standards and Samples be added in duplicate to the Microelisa Stripplate.1. All reagents needed for the assays were before starting assay procedure. 2. Standards (50 µL) were added in duplicate into the standard well. 3. Sample (25 µL) was added in to sample well and blank well was left empty. 4. HRP-conjugate reagent (50 µL) and Progesterone biotin reagent (50 µL) were added to each well and cover with an adhesive strip and incubate for 60 minutes at 37°C. 5. The liquid content of each well was aspirated and washed with wash Solution (400 µL); this process was repeated four times for a total of five washes. For good performance, the plate was inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well. 6. chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, gently mix and incubated for 15 minutes at 37°C, the plate was protected from light. 7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow. 8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

The standard curve was generated by plotting the average O.D. (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding level of progesterone concentration on the horizontal (X) axis. The standard curve was used to determine the unknown progesterone concentration in the sample.

3.10.4 Determination of luteinizing hormone (LH) concentration

Enzyme immunoassay for the quantitative determination of LH in serum, blood plasma, and other biological fluids was used for the study (Monobind, USA)[35].

Test principle:

The kit utilizes an enzyme-linked immunosorbent assay (ELISA) based on the double antibody sandwich principle to analyze the LH level in the sample. The Microelisa strip plate provided in the kit is coated with LH antibody, creating a solid-phase antibody. LH is added to the wells and binds with LH antibody labeled with HRP, forming an antibody-antigen-enzyme-antibody complex. After thorough washing to remove any unbound enzyme, Chromogen Solution A and Chromogen Solution B are added, resulting in a color change to blue. Subsequently, the addition of acid causes the color to change to yellow. The extent of the color change was measured spectrophotometrically at a wavelength of 450 nm. The activity of LH in the samples was then determined by comparing the optical density (O.D.) of the samples to the standard curve[35].

Standards

S0, S1, S2, S3, S4 and S5 were 0, 5, 25, 50, 100, and 200 IU/L (mIU/mL) LH concentration respectively

Preparation of wash solution:

Dilute wash solution with distilled or deionized water 1:20.

Assay procedure:

All Standards and Samples be added in duplicate to the Microelisa Stripplate.1. All reagents needed for the assays were before starting assay procedure. 2. Standards (50 µL) were added in duplicate into the standard well. 3. Sample (50 µL) was added in to sample well and blank well was left empty. 4. HRP-conjugate reagent (100 µL) was added to each well and cover with an adhesive strip and incubate for 60 minutes at 37°C. 5. The liquid content of each well was aspirated and washed with wash Solution (400 µL); this process was repeated four times for a total of five washes. For good performance, the plate was inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well. 6. chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, gently mix and incubated for 15 minutes at 37°C, the plate was protected from light. 7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow. 8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the six standards on the vertical (Y) axis versus the concentration of LH concentration on the horizontal (X) axis. The standard curve was used to determine the unknown LH activity in the sample.

3.10.5 Estradiol (E2) determination in the serum sample

ELISA kit for the determination of E2 concentration (Monobind, USA) in serum, plasma, and other biological fluids was used[36].

Standards

S0, S1, S2, S3, S4, S5 and S6 were 0, 20, 100, 250, 500, 1500 and 3000 pg/mL E2 concentration respectively

Preparation of wash solution:

Dilute wash solution with distilled or deionized water 1:20.

Assay procedure:

All Standards and Samples be added in duplicate to the Microelisa Stripplate.1. All reagents needed for the assays were before starting assay procedure. 2. Standards (50 µl) were added in duplicate into the standard well. 3. Sample (25 µL) was added in to sample well and blank well was left empty. 4. HRP-conjugate reagent (50 µL) and E2 biotin reagent (50 µL) were added to each well and cover with an adhesive strip and incubate for 60 minutes at 37°C. 5. The liquid content of each well was aspirated and washed with wash Solution (400 µL); this process was repeated four times for a total of five washes. For good performance, the plate was inverted and blotted it against clean paper towels to ensure complete removal of liquid in the well. 6. chromogen solution A (50 µL) and chromogen solution B (50 µL) were added to each well, gently mix and incubated for 15 minutes at 37°C, the plate was protected from light. 7. Stop solution (50 µL) was added to each well. The colour in the wells should change from blue to yellow. 8. The Optical Density (O.D.) of the content of the well was read at 450 nm using a microtiter plate reader within 15 minutes.

Calculation of results

The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding level of E2

concentration on the horizontal (X) axis. The standard curve was used to determine the unknown E2 concentration in the sample.

3.11 Gas Chromatography-Mass Spectroscopy (Gc-Ms) Analysis of *Citrus sinesis* peel Extracts

The methanol, acetone and n-hexane extracts of CSP were initially transformed into a gaseous form using Gas Chromatography (GC). Subsequently, they were introduced into the mobile phase, typically composed of gases like helium or argon. The mobile phase carries the sample until it reaches the stationary phase present in the GC column. The stationary phase consists of a specially designed chemical that selectively interacts with specific compounds within the sample, resulting in their separation[3,4]. This separation occurs due to the gradual heating of the GC column, known as ramping, causing compounds with lower boiling points to elute first. Additionally, the pressure of the mobile phase can be adjusted to optimize the separation process. The interactions between the sample compounds and the stationary phase also play a role, with weaker interactions causing faster dissociation and earlier elution. The time taken for a compound to move from its injection point to its elution from the GC column is known as its retention time. Compounds with lower molecular weights elute earlier than those with higher molecular weights due to differences in boiling points[4].

Once the elution process is finished, the compounds undergo either electron ionization (EI) or chemical ionization (CI), resulting in the generation of charged species. These charged species then enter a mass spectrometer for mass analysis, where their unique mass (m) and charge (z) information are recorded as numerical m/z ratios. These values are commonly represented as ion peaks. Subsequently, the recorded values are compared to pre-existing databases of known mass

spectra using specialized software programs. Spectra that match the recorded values are identified and characterized accordingly. Mass spectrometry analysis plays a crucial role in determining the molecular weight and formula of compounds, as well as providing information about their functional groups[3,4].

GC-MS samples frequently consist of complex mixtures containing unstable, volatile, and potentially impure compounds, which may necessitate additional treatment before their introduction into the gas chromatograph. Various manual and automated techniques are commonly employed for sample extraction prior to gas chromatography. The choice of extraction method depends on the desired selectivity during sample preparation and the initial cleanliness of the samples[4].

3.12 *In-silico* Screening of the Phytochemicals in the Extracts of *Citrus sinesis* peel

In this study, the bioactive components present in citrus sinesis peel were evaluated using computer-based screening (in-silico) against specific receptor targets associated with BPH disease and its treatment. The activity of the citrus sinesis peel extracts in relation to hyperplasia potential was assessed by targeting 5α-reductase.

3.13 Preparation of Ligands

The library of compounds was built by drawing compounds identified to be present in MECSP and HECSP via GC analysis using Marvin sketch (version 20.4) and Chem Draw Professional (20.1.1). The compounds were prepared using PyRx. The crystal structures of the target proteins were obtained were obtained from the RCSB protein data bank (PDB).

The PDB format of the structures were uploaded to Chimera 1.14 workspace and the non-standard residues including ions, water and bounded ligands were first removed. The proteins were structurally minimized at 200 steepest descent steps, 0.02 steepest decent steps size (A), 10 conjugate gradient steps, 0.02 conjugate gradient steps size (A), and 10 update intervals, using the structure editing wizard Chimera 1.14. Furthermore, solvents were removed, hydrogen bonds were added, charges were assigned using Gasteiger force field and histidine was set for the protonation state.

3.14 Molecular Docking

The ligands were constructed using Marvin Sketch and Chem Draw. Molecular docking of the prepared ligands and proteins were performed using AutoDock vina in the PyRx workspace. Grid space was set by targeting important amino acid residues involved in interaction of the proteins with compounds previously reported to have affinity for the protein. Grid box size x = 26.4840A, y = -0.4210A and z = 59.3939A

The output files were uploaded to Discovery Studio workspace for post docking analysis of the protein-ligand complex. The molecular interactions were generated Discovery Studio 2020[5].

3.15 Assessment of Absorption, Distribution, Metabolism, Excretion (ADME) and Toxicity Profiles of Lead Phytochemicals

The (ADME) Assessment of Absorption, Distribution, Metabolism, Excretion) and Toxicity profiles of the lead phytochemicals were respectively determined according to the procedure described Swiss ADME and PROTOX-II web server. The canonical smiles for the ligands were fetched from Marvin sketch[5].

3.16 Statistical Analysis

The data were analyzed with Microsoft excel 2013, and GraphPad prism 6.0. One way analysis of variance (ANOVA) with multiple test comparison test was used to determine the differences in the mean of each parameter across group at 5% significance level ($p < 0.05$)

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Chapter Four

Results and Discussion of Findings

4.1 The effects of MECSP and HECSP on relative organ weights (g/100 g rat) in Testosterone-induced Benign prostate hyperplasia rats

Table 4.1 shows the relative organ weight of BPH rats. Animals with BPH exhibited a higher relative prostate weight when compared to the control groups (normal control and castrated group) ($p < 0.05$). Conversely, animals treated with MECSP and HECSP demonstrated a reduction in relative prostate weight and liver weight when compared to the BPH group ($p < 0.05$).

Table 4.1: The effect of MECSP and HECSP on relative organ weights (g/100 g rat) in Testosterone-induced Benign prostate hyperplasia rats

Groups	Prostate	Liver
Control	0.11± 0.05	2.39 ± 0.30
Castrated Control	0.04 ± 0.01	2.64 ± 0.10
Castrated Control + T.P (BPH)	0.22±0.10$^{\alpha, \gamma}$	3.31 ± 1.10 $^{\alpha, \gamma}$
BPH + 250mg/kg bw MECSP	0.17± 0.05 $^{\alpha, \beta, \gamma}$	2.71 ± 1.05 $^{\alpha, \beta, \gamma}$
BPH + 500mg/kg bw MECSP	0.13± 0.03 $^{\alpha, \beta, \gamma}$	2.24 ± 0.05 $^{\alpha, \beta, \gamma}$

BPH + 250mg/kg bw HECSP	$0.18 \pm 0.02^{\alpha, \beta, \gamma}$	$2.50 \pm 0.12^{\alpha, \beta, \gamma}$
BPH + 500mg/kg bw HECSP	$0.17 \pm 0.01^{\alpha, \beta, \gamma}$	$2.29 \pm 0.16^{\alpha, \beta, \gamma}$
BPH+ Dutasteride	$0.11 \pm 0.10^{\beta}$	$2.53 \pm 0.21^{\beta}$

Results were computed as mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

4.2: The effects of MECSP and HECSP on the Prostate and Serum 5-α Reductase concentration in Testosterone-induced Benign prostate hyperplasia rats

MECSP and HECSP caused notable a decrease ($p < 0.05$) in the prostate concentration of 5-α reductase enzyme (5-αR) which was initially increased in testosterone-induced benign hyperplasia rats (Figure 4.1). Serum 5-α reductase enzyme concentration was observed to decrease in untreated BPH rats when compared with control ($p < 0.05$), the concentration of the enzyme was increased when the rats that were treated with 500 mg/kg HECSP (Figure 4.2).

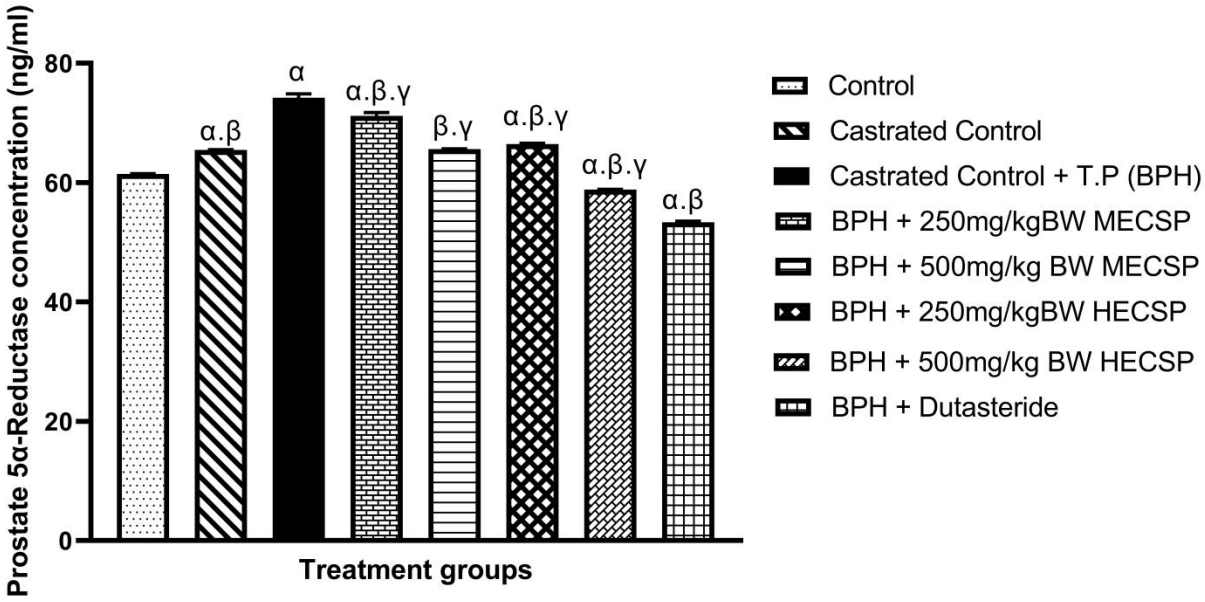

Figure 4.1: The effect of MECSP and HECSP on Prostate 5-α Reductase concentration in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

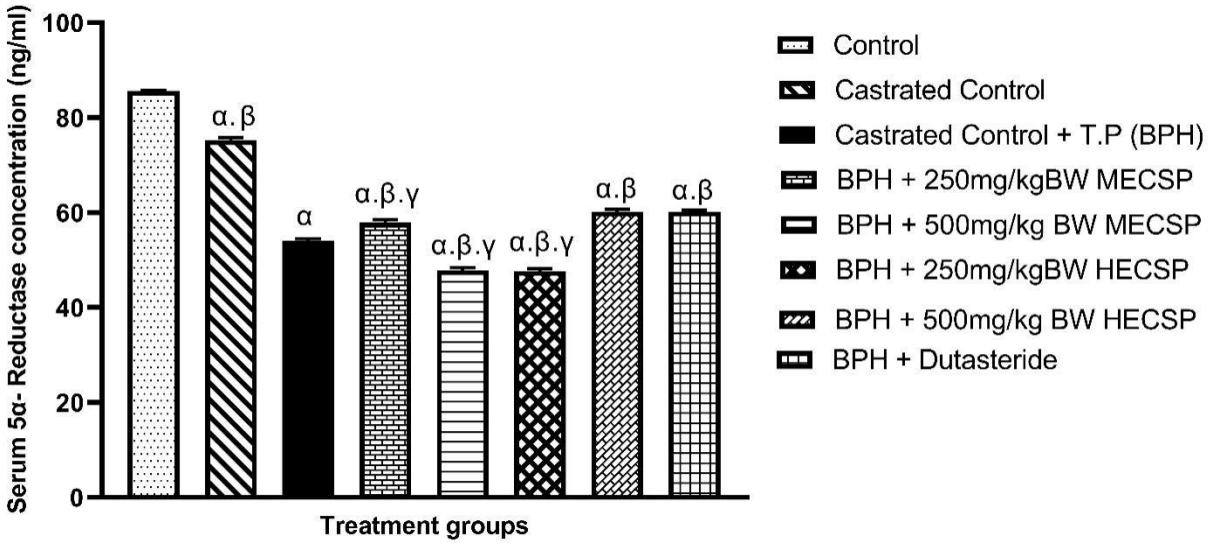

Figure 4.2: The effect of MECSP and HECSP on Serum 5-α Reductase concentration in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

4.3: The effects of MECSP and HECSP on Liver function markers in Testosterone-induced Benign prostate hyperplasia rats

Serum alanine aminotransferase activity was greatly raised in testosterone-induced benign prostate hyperplasia rats. However, those hyperplasia rats treated with CSP extract reduced the activity, with n-Hexane extract showing much decrease. Serum aspartate aminotransferase activity was slightly increased in untreated hyperplasia rats, this increased activity was reduced in all the treatment groups, with methanol extract group showing great decrease.

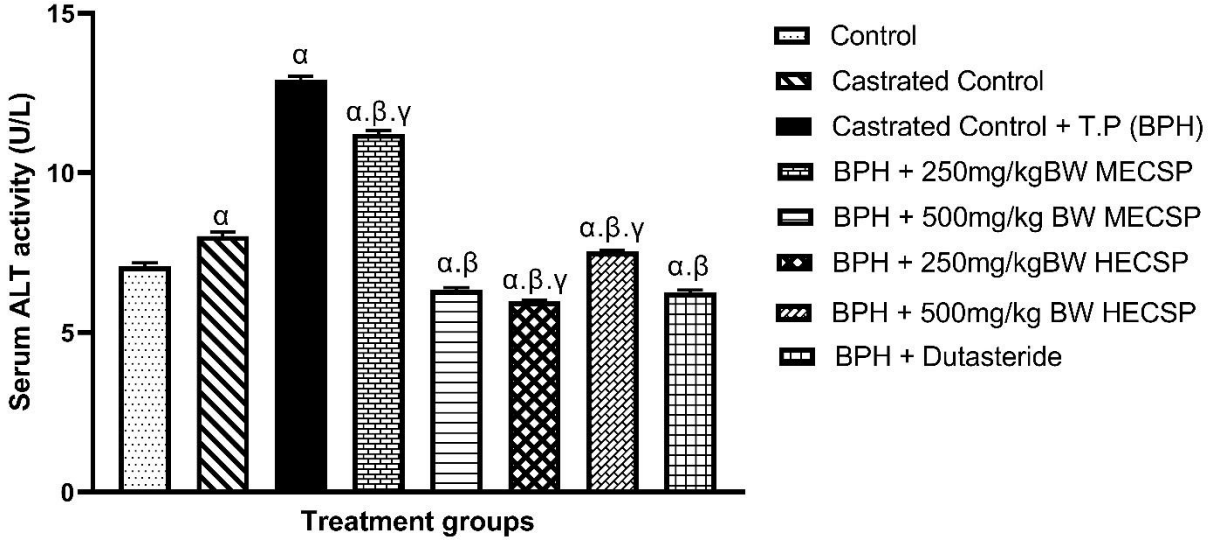

Figure 4.3: The effect of MECSP and HECSP on Serum ALT activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

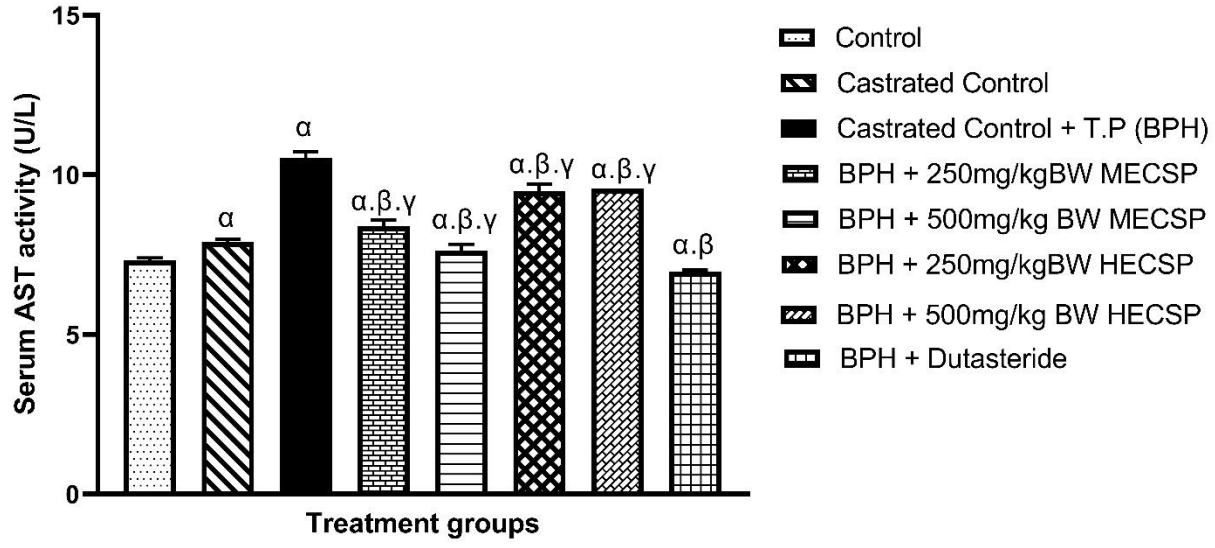

Figure 4.4: The effect of MECSP and HECSP on Serum AST level in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

4.4: The effects of MECSP and HECSP on Oxidative stress markers in Testosterone-induced Benign prostate hyperplasia rats

There was no marked change in serum SOD activity of BPH rats treated with CSP extracts and untreated BPH rats when compared to the control group and the treatment groups. There was no notable difference in the activity of liver GSH in untreated BPH rats compared to the control group, however, rats treated with CSP extracts had increase liver GSH activity ($p < 0.05$) like those treated with dutasteride (standard drug). Increase in the activity of prostate GSH was observed in the untreated BPH when compared with the control, however, rats treated with CSP extracts was able to decrease the activity. MECSP and HECSP caused notable a decrease ($p < 0.05$) in prostate and liver activity of catalase which was initially increased in testosterone-induced BPH rats (Figure 15). Liver MDA concentration was observed to increase in untreated BPH rats when compared with control ($p < 0.05$), the concentration was markedly lowered when the rats that were treated with 250 and 500 mg/kg MECSP, however, the concentration was further raised when the rats were treated with 250 and 500 mg/kg HECSP like those treated with dutasteride (standard drug). Prostate MDA concentration was increased in the untreated BPH rats when compared with the control ($p < 0.05$), the concentration of MDA was decreased in rats treated with CSP extracts, with high dose of HECSP showing much decrease. GPx activity was slightly raised in the liver homogenate when compared with the control, however, rats treated with CSP extracts reduced the activity, with 250 mg/kw HECSP showing huge decrease.

Table 4.2: The effect of MECSP and HECSP on total protein concentration in the serum and tissues of testosterone-induced Benign prostate hyperplasia

Groups	Serum (g/L)	Prostate (g/L)	Liver (g/L)
Control	27.51±1.02	13.17±0.31	25.22±3.33
Castrated Control	29.86±2.62	15.02±1.38	13.41±2.07
Castrated Control + T.P (BPH)	38.04±4.53$^{\alpha}$	8.01±6.37$^{\alpha}$	19.70±3.33$^{\alpha}$
BPH + 250mg/kg bw MECSP	47.13±1.04$^{\alpha,\beta,\gamma}$	10.25±2.52$^{\alpha,\beta,\gamma}$	18.16±1.48$^{\alpha,\beta,\gamma}$
BPH + 500mg/kg bw MECSP	48.16±2.35$^{,\beta,\gamma}$	9.70±3.47$^{\alpha,\beta,\gamma}$	16.57±4.37$^{\alpha,\beta,\gamma}$
BPH + 250mg/kg bw HECSP	47.16±2.12$^{\alpha,\beta,\gamma}$	7.81±3.49$^{\alpha,\beta,\gamma}$	13.46±2.36$^{\alpha,\beta,\gamma}$
BPH + 500mg/kg bw HECSP	45.36±1.57$^{\alpha,\beta}$	4.08±0.67$^{\alpha,\beta}$	15.70±2.45$^{\alpha,\beta}$
BPH+ Dutasteride	38.05±0.20$^{\alpha}$	6.38±2.33$^{\alpha,\beta}$	11.88±2.25$^{\alpha,\beta}$

Results were computed as mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

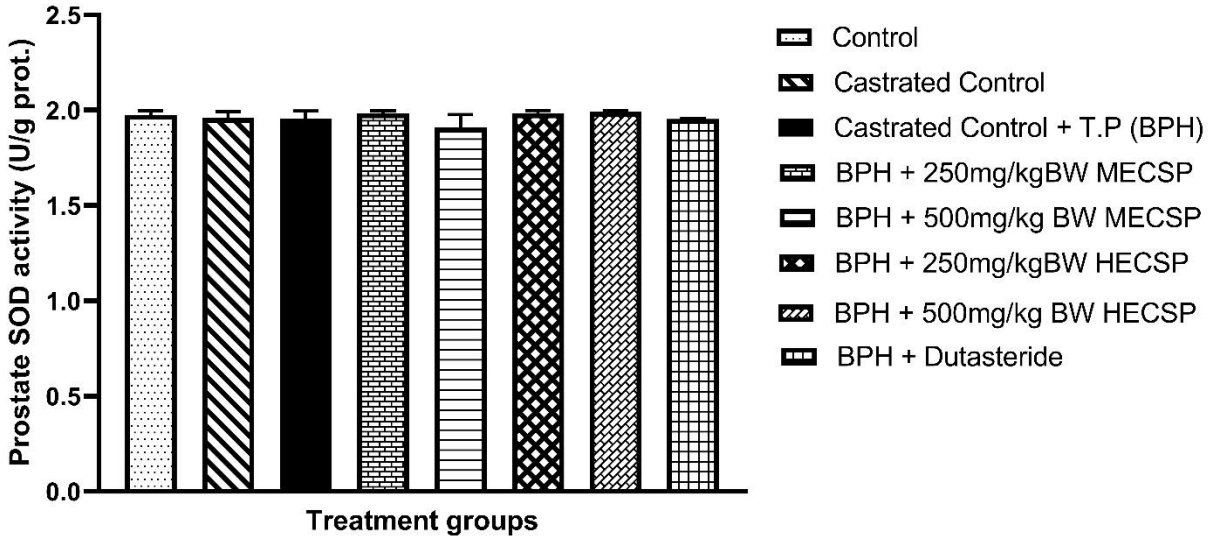

Figure 4.5: The effect of MECSP and HECSP on Prostate SOD level in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

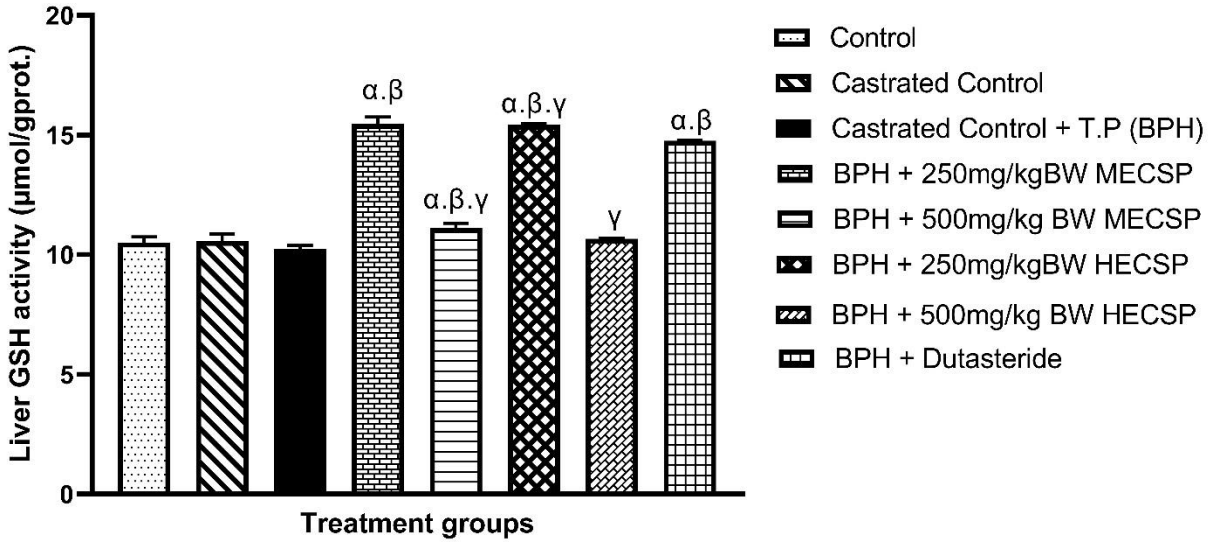

Figure 4.6: The effect of MECSP and HECSP on Liver GSH activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

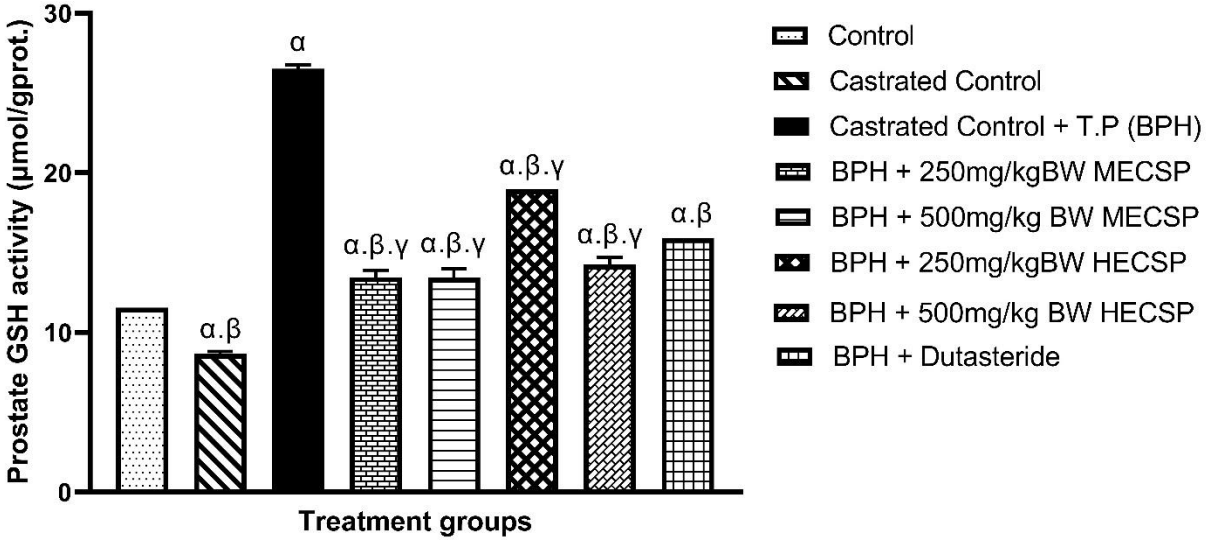

Figure 4.7: The effect of MECSP and HECSP on Prostate GSH activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

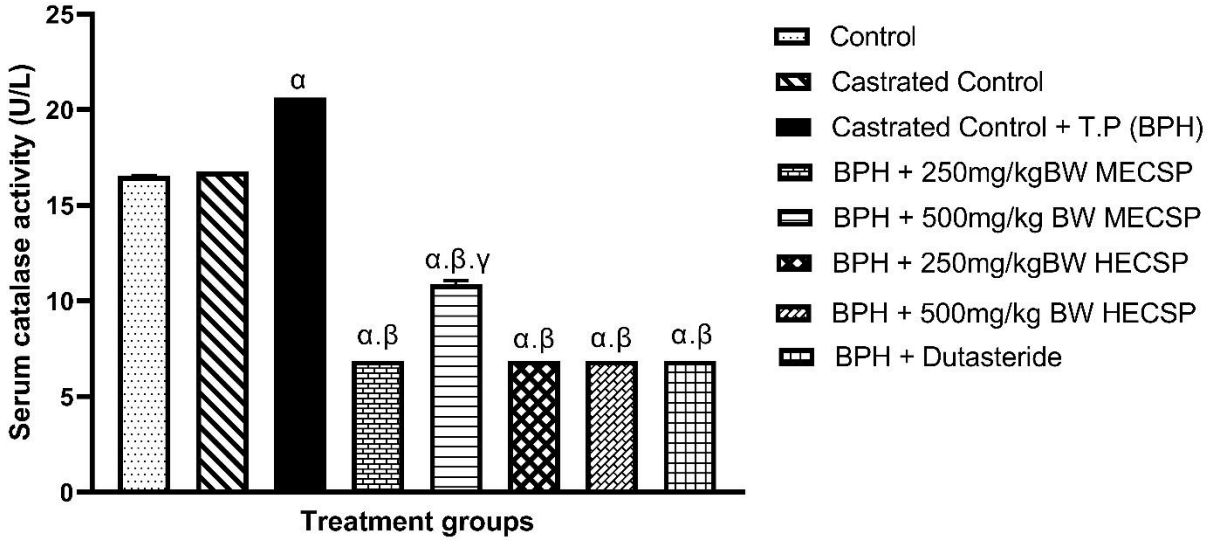

Figure 4.8: The effect of MECSP and HECSP on Serum catalase activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

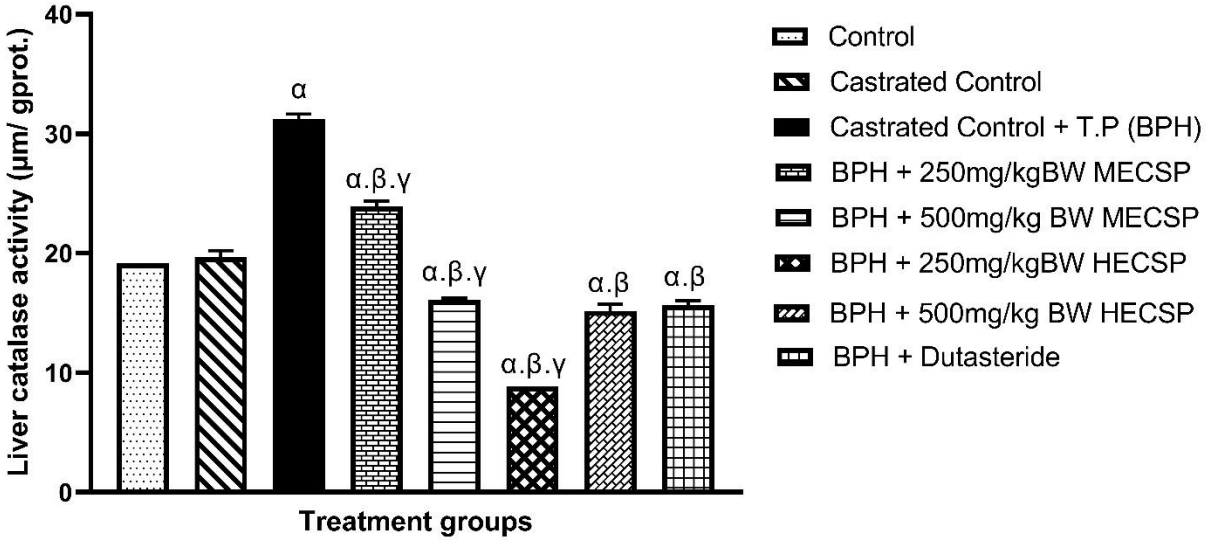

Figure 4.9: The effect of MECSP and HECSP on Liver catalase activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

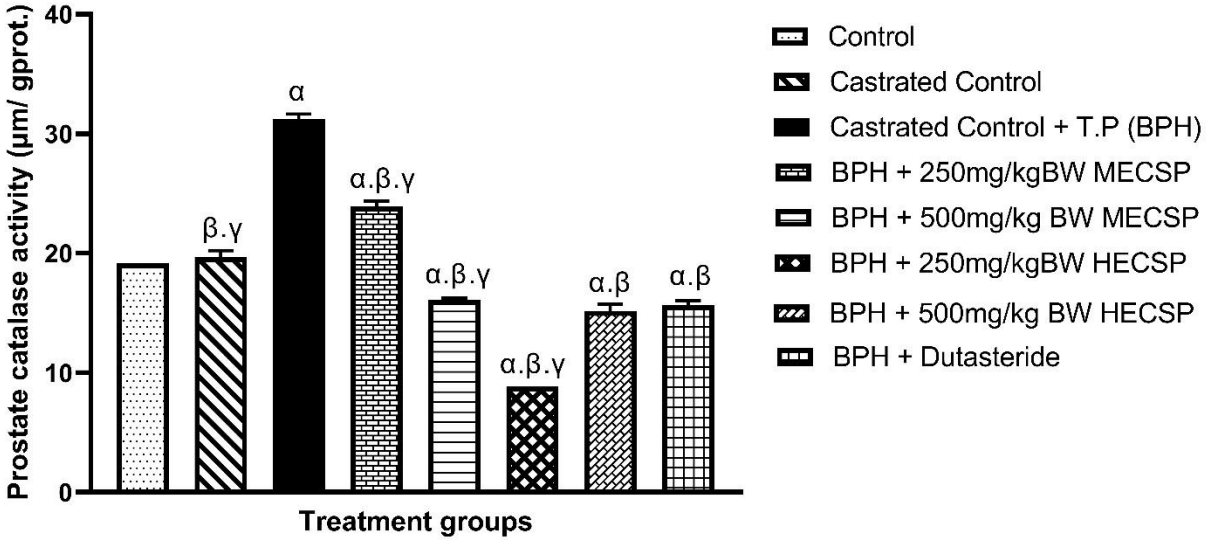

Figure 4.10: The effect of MECSP and HECSP on Prostate catalase activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

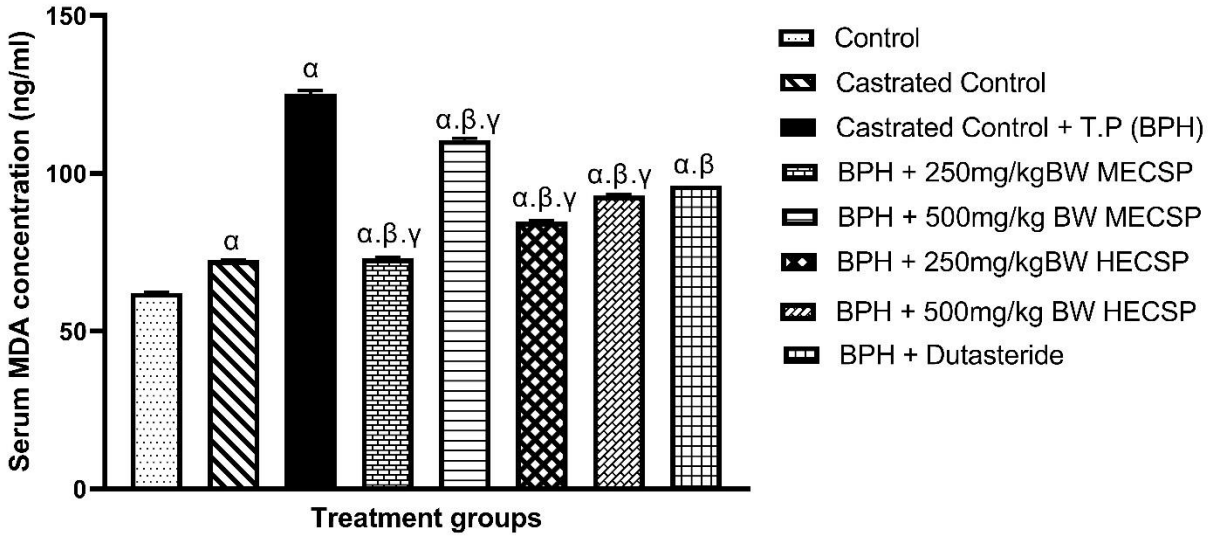

Figure 4.11: The effect of MECSP and HECSP on Serum MDA concentration in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

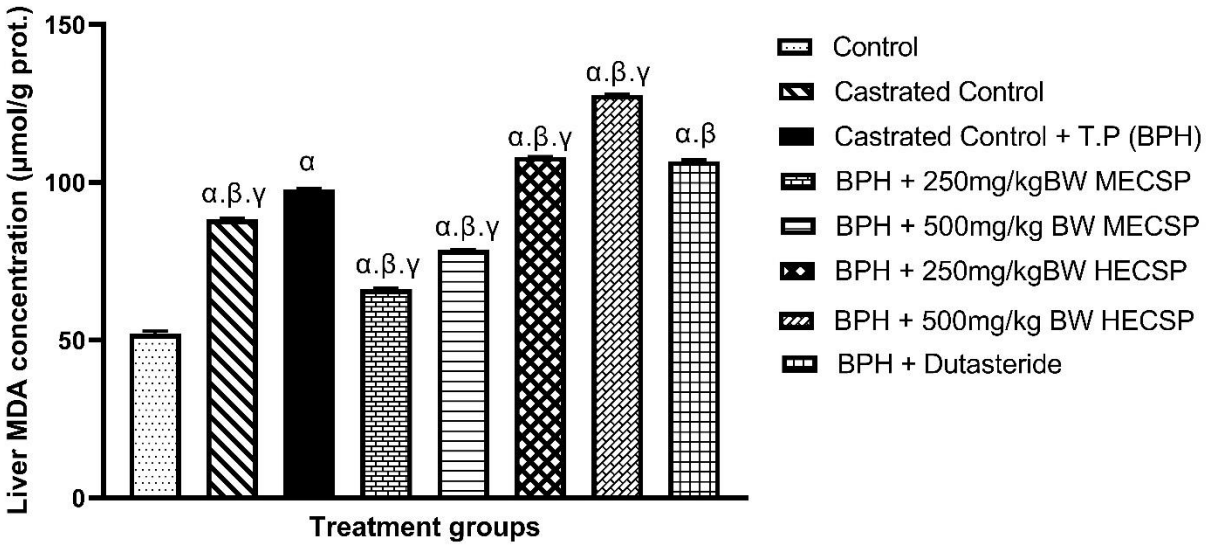

Figure 4.12: The effect of MECSP and HECSP on Liver MDA concentration in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

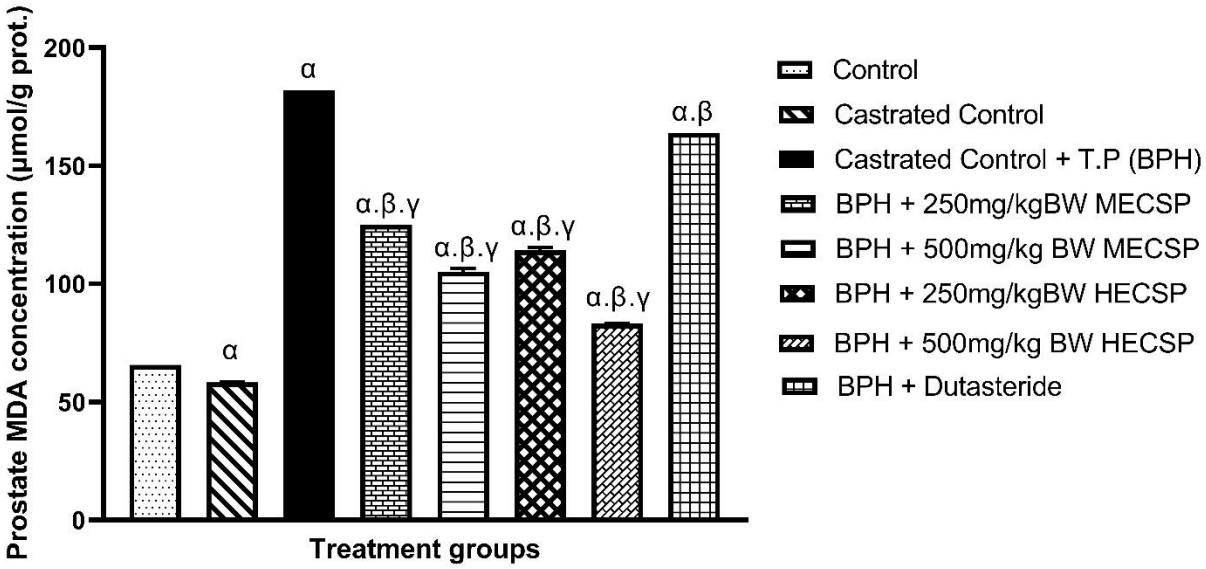

Figure 4.13: The effect of MECSP and HECSP on Prostate MDA concentration in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

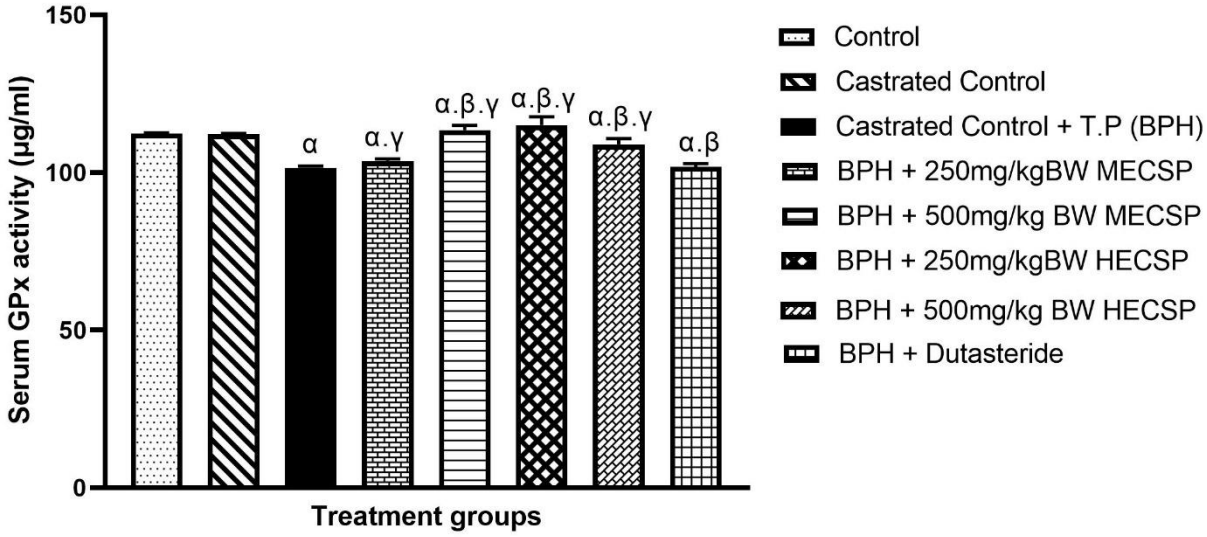

Figure 4.14: The effect of MECSP and HECSP on Serum GPx activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

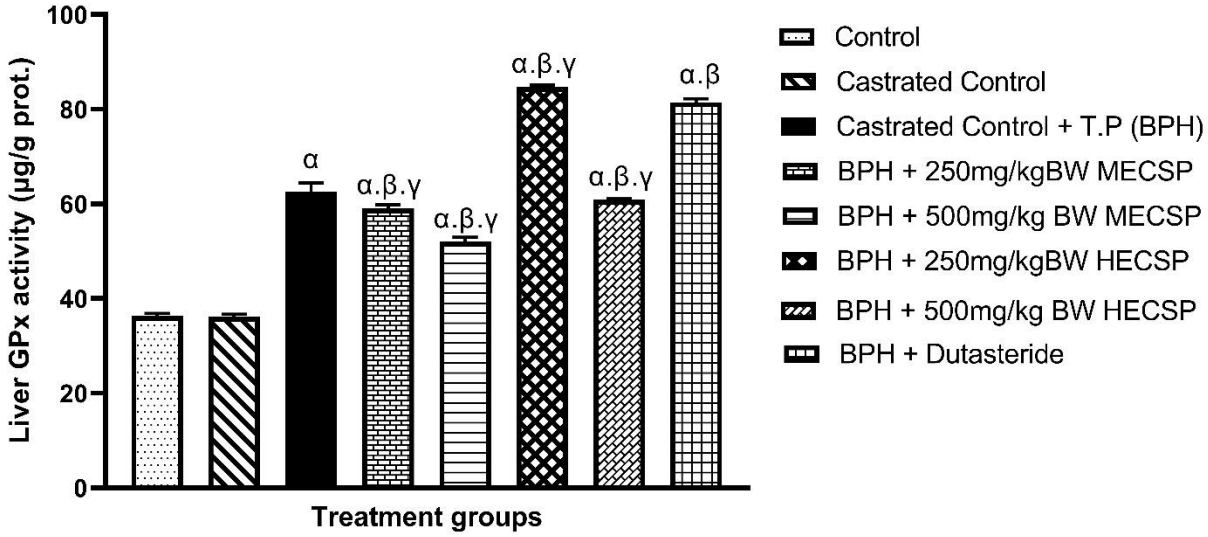

Figure 4.15: The effect of MECSP and HECSP on Liver GPx activity in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

4.5: The effects of MECSP and HECSP on Inflammatory markers in Testosterone-induced Benign prostate hyperplasia rats

PPAR-α was increased in untreated BPH rats, however BPH treated with 250mg/kg, 500mg/kg MECSP and 500mg/kg HECSP had decreased concentration when compared to untreated BPH group and the control group. Serum level of NF Kβ was slightly increased in untreated BPH group, BPH treated with 250mg/kg and 500mg/kg MESCP showed decrease, while BPH treated with 500mg/kg increased serum NF Kβ level.

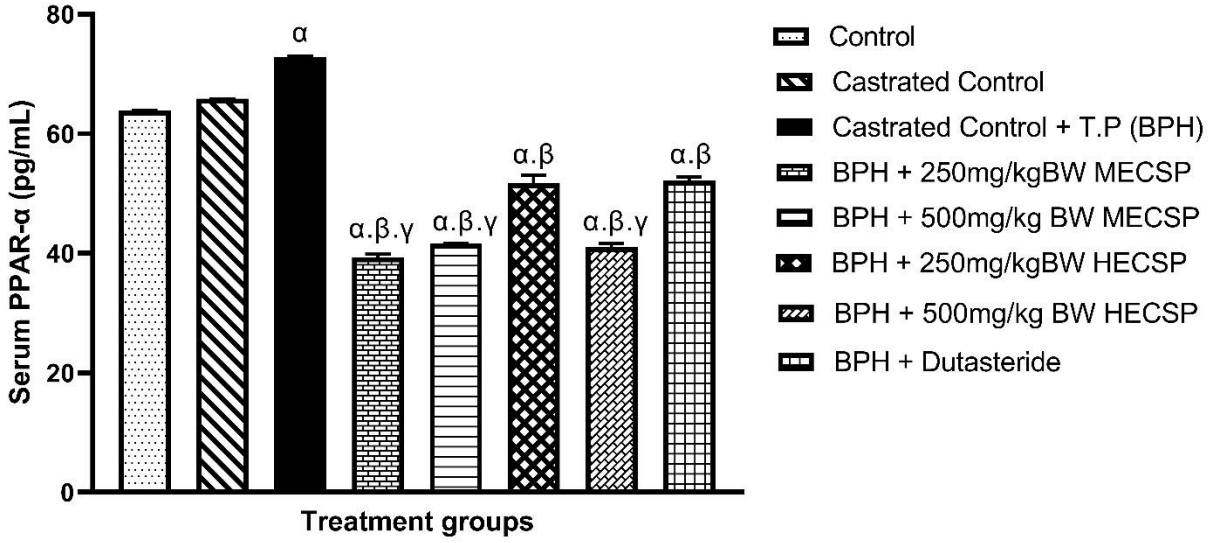

Figure 4.16: The effect of MECSP and HECSP on serum level of PPAR-α in Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

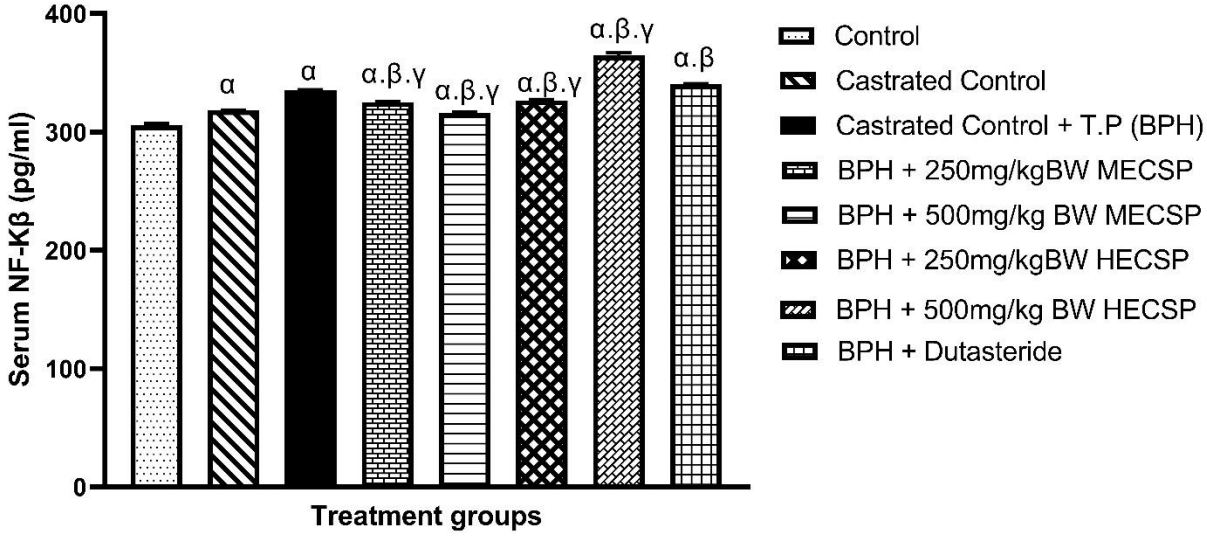

Figure 4.17: The effect of MECSP and HECSP on level of NF-Kβ in the serum of Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

4.6: The effects of MECSP and HECSP on Sex hormone concentration in Testosterone-induced Benign prostate hyperplasia rats

Serum testosterone concentration was observed to decrease in untreated BPH rats when compared with control ($p < 0.05$), the concentration of the enzyme was markedly increased when the rats were treated with MECSP and HECSP, however notable increase was shown in rats treated with 500mg/kg HECSP. Similar trend was observed in serum progesterone level with 250mg/kg and 500mg/kb HECSP showing effective increase when compared with MECSP.

Figure 4.4c shows the effect of MECSP and HECSP on luteinizing hormone (LH) concentration in the serum of testosterone-induced Benign prostate hyperplasia rats. Serum LH concentration was high in untreated BPH rats ($p<0.05$) while the concentration was more increased in the treatment groups except BPH rats treated with 500 mg/kg MECSP. Significant increase in serum estradiol concentration was observed in untreated BPH rats while the concentration was decreased in the treatment groups, with 500mg/kg HECSP showing much decrease. There was no significant variation in the concentration of follicle stimulating hormone in the serum of untreated hypertensive rats when compared to control, however, notable increased was observed in the treatment groups.

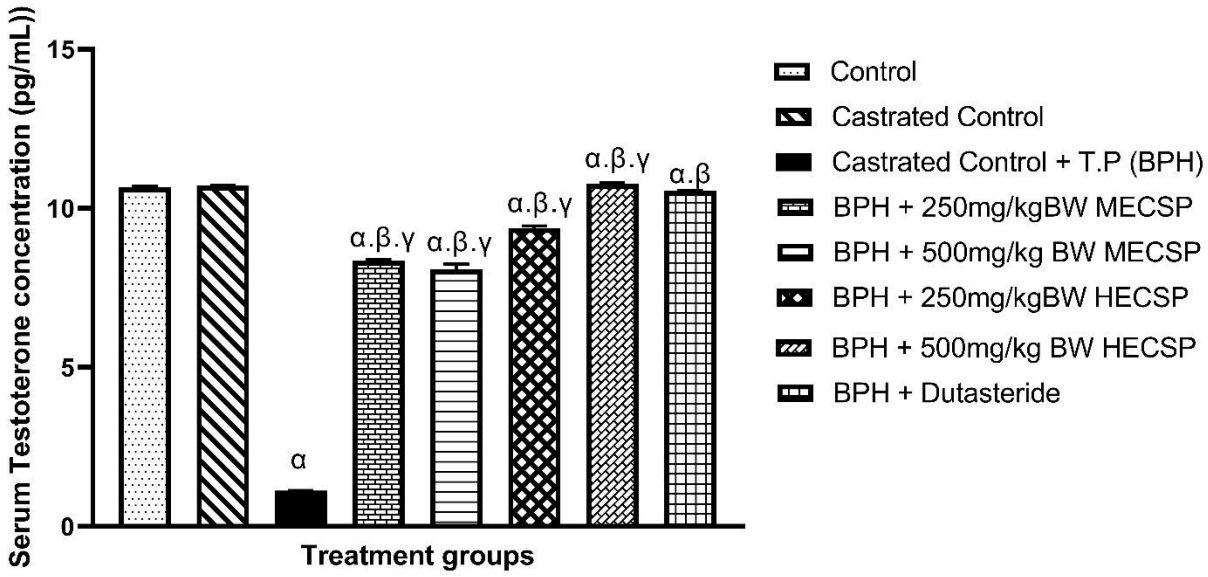

Figure 4.18: The effect of MECSP and HECSP on serum Testosterone concentration of Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

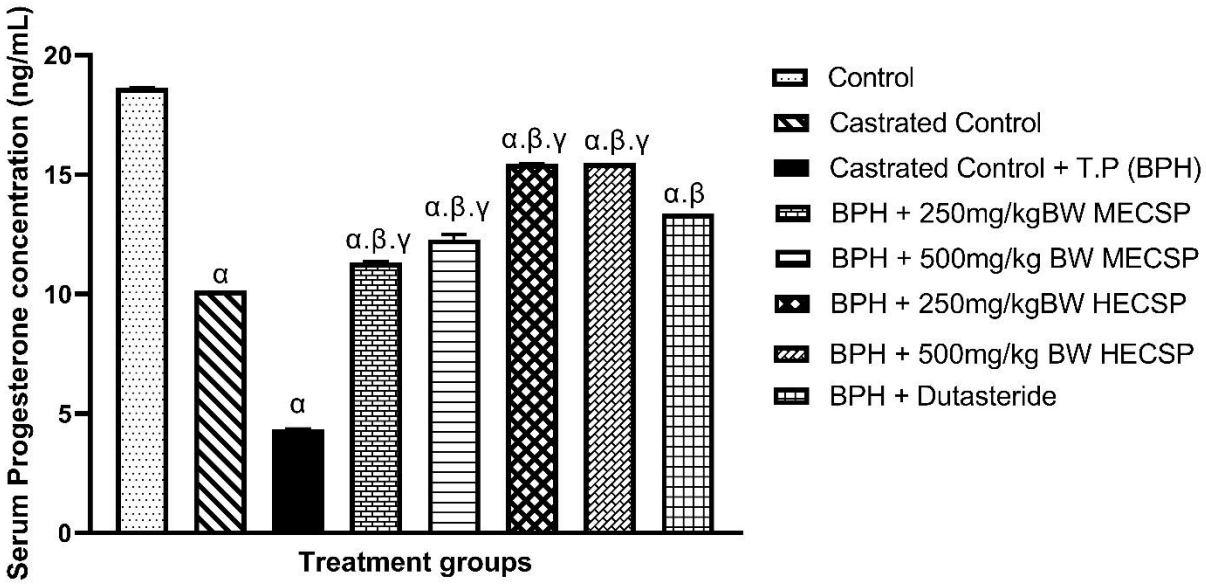

Figure 4.19: The effect of MECSP and HECSP on serum Progesterone concentration of Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

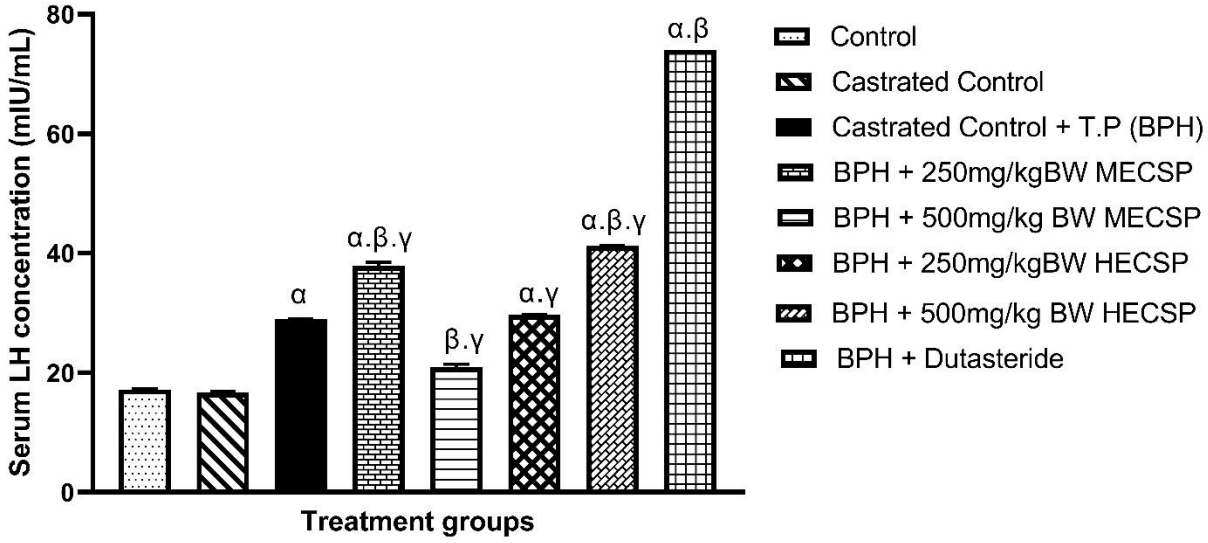

Figure 4.20: The effect of MECSP and HECSP on serum Luteinizing hormone concentration of Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

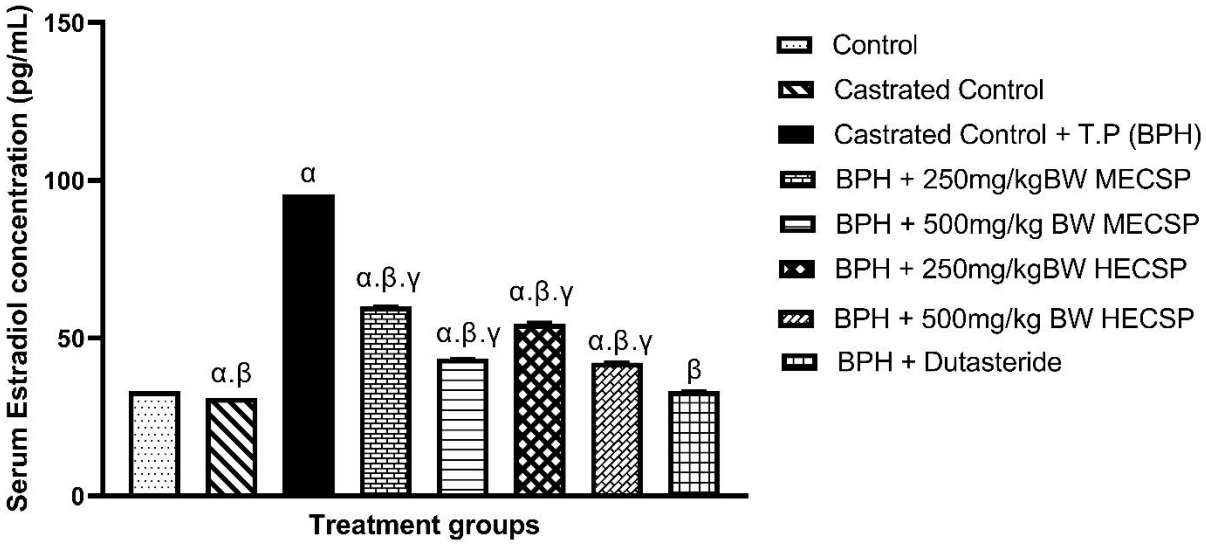

Figure 4.21: The effect of MECSP and HECSP on serum Estradiol hormone concentration of Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

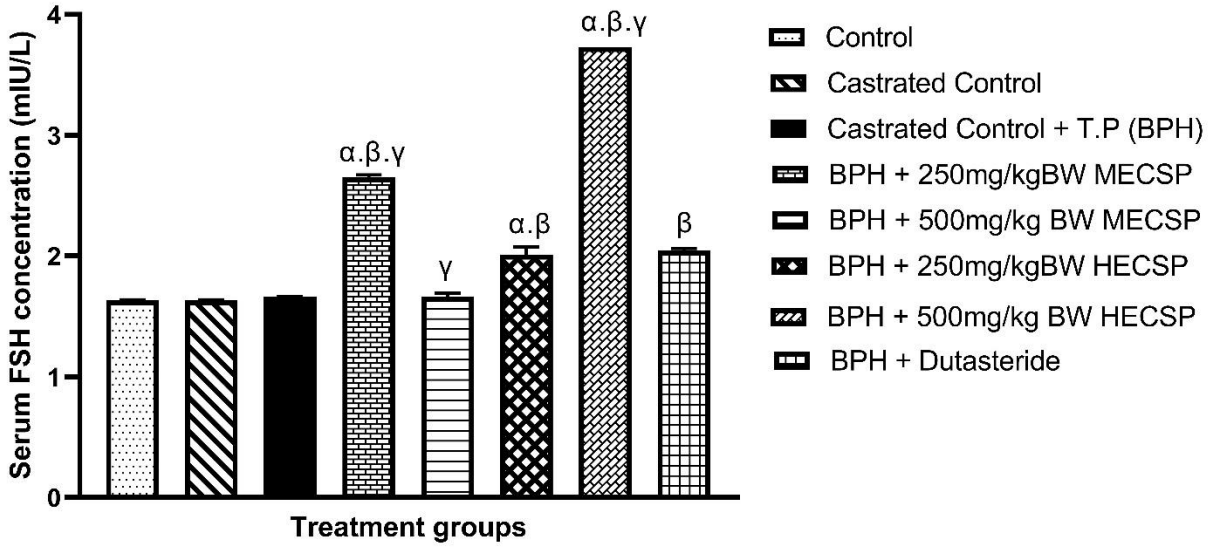

Figure 4.22: The effect of MECSP and HECSP on serum Follicle stimulating hormone concentration of Testosterone-induced Benign prostate hyperplasia rats. Bars represent mean ± standard deviation (n=6). Superscript α: parameter is significant ($p < 0.05$) when compared to control at 95% confidence level. Superscript β: parameter is significant ($p < 0.05$) when compared to untreated BPH group at 95% confidence level. Superscript γ: parameter is significant ($p < 0.05$) when compared to Dutasteride treated group at 95% confidence level.

Source: Author's Analysis, 2023

4.7: The effects of MECSP and HECSP Histopathology of the tissues of Testosterone-induced Benign prostate hyperplasia rats

The photomicrographs of the section of liver (X100 and X400) and prostate (X100 and X400) were presented in fig. 4. 23, fig. 4.24, fig. 4.25 and fig. 4.26, respectively. Disseminated microvesicular steatosis and mild disseminated infiltration of zone 2 (black arrow) were noticed in BPH rats. There was inflamed section of the prostate of BPH rats and rats treated with 250 mg/kg MECSP when related to other groups. The control groups showed no significant lesion.

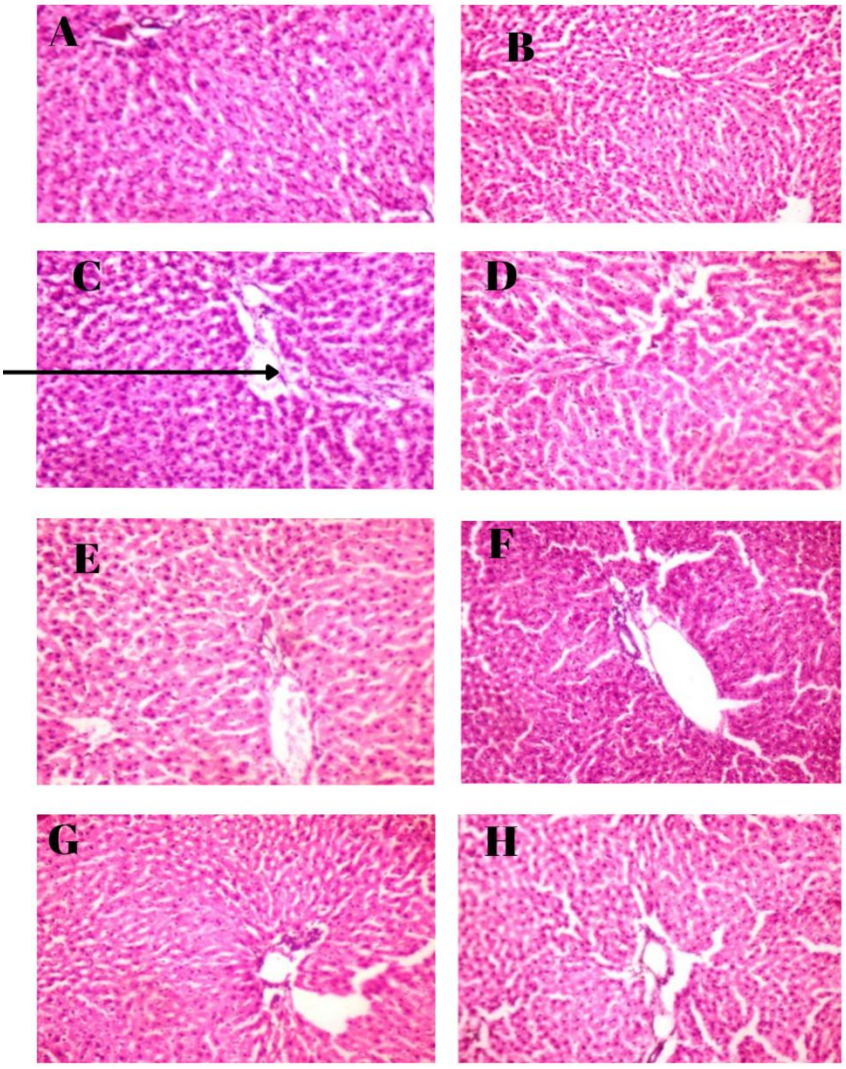

Figure 4.23: The effects of MECSP and HECSP Histopathology of the liver tissues of Testosterone-induced Benign prostate hyperplasia rats

Photomicrograph of the section of liver tissue (X100). A (control), B (castrated control), C (untreated BPH rats), D (BPH + 250mg/kg bw MECSP), E ((BPH + 500mg/kg bw MECSP), F ((BPH + 250mg/kg bw HECSP), G ((BPH + 500mg/kg bw HECSP), and H ((BPH + Dutasteride).

Source: Author's Analysis, 2023

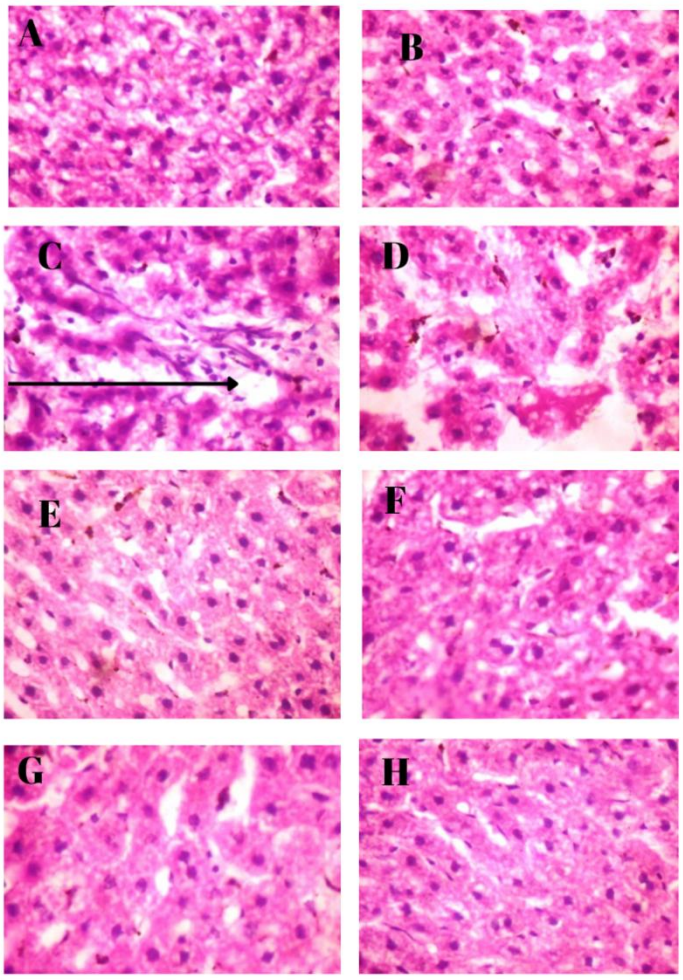

Figure 4.24: The effects of MECSP and HECSP Histopathology of the liver tissues of Testosterone-induced Benign prostate hyperplasia rats

Photomicrograph of the section of liver tissue (X400). A (control), B (castrated control), C (untreated BPH rats), D (BPH + 250mg/kg bw MECSP), E ((BPH + 500mg/kg bw MECSP), F ((BPH + 250mg/kg bw HECSP), G ((BPH + 500mg/kg bw HECSP), and H ((BPH + Dutasteride).

Source: Author's Analysis, 2023

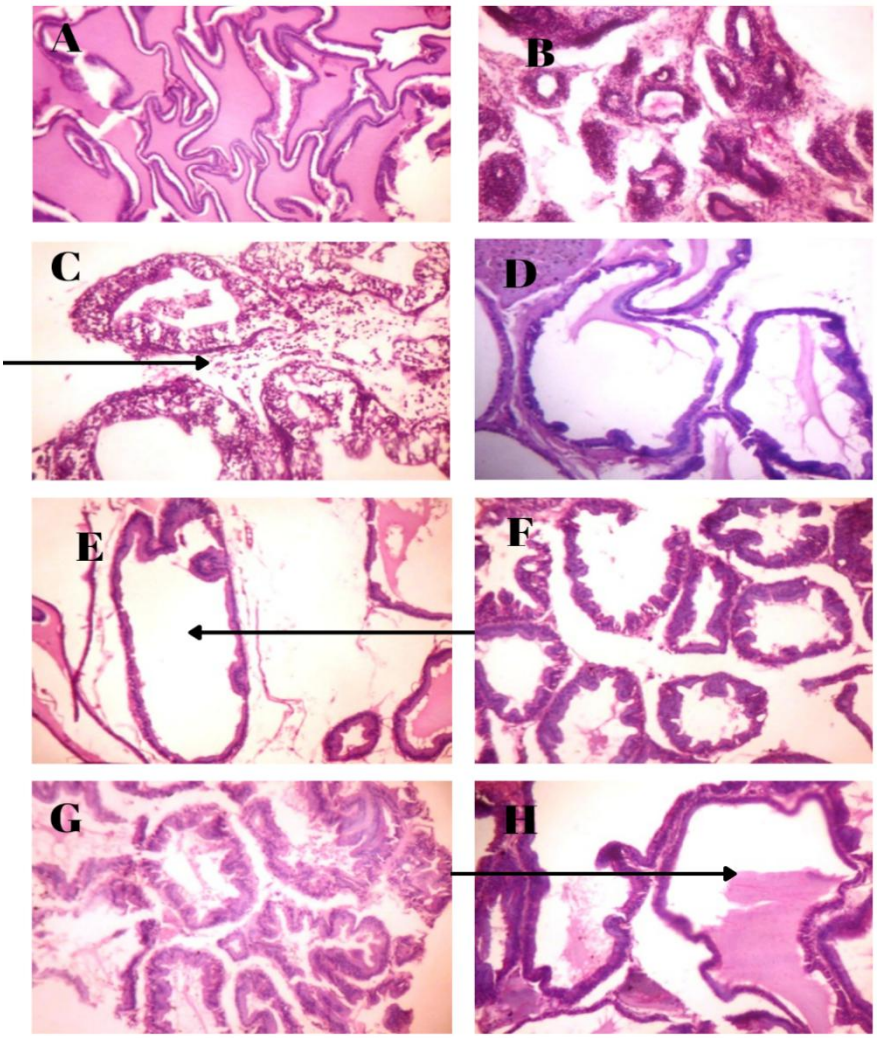

Figure 4.25: The effects of MECSP and HECSP Histopathology of the prostate tissues of Testosterone-induced Benign prostate hyperplasia rats

Photomicrograph of the section of liver tissue (X100). A (control), B (castrated control), C (untreated BPH rats), D (BPH + 250mg/kg bw MECSP), E ((BPH + 500mg/kg bw MECSP), F ((BPH + 250mg/kg bw HECSP), G ((BPH + 500mg/kg bw HECSP), and H ((BPH + Dutasteride).

Source: Author's Analysis, 2023

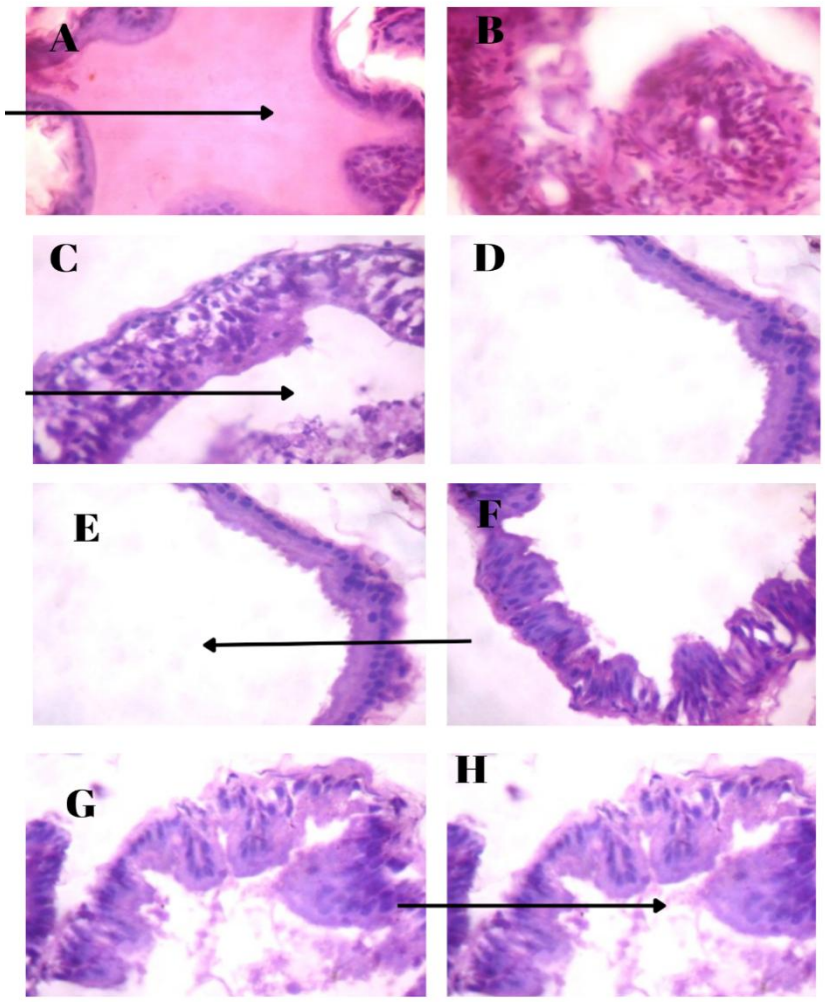

Figure 4.26: The effects of MECSP and HECSP Histopathology of the prostate tissues of Testosterone-induced Benign prostate hyperplasia rats

Photomicrograph of the section of liver tissue (X400). A (control), B (castrated control), C (untreated BPH rats), D (BPH + 250mg/kg bw MECSP), E ((BPH + 500mg/kg bw MECSP), F ((BPH + 250mg/kg bw HECSP), G ((BPH + 500mg/kg bw HECSP), and H ((BPH + Dutasteride).

Source: Author's Analysis, 2023

4.8: Gas column mass chromatography (GC-MS) Analyses of MECSP and HECSP

GC-MS analyses of MECSP and HECSP revealed the presence of forty (40) and thirty-four (34) active compounds or phytochemicals in each of the extracts as shown in Tables 4.3 and 4.4 respectively. The respective chromatograms are represented by appendix VI and VII. The structures of some of the compounds detected in MECSP and HECSP are shown in appendix VIII, IX, X and X1.

4.9: Molecular docking of bioactive compounds

The results of the molecular docking of the lead phytochemicals in the methanol extracts of *citrus sinesis* peel and n-hexane extracts of *citrus sinesis* peel against receptor targets (5α-reductase (7WI), prostate specific membrane antigen (PSMA) involved in progression of BPH are shown in Tables 4.5 and 4.6. The bioactive constituents of the extracts showed effective interactions with the receptor target used in the study and in most cases, exhibited stronger binding affinity compared to the standard antihyperplasia used, dutasteride and finasteride. The dimensional structures showing the binding interactions of the lead phytochemicals with the receptor target are represented by figures 4.28 to 4.40.

Table 4.7 shows that 2 Cholestan-7-one, cyclic 1,2-ethanediyl acetal, (5.alpha.), 4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-, 4H-1-Benzopyran-4-one, 2,3-dihydro-5,6,7-trimethoxy-2-(4-methoxyphenyl)-, Vitamin E, 4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-2,3-dihydro-3-hydroxy-5,7-dimethoxy-3-phenyl- are the lead compounds in MECSP.

Table 4.8 shows that 4H-1-Benzopyran-4-one,2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-,Alantolactone, 4.alpha.,4A.alpha.-epoxy-, 4-(2-Furamido)phenyl 2-furoate, 4h-1-benzopyran-4-

one, 2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxy-, Bis(2-ethylhexyl) phthalate, Dibutyl phthalate and Phytol are the lead compound for HECSP.

Table 4.9 shows the drugability profiles of the lead compounds for MECSP and HECSP.

Table 4.3: Bioactive compounds detected in Methanol Extracts of *Citrus Sinesis* Peel through Gas column mass chromatography (GC-MS)

PK	RT	% Abundance	Bioactive Compounds
1	8.190	16.38	Glycerin
2	9.970	5.17	2,4-Cycloheptadien-1-one, 2,6,6-trimethyl-
3	10.823	21.16	Benzofuran, 2,3-dihydro-
4	11.085	9.51	2-Methoxy-4-vinylphenol
5	11.327	9.91	1,2-Cyclohexanediol, 1-methyl-4-(1-methyleth
6	13.145	5.93	15-Hexadecenoic acid, 14-hydroxy-15-methyl
7	13.260	3.09	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dime
8	13.648	4.15	3',5'-Dimethoxyacetophenone
9	13.690	9.17	9-Octadecenoic acid, 12-hydroxy-
10	14.208	8.35	Megastigmatrienone
11	14.676	26.40	Ethyl. alpha.-d-glucopyranoside
12	15.291	21.02	3,4-Altrosan
13	16.624	2.35	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien
14	16.749	3.80	Hexadecanoic acid, methyl ester
15	17.128	5.49	n-Hexadecanoic acid
16	17.383	3.74	Pentacontanoic acid, ethyl ester
17	17.492	2.98	1-Nonadecene
18	18.067	10.19	Cyclopentanecarboxylic acid, pentyl ester
19	18.307	1.86	12,15-Octadecadienoic acid, methyl ester

20	18.373	2.34	11-Octadecenoic acid, methyl ester
21	18.558	1.06	Phytol
22	18.692	2.82	9,12-Octadecadienoic acid (Z,Z)-
23	18.809	9.82	2,2',2"-Nitrilotriethanol, triethyl ether
24	18.975	4.63	Undecanoic acid, 10-bromo-
25	19.329	3.20	1-Docosene
26	21.324	5.27	Vitamin E
27	21.504	8.87	4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyph
28	21.742	3.53	4H-1-Benzopyran-4-one, 5,6,7-trimethoxy-2-(
29	21.874	12.88	Floxuridine
30	22.124	3.45	Bis(2-ethylhexyl) phthalate
31	22.200	6.17	3-N-Nitroso-solanocapsine
32	22.342	7.26	cis-9-Hexadecenoic acid, trimethylsilyl ester
33	22.472	3.72	Hexadecanoic acid, tetradecyl ester
34	22.733	7.58	Cholestan-7-one, cyclic 1,2-ethanediyl acetal,
35	23.666	2.16	Succinic acid, 3,7-dimethyloct-6-en-1-yl nonyl
36	23.726	2.00	2,6-Octadien-1-ol, 3,7-dimethyl-, propanoate,
37	23.949	3.84	Cyclohexane, 3,4-bis(1-methylethenyl)-1,1-di
38	24.462	7.26	Silane, dimethyl(dimethylpentyloxysilyloxy)te
40	24.983	7.79	4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-

Table 4.4: Bioactive compounds detected in n-Hexane Extracts of *Citrus Sinesis* Peel through Gas column mass chromatography (GC-MS)

PK	RT	% Abundance	Bioactive Compounds
1	5.508	4.39	4-(2-Furamido)phenyl 2-furoate
2	5.944	10.68	2-Furanmethanol
3	6.700	11.89	1H-Pyrazole, 3-ethoxy-5-methyl-
4	6.981	9.87	2-Furancarboxaldehyde, 5-methyl-
5	7.150	6.76	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-on
6	8.324	11.61	5,6-Dihydro-5-methyluracil
7	9.334	11.63	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6
8	9.849	7.28	Thiophene, 2,3-dihydro-
9	10.018	15.61	Furazan-3-carboximide acid hydrazide, 4-amin
10	10.353	13.01	5-Hydroxymethylfurfural
11	10.875	16.53	Cyclohexanone, 2-ethyl-4-methoxy-
12	11.042	8.27	N,N,N'-Trimethyl-1,4-phenylenediamine
13	11.268	7.22	Pentanoic acid, 2,2-dimethyl-, 2-hydroxyethyl
14	11.476	6.42	Pyrazine, 3,5-dimethyl-2-propyl-
15	13.449	28.09	d-Glycero-d-galacto-heptose
16	14.304	5.17	Propylamine, N-[9-borabicyclo[3.3.1]non-9-yl
17	14.855	11.25	Ethyl .alpha.-d-glucopyranoside
18	16.209	38.27	3-O-Methyl-d-glucose
19	16.958	2.50	Dibutyl phthalate

20	17.130	3.09	n-Hexadecanoic acid
21	17.374	2.00	Hexadecanoic acid, ethyl ester
22	18.554	1.98	Phytol
23	18.691	2.47	9,12-Octadecadienoic acid (Z,Z)-
24	18.746	4.31	Oleic Acid
25	18.906	2.24	Ethyl 9.cis.,11.trans.-octadecadienoate
26	18.971	3.14	(E)-9-Octadecenoic acid ethyl ester
27	20.624	1.97	erythro-9,10-Dibromopentacosane
28	20.679	3.55	9-Octadecenamide, (Z)-
29	21.574	11.03	4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphe
30	22.122	1.94	Bis(2-ethylhexyl) phthalate
31	23.290	6.12	Alantolactone, 4.alpha.,4A.alpha.-epoxy-
32	24.469	6.38	Silane, dimethyl(dimethylpentyloxysilyloxy)te
33	24.617	5.71	2-(5,7-Di-tert-butyl-benzo[1,3]oxathiol-2-ylid
34	24.946	5.36	4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol

Table 4.5: Molecular docking scores of the phytochemicals from MECSP against selected receptors involved in some BPH pathways

Ligands	Canonical smile
Dutasteride (Standard Drug)	CC12CCC3C(C1CCC2C(=O)NC4=C(C=CC(=C4)C(F)(F)F
Glycerin	C(C(CO)O)O
2,4-Cycloheptadien-1-one, 2,6,6-trimethyl-	CC1=CC=CC(CC1=O)(C)C
Benzofuran, 2,3-dihydro-	C1COC2=CC=CC=C21
2-Methoxy-4-vinylphenol	COC1=C(C=CC(=C1)C=C)O
1,2-cyclohexanediol, 1-methyl-4-(1-methylethyl)-	CC(C)C1CCC(C(C1)O)(C)O
15-Hexadecenoic acid, 14-hydroxy-15-methyl	CC(=C)C(CCCCCCCCCCCCC(=O)O)O
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	CC1=CC2C(CCC(=C2CC1)C)C(C)C
3',5'-Dimethoxyacetophenone	CC(=O)C1=CC(=CC(=C1)OC)OC
9-Octadecenoic acid, 12-hydroxy-	CCCCCCC(CC=CCCCCCCCC(=O)O)O
Megastigmatrienone	CC1=CC(=O)CC(C1C=CC=C)(C)C
Ethyl alpha-d-glucopyranoside	CCOC1C(C(C(C(O1)CO)O)O)O
3,4-Altrosan	C(C1C2C(O2)C(C(O1)O)O)O
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	CC(C)(C)C1=CC2(CCC(=O)O2)C=C(C1=O)C(C)(C)C
Hexadecanoic acid, methyl ester	CCCCCCCCCCCCCCCC(=O)OC

n-Hexadecanoic acid	CCCCCCCCCCCCCCCC(=O)O
Pentacontanoic acid, ethyl ester	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
1-Nonadecene	CCCCCCCCCCCCCCCCCCC=C
Cyclopentanecarboxylic acid, pentyl ester	CCCCCOC(=O)C1CCCC1
12,15-Octadecadienoic acid, methyl ester	CCC=CCC=CCCCCCCCCCCC(=O)OC
11-Octadecenoic acid, methyl ester	CCCCCCC=CCCCCCCCCCC(=O)OC
Phytol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C
9,12-Octadecadienoic acid (Z,Z)-	CCCCCC=CCC=CCCCCCCCC(=O)O
2,2',2"-Nitrilotriethanol, triethyl ether	CCOCCN(CCOCC)CCOCC
Undecanoic acid, 10-bromo-	CC(CCCCCCCCC(=O)O)Br
1-Docosene	CCCCCCCCCCCCCCCCCCCCCC=C
Vitamin E	CC1=C(C2=C(CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C
4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-2,3-dihydro-3-hydroxy-5,7-dimethoxy-3-phenyl-	COC1=C(C=C(C=C1)C2C(C(=O)C3=C(O2)C=C(C=C3OC
4H-1-Benzopyran-4-one, 2,3-dihydro-5,6,7-trimethoxy-2-(4-methoxyphenyl)-	COC1=CC=C(C=C1)C2CC(=O)C3=C(C(=C(C=C3O2)OC)
Floxuridine	C1C(C(OC1N2C=C(C(=O)NC2=O)F)CO)O
Bis(2-ethylhexyl) phthalate	CCCCC(CC)COC(=O)C1=CC=CC=C1C(=O)OCC(CC)CC
3-N-Nitroso-solanocapsine	CC1CC2(C(C(C3C(O2)CC4C3(CCC5C4CCC6C5(CCC(C6
(9E)-9-hexadecenoic acid, trimethylsilyl ester	CCCCCCC=CCCCCCCCC(=O)O[Si](C)(C)C
Hexadecanoic acid, tetradecyl ester	CCCCCCCCCCCCCCCC(=O)OCCCCCCCCCCCCCC
Cholestan-7-one, cyclic 1,2-ethanediyl acetal,	CC(C)CCCC(C)C1CCC2C1(CCC3C2C4(CC5C3(CCCC5)(

(5.alpha.)-	
succinic acid, 3,7-dimethyloct-6-en-1-yl nonyl ester	CCCCCCCCCCOC(=O)CCC(=O)OCCC(C)CCC=C(C)C
2,6-octadien-1-ol, 3,7-dimethyl-, propanoate	CCC(=O)OCC=C(C)CCC=C(C)C
cyclohexane, 3,4-bis(1-methylethenyl)-1,1-dimethyl-	CC(=C)C1CCC(CC1C(=C)C)(C)C
Silane, dimethyl(dimethylpentyloxysilyloxy)tetradecyloxy-	CCCCCCCCCCCCCCO[Si](C)(C)O[Si](C)(C)OCCCCC
4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-	COC1=CC2=C(C(=C1)OC)C(=O)C=C(O2)C3=CC4=C(C=

Table 4.6: Molecular docking scores of the phytochemicals from HECSP against selected receptors involved in some BPH pathways

Ligands	Canonical smile
Dutasteride (Standard Drug)	CC12CCC3C(C1CCC2C(=O)NC4=C(C=CC(=C4)C(F)(F)F
4-(2-Furamido)phenyl 2-furoate	C1=COC(=C1)C(=O)NC2=CC=C(C=C2)OC(=O)C3=CC=
2-Furanmethanol	C1=COC(=C1)CO
1H-Pyrazole, 3-ethoxy-5-methyl-	CCOC1=NNC(=C1)C
2-Furancarboxaldehyde, 5-methyl-	CC1=CC=C(O1)C=O
2,4-dihydroxy-2,5-dimethyl-3(2h)-furan-3-one	CC1=C(C(=O)C(O1)(C)O)O
5,6-Dihydro-5-methyluracil	CC1CNC(=O)NC1=O
4h-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	CC1=C(C(=O)C(CO1)O)O
Thiophene, 2,3-dihydro-	C1CSC=C1
4-Amino-1,2,5-oxadiazole-3-carbohydrazonamide	C1(=NON=C1N)C(=NN)N

5-Hydroxymethylfurfural	C1=C(OC(=C1)C=O)CO
Cyclohexanone, 2-ethyl-4-methoxy-	CCC1CC(CCC1=O)OC
N,N,N'-Trimethyl-1,4-phenylenediamine	CNC1=CC=C(C=C1)N(C)C
pentanoic acid, 2,2-dimethyl-, 2-hydroxyethyl ester	CCCC(C)(C)C(=O)OCCO
Pyrazine, 3,5-dimethyl-2-propyl-	CCCC1=NC=C(N=C1C)C
d-Glycero-d-galacto-heptose	C(C(C(C(C(C(C(C=O)O)O)O)O)O)O)O
Propylamine, N-[9-borabicyclo[3.3.1]non-9-yl	B1(C2CCCC1CCC2)NCCC
Ethyl .alpha.-d-glucopyranoside	CCOC1C(C(C(C(O1)CO)O)O)O
3-O-Methyl-d-glucose	COC(C(C=O)O)C(C(CO)O)O
Dibutyl phthalate	CCCCOC(=O)C1=CC=CC=C1C(=O)OCCCC
n-Hexadecanoic acid	CCCCCCCCCCCCCCCC(=O)O
Hexadecanoic acid, ethyl ester	CCCCCCCCCCCCCCCC(=O)OCC
Phytol	CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C
9,12-Octadecadienoic acid (Z,Z)-	CCCCCC=CCC=CCCCCCCCC(=O)O

Oleic Acid	CCCCCCCCC=CCCCCCCCC(=O)O
Ethyl 9.cis.,11.trans.-octadecadienoate	CCCCCCC=CC=CCCCCCCCC(=O)OCC
(E)-9-Octadecenoic acid ethyl ester	CCCCCCCCC=CCCCCCCCC(=O)OCC
erythro-9,10-Dibromopentacosane	CCCCCCCCCCCCCCCCC(C(CCCCCCCC)Br)Br
9-Octadecenamide, (Z)-	CCCCCCCCC=CCCCCCCCC(=O)N
4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxy-	COC1=C(C=C(C=C1)C2=C(C(=O)C3=C(O2)C=C(C=C3O
Bis(2-ethylhexyl) phthalate	CCCCC(CC)COC(=O)C1=CC=CC=C1C(=O)OCC(CC)CC
Alantolactone, 4.alpha.,4A.alpha.-epoxy-	CC1CCCC2(C13C(O3)C4C(C2)OC(=O)C4=C)C
Silane, dimethyl(dimethylpentyloxysilyloxy)tetradecyloxy-	CCCCCCCCCCCCCCO[Si](C)(C)O[Si](C)(C)OCCCCC
2-(5,7-Di-tert-butyl-benzo[1,3]oxathiol-2-ylid	COC1=CC2=C(C(=C1)OC)C(=O)C=C(O2)C3=CC4=C(C=
4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-	

Table 4.7: Potential Lead phytochemicals in MESCP

Ligands	Binding Affinity (K/cal) 5-αR	Binding Affinity (K/cal) PSMA
Cholestan-7-one, cyclic 1,2-ethanediyl acetal, (5.alpha.)-	-10.2	-6.8
4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-	-9.4	-5.9
4H-1-Benzopyran-4-one, 2,3-dihydro-5,6,7-trimethoxy-2-(4-methoxyphenyl)-	-9.1	-8.0
Vitamin E	-8.9	-7.8
4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-2,3-dihydro-3-hydroxy-5,7-dimethoxy-3-phenyl-	-8.8	-5.9
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	-8.6	-7.8
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	-8.1	-7.6
Floxuridine	-7.5	-7.9
cyclohexane, 3,4-bis(1-methylethenyl)-1,1-dimethyl-	-7.4	-7.1
Megastigmatrienone	-7.3	-6.5
succinic acid, 3,7-dimethyloct-6-en-1-yl nonyl ester	-7.1	-7.5
Phytol	-7.1	-7.2

Table 4.8: Potential Lead phytochemicals in HESCP

Ligands	Binding Affinity (K/cal) 5-αR	Binding Affinity (K/cal) PSMA
4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-	-9.2	-9.4
Alantolactone, 4.alpha.,4A.alpha.-epoxy-	-8.9	-8.8
4-(2-Furamido)phenyl 2-furoate	-8.4	-9.2
4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxy-	-8.3	-7.7
Bis(2-ethylhexyl) phthalate	-8.1	-7.9
Dibutyl phthalate	-7.2	-6.9
Phytol	-7.2	-7.0

Table 4.9: Drugability Profiles of Phytochemicals from MECSP and HECSP

Ligands	Synonym	Extracts	Molecular Weight	Hydrogen Bound Acceptor	Hydrogen Bound Donor	Lipophilicity	Viola Sco
Cholestan-7-one, cyclic 1,2-ethanediyl acetal, (5.alpha.)-	ATYGMLMJIQNUHX-UHFFFAOYSA-N	MECSP	430.717	2	0	7.8508	1
4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-	55320-05-3	MECSP	436.46	7	1	3.9252	0
4H-1-Benzopyran-4-one, 2,3-dihydro-5,6,7-trimethoxy-2-(4-methoxyphenyl)-	2569-77-9	MECSP	344.363	6	0	3.4275	0
Vitamin E	alpha-Tocopherol	MECSP	430.717	2	1	8.84026	1
4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-2,3-dihydro-3-hydroxy-5,7-dimethoxy-3-phenyl-	89029-12-9	MECSP	326.304	6	0	3.2059	0
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	82304-66-3	MECSP	276.376	3	0	3.5899	0
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-	Cadina-1(10),4-diene	MECSP	204.357	0	0	4.7252	0

dimethyl-1-(1-methylethyl)-, (1S-cis)-							
Floxuridine	floxuridine	MECSP	246.194	7	3	-1.6836	0
cyclohexane, 3,4-bis(1-methylethenyl)-1,1-dimethyl-	Cyclohexane, 3,4-bis(1-methylethenyl)-1,1-dimethyl-	MECSP	192.346	0	0	4.5811	0
Megastigmatrienone	Megastigmatrienone	MECSP	190.286	1	0	3.2901	0
succinic acid, 3,7-dimethyloct-6-en-1-yl nonyl ester	YCHMPZZWGODQJG-UHFFFAOYSA-N	MECSP	382.585	4	0	6.3762	1
Phytol	Phytol	MECSP	296.539	1	1	6.3641	1
4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-	Quercetin pentamethyl ether	HECSP	372.373	7	0	3.503	0
Alantolactone, 4.alpha.,4A.alpha.-epoxy-	NSC250681	HECSP	248.322	3	0	2.4518	0
4-(2-Furamido)phenyl 2-furoate	4-(2-Furoylamino)phenyl 2-furoate	HECSP	297.266	6	1	3.3441	0
4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxy-	89029-12-9	HECSP	326.304	6	0	3.2059	0
Bis(2-ethylhexyl) phthalate	Bis(2-ethylhexyl) phthalate	HECSP	390.564	4	0	6.433	1

Dibutyl phthalate	dibutyl phthalate	HECSP	278.348	4	0	3.6004	0
Phytol	Phytol	HECSP	296.539	1	1	6.3641	1

4.10 Discussion

Natural products have proven to be valuable resources for discovering of new drugs[1,2,3]. Citrus fruits contain myriad of phytochemicals with medicinal uses. Benign prostatic hyperplasia is a condition that occurs when the cell count of the stromal and epithelial cells within the prostate gland's transition zone increases causing the narrowing of the urethra and the inability to fully empty the bladder[4].

The relative prostate weight is an important marker for monitoring the progression of BPH. Previous studies haves observed an elevated relative prostate weight in animals with BPH[2]. Treatment with Dutasteride or other agents used for BPH management has been shown to decrease the relative prostate weight[5]. In this study, animals with BPH exhibited a higher relative prostate weight when compared to the control groups (normal control and castrated group) ($p<0.05$). Conversely, treatment with 250 mg/kg bwt of MECSP, 500mg/kg bwt of MECSP, 250mg/kg bwt of HECSP, 500mg/kg bwt of MECSP and Dutasteride significantly reduced ($p<0.05$) relative prostate weight. Treatment with 500 mg/kg bwt of MECSP had the highest reduction in weight, 0.13 ±0.03 which was near the value for Dutasteride 0.11 ±0.01 (Table 4.1).

Androgens are essential for the development of prostate growth. Among the androgens, dihydrotestosterone (DHT) is the most important enzyme. DHT is converted from testosterone in the prostate by 5 α -reductases, which are enzymes involved in steroid metabolism, and it interacts with the androgen receptor (AR) with a higher affinity than testosterone. 5 α –reductase is located primarily in the intracellular membrane in prostatic stromal and basal cells. In this study, 5α-reductase activity was increased in BPH rats,

subsequently treatment with 250 mg/kg bwt of MECSP, 500mg/kg bwt of MECSP, 250mg/kg bwt of HECSP, 500mg/kg bwt of MECSP and Dutasteride reduced the activity. Treatment with 500 mg/kg bwt of HECSP had the highest reduction (Figure 4.2).

The activities of ALT and AST in the serum of BPH rats exhibited a significant increase when compared to the control and castrated rats. An increase in the activities of AST and ALT in BPH rats indicates liver dysfunction or damage. AST and ALT are enzymes primarily found in liver cells, and their elevated activities in the bloodstream are commonly used as markers of liver injury. Therefore, an increase in AST and ALT activities suggests that BPH may have an impact on liver function or cause liver damage in rats. This result corroborates with the findings of R.I. Uroko & F.A. Adamude[5,6], that the increased levels of ALT and AST in the BPH rats suggest that their liver functions are compromised, potentially impacting the normal processing of androgenic hormones like Testosterone and DHT, which play a role in the development of BPH. Additionally, aside from affecting the metabolism of androgenic hormones, impaired liver function could hinder other essential liver processes such as synthesizing large molecules, transforming foreign substances, and eliminating waste products[5]. However, treatment with 250 mg/kg bwt of MECSP, 500mg/kg bwt of MECSP, 250mg/kg bwt of HECSP, 500mg/kg bwt of MECSP and Dutasteride significantly reduced ($p<0.05$) AST and ALT activities as shown in figure 4.4 and 4.5

Oxidative stress plays a crucial role in the development and progression of BPH, leading to increased susceptibility to DNA damage in BPH rats due to faster cell turnover and fewer DNA repair enzymes. In this study, elevated levels of MDA were observed in the prostate and serum of BPH rats when compared to the control and castrated control group. This finding aligns with the report of O.A Adaramoye & T.D Oladipo[8] which documented increase levels of MDA in

BPH rats. However, treatment with 250mg/kg and 500mg/kg of MECSP resulted in a significant decrease in MDA levels in the prostate and serum. This protective effect is likely attributed to the antioxidant properties of MECSP, which mitigate oxidative damage by inhibiting the formation of free radicals and suppressing lipid peroxidation.

There was no marked change in the serum SOD activity of BPH rats when compared to the control group and the treatment groups. This finding disagrees with a study by Fang C, & Wu. L that reported that BPH increases SOD activity.

Catalase is an enzyme that has vital function in the decomposition of hydrogen peroxide (H_2O_2) to give water (H_2O) and oxygen (O_2). It is found in the peroxisomes of cells and is involved in safeguarding the against detrimental impacts of reactive oxygen species (ROS). Catalase is highly efficient in its reaction with hydrogen peroxide, making it a significant antioxidant enzyme, catalase plays a pivotal role in the body's defense against oxidative stress. In this study, excessive increase in catalase activity was observed in BPH rats when compared to the control and castrated control. At both administered doses of MECSP and HECSP, there was a significance decrease ($p < 0.05$) in prostate and liver activity of catalase. This result does not correlate with the previous findings that reported decrease catalase activity in BPH rats[9,10]. An excessive increase in catalase activity observed from this study may suggests an imbalance in the antioxidant defense system, potentially indicating an underlying pathological process or physiological disturbance. Further investigation is required to determine the specific cause and implications of the excessive increase in catalase activity.

There was no notable difference in the activity of liver GSH in untreated BPH rats compared to the control group, however, excessive increase in the activity of prostate GSH was observed in

the untreated BPH when compared with the control. Treatment with the two doses of MECSP and HECSP was able to decrease the activity.

The transcription factor NF-κB serves as a crucial connection between oxidative stress and inflammatory processes. In this particular study, the level of NF-κB was assessed in the serum. An elevated level of NF-κB was observed in BPH rats when compared to the control groups. This finding is consistent with the report by Almukadi H and Eid BG[13], which also indicated an increase in NF-κB level associated with BPH. However, when the BPH rats were treated with MECSP and HECSP, there was a significant decrease in nuclear NF-κB levels compared to the BPH group. This suggests that the administration of MECSP and HECSP led to a notable reduction in the activation of NF-κB within the nucleus. PPAR-α was increased in the untreated BPH rats, however treatment with 250mg/kg, 500mg/kg MECSP and 500mg/kg HECSP caused a decreased concentration when compared to untreated BPH group.

In addition, Testosterone concentration in the serum was markedly decreased in BPH rat when compared to the control groups. This decrease could be as a result of the increased concentration of 5α-reductase, which enhanced the conversion of testosterone to dihydrotestosterone (DHT). DHT is a potent androgen that plays a role in stimulating the growth and proliferation of prostate cells[11]. However, treatment with the two doses of MECSP and HECSP caused increase in the concentration of testosterone. Similar trend was observed in serum progesterone concentration with 250mg/kg and 500mg/kb HECSP showing effective increase when compared with MECSP.

Furthermore, Serum LH concentration was high in untreated BPH rats when compared to the control groups ($p<0.05$) while the concentration was more increased in all the treatment groups

except in the group treated with 500 mg/kg MECSP. Significant increase in serum estradiol concentration was observed in untreated BPH rats while the concentration was decreased in the treatment groups, with 500mg/kg HECSP showing much decrease. There was no significant variation in the concentration of follicle stimulating hormone in the serum of untreated hyperplasia rats when compared to control, however, notable increased was observed in all the treatment groups.

The bioactive compounds present in CSP extracts have been established to be responsible for the pharmacological effects elicited by the extracts. In this study, GC-MS analysis of each of the methanol and n-hexane extract of *Citrus sinesis* peel contained a number of biologically active compounds. Certain target receptors (proteins) have been reported to be implicated in hyperplasia pathway; these include 5α-reductase and protein specific membrane antigen.

This study employs *in silico* assay to evaluate compounds identified through GC to be present in MECSP and HECSP as potential inhibitors for these proteins.

The ligands present in MECSP and HESCP displayed binding affinities similar to a well-known drug "Dutasteride" which have been used in the management of BPH. The binding affinity of these compounds for the selected targets indicates their inhibitory potential against these biomolecules implicated in the progression of BPH. 2 Cholestan-7-one, cyclic 1,2-ethanediyl acetal, (5.alpha.), 4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-, 4H-1-Benzopyran-4-one, 2,3-dihydro-5,6,7-trimethoxy-2-(4-methoxyphenyl)-, Vitamin E, 4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-2,3-dihydro-3-hydroxy-5,7-dimethoxy-3-phenyl- are the lead compounds in MECSP.

4H-1-Benzopyran-4-one,2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-,Alantolactone, 4.alpha.,4A.alpha.-epoxy-, 4-(2-Furamido)phenyl 2-furoate, 4h-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxy-, Bis(2-ethylhexyl) phthalate, Dibutyl phthalate and Phytol are the lead compound for HECSP.

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Chapter Five

Conclusion

5.1 Summary of Findings

In summary, the treatment of BPH rats with CSP extracts reduced the size of the prostate and the liver. MECSP and HECSP caused notable a decrease ($p < 0.05$) in the prostate concentration of 5-α reductase enzyme (5-αR) which was initially increased in testosterone-induced benign hyperplasia rats. Serum 5-α reductase enzyme concentration was observed to decrease in untreated BPH rats when compared with control ($p < 0.05$), the concentration of the enzyme was increased when the rats that were treated with 500 mg/kg HECSP.

Serum alanine aminotransferase activity was greatly raised in testosterone-induced benign prostate hyperplasia rats. However, those hyperplasia rats treated with CSP extract reduced the activity, with n-Hexane extract showing much decrease. Serum aspartate aminotransferase activity was slightly increased in untreated hyperplasia rats, this increased activity was reduced in all the treatment groups, with methanol extract group showing great decrease.

MECSP and HECSP caused notable decrease ($p < 0.05$) in prostate and liver activity of catalase which was initially increased in testosterone-induced BPH rats. Liver MDA concentration was observed to increase in untreated BPH rats when compared with control ($p < 0.05$), the concentration was markedly lowered when the rats that were treated with 250 and 500 mg/kg

MECSP, however, the concentration was further raised when the rats were treated with 250 and 500 mg/kg HECSP like those treated with dutasteride (standard drug). Prostate MDA concentration was increased in the untreated BPH rats when compared with the control ($p< 0.05$), the concentration of MDA was decreased in rats treated with CSP extracts, with high dose of HECSP showing much decrease.

Serum testosterone concentration was observed to decrease in untreated BPH rats when compared with control ($p < 0.05$), the concentration of the enzyme was markedly increased when the rats were treated with MECSP and HECSP, however notable increase was shown in rats treated with 500mg/kg HECSP. Similar trend was observed in serum progesterone level with 250mg/kg and 500mg/kb HECSP showing effective increase when compared with MECSP. Serum LH concentration was high in untreated BPH rats ($p<0.05$) while the concentration was more increased in the treatment groups except BPH rats treated with 500 mg/kg MECSP. Significant increase in serum estradiol concentration was observed in untreated BPH rats while the concentration was decreased in the treatment groups, with 500mg/kg HECSP showing much decrease. There was no significant variation in the concentration of follicle stimulating hormone in the serum of untreated hypertensive rats when compared to control, however, notable increased was observed in the treatment groups.

Molecular docking shows that interaction of compounds from MESCP with 5α-reductase and Prostrate specific membrane antigen Cholestan-7-one, cyclic 1, 2-ethanediol acetal, (5.alpha.) and Vitamin E had the highest binding affinities for 5α-reductase and 4H-1-Benzopyran-4-one, 2-(1,3-benzodioxol-5-yl)-5,7-dimethoxy-and Vitamin E had the highest binding affinity for prostate-specific membrane antigen.

5.2 Conclusion

This study showed that methanol and n-Hexane extracts of Citrus sinesis peel was able to reverse the complications observed in BPH rats. Toxicity assessment of the lead compounds in the extracts showed high level of safety. The two extracts apparently possessed bioactive compounds capable of demonstrating mitigating effects against BPH. CSP extracts elicited protective effects on BPH via restoring the relative organ weight, 5α-reductase activity, biomarkers of oxidative stress, inflammatory biomarkers, and hormonal parameters. These extracts contain bioactive compounds that might be chemo preventive against Benign prostate hyperplasia.

5.3 Recommendations

Citrus sinesis peel extract can be taken as concoction for management of BPH. Also, the lead compounds could serve as a promising drug in the management of BPH.

5.4 Contribution to Knowledge

The findings from this study could be utilised for further research. This study has provided a new set of data on the management and treatment of BPH

5.5 Suggested Areas for Further Research

There is need to further study the antihyperplasia efficacy of some bioactive compounds found in MECSP and HECSP. Also, there is need to further study the combination therapy of CSP extracts with a known drug.

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Appendix I

Picture of *Citrus sinesis* Peel

Source: Author's Fieldwork, 2023

Appendix II

Picture of *Citrus sinesis* Peel Powder

Source: Author's Lab work, 2023

Appendix III

Picture of Filtration Process after Sieving with Muslin Cloth

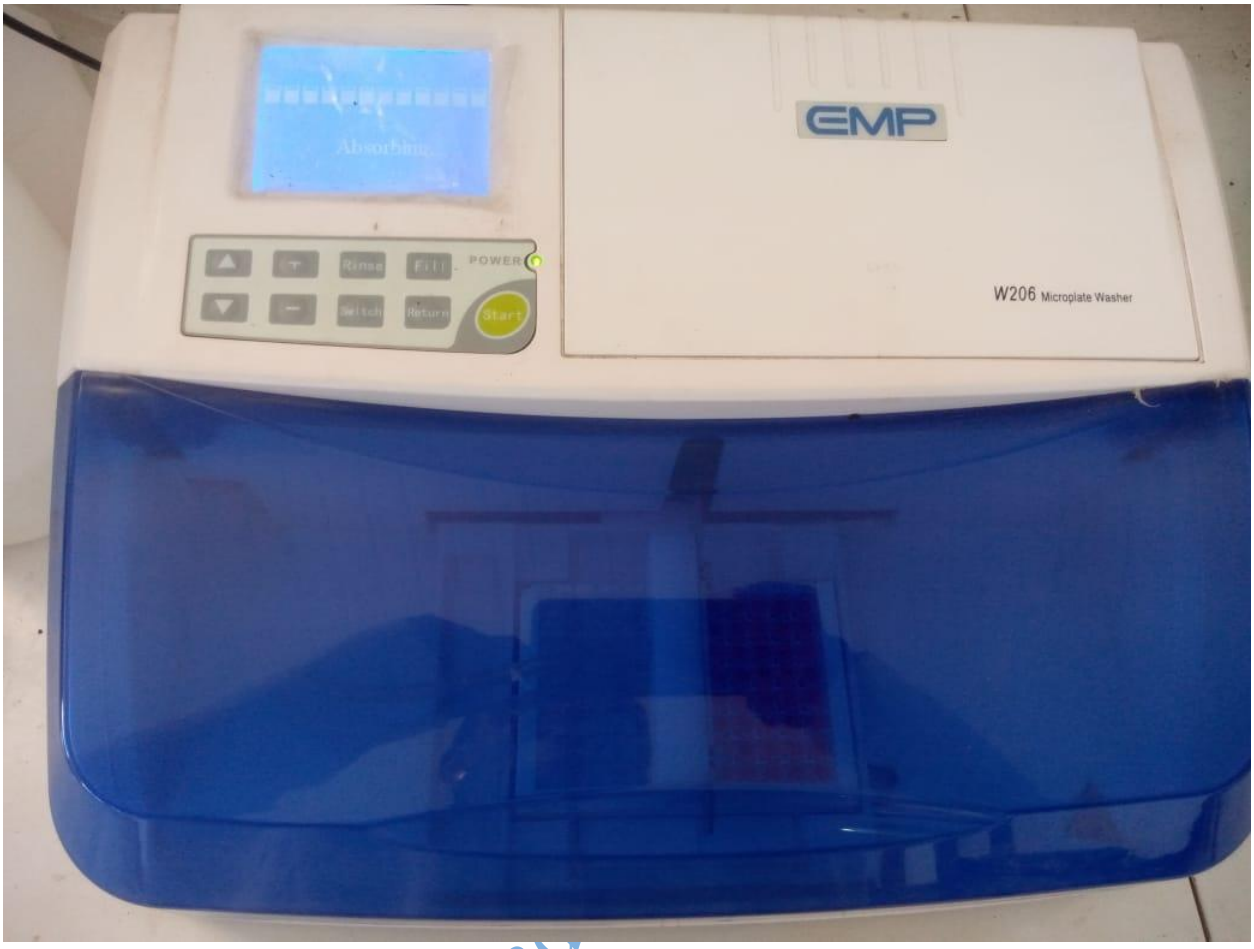
Source: Author's Analysis, 2023

Appendix IV

ELISA Microplate Washer

Source: Author's Analysis, 2023

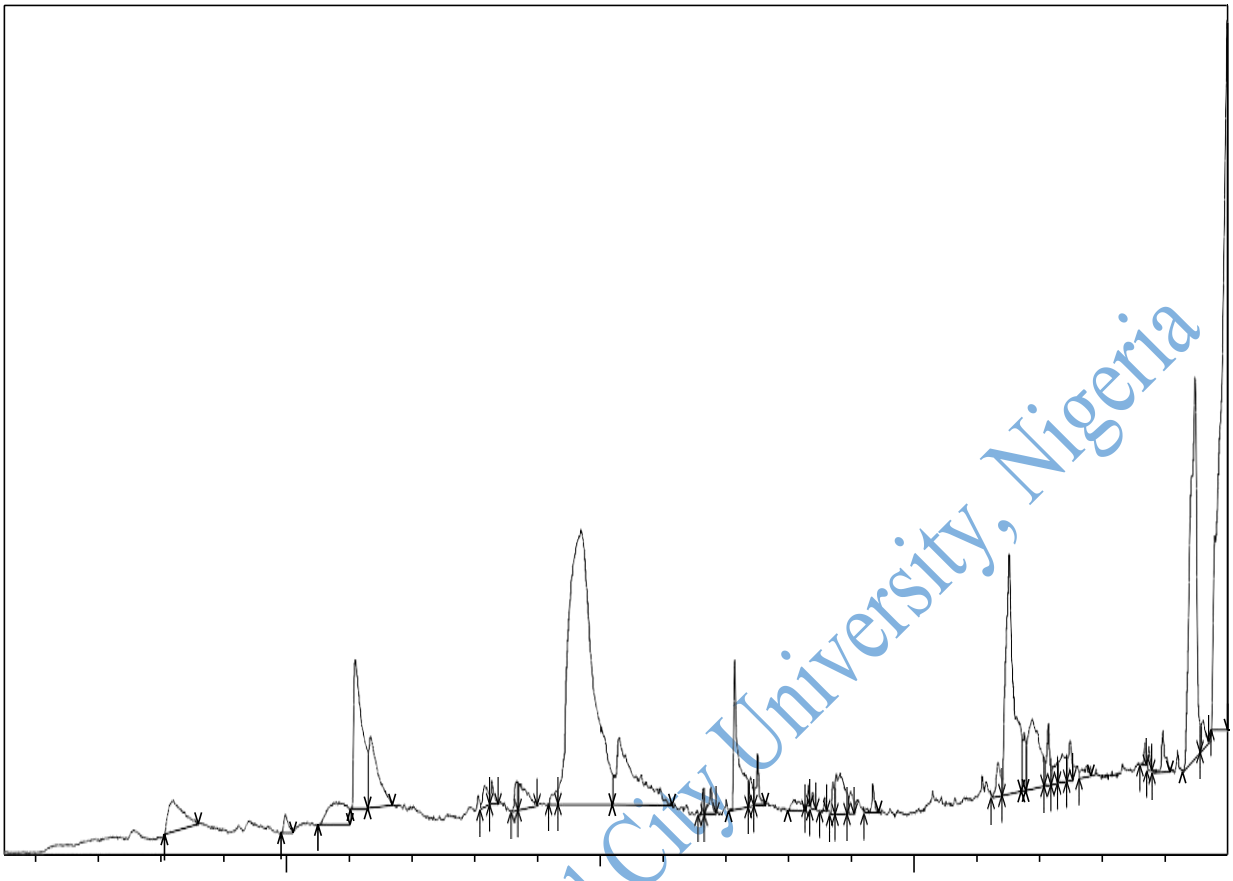
Appendix V



ELISA Plate Reader used for this Research.

Source: Author's Analysis, 2023

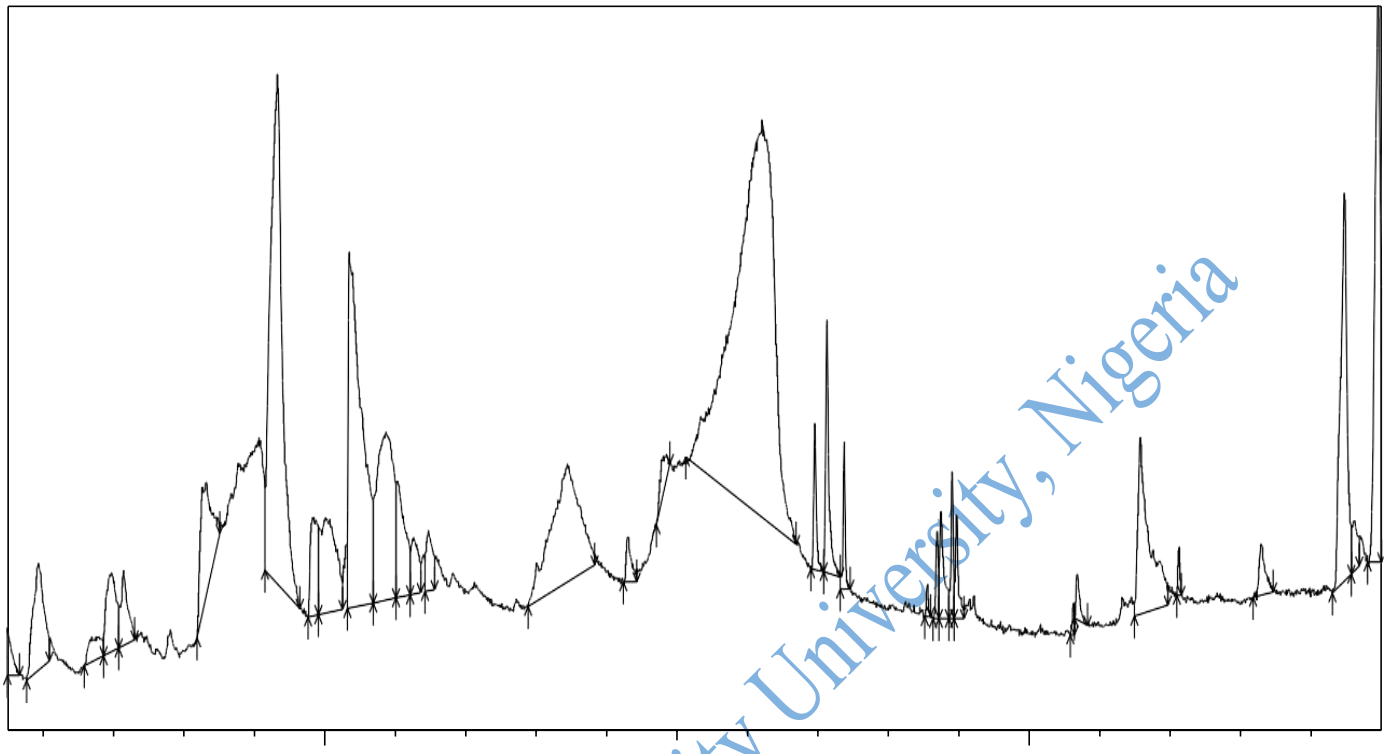
Appendix VI



Chromatograms and Structure of Methanol Extract of *Citrus sinesis* Peel

Source: Author's Analysis, 2023

Appendix VII

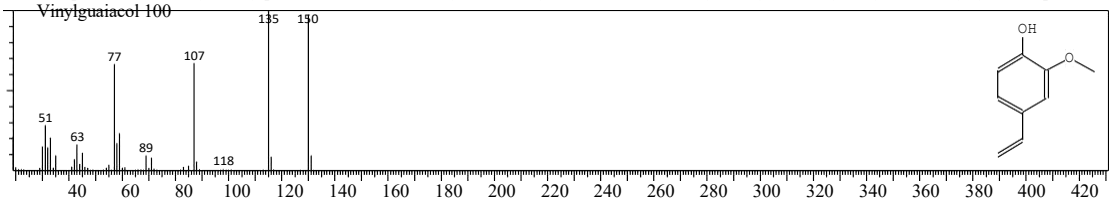


Chromatograms and Structure of n-Hexane Extract of *Citrus sinesis* Peel

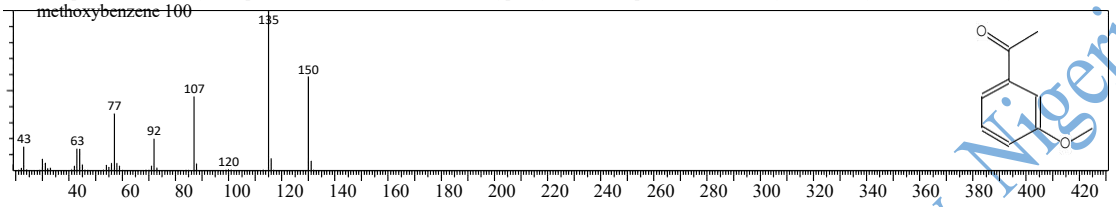
Source: Author's Analysis, 2023

Appendix VIII

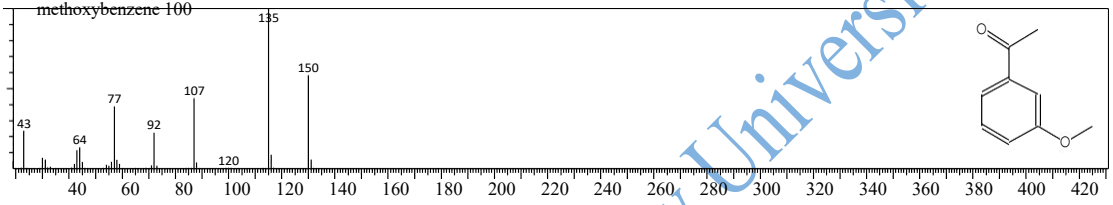
Hit#:1 Entry:15257 Library:NIST11.lib
SI:92 Formula:C9H10O2 CAS:7786-61-0 MolWeight:150 RetIndex:1293
CompName:2-Methoxy-4-vinylphenol $$ Phenol, 4-ethenyl-2-methoxy- $$ Phenol, 2-methoxy-4-vinyl- $$ 4-Hydroxy-3-methoxystyrene $$ p-Vinylguaiacol 100



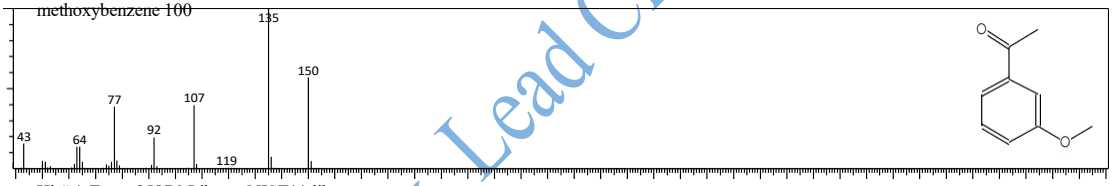
Hit#:2 Entry:15258 Library:NIST11.lib
SI:88 Formula:C9H10O2 CAS:586-37-8 MolWeight:150 RetIndex:1218
CompName:3-Methoxyacetophenone $$ Ethanone, 1-(3-methoxyphenyl)- $$ Acetophenone, 3'-methoxy- $$ 3-Acetylanisole $$ 1-Acetyl-3-methoxybenzene 100



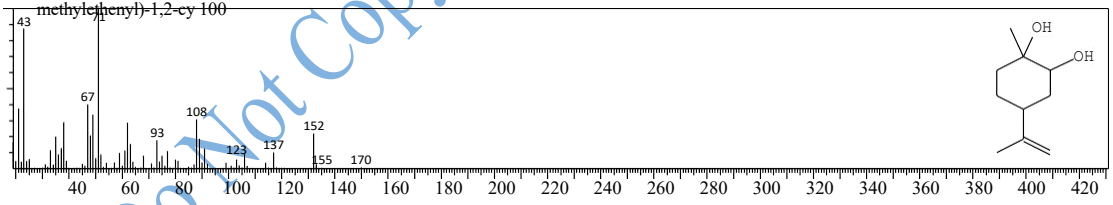
Hit#:3 Entry:8939 Library:NIST11s.lib
SI:88 Formula:C9H10O2 CAS:586-37-8 MolWeight:150 RetIndex:1218
CompName:3-Methoxyacetophenone $$ Ethanone, 1-(3-methoxyphenyl)- $$ Acetophenone, 3'-methoxy- $$ 3-Acetylanisole $$ 1-Acetyl-3-methoxybenzene 100



Hit#:4 Entry:8938 Library:NIST11s.lib
SI:87 Formula:C9H10O2 CAS:586-37-8 MolWeight:150 RetIndex:1218
CompName:3-Methoxyacetophenone $$ Ethanone, 1-(3-methoxyphenyl)- $$ Acetophenone, 3'-methoxy- $$ 3-Acetylanisole $$ 1-Acetyl-3-methoxybenzene 100



Hit#:1 Entry:25876 Library:NIST11.lib
SI:81 Formula:C10H18O2 CAS:1946-00-5 MolWeight:170 RetIndex:1346
CompName:1,2-Cyclohexanediol, 1-methyl-4-(1-methylethenyl)- $$ 4-Isopropenyl-1-methyl-1,2-cyclohexanediol # $$ 1-Methyl-4-(1-methylethenyl)-1,2-cy 100



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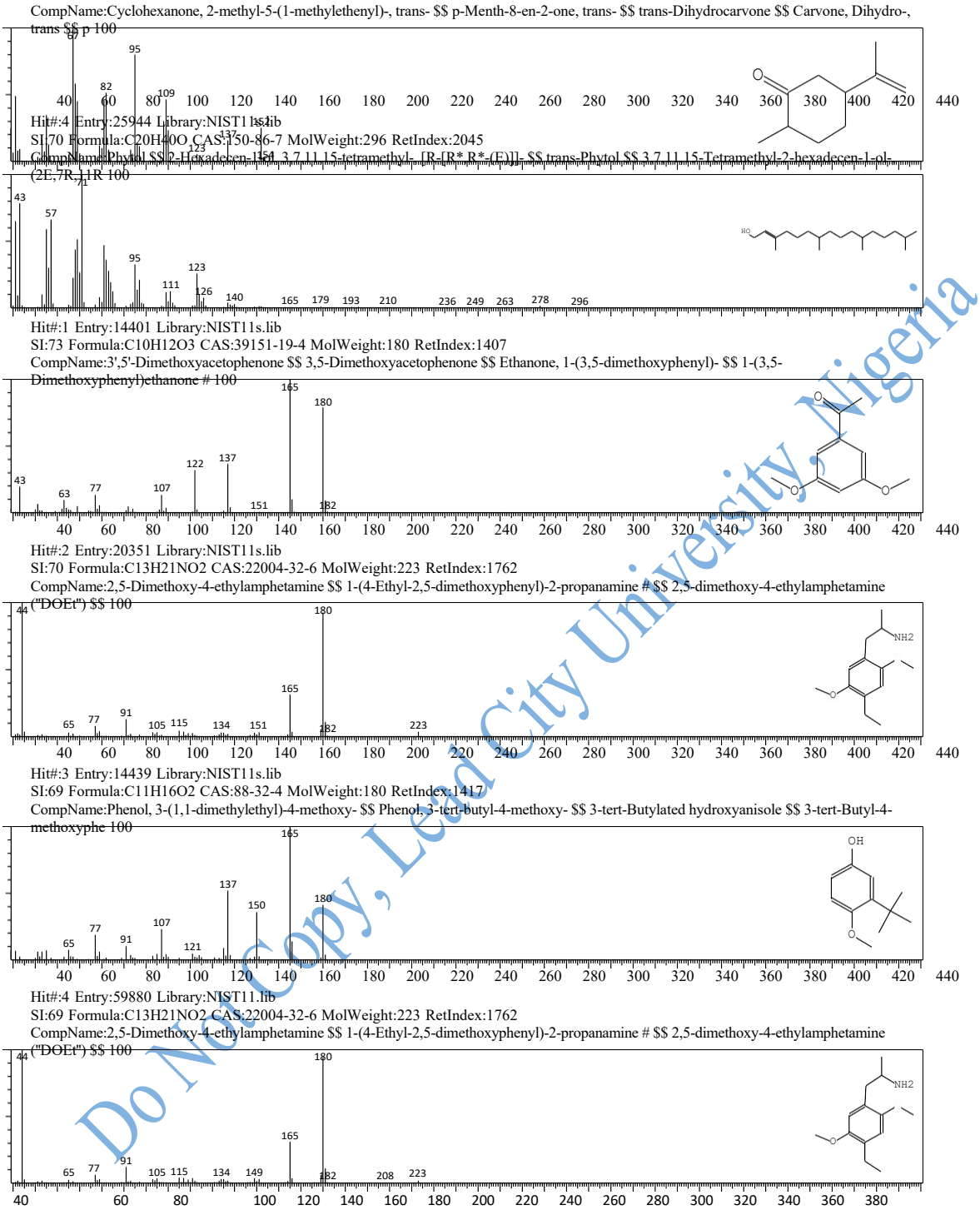
SI:71 Formula:C8H14O CAS:162239-52-3 MolWeight:126 RetIndex:865
CompName:7-Oxabicyclo[4.1.0]heptane, 1,5-dimethyl- $$ 1,5-Dimethyl-7-oxabicyclo[4.1.0]heptane # $$ 100

Chromatograms and Structures of Some of the Bioactive Compounds detected in MESCP

Source: Author's Analysis, 2023

Appendix IX

SI:70 Formula:C10H16O CAS:5948-04-9 MolWeight:152 RetIndex:1179

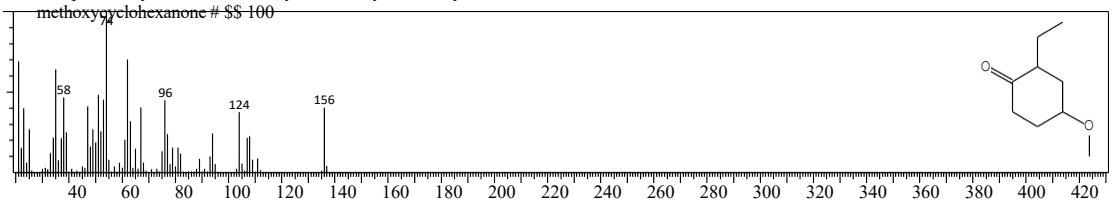


Chromatograms and Structures of Some of the Bioactive Compounds detected in MESCP

Source: Author's Analysis, 2023

Appendix X

SI:62 Formula:C9H16O2 CAS:13482-27-4 MolWeight:156 RetIndex:1188
CompName:Cyclohexanone, 2-ethyl-4-methoxy- $$ 2-Ethyl-4-methoxycyclohexanone # $$ 100



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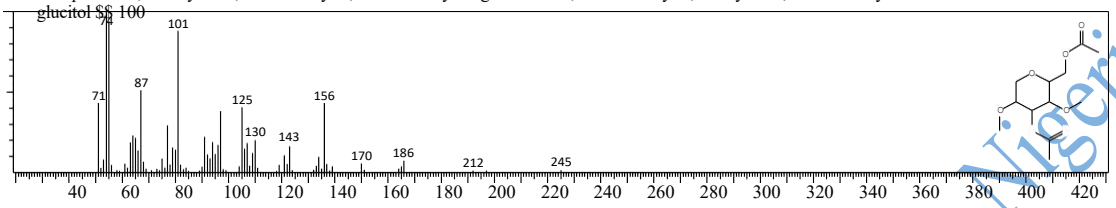
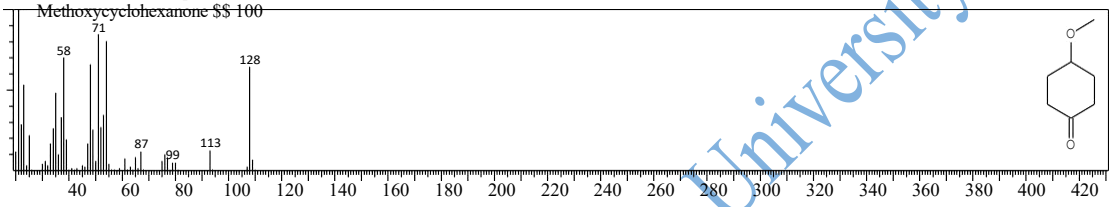
SI:60 Formula:C12H20O7 CAS:97275-53-1 MolWeight:276 RetIndex:1737
CompName:1,5-Anhydro-3,6-di-O-acetyl-2,4-di-O-methyl-D-glucitol $$ 3,6-di-O-Acetyl-1,5-anhydro-2,4-di-O-methyl-D-glucitol $$ 100



Hit#:3 Entry:7114 Library:NIST11.lib
SI:60 Formula:C7H12O2 CAS:13482-23-0 MolWeight:128
RetIndex:1028CompName:Cyclohexanone, 4-methoxy- $$ 4-Methoxycyclohexanone $$ 100



Hit#:1 Entry:15313 Library:NIST11.lib
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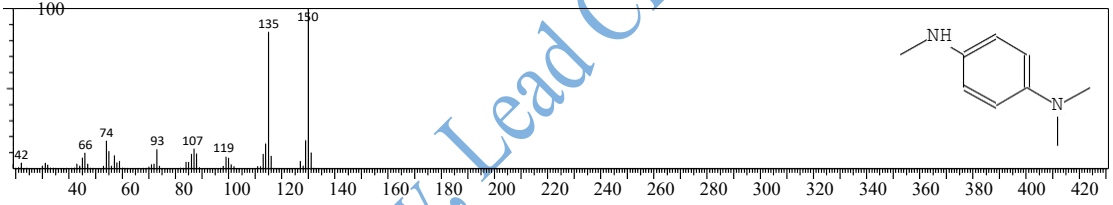
RetIndex:1254CompName:N,N,N'-Trimethyl-1,4-phenylenediamine
100



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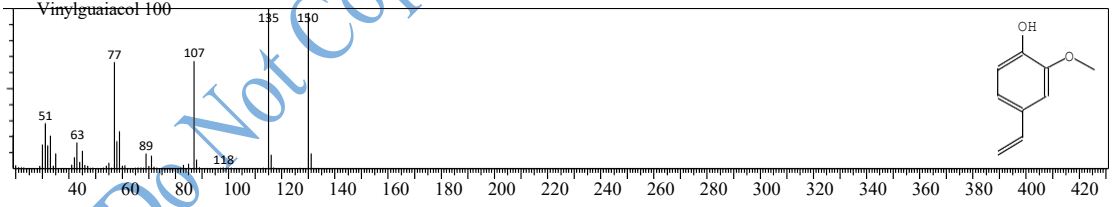
CompName:2-Methoxy-4-vinylphenol $$ Phenol, 4-ethenyl-2-methoxy- $$ Phenol, 2-methoxy-4-vinyl- $$ 4-Hydroxy-3-methoxystyrene $$ p-Vinylguaiacol 100

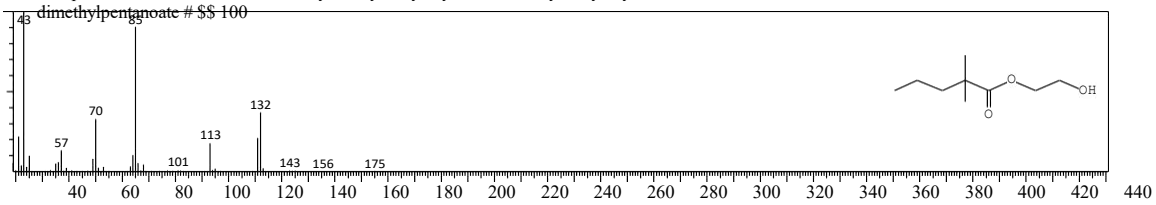


Chromatograms and Structures of Some of the Bioactive Compounds detected in MESCP

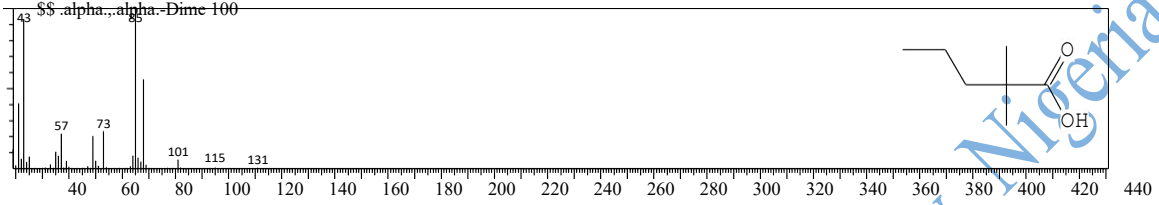
Source: Author's Analysis, 2023

Appendix XI

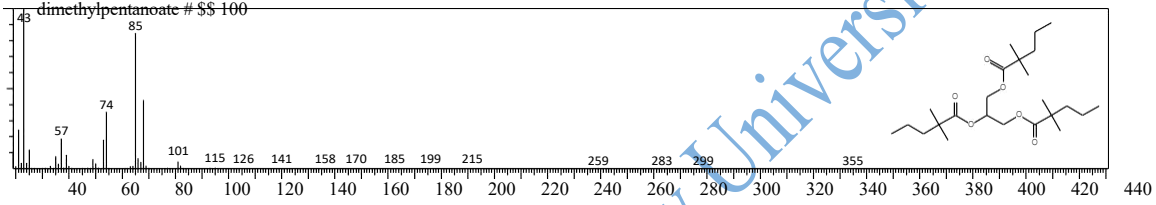
Hit#:1 Entry:27947 Library:NIST11.lib
SI:71 Formula:C9H18O3 CAS:0-00-0 MolWeight:174 RetIndex:1241
CompName:Pentanoic acid, 2,2-dimethyl-, 2-hydroxyethyl ester $$ 2-Hydroxyethyl 2,2-dimethylpentanoate # $$ 100



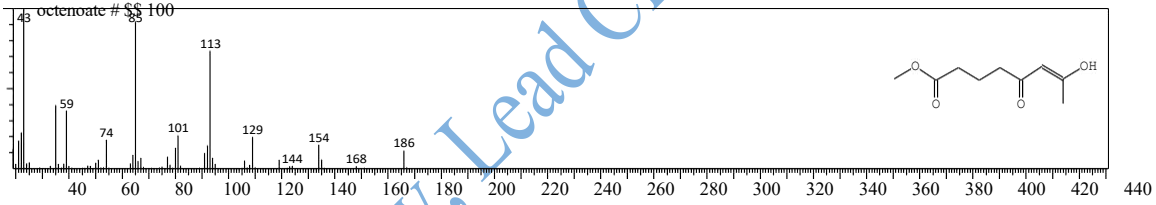
Hit#:2 Entry:5569 Library:NIST11s.lib
SI:68 Formula:C7H14O2 CAS:1185-39-3 MolWeight:130 RetIndex:989
CompName:2,2-Dimethylvaleric acid $$ Pentanoic acid, 2,2-dimethyl- $$ Valeric acid, 2,2-dimethyl- $$ 2,2-Dimethylpentanoic acid $$ .alpha.,.alpha.-Dime 100



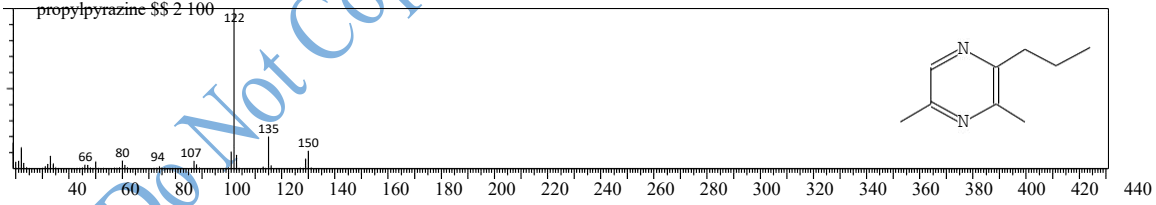
Hit#:3 Entry:191518 Library:NIST11.lib
SI:65 Formula:C24H44O6 CAS:57346-62-0 MolWeight:428 RetIndex:2591
CompName:Pentanoic acid, 2,2-dimethyl-, 1,2,3-propanetriyl ester $$ 2,3-Bis[(2,2-dimethylpentanoyl)oxy]propyl 2,2-dimethylpentanoate # $$ 100



Hit#:4 Entry:34836 Library:NIST11.lib
SI:65 Formula:C9H14O4 CAS:0-00-0 MolWeight:186 RetIndex:1344
CompName:5,7-Dioxooctanoic acid, methyl ester $$ Methyl (6E)-7-hydroxy-5-oxo-6-octenoate # $$ 100



Hit#:1 Entry:15301 Library:NIST11.lib
SI:72 Formula:C9H14N2 CAS:32350-16-6 MolWeight:150 RetIndex:1206
CompName:Pyrazine, 3,5-dimethyl-2-propyl- $$ 2,6-Dimethyl-3-propylpyrazine $$ Pyrazine, 2,6-dimethyl-3-propyl- $$ 3,5-Dimethyl-2-propylpyrazine $$ 2 100



Chromatograms and Structures of some of the Bioactive Compounds detected in MESCP

Source: Author's Analysis, 2023

Bio-data

A. PERSONAL DATA

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Place of Birth	Ibadan
Nationality	Nigerian
Next of Kin	Miss Oladayo Samuel
Address	No 16, Ifedayo Estate, Monijesu Street, Soka, Ibadan.

B. EDUCATION BACKGROUND

Educational Institutions Attended with Dates and Qualifications

- Lead City University {**MSc. Biochemistry**} 2021- 2023
- Lead City University {**BSc. Biochemistry**} 2015-2019
- WESLEY College of Science, Elekuro, Ibadan.
  (**Senior Secondary School Certificate**) 2012 – 2015
- Genius Royal Academy, Ibadan. 2009 - 2012
- Agbala Holy Michael, Nur. & Pri. School, Sanyo, Ibadan.
  (**Primary School Leaving Certificate**) 2003 – 2009

C. WORK EXPERIENCE WITH DATES

- Lead City University, Ibadan. (Graduate Assistant) 2021 - till date
- Chrisland University 2019 - 2020
  Ajebo, Abeokuta. (Biochemistry Technologist Assistant) (NYSC).
- MYJOY Food Industry Limited, Ibadan, Oyo State. (Intern) 2018
- JOLLABEL Group of Schools
  Alaagbole Area, Asoore, Berger, Ogun State 2017

D. AWARDS AND FELLOWSHIP Nil

E. MEMBERSHIP OF ACADEMIC PROFESSIONAL BODIES

- Chemical Science of Nigeria (CSN)
- Nigerian Society for Biochemistry and Molecular Biology ((NSBMB)

F. PUBLICATIONS

1. Asejeje, F. O., Ajayi, B. O., Abiola, M. A., **Samuel, O.M**, Asejeje, G. I., Ajiboye, E. O., & Ajayi, A. M. (2022). Sodium benzoate induces neurobehavioral deficits and brain oxido-inflammatory stress in male Wistar rats: Ameliorative role of ascorbic acid. *Journal of Biochemical and Molecular Toxicology*, *36*(5), e23010. http://dx.doi.org/10.1002/jbt.23010

2. Arojojoye O. A, Nwaechefu O. O, Adeosun A. M., **Samuel O. M**. (2022). Sub-chronic exposure to Leachate from Ojota, Lagos is associated with Liver and kidney toxicity in Rats. *FUDMA JOURNAL OF SCIENCES*, *6*(1), 308-313. https://doi.org/10.33003/fjs-2022-0601-829

3. Taiwo, O. A., Dosumu, O. A., Akomolafe, O. V., Oni, E. O., Adefuye, A. O., Shofunde, A. A., **Samuel O.M**, & Ojo, O. A. (2022). Occupational exposure in automobile repair workshops: toxicological effects of contaminated soil in Wistar rats. *Toxicology Research*. https://doi.org/10.1093/toxres/tfac030

Thesis and Dissertation

Assessment of the role of Vitamin C on sodium benzoate- induced toxicity on brain and liver of male Wistar rats. **(B.Sc.)**

G. REFERENCES

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…… ……

Signature **Date**

The University Compliance Certification

This is to certify that this Thesis was written by **Omolola Mary, SAMUEL with Matric Number LCU/PG/002341** of the Department of Chemical Sciences, Faculty of Natural and Applied Sciences, Lead City University, Ibadan and it is in full compliance with the approved University format and style.

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Signature **Date**