# BENIGN PROSTATIC HYPERPLASIA ATTENUATION AND CYTOTOXIC EFFECTS OF *Lonchocarpus griffonianus* G. DON (FABACEAE) STEM AND ROOT BARKS

BY

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DEPARTMENT OF PHARMACOGNOSY
FACULTY OF PHARMACY

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BENIN CITY
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A THESIS WRITTEN IN THE DEPARTMENT OF PHARMACOGNOSY AND SUBMITTED TO THE POSTGRADUATE STUDIES IN IN FULFILLMENT OF THE REQUIREMENT FOR THE DOCTOR OF PHILOSOHPY DEGREE (PhD) IN PHARMACOGNOSY OF THE UNIVERSITY OF BENIN, BENIN-CITY, NIGERIA

AUGUST, 2023

**CERTIFICATION**

This is to certify that this project was carried out by DANIEL AKPE-EFIAK AMBE of the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin-city, Nigeria.

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Dr (Mrs) Josephine O. Ofeimun Date

Ag. Head of Department

**DEDICATION**

I dedicate this work to the Eternal God that granted me the grace to finish this work, and my beautiful and talented wife Pharm. (Mrs.) Ima Daniel Ambe, my gifted children (Prosperity Daniel Ambe, Akpe Daniel Ambe (Jnr) and Love Daniel Ambe).

To my late parents, Chief Akpe Daniel Ambe and Mrs. Arit Akpe Daniel Ambe

**ACKNOWLEDGEMENTS**

I am deeply grateful to the Almighty God for his benevolence and grace sufficient for me to finish the work. I return all the glory to him.

I am also grateful to my supervisor for sharing a part of his knowledge and experience with me. Through his tutelage, I am now a better person than I was before. Thank you, Prof.

I appreciate Dr (Mrs.) Josephine Ofeimun for her teachings, guidance, robustness and perfection during the program.

Prof. (Dr) Iqbal Choudhary's contributions from the International Center for Biological and Chemical Sciences are deeply acknowledged for analyzing (Cytotoxic and NMR analysis) my samples free of charge.

The University of Uyo is also acknowledged for allowing me to acquire more degrees. Thank you, Prof. Akeneren Essien, Prof. Olorunfemi Eseyin, Prof. Paul Nwafor and many others too numerous to mention.

Special appreciation goes to the Department of Pharmacognosy, University of Benin staff. My wonderful friends Mr Ugwu Kingsley, Mrs Florence Adesina, Dr Osamuyi Uwunarongie, Dr Rose Imade, Mallam Abdul-hafiz Sanni, "High Chief" Chijoke Samuel and others I have failed to mention due to space constraints.

I want to acknowledge the contributions of Dr Emmanuel Odion, the acting Head of the Department of Pharmaceutical Chemistry. He has been a wonderful mentor, a father, and a light in the dark for me and others. May the Almighty God reward you both on Earth and in Heaven. Amen.The patience, prayers and tolerance of my wife and children are deeply acknowledged and appreciated. Those cold and long nights, loneliness and many more things you all fought without letting go due to my unavoidable absence, during the course of the program, have made me who I am today. I will not take these sacrifices for granted. May God grant you all eternal grace to reap the fruit of this seed. Amen.

Thank you Mrs. Emem Bassey Edet for your sacrifices when I was on the bench. You took the pain comes sunshine and during rainfall to get samples and lots more. May the Lord reward you bountifully. Amen.

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**ABSTRACT**

Rising incidences of benign and cancerous tumours, such as benign prostatic hyperplasia (BPH) and prostate cancer, coupled with the unpleasant side effects of current therapy, suggest a need to search for new drug molecules. The stem bark of *Lonchocarpus griffonianus G. Don (Fabaceae)* is an important medicinal plant used in Nigeria to treat BPH and other tumour-related ailments. No pharmacological study on the use of the plant for treating BPH has been reported. This study aimed to investigate the protective effect of *L. griffonianus* (LG) on BPH.

Two organs (stem and root barks) of LG were identified, collected, pulverized and extracted with absolute methanol (99 %) using a Soxhlet extractor. Comparative preliminary biological evaluations were done on the *L. griffonianus* stem bark (LGSB) extract and root bark (LGRB) extracts using two benchtop assays (cytotoxic and antiproliferative). The acute toxicity of the LG stem bark extract was done using a modified Lorke's method. The extract was subjected to Vacuum Liquid Chromatography (VLC) and Gravity Column Chromatography (GCC) to obtain two isolated compounds, LO1 and LO2. The compounds were subjected to MS and 1D NMR analysis for identification. The isolated compounds (LO1 and LO2) were subjected to cytotoxic evaluation on human prostate (PC3) and uterine cervical cancer (Hela) cell lines using a 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Anti-BPH evaluation was done on the extract and LO1 using testosterone-induced BPH in the rat model. BPH was induced by the administration of testosterone propionate (4 mg/kg, s.c., in olive oil) for 28 days. LGSB extract (100, 200 and 400 mg/kg), LO1 (5 mg/kg), LGSB extract (200 mg/kg)+finasteride (5 mg/kg) and finasteride (5 mg/kg) were orally administered daily. On day 29, the rats were

sacrificed under anaesthesia and blood was collected via the abdominal aorta. The collected blood was centrifuged, and the serum was separated. The serum was analyzed for biochemical parameters such as prostate-specific antigen (PSA), testosterone and estradiol. The prostate was harvested for histological examination. The wet weight and volume of the prostate were taken. The prostate index (PI) was calculated. All data were expressed as mean ± SEM (standard error of the mean) and were compared using analysis of variance (ANOVA),

The result of preliminary evaluations indicated that the LGSB extract has a higher activity (100 ± 0.00% mortality at 80 µg/mL) than the LGRB extract (3.33 ± 1.29% at 80 µg/mL). Acute toxicity results revealed no mortality in both phases after oral administration with LD50>5000 mg/kg. LO1 and LO2 significantly inhibited the multiplication of PC3 and Hela cells *in vitro*. The LGSB extract treatment significantly ($p<0.0001$) reduced prostate weight, volume and prostate index in rats induced with testosterone. There was a significant ($p<0.001$ and $p<0.0001$) lowering of testosterone in the serum of the animals. The dose of 100 and 400 mg/kg body weight of the extract caused a 79.26% (0.28 ± 0.06) reduction in the testosterone concentration of rats serum. The Prostate-Specific Antigen (PSA) level in the serum was significantly ($p<0.01$ and $p<.001$) reduced in comparison with the negative control. The dose of 400 mg/kg body weight of the extract caused a 70.28% (0.74 ± 0.18) reduction in the PSA of rats. There was no significant difference in the estradiol result obtained for the study. The histopathological presentation revealed that the extract ameliorated testosterone's effect on prostate histomorphology's architecture. The results of the study demonstrated that the crude extract and lupeol isolated from the extract attenuated rat prostate enlargement. Two bioactive phytosterols (LO1 and LO2) were characterized as lupeol and β-sitosterol and showed cytotoxic activity against human prostate and uterine cervical cancer cell lines. The ethnomedicinal usage of *L. griffonianus* for the treatment of BPH was validated by this study.

# CHAPTER ONE

## 1.0 INTRODUCTION AND LITERATURE REVIEW

### 1.1 Historical perspective of natural products

A clay tablet from Mesopotamia (2600 B.C.) contains the most ancient records of natural products in the form of essential oils from *Cupressus sempervirens* L. (Cupressaceae) which is still useful today as a cough suppressant (Cragg and Newman, 2005). The Ebers Papyrus (2900 B.C.), an equivalent of the modern-day drug codex, is an Egyptian document containing more than 700 medicinal plants. The Materia Medica (1100 B.C.) was authored by Pedonius Dioscorides, It is a prescription document listing 52 naturally derived products written. He was a Greek doctor who collated herbal remedies according to subheads such as collection, storage and functions. This knowledge was preserved by the monks in England, Ireland, France and Germany but the Arabs introduced private pharmacies in the 8$^{th}$ century. *Canon Medicinae*, written by Avicenna, a pharmacist and philosopher who also prague e medicine contributed to the Arab's success in the establishment of the ancient pharmaceutical practice (Cragg and Newman, 2005).

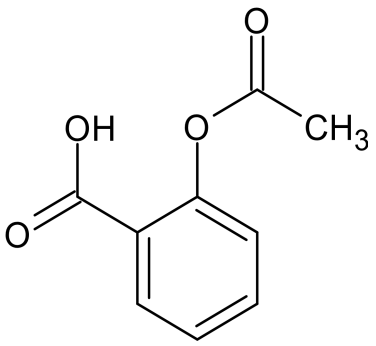
### 1.2 Natural products from medicinal plants

Herbal medicine contains myriads of compounds some of which can be active or non-active, others can be toxic and require to be eliminated. The identification of active molecules is essential for health and availability assurance. Some techniques have been developed to identify and isolate bioactive compounds from medicinal plants, and one such technique is bioassay-guided isolation methods which have yielded a high success rate in the discovery of lead compounds. In this technique extract from herbal medicine is partitioned into fractions and monitored with bioassay methods for activity. The solvent fractions usually contain compounds of similar properties of which the amount of the active principle is a direct reflection of the biological activity as well. This step is repeated until the compound responsible for the observed activity is isolated and characterized, and could further be developed into pharmaceutical products (Wolfender *et al.*, 2019). There are several examples of herbal medicines that have been translated into modern pharmaceutical products. The technique of bioassay-guided fractionation is cumbersome, but it is also heavily reliant on the availability of a suitable assay. Reports in the literature suggested the essence of data mining in ethnomedicine to identify bioactive metabolites, but these are still subject to the fact that the biological effects of a plant extract vary quantitatively according to the relative components exerting the biological action. This is the concept of quantitative composition-activity relationship. (Chang *et al.*, 2006; Wang *et al.*, 2008). Natural products as sources of drug leads have witnessed the greatest breakthrough in scientific advancement in recent times. They continue to be a source of many diverse bioactive molecules as compared to other sources of drug leads such as combinatorial chemistry (Cragg and Newman, 2005;Mishra and Tiwari, 2011). About 10% of the global natural sources of bioactive molecules have been examined for possible drug lead, therefore, the possibility of drug discovery from

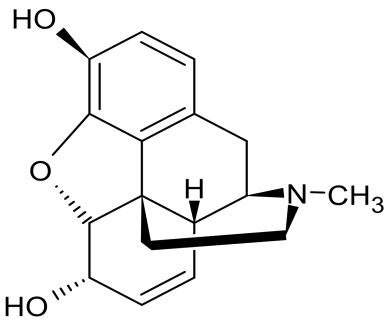
biodiversity remains very high. Natural products used for therapeutic purposes have been in practice for ages. Some medicinal effects were discovered by man in ethnomedicine by trial and error over centuries while sourcing herbs to treat diseases (Kinghorn *et al.*, 2011). The biosynthesis of natural products is exclusive to an organism or species and is called "secondary metabolism". Natural products are not required for the existence of an organism but are products of stress factors (Dewick, 2002; Colegate and Molyneux, 2007). The biosynthesis of natural products in plants is through important pathways such as the photosynthetic pathway. Others are glycolysis and the Krebs cycle (Dewick, 2002). Intermediate pathways including Acetyl coenzyme A, Mevalonic acid, Shikimic acid, and 1-deoxyglucose-5-phosphate are the basic building blocks required to synthesize natural products. Although limited, arrays of compounds that can be formed are infinite. Alterations in the biosynthetic pathways can be due to physical, chemical or artificial stresses (Sarker *et al.*, 2006). Some of these compounds with unique chemical structures confer numerous biological activities on natural products. For instance, the anticancer drug paclitaxel was discovered from an analysis of the plant known as *Taxus brevifolia* Nutt. (Taxaceae) in 1962 and was licensed for the treatment of breast cancerous tumours (Cragg, 1998). Acetylsalicylic acid was synthesized from a starting material known as salicin that was isolated from the plant: *Salix alba L. (Salicaceae)*. Studies on *Papaver somniferum* L. (Papaveraceae) led to the isolation of morphine, an important opioid used for the control of pain. The United States Food and Drug Administration (FDA) licensed a compound known as quinine in 2004. It was an antimalarial drug derived while working on the bark of *Cinchona succirubra Pav.* (Rubiaceae) (DerMarderosian and Beutler, 2002). Pilocarpine, an alkaloidal compound obtained from *Pilocarpus jaborandi* Holmes (Rutaceae) is registered for

the treatment of glaucoma. The compounds known as calanolide A and calanolide B gotten from the *Calophyllum anigerum* Miq (Clusiaceae) are credited with activity and presently, they have scaled through phase II clinical trial as an anti-HIV agent.

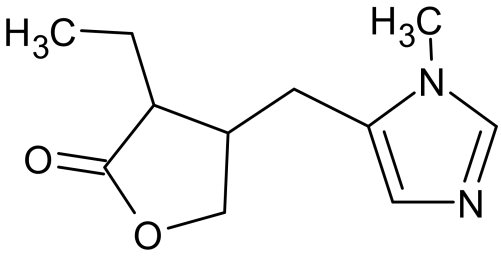
(Aniszewski, 2007).



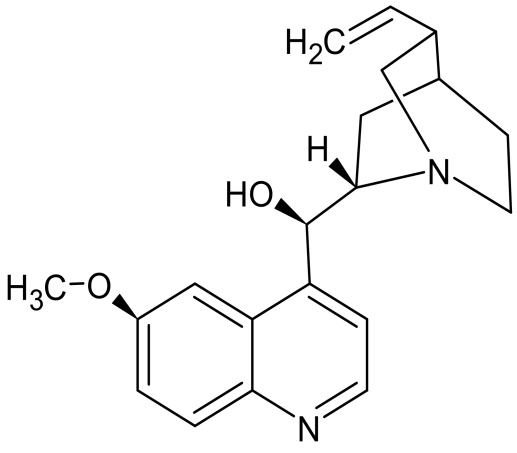

Acetylsalicylic acid




Morphine

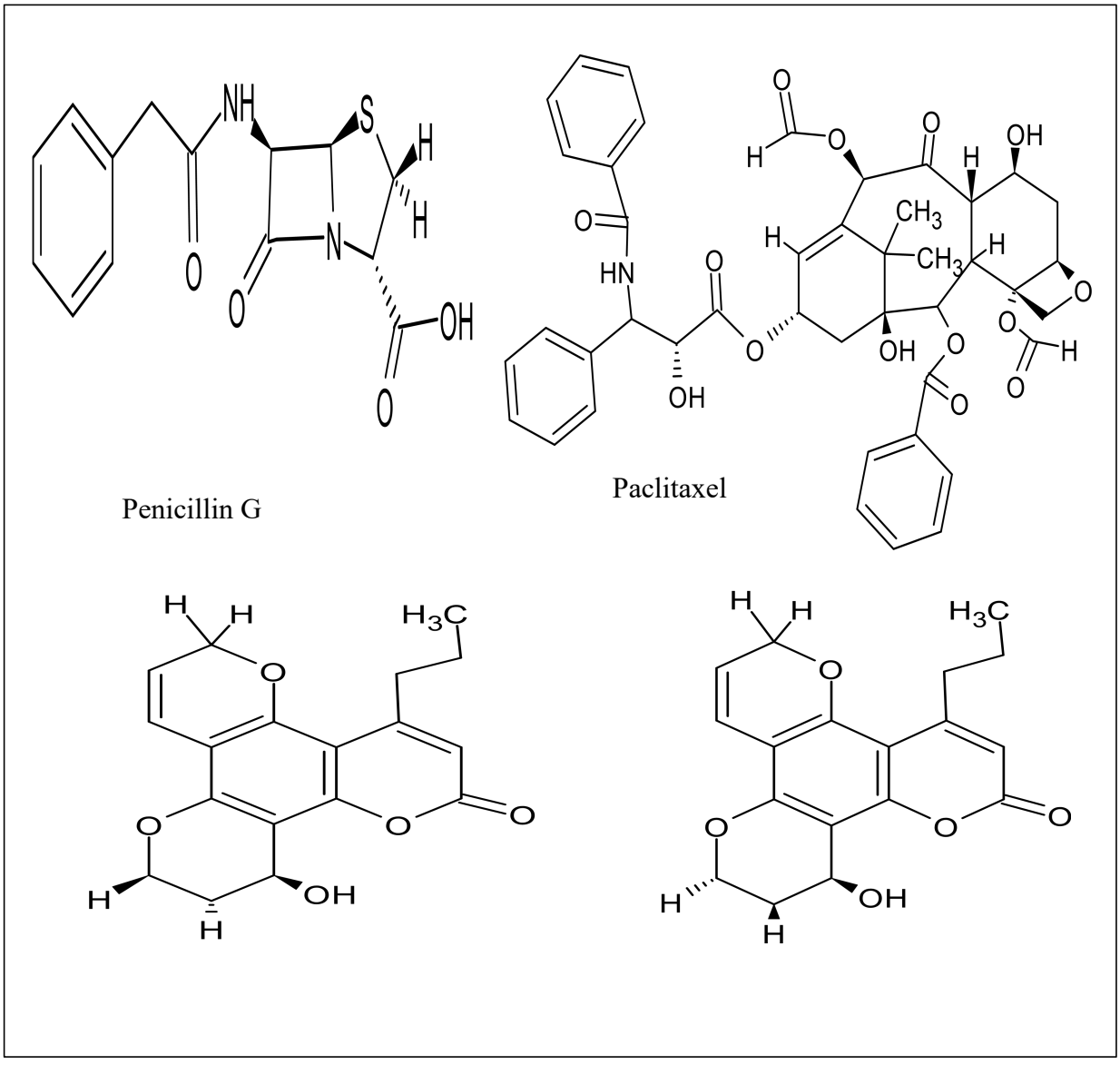



Pilocarpine



Quinine

**Figures 1.1**: structures of some compounds isolated from medicinal plants used at various times to treat different disease conditions.



Calanolide A Calanolide B

**Figure 1.2**: structures of some compounds isolated from medicinal plants used at various times to treat different disease conditions.

### 1.3 Cancer

Cancer can be described as a conglomeration of ailments that have the capability of affecting any tissue of the human system. They arise when renegade cells at any section of the body escape control mechanisms and continue to proliferate irrepressibly with a loss of boundary restrictions (Weinberg, 1996). This group of ailments remain at the forefront of killer diseases before seventy years of age. According to Global Cancer Observatory (GLOBOCAN) estimates, about 19.3 million new cancer occurrences occurred in 2020 with about 10 million mortalities recorded globally. The African continent alone accounted for 5.7% of this incidence. Statistics have shown that by 2040, there may be a staggering 47% increment in cancer incidence from these estimates globally (Sung *et al.*, 2021). Two major types have been identified: Tumor (benign) and malignant cancers (Selchick, 2022).

#### 1.3.1 Tumor

Tumours are cancerous cells restricted to a specific location in the human body. They occur due to the loss of the apoptosis control mechanism, so they continue to grow in an uncontrollable

fashion. They tend not to migrate to other locations in the body. Examples include BPH in men and uterine fibroid in women (Selchick, 2022).

### 1.3.2 Cancerous tumor

Cancers are cancerous cells that are highly invasive. Irrespective of the originator site, it can migrate to other locations and retain the power to attack and change the cellular function of the cells that have been overtaken. This type of cancer is usually difficult to manage and the prognosis outcome is not always good (Weinberg, 1996).

## 1.4 Causes of tumours and cancerous tumours

The subject of cancer causes has remained intriguing for ages. Studies have shown that most cancers are a result of exposure to environmental factors rather than genetic involvement. (Blackadar, 2016)

### 1.4.1 Occupational causes due to exposure to susceptible chemicals

A report of high cancer cases in coal tar distillers, miners, and upon x-ray exposure called for attention to occupational risk associated with the development of cancer on a short or long-term basis. Dye workers in Germany were observed to suffer from bladder cancer. Ortho-toluidine is used to synthesize dyes and herbicides. It was reported that 73 workers with previous exposure to the chemical over a decade had bladder cancer (Ward *et al.*, 1991).

Exposure to silica dust causes elevated risk of having lung cancer. Silica dust exposure in manufacturing plants was found to positively correlate with the development of lung cancerous tumour within thirty years (Costello and Graham, 1988).

Diesel exhaust emission inhalation is also reported to be the cause of elevated risk of lung cancerous tumour development in persons in contact with a diesel operated machinery (Garshick *et al.*, 2004).

Tetraoxosulfate (VI) acid is an important acid that is used both in the laboratory and in industries. Mist inhalation of sulfuric acid is linked to laryngeal cancer cases. A study revealed a latency period of about 9.5 years with an incidence rate of 2.30 (Steenland *et al.*, 1988)

Formaldehyde exposure has been linked to the occurrence of nasopharyngeal carcinoma in factory workers that were exposed to formaldehyde after a period of 35 years on average (Hauptmann *et al.*, 2004).

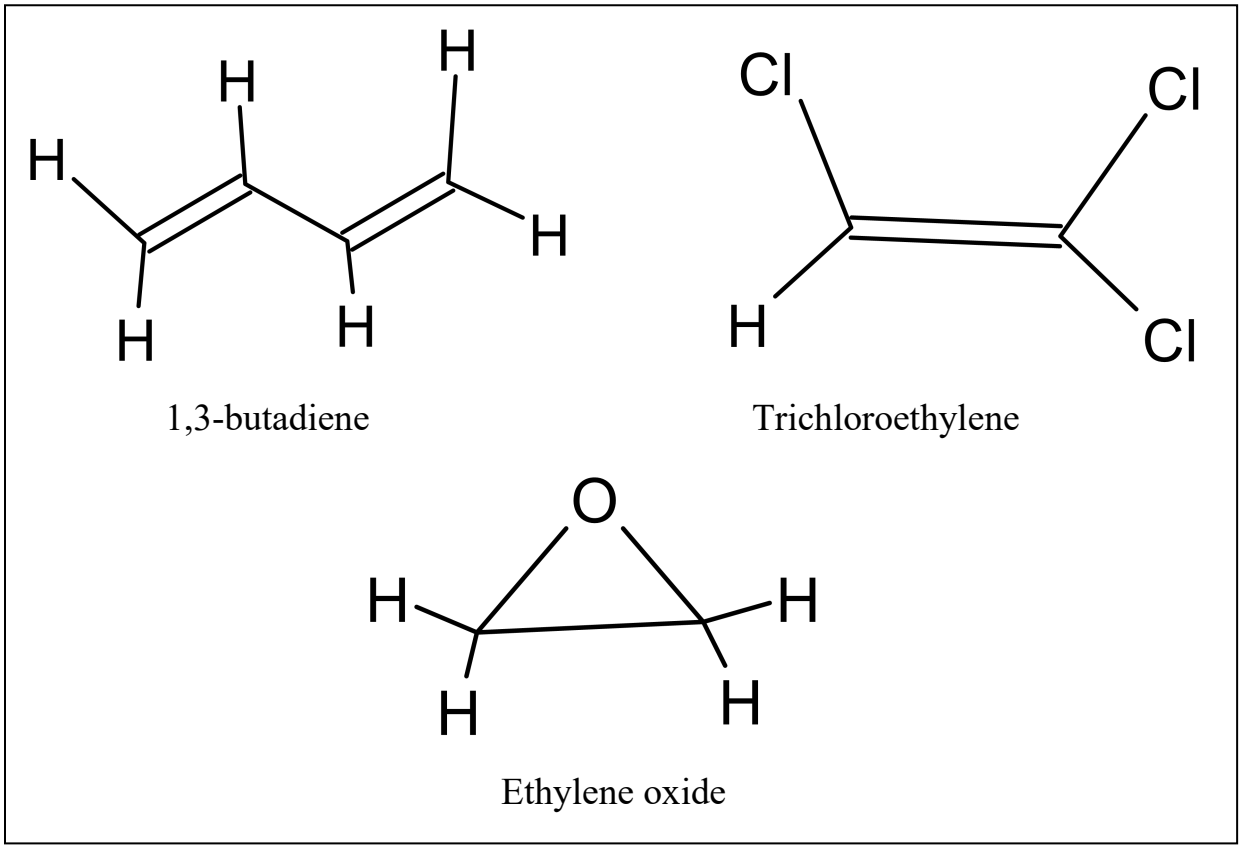



**Figure 1.3**: Structures of some compounds that can cause tumour or cancerous tumour.

1,3-butadiene is used to manufacture polymers and synthetic rubbers in plastic industries. This chemical is known to stimulate the development of non-Hodgkin's lymphoma (Grosse *et al.*, 2007). The dry-cleaning company makes use of Trichloroethylene as a solvent for the removal of grease from metallic surfaces. This solvent is reported to cause renal cell cancer (Guha *et al.*, 2012). Ethylene oxide is a sterilizing agent deployed in facilities that manufacture sterile items for medical uses. This compound is known to cause lymphatic and haematopoietic cancers (Loprieno, 1975).

### 1.4.2 Virus and parasite as causes of cancer

i. **Human papillomavirus**

Cervical cancer is a result of infection by the Herpes simplex virus type 2. (Zur Hausen, 1996). HPV-16 and 18 produces proteins (E5, E6, and E7) having carcinogenic activities (DiMaio and Petti, 2013; Tommasino, 2014). E6 activates chromosomal aberration by coupling to p53. This disrupts normal function leading to cellular degradation (Scheffner *et al.*, 1990). E7 interferes with the regular role of retinoblastoma proteins which results in the inactivation of such proteins (Boyer *et al.*, 1996; McLaughlin-Drubin and Münger, 2009). Epstein reported the association of virus particles (Epstein Barr-virus) with the occurrence of tumours in the abdomen and jaw of local minors in Uganda (Burkitt, 1983).

ii. **Ultraviolet radiation**

A cyanotic redness on sailors' skin caused by sun exposure was described by skin disease expert Dr. Paul Unna. He detailed the histology development of the lesion into a cancerous tumor. The sun's impact on the skin triggered this example of adult skin cancer (Unna, 1894).

iii. **Fungal infection**

***Aspergillus flavus***

The fungus *Aspergillus flavus* is known to express a toxin known as Aflatoxin. Aflatoxin B1 is carcinogenic and induces liver tumours in rats without cirrhosis. Primates have been observed to suffer from liver tumours as a result of exposure to Aflatoxin. A common food item that is easily contaminated by Aflatoxin is groundnuts, which must be consumed with caution (Groopman *et al.*, 1996).

iv. **Bacteria**

A spiral-like Bacterium was found in the stomach samples by Dr. Robin Warren. This bacterium, *Helicobacter pylori*, was shown to be present in 80% of individuals with gastric ulcers and 100% of those with duodenal ulcers. The organism has been observed to appear less often than in regular tissues when stomach ulcers develop into cancer (Kang *et al.*, 2006)

### 1.4.3 Wood dust and alcohol

Wood workers were observed to have an increased occurrence of nasal cancer. This effect was largely seen in those exposed to hardwood dust (Nylander and Dement, 1993) . High alcohol consumption is also observed to cause cancer of the oesophagus (Pöschl and Seitz, 2004).

## 1.5 Treatment options for cancer

### 1.5.1 Drug treatment

Pharmacological therapy is essential to eliminate malignant cells that have metastasized. Chemotherapy, which kills most of the rapidly dividing cells, including both malignant and healthy cells, has recently been the cornerstone of pharmaceutical therapy. To generate additive or synergistic effects, cytotoxic medications are most effective when administered in combination. Success has come with the proviso that medications must be able to be combined at their individual therapeutic dosages without causing unwanted side effects. Combined chemotherapy with cytotoxic agents has been justified by the co-administration of medications with various molecular mechanisms, enhancing tumor cell death while lowering the risk of developing drug resistance and decreasing overlapping effects (Al-Lazikani *et al.*, 2012).

### 1.5.2 Radiotherapy

Radiation therapy is the most-effective cytotoxic therapy available for the treatment of localized solid cancers. The success of this approach is exemplified by the fact that about 60% of patients with cancer continue to receive curative radiation therapy. Clinical radiation treatment involving physical dose delivery methods has dramatically increased during the last 20 years. There have been significant advancements in computer-aided, 3D therapy scheduling techniques with high-precision equipment which monitor organ motion throughout delivery. Usually, ionizing radiation is used to cause DNA damage as a kind of anticancer therapy since it can result in fatal lesions to the cancerous cells (Schaue and McBride, 2015).

### 1.5.3 Surgical intervention

Surgery's importance in the treatment of cancer patients is for the local management of the illness and for instance with mixed histology. Achieving local control over the primary site is an important benefit of surgical intervention. In circumstances when the preoperative medical diagnosis is unclear, surgery is also done. There is little doubt that surgery has a place in the holistic treatment of early tumours lacking lymph node involvement (Inoue *et al.*, 2012).

### 1.5.4 Immunotherapy

Immunotherapies function by obstructing vital metabolic processes or mutant proteins necessary for the continued existence and proliferation of malignant cells. In molecularly defined individual subgroups, these medications can stop the growth of tumours and cause startling regressions. Concurrent with these developments in the study of oncogenic pathways, immunotherapy has also been shown to increase the life expectancy of cancer patients (Vanneman and Dranoff, 2012).

### 1.5.5 Alternative medicine in cancer management

Current statistical analysis has shown that about 87% of people with cancer disease has taken a minimum of one type or form of alternative medication (AM) or therapy. Alternative medicine refers to the use of non-conventional approaches to cure diseases. Such practices make use of and are not limited to formulations including biological-based medications (vitamins), mind-body attunements (yoga), body manipulations (chiropractic), energy therapies (reiki) and alternative medical systems (natural products and traditional medicine). The health benefits of using AM as outlined by cancer patients include the amelioration of side effects due to chemotherapy, improvement of life quality, immune booster, curative effects and re-occurrence

prevention. Sometimes, the involvement of AM usually forms part of a get-well-soon program (Balneaves *et al.*, 2022)

### 1.6. Reproductive organ related tumour and cancer

These are tumours and cancerous diseases that occur in the reproductive organs of both male and female human beings. They usually affect the breast, uterine cervix, vulva, endometrium, ovaries, prostate, testicle, and penis. Prostate and uterine cervix remain the highest cause of mortality in this category (Kim *et al.*, 2017; Sung *et al.*, 2021; Niu *et al.*, 2022; Gadiraju *et al.*, 2022)**.**

#### 1.6.1 Epidemiology of BPH, prostate and uterine cervix cancer

In the year two thousand two hundred and twenty-two, GLOBOCAN reported that cancer of the reproductive organs accounted for 15.1% of new cancer cases. Relative mortality of 11.2% compared to all fatal cancer cases was due to reproductive organ-related carcinoma. The highest diagnosed cases for the year in question for males were prostate cancer and uterine cancer for females. The incidence rate for prostate cancer was 7.3% and 3.1% for uterine cancer. Mortality for both cases was estimated to be 3.8% (Sung *et al.*, 2021).

Global estimates indicated a 105% increment in the incidence rate from 1990 to 2019. Age-specific incidence reached the zenith between the age bracket of 65-69 and then declined. There is strong evidence that hereditary is equally implicated in the occurrence of BPH. Men of African ancestry show higher chances of developing the disease (Conti *et al.*, 2021). Population growth in the western sub-Saharan region of Africa caused a 122.3% increase in the incidence rate of BPH. These statistics are staggering but could be tremendous in reality since BPH cases are usually under-reported (Xu *et al.*, 2021)

### 1.7 Benign Prostatic Hyperplasia (BPH)

It is a pathologically induced prostate gland growth that results in lower urinary tract symptoms (LUTS). This growth is as a consequence of the proliferation of the stromal and epithelial cells of the transitional region of the prostate gland. This enlargement causes a compression attack on the urethra that leads to obstruction of urine flow. The resultant effect of this is overactivity or dampened contraction of detrusor muscle. BPH and Bladder Outlet Obstruction present symptoms such as urinary stream , splitting of the urinary stream, hesitancy, straining to void, terminal dribbling, obstruction, increased frequency, urgency and incontinence (Roehrborn, 2008; Thorpe and Neal, 2003).

#### 1.7.1 Pathophysiology of BPH

BPH has both stationary and active features of the disease. The stationary component is due to the compression by the enlarged prostate gland resulting in periurethral and bladder outlet obstruction. This gives rise to voiding symptoms and flow obstruction. Dynamic components result from the tension exerted on the urethra by the smooth muscle of the prostate, the exerted tension results in increased urine resistance (Ng and Baradhi, 2022).

##### 1.7.1.1 Role of androgen

The development of BPH is only possible with the involvement of androgens produced by the testis. A nuclear membrane-bound steroidal enzyme 5-α-reductase 2 (also known as 3-oxo-5-α-

steroid-4-dehydrogenase-2), converts testosterone into dihydrotestosterone (DHT), which accounts for about 90 per cent of the androgen in the prostate. The intraprostatic DHT, even with ageing, remains significantly high (Roehrborn, 2008).

Growth factors depending on DHT are responsible for the interactions between the prostate's stroma and epithelium (Foster, 2000). The pathological development of BPH, even though not fully understood, has to do with the interference of the hormone called DHT-related homeostasis of cell growth and apoptosis which favours the former over the latter (Griffiths *et al.*, 1997;Carson and Rittmaster, 2003). Growth factors activated by DHT, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF) and insulin-like growth factors (IGFs), regulate cellular proliferation in the prostate. The production of transforming growth factor β (TGFβ), which is responsible for the modulation of apoptosis, is controlled by the hormone called DHT. The general effects, and not increased levels or activities of these growth factors, lead to BPH (Kim *et al.*, 1996; Niu *et al.*, 2001).

#### 1.7.1.2 Role of estrogen

It's vital to take into account both the effects of estrogen on the prostate when discussing the pathophysiology of BPH. It has been demonstrated that estrogen stimulates the development of stromal cells originating from both healthy and BPH prostate tissue. Through the action of the estrogen receptor, estradiol promotes the proliferation of prostate stromal cells whereas it does not affect the epithelial cells. According to several reports, androgen and estrogen work together to promote prostate cell development (Ho and Habib, 2011).

#### 1.7.1.3 Inflammation

The involvement of infective agents such as bacteria and viruses has been linked to developing BPH with an inflammatory origin. During the inflammatory situation, the stromal cells stimulate the expression of proinflammatory cytokines and chemokines that activate prostatic prague e resulting in BPH (Chughtai *et al.*, 2011). The infiltration of induced T-lymphocyte cells in the BPH tissues secret numerous growth factors that cause prostatic stromal and glandular hyperplastic growth, confirms inflammatory involvement in BPH pathogenesis. Recently, studies have shown the paths of precise inflammatory factors and their potential role in the pathogenesis of BPH. High levels of IL 2, IL 4, IL 7, IL 17, IFNγ, and their corresponding receptors have been identified in BPH tissue. IL2, IL 7 and IFNγ have been demonstrated to activate the proliferation of stromal cells *in vitro*. Chronic inflammation in BPH has been linked with the upregulation of cyclooxygenase 2 in the glandular epithelium, leading to the expression of proinflammatory prostaglandins, which resulted in the enlargement of prostatic tissues (Robert *et al.*, 2009).

### 1.7.2 Risk factors BPH

#### 1.7.2.1 Obesity

Adipose tissue level is known to be directly proportional to prostate volume (Parsons *et al.*, 2013). It was reported that every 0.05 rise in the abdominal obesity parameter was linked with a 10% rise in the risk of BPH ($P < 0.003$) (Kristal *et al.*, 2007). Likewise, elevated fasting blood glucose levels and diabetic conditions were positively correlated with prostate enlargement (Parsons *et al.*, 2006). Systemic inflammation was advanced as the possible molecular explanation of the link between obesity and BPH. Also, the stimulus responsible for the initiation

of prostate cancer was found to be inflammation, which, of course, BPH may be considered as a nonmalignant version that is initiated by oxidation and inflammatory factors (De Nunzio *et al.*, 2012)

#### 1.7.2.2 Diet

There is no clear-cut evidence of diet involvement in the pathophysiology of BPH. It was previously reported that milk and dairy products increased the risk of BPH, which was later discovered not to be involved (Lagiou *et al.*, 1999). However, it is known that some macro and micronutrients lower the possibility of having BPH. A higher risk of developing BPH was observed with some frequently consumed diets like cereals, bread, eggs and poultry, while a lower risk was observed for consuming legumes, cooked vegetables, and citrus fruits (Bravi *et al.*, 2006). Furthermore, fruits and diets that are low in fats and have high fibre content are generally known to reduce the propensity of future development of BPH (Denis *et al.*, 1999).

#### 1.7.2.3 Genetics

The contribution of genetics in the occurrence and severity of prostate cancer is the most studied to date. However, a single nucleotide polymorphism has been implicated in the possibility of BPH development. It was also reported that people with risk allele ‘C’ had a higher risk for BPH. It is on record that first-degree family member of men who have suffered the BPH disease have a fourfold likelihood of developing BPH (Jiao *et al.*, 2013; Qi *et al.*, 2013).

### 1.7.3 Diagnosis

Essential investigation tools are available for clinicians to diagnose BPH. There are several published guidelines to use when investigating cases related to BPH (McVary *et al.*, 2011).

#### 1.7.3.1 Physical examination of the prostate

A digital rectal examination (DRE) is an important diagnostic tool. This should be done with an emphasis as regards the urinary tract. The suprapubic area must be observed for symptoms associated with bladder distension. The penis could be observed for signs of phimosis. Another symptom to look out for is meatal stenosis or strange lesions on the penis. DRE assists in the assessment of prostate gland volume. Even though lower in accuracy when compared to Transrectal Ultrasonography (TRUS), it also assists in the determination of the outline and uniformity of the prostate gland. Firm or hard regions or nodules can also be readily determined using this method, which also helps to rule out the likelihood of developing prostate cancer (Gratzke *et al.*, 2015).

#### 1.7.3.2 Urinalysis

Urine samples can be examined to identify markers linked to metabolic ailment, renal disease and urinary tract infections. The European Urological Association guidelines (Gratzke *et al.*, 2015) reported that the evidence for the use of urinalysis might be limited, but the benefits outweigh the costs. Investigations like cystoscopy should be done if haematuria is observed. Leukocytes and nitrite indicate infection, which should be treated to improve prognosis (Davis *et al.*, 2012).

#### 1.7.3.3 Prostate-specific antigen test

It is in the report that there is a positive link between the level of PSA and prostate volume. A community-based study in the Netherlands showed a 72% chance of having a prostate volume of less than 30 mL when the serum PSA level was between the range of 2.1–2.5 ng per ml. The more the increase in the PSA level, the higher the likelihood of an enlarged prostate (Bohnen *et al.*, 2007).

#### 1.7.3.4 Renal function test

A renal function test is usually done when renal insufficiency is suspected by measuring serum levels of creatinine and urea (Gratzke *et al.*, 2015). Urinary retention, hydronephrosis and renal insufficiency usually occur in patients with obstructive symptoms due to BPH (Gerber *et al.*, 1997). Renal insufficiency affects 11% of elderly people with symptoms of the lower urinary tract, according to data (Gerber *et al.*, 1997). With successive declines in GFR and renal blood flow, aging may also be to blame for the loss of kidney functionality (Weinstein and Anderson, 2010).

#### 1.7.3.5 Urinary tract imaging.

Urinary tract imaging is necessary for patients with a urinary tract infection, urolithiasis, renal insufficiency, or haematuria. There are several methods by which the prostate can be imaged, such as Transrectal Ultrasound Scan (TRUS), Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) (Gratzke *et al.*, 2015). Amongst the methods mentioned above, TRUS, or transabdominal ultrasonography, is the most widely used and easily performed. TRUS is the best for assessing prostate volume (Loch *et al.*, 2007). TRUS is also pivotal in determining the extent of intravesical prostatic protrusion, the length between the bladder neck and the tip of the

prostate median lobe. However, patients with a history of haematuria should be investigated appropriately per guidelines (Davis *et al.*, 2012). Patients with suspected anatomical urethral stricture should be investigated using urethrocystoscopy because such investigations can provide useful information capable of changing the course of treatment (Stravodimos *et al.*, 2009).

### 1.7.4 Management of Benign Prostatic Hyperplasia

#### 1.7.4.1 Watchful waiting

Patients with mild and no complicating symptoms can be managed with watchful waiting, which includes providing advice about lifestyle changes that can assist in ameliorating symptoms. These lifestyle changes entail advice about the volume, type and timing of liquids taken, avoidance of caffeine, abstinence from alcohol, and control of stool with avoidance of constipation (Gratzke *et al.*, 2015). Over-the-counter medications for the treatment of common cold should be avoided because they can worsen symptoms due to their α-adrenergic agonist activity at the bladder and neck, which may result in urinary retention (Verhamme *et al.*, 2008).

#### 1.7.4.2 Pharmacotherapy

Medical therapy is important to alleviate lower urinary tract symptoms, reduce the possibity of progression and enhance the quality of life. Several classes of medical treatments are available for treating lower urinary tract symptoms due to BPH, including those used to alleviate Bladder Outlet Obstruction (BOO) and treat bladder overactivity. The predominant receptor in the prostate stromal smooth muscle is α-1-Aadrenoceptor several medical treatment options target this and other adrenergic receptor subtypes (α-1-B-adrenoceptor and α-1-D-adrenoceptor).

Inactivation of these α-1-A-adrenoceptors (also present in the bladder neck) results in smooth muscle relaxation and subsequent improvement of symptoms (Lepor, 2007).

**1) Selective α-1-adrenergic antagonists**

Tamsulosin and Silodosin are the first-line drugs available for treating men with lower urinary tract symptoms. Tamsulosin displays a selective coupling or affinity for the α-1-A- adrenoceptor against the α-1-B-adrenoceptor. In contrast, it displays no selective affinity for the α1Aadrenoceptor against the α-1-D-adrenoceptor (Foglar *et al.*, 1995). Silodosin shows 162/1 selectivity for α1Aadrenoceptors against α1B adrenoceptors106. Both drugs can rapidly improve symptoms within three to four days of the commencement of treatment, and the effects can last for about 12 months. The effects of treatments are increased bladder capacity and decreased detrusor overactivity (Yamanishi *et al.*, 2010).

**2) 5-α-reductase inhibitors**

Finasteride and Dutasteride, known as 5-α-reductase inhibitors, inhibit the conversion of testosterone to DHT, which results in a reduction in the size of the prostate. The effects of these agents are observable between four to six weeks of continuous administration. The maximal effect is observed between three to six months of treatment (Gratzke *et al.*, 2015).

**3) Targeting different receptors with combination therapy**

Combining medications that elicit effects by targeting different pathways can be very useful. This effect can be seen in the combination of Dutasteride and tamsulosin, which has been used to improve voiding symptoms and the risk of disease progression (Roehrborn *et al.*, 2010; Regadas

*et al.*, 2013). Studies have shown that combining different agents, such as PDE5 inhibitors, α1adrenergic antagonists and antimuscarinic agents at various levels, significantly improved the symptoms of BPH (Filson *et al.*, 2013).

#### 1.7.4.3 Surgical intervention

The gold standard surgical treatment for BPH has been TURP. Even though TURP has been the most studied and prague e procedure for decades, the complication rate for this intervention is about 20%. Some transurethral procedures are under development to treat BPH through ablation, resection, enucleation or compression to curb the complication challenges of TURP. One of these novel technologies is Aquablation, which involves using a high-pressure water jet and surgical laser to treat BPH (Faber *et al.*, 2015). The water jet is mechanically controlled to deliver a high-energy jet to the desired prostate area to cause surface haemostasis to ablate the tissue, resulting in the removal of obstructing tissue. Another is Prostate Artery Embolization (PAE), whereby the inferior vesicle artery is ablated using hydrophilic micro-catheters and polyvinyl alcohol (Somani *et al.*, 2014). This alcohol contains microparticles that cause occlusion of the vascular bed. This occlusion results in the ischaemic necrosis of the targeted prostate region leading to shrinkage and alleviation of symptoms (Camara-Lopes *et al.*, 2013).

### 1.7.5 Emerging therapies

#### 1.7.5.1 β3-adrenoceptor agonists

Human prostatic stromal tissues express more of β3-adrenoceptor than β1 and β2. The excitation of these receptors ameliorates the alpha1 adrenoceptor-initiated contractions. B3-adrenoceptor

would be explored as a valuable drug target for future drug development. Nevertheless, the use of beta blockers elevates the risk of the occurrence of BPH. This proves that coupling otherwise will elicit a favourable therapeutic response (Haynes and Hill, 1997; Meigs *et al.*, 2001).

#### 1.7.5.2 muscarinic receptor antagonists

The literary belief that inhibition of detrusor muscles by muscarinic antagonists poses a challenge to using this class of agents for treating BPH. Side effects such as urinary retention and passing urine constriction are uncommon. To this end, their use for treating BPH is usually restricted. Current clinical trials include muscarinic antagonists such as Solifenacin, Oxybutynin and Tolterodine, and alpha-one adrenoceptor antagonists, such as Doxazosin and Tamsulosin, have shown significant amelioration of storage capacity deficit symptoms. Lower urinary tract symptoms treatment using this method was efficient (Athanasopoulos, 2010; Chapple, 2010).

#### 1.7.5.3 CB1-cannabinoid receptors

Cannabinoid receptors have been expressed on the epithelial region of the human prostatic tissue. According to an investigation, excitation of these receptors in rats resulted in the prevention of contraction of the rat prostate. Stimulation of CB1 cannabinoid receptors has been shown to inhibit contractions of the prostate gland. Necessary enzymes that degrade endocannabinoids are also present in the human prostate, including CB1 and CB2 receptors in the stroma area. These receptors inhibit contraction (Ruiz-Llorente *et al.*, 2003; Tokanovic *et al.*, 2007).

#### 1.7.5.4 Prostanoid receptor EP2

Inflammatory processes have been implicated in the pathogenesis of BPH. The activity of prostaglandins on the prostate has not been thoroughly analyzed, but some prostaglandins have been known to cause prostate contraction. Precisely prostaglandin E2 exhibit an inhibitory effect on the contractility of the rat prostate via a prostanoid receptor of the EP2 subtype (Tokanovic *et al.*, 2010). The nomenclature of prostaglandin was coined from the word prostate because it was isolated from seminal fluid for the first time and was thought to originate from the prostate (Bergström *et al.*, 1968; Kitada and Kumazawa, 1987; Najbar-Kaszkiel *et al.*, 1997). Although prostanoids have pervasive effects all over the body, they could still serve as a drug target for future drug development (Narumiya *et al.*, 1999).

#### 1.7.5.5 Endothelin ETB receptor

The peptide endothelin-1 is known to be secreted by the glandular epithelium of the human prostate. *In vitro* experiments were able to cause the prostate epithelium cells derived from human to secret endothelin-1. Man, the contractile action of this peptide on human BPH tissue is known to be mediated through Endothelin B (ETB). Scientific evidence has proven that this peptide stimulates the growth of prostatic smooth muscle cells facilitated through Endothelin A (ETA) and ETB receptors. These peptides' proliferative and contractile nature indicates their importance in drug development for BPH treatment (Langenstroer *et al.*, 1993; Webb *et al.*, 1995; Saita *et al.*, 1998; Walden *et al.*, 1998).

#### 1.7.5.6 Phosphodiesterase PDE5 receptor

Phosphodiesterase (PDE) is an important drug target that could be explored for treating BPH. Literature has reported 14 isozymes of phosphodiesterase, the most significant being the PDE4A,

PDE4B, PDE5A and PDE11A4. Scientific evidence has proven that activating the pathways involving these enzymes has caused a relaxation of the prostate smooth muscle. A mechanism believed to be through the activation of potassium channels (Drescher *et al.*, 1994; Stacey *et al.*, 1998; Yuasa *et al.*, 2000;; Fawcett *et al.*, 2000; Cook and Haynes, 2004; Haynes and Cook, 2006; Kedia *et al.*, 2006; Oger *et al.*, 2009; Fibbi *et al.*, 2010;). They are also believed to control the growth of human prostatic cells (Guh *et al.*, 1998; Adolfsson *et al.*, 2002). Actually, the potency of PDE5 inhibitor treatment either in combination or single therapy remains vague (Kaplan and Gonzalez, 2007; Kaplan and Hatzichristou, 2007).

#### 1.7.5.7 RhoA/Rho kinase

RhoA is a G-protein linked to α1-adrenoceptors. This G-protein activates Rho kinase to excite actin filaments into a state of contraction. RhoA and Rho have been characterized in the human prostate and have also been found to decrease cell proliferation. This critical pathway can be a helpful drug target for treating BPH since it can control growth and contraction during BPH. This assertion is yet to be confirmed through a clinical trial. Specific plant metabolites such as isoflavones useful in the treatment of BPH have been proven to work through this pathway (Rees *et al.*, 2003; Takahashi *et al.*, 2007; Seok *et al.*, 2008).

#### 1.7.5.8 Nitric oxide

During BPH development, there is usually a reduction in the nitrergic receptors found in the stroma tissue of the human prostate (Bloch *et al.*, 1997). Scientific studies have proven the attenuation effect of nitric oxide donors on contractions induced by noradrenaline on smooth muscles in human prostate tissue. This activity is believed to be excited through cGMP

production and coupling of the KATP channels (Hedlund *et al.*, 1997). The use of this pathway has been studied in clinical trials, which has indicated a significant improvement in symptoms of BPH (Klotz *et al.*, 1999).

### 1.7.6 Plants with anti-BPH activity

Some plants have been identified to have anti-BPH activity. These include:

#### 1.7.6.1 *Serenoa repens* W. Bartram (Arecaceae)

*Serenoa repens* also called saw palmetto is in the Arecaceae plant family. This plant is a palm that grows in abundance in the West Indies and the United States of America. The fruits of this plant are rich in β–Sitosterol, cycloartenol, oleic, caprylic, flavonoids, volatile oil and polysaccharides. It is used to treat BPH (Bent *et al.*, 2006).

Moreover, it has been widely researched as an acclaimed activity. Amongst the constituents, sterols and fatty acids are believed to exhibit antagonistic effects on 5-α-reductase, inhibiting seroconversion of testosterone to dihydrotestosterone, and activating the prostate gland growth. Apart from the mechanism of action, as mentioned earlier, several other probable mechanisms may be implicated. Such a mechanism may involve the effect of estrogen receptors in the gland and the

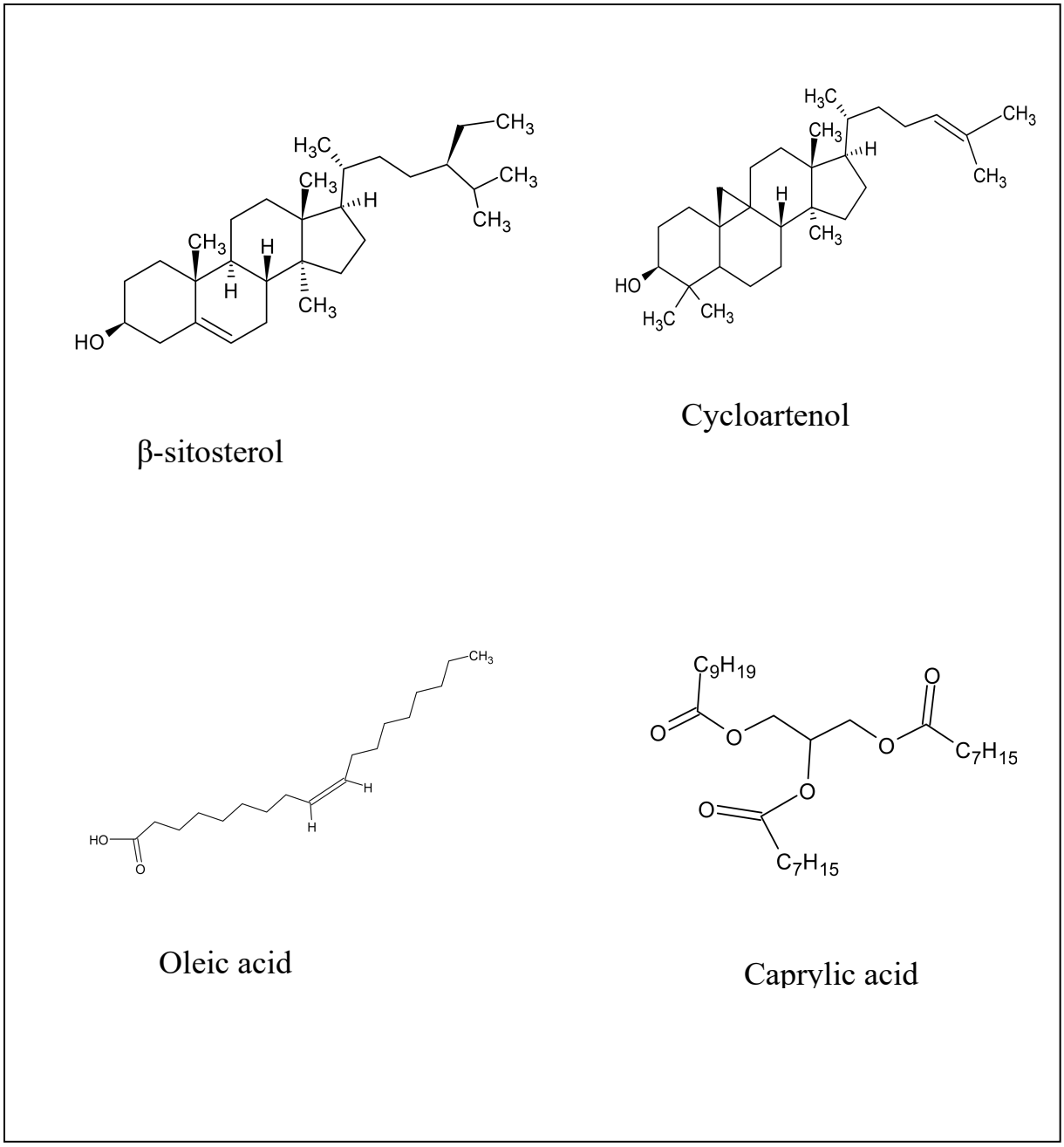



**Figure 1.4**: structures of some compounds with anti-BPH activity

anti-inflammatory and spasm dampening effects on the bladder detrusor muscle. Lately, scientists have been able to analyze the activity of *Serenoa repens* on the upregulation of genes associated with inflammation using cell cultures of human prostate carcinoma (Capasso *et al.*, 2003; Silvestri *et al.*, 2013). The attenuation effects of *S. repens* on prostatic cell proliferation may be due to the suppression of inflammatory mediators and the excitation of nuclear factor-kappa B expressed on the gland. Studies conducted in Italy to ascertain the efficacy of herbal remedies using saw palmetto on patients with BPH have shown it was effective in reducing prostate enlargement and urination. When saw palmetto was compared to tamsulosin, it was seen to have equal potency, and when combined with nettle, it was observed to have the same potency as finasteride. The plant is also reported to alleviate dysuria in senior citizens having BPH (Mantovani, 2010; Azimi *et al.*, 2012; Bertaccini *et al.*, 2012). Others have reported improvement in the peak urine flow and reduction in nocturia frequency compared to the control, and a contrary view by Tacklind *et al.* (2009) stating the non-improvement by saw palmetto on Lower Urinary Tract Symptoms (LUTS) and prostate size (Wilt *et al.*, 1998; Boyle *et al.*, 2004; Tacklind *et al.*, 2009; Mouraviev and McDonald, 2018). Clinical trials displayed that the side effects were reversible, such as stomach upset, prague , nausea, headache, allergy, and reduction of libido (Agbabiaka *et al.*, 2009).

#### 1.7.6.2 *Pygeum africanum* Hook. f. (Rosaceae)

*Pygeum africanum* (Rosaceae) is a tree about thirty meters tall and indigenous to Africa. The bark contains Secondary metabolites such as β-Sitosterol and Ursolic acid. A tea made from this plant has been used for ages to treat lower urinary tract infection in Africa. Several mechanisms

are used to explain the effects of *P. africanum* in treating BPH, such as inhibition of prostatic fibroblast proliferation, anti-inflammatory effect, blockade of 5-α-reductase, and suppression of prolactin quantity (Capasso *et al.*, 2003).

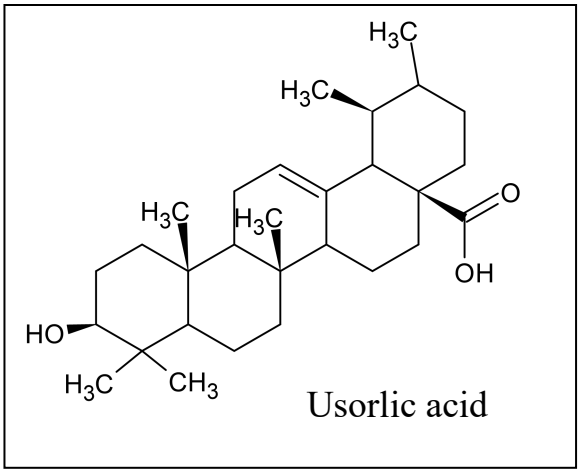



**Figure 1.5**: The structure of usorlic acid.

### 1.7.6.3 *Urtica dioica* L. (Urticaceae)

This plant is commonly called nettle, it belongs to the Urticaceae plant family. The roots employed to treat BPH are known to contain secondary metabolites that inhibit sex hormones and prevent the coupling of growth factors and their receptor, thus stopping prostate enlargement. The root is known to contain sterols and stero-glycosides, gly-proteins, polysaccharides and fatty acids. Nettle inhibits the proteolytic enzyme and demonstrates anti-inflammatory activity, and drives genitourinary tract inflammatory events. The plant is known to be a weak inhibitor of 5-α-reductase. A significant reduction in prostate size in BPH patients treated for six months with the plant is on the literature (Melo *et al.*, 2002; Safarinejad, 2005; Ghorbanibirgani *et al.*, 2013).

#### 1.7.6.4 *Secale pragu* L. (Poaceae)

The plant belongs to the Poaceae family. Pollens from *S. pragu* are digested microbially and extracted with water and acetone. The crude extract is made up of water-loving and acetone–loving fractions. The acetone soluble part is made up sterols. The secondary metabolites in these fractions remain vague, but several mechanisms have been proposed for the activity against BPH. Such mechanisms include 5-α-reductase antagonism, antagonism of α-adrenergic receptors and anti-inflammatory effects. Research has shown that the extract has activity against Lower Urinary Tract Symptoms (LUTS) (MacDonald *et al.*, 2000). The side effects of rye grass pollen are mild and well tolerated and rarely cause gastrointestinal side effects. The extract reduces nocturia symptoms (Wilt *et al.*, 1999; MacDonald *et al.*, 2000).

#### 1.7.6.5 *Cucurbita pepo* L. (Cucurbitaceae)

This is an annual prostrate plant indigenous to the USA. The plant is known as Pumpkin in the Cucurbitaceae family. The seeds have been known to contain $\Delta_7$-sterols (avenasterol and spinasterol, $\Delta_5$-sterol (sitosterol), fatty oils (oleic acid) and zinc. The $\Delta_7$-sterols are responsible for significantly reducing increased levels of DHT in BPH patients. Pumpkins exert a contractile activity on the bladder detrusor muscle. There are reports documenting that pumpkin seed ameliorates the LUTS. While some show no effect on prostate size. Some investigations showed that pumpkin seeds contain zinc that assists to shrink prostatic enlargement. How these mechanisms work remains equivocal (Blumenthal, 1998).

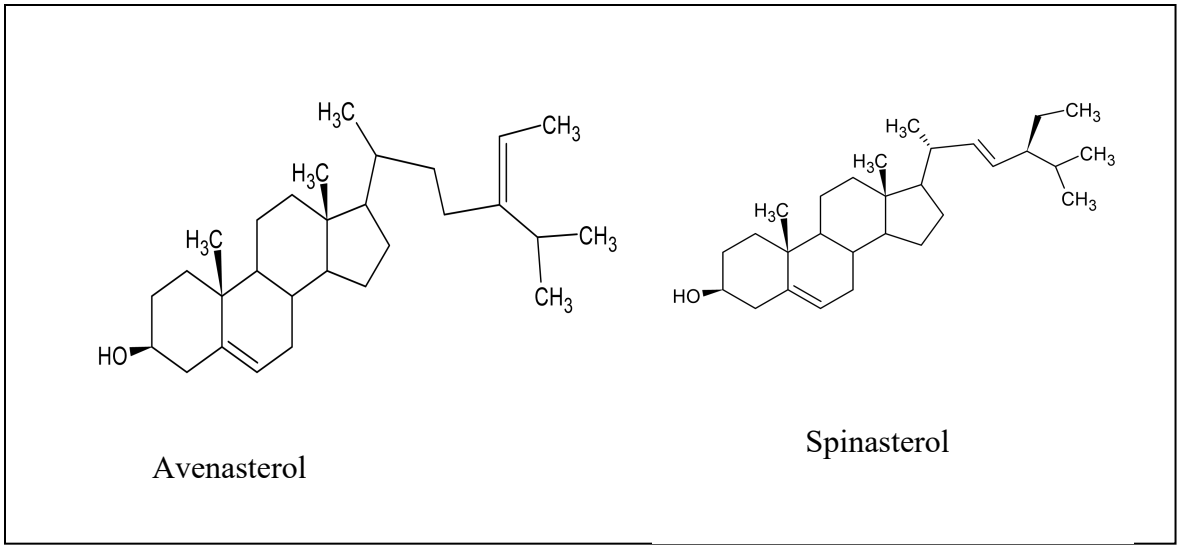



**Figure 1.6**: Structures of some compounds with anti-BPH activity

### 1.7.6.6 *Glycine max* L. Merr. (Fabaceae)

This is a herbaceous plant native to East Asia and belongs to the Fabaceae plant family. The beans from this plant contain isoflavones which are joint supplements for treating BPH (Wong *et al.*, 2008). Research has shown that isoflavones act on 5 alpha-reductase and 5 diphospho-glucuronosyltransferase to exert a reduction activity on the glandular epithelium. Other target enzymes that isoflavones inhibit are 17-hydroxysteroid dehydrogenase, aromatase and estrogen receptors in the stromal cell of the prostate, thus blocking prostate tissue enlargement (Pagano *et al.*, 2014). Large consumption of soy milk has been shown to reduce the occurrence of BPH (Jacobsen *et al.*, 1998; Morton *et al.*, 1997).

### 1.7.7 Isolated plant secondary metabolites with anti-BPH activity

#### 1.7.7.1 Lycopene

Chemically lycopene is a member of the secondary plant metabolites known as carotenoids. It is a fat-soluble natural pigment found in fruits and vegetables such as tomatoes, watermelon, papaya, pink grapefruit, and pink guava. *Solanum lycopersicum* L. (Solanaceae) is the basic nutritional supply of lycopene. Lycopene is sequestrated in the gland (Clinton *et al.*, 1996), meanwhile the sequestration process is equivocal. Chronic ailments Cancer inclusive is connected to oxidative stress pathogenesis. Lycopene is known to possess high antioxidant effect, which is a factor in the prevention of BPH. This vital carotenoid inhibits *in vitro* normal prostatic epithelial cells and stimulates apoptosis in hyperplastic prostate tissue (Obermüller-Jevic *et al.*, 2003). Furthermore, it promotes cell death in BPH tissue (Bowen *et al.*, 2002). Another mechanism could be the blockade of 5-α-reductase and interleukin-6 signalling (Herzog *et al.*, 2005).

#### 1.7.7.2 β-Sitosterol

β-Sitosterol belongs to a class of plant compounds referred to as phytosterols. It is mainly extracted from a South African plant called *Cynodon plectostachyus* (K. Schum) Pilg. (Poaceae). B-Sitosterol is reported to ameliorate Lower Urinary Tract Symptoms (LUTS) in patients with BPH (Wilt *et al.*, 1999).

### 1.7.8 Triterpenoids with antitumor activity

Recently, reports have shown that triterpenes possess inhibitory effects on tumour proliferation *in vitro* and *in vivo* settings. The following classes of triterpenes with their examples exhibit antitumor activity.

**1.7.8.1 Lupane-type pentacyclic triterpenoid**

Lupeol is a pentacyclic triterpene that is obtainable from natural sources such as cucumber, cabbage and pepper. Several studies have shown that lupeol possesses an array of biological effects including anti-inflammatory and antitumor effect geared towards prostate cancer, skin cancerous tumour and breast cancerous tumour. The mechanism of action could be due to the activation of pathways including nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt). Furthermore, other evidence has also demonstrated the possibility of an antitumor effect through the regulation of Bax/Bcl-2 (Saleem, 2009; Saleem *et al.*, 2008, 2004)

Betulinic acid and related molecules are members of the lupane-type pentacyclic triterpenoids. They are known to inhibit the growth of human melanoma, nerve tumour and children's malignant brain tumour cells.(Eiznhamer and Xu, 2004) *In vitro* analysis of this molecule has revealed that the compound inhibits the proliferation of HeLa and human leukaemia cell line (HL-60 (Bi *et al.*, 2012, 2007).

**1.7.8.2 Oleanane-type pentacyclic triterpenoids**

Oleanolic acid is a typical example of triterpenoids in this class of compounds. They occur extensively in flowering plants including ginseng, clove, liquorice, and panax studies in nude mice have revealed that they possess intense antitumor activity (Jutooru *et al.*, 2010). Soybean saponins are oleanane-type molecules with antitumor effect target at the proliferation of uterine

cancerous tumour cells and colon cancer (HCT-15) cells through the induction of early apoptosis and autophagy (Daveby *et al.*, 1998; Ellington *et al.*, 2005; Xiao *et al.*, 2007; Zhang *et al.*, 2013). 3-O-β-d-glucopyranosyl-hederagenin 23-O-α-d-ribofuranoside gotten from the root of *Pulsatilla vulgaris* Mill. (Ranunculaceae) inhibits the growth of tumour (Zhang *et al.*, 2013).

**1.7.8.3 Ursane-type pentacyclic triterpenoids**

Ursolic acid is a typical example of Ursane-type pentacyclic triterpenoids. It is commonly found in several plants including *Paulownia tomentosa* Steud (Paulowniaceae) and G*ardenia gummifera* L.f. (Rubiaceae). Ursolic acid is known to suppress the proliferation of the human tongue cancerous tumour cell line (TSCC-α ; HL-60). The compound also upregulates the apoptosis of breast cancer (MCF-7) (Manu and Kuttan, 2008; Wang *et al.*, 2012, 2012)).

The following compounds: 3α, 6α, 30-trihydroxy-urban-28-oic acid, 3α, 30-dihydroxy-6-oxo-urban-28-oic acid and 3α, 6α, α, 30-tetrahydroxy-urban-28-oic are isolated from *Paralasianthus hiananensis* (Merr.) H.Zhu (Rubiaceae) are known to attenuate the progress of tumour cells development (Zhang *et al.*, 2013). 3β, 23-dihydroxy-20α(H)-urs-12-en-28-oic acid and 3β, 19A, 23-trihydroxy-20α(H)-urs-12-en-28-oic acid 3β-O-α-l-arabinopyranoside are dual new compounds gotten from the root of medicinal tree known as *Ilex cornuta* Lindl. and Paxton (Aquifoliaceae) . They exhibit potent antitumor activity (Wang *et al.*, 2014).

**1.7.8.4 Cork-type pentacyclic triterpenoids**

The compound known as celastrol is a typical example of cork-type pentacyclic triterpene compounds with demonstrable cytotoxic effect on numerous human cancerous tumour cell lines. The mechanism of action in which the compound works is vague (Yang *et al.*, 2006).

#### 1.7.8.5 Lanostane-type tetracyclic triterpene compounds

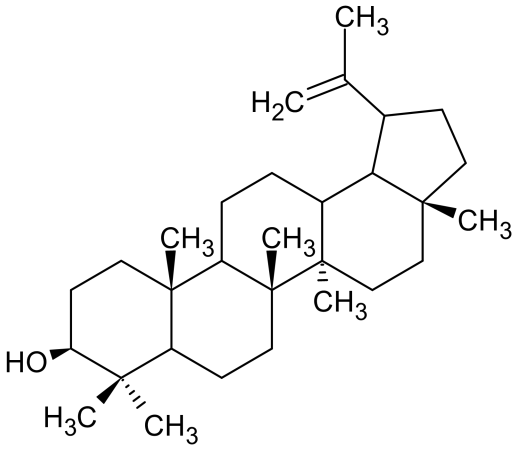
Ganoderic acid D is an example of a compound in this class. It is isolated from *Ganoderma lucidum* (Curtis) P. (Ganodermataceae). The study has shown that it suppresses the growth of uterine cervical cancer cells, with 50% minimum inhibition concentration of 17.3 μmol/L, through program cell death. The cell cycle apprehension phase is in the G2/M as a mechanism of action. Ganoderiol F, also isolated from the same fungus inhibits the proliferation of human tumour cells (Chang *et al.*, 2006; Yue *et al.*, 2008). Sterols isolated from *Poria cocos* Wolf. (Polyporaceae), upregulates the program cell death of tumour cells via the initiation of the caspase-3 pathway and the arrest of G2/M phase (Kang *et al.*, 2006).

Ananosic acid B and Ananosic acid C, isolated from *Kadsura longepedunculata* Finet. (Schisandraceae) and presented growth suppression of uterine cervical cancer cell lines.

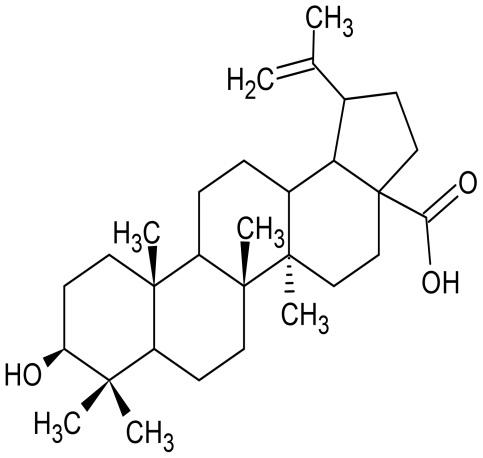
Eco-coccinic acids A, B, C, D and E isolated from the roots cuttings of *Kadsura coccinea* (Lem.) A.C.Sm. (Schisandraceae), another type of compound in this class also displayed growth-suppressive effects on HL-60 cells (Wang *et al.*, 2008).

Triterpene compounds gotten from *Daedalea dickinsii*, is known to exert prague e on the HL-60 cell lines. They act via the induction of the target cell inter-nucleosomal DNA fragmentation (Yoshikawa *et al.*, 2005).

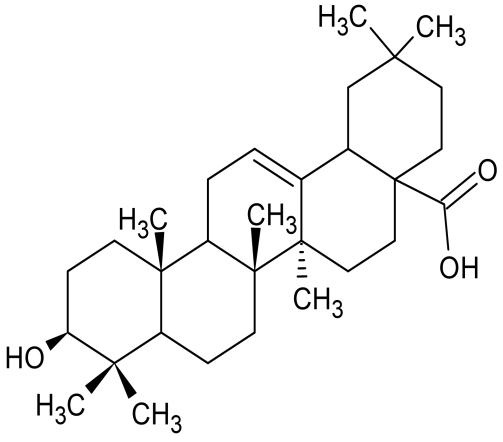
Inonotsuoxides A is a compound that was gotten from the sclerotia of *Inonotus obliquus* with a potent antitumor-enhancing activity on cell lines from lymphoma patients (Nakata *et al.*, 2007).



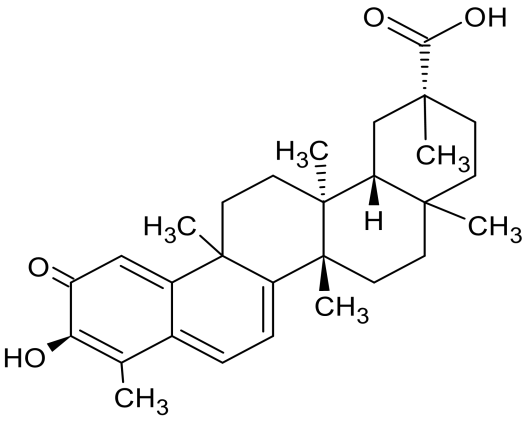

Lupeol




Betulinic acid

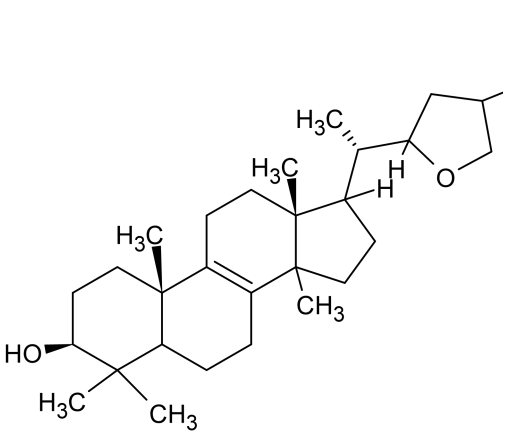



Oleanolic acid

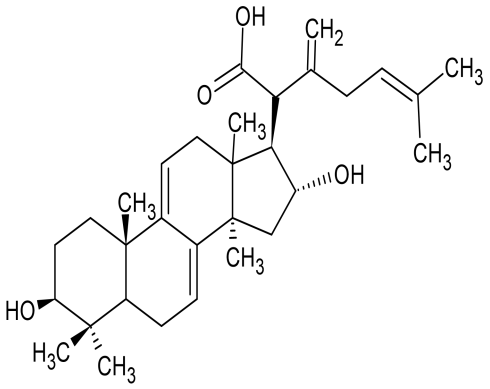



Celastro

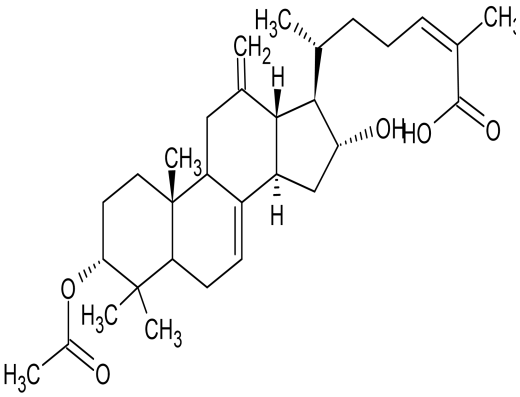
**Figure 1.7**: Structures of some triterpene compounds with antitumor activity.



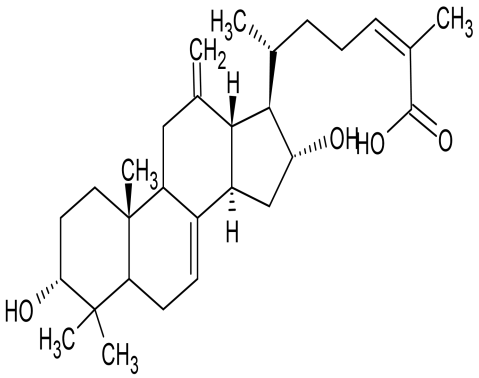

Inonotsuoxides A




Dihydrotrametenolic acid

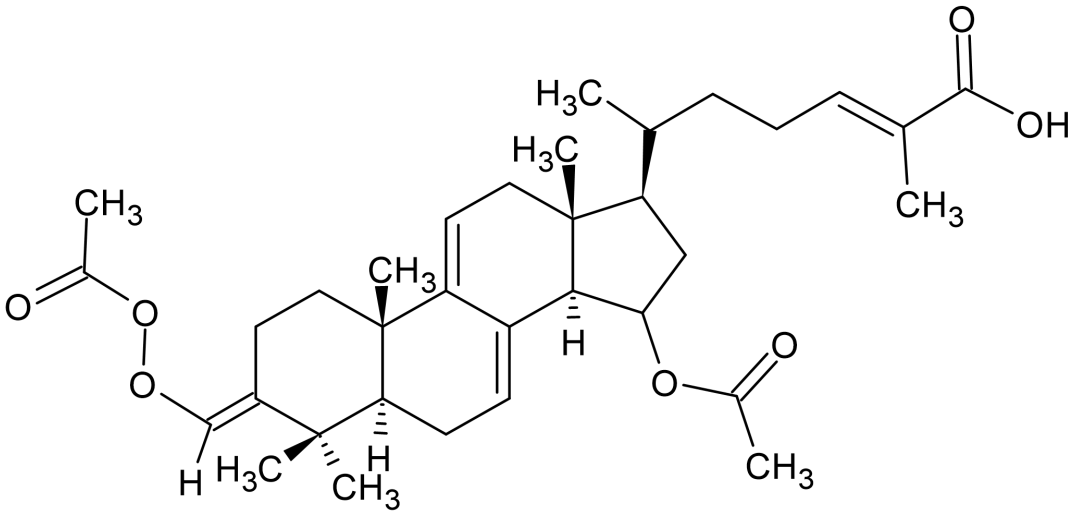



Ananosic acid B

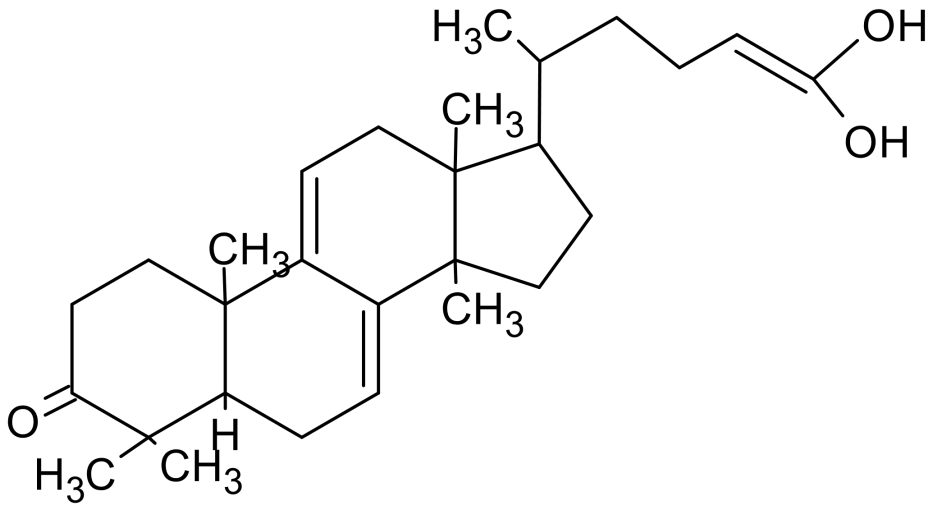



Ananosic acid C

**Figure 1.8**: Structures of some triterpene compounds with antitumour activity




Ganodermic acid



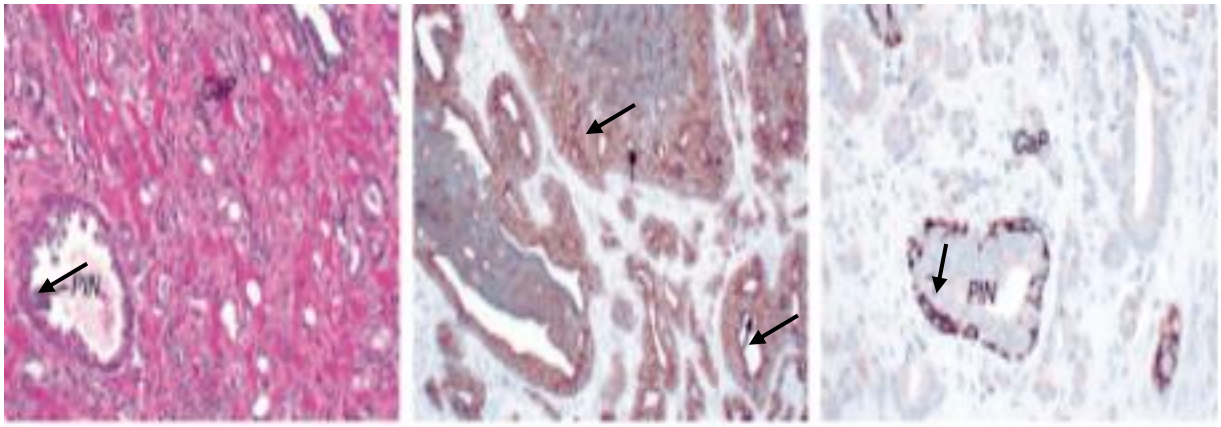
Ganoderiol F

**Figure 1.9**: Structures of some triterpene compounds with antitumour activity

## 1.8 Prostate carcinoma

### 1.8.1 Pathophysiology of prostate carcinoma

A type of lesion known as Prostate Intra-lesion Neoplasia (PIN) is highly implicated in the development of prostate cancer. (Plate 1A–C). Four different types of PIN lesion are identified as seen in figure 2.3. The four common types are tufting, micropapillary, cribiform and flat. Low-grade and high-grade types of PIN have been described. High-grade form is known to




precede cancerous tumour (Abate-Shen and Shen, 2000a).

**Figure 1.10 a-c:** Histopathological features showing PIN (Abate-Shen and Shen, 2000a).

Several lines of evidence implicate High-Grade PIN (HGPIN) as a preneoplastic lesion in humans. Initially, PIN lesions are spotted in the peripheral zone quite close to invasive carcinoma. Then followed by the appearance of HGPIN lesions which may take up to ten years

of latency period before the appearance of carcinoma. PIN lesions are known to secret low levels of PSA as such can be diagnosed through a biopsy. Proliferative Inflammatory Atrophy (PIA) known to be a precursor to PIN is an early step in prostate carcinogenesis. Once the initiation step is locked in, then several chromosomal aberrations such as the ones involving chromosome 8p and Androgen Receptor (AR) protein are set in motion to drive the progression to a full blown carcinoma (Abate-Shen and Shen, 2000a).

### 1.8.2 Risk factors

Smoking, nutrition, weight and exercise could be referred to as lifestyle factors which are modifiable. Studies have identified a strong correlation between these factors and the development of prostate carcinoma. The $6^{th}$ power of age, racial variation, genetic factors, male pattern baldness, digit length and Urinary tract infections are other commom etiology of prostate cancerous tumour (Cuzick *et al.*, 2014).

### 1.8.3 Drugs used for the management of prostate cancer

#### 1.8.3.1 Cabazitaxel

Cabazitaxel is a recently introduced drug as a second line treatment after docetaxel failure for metastatic castrate resistant prostate cancer (MCRPC) in men. It exert its effect by coupling to different sites on intracellular β-tubulin subunit of microtubule and promote the get-together of tubulin into microtubules. These microtubule bundles impair the natural dynamics of microtubules and appear in the mitotic phase of the cell cycle leading to mitotic block and apoptosis of the cancer cell (Abidi, 2013).

#### 1.8.3.2 Abiraterone Acetate

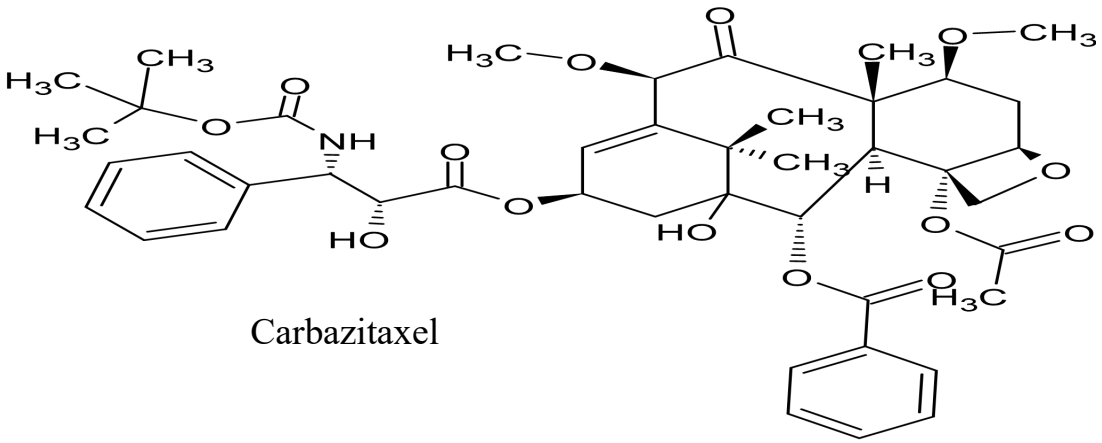
This compound is a prodrug that is licensed for the treatment of prostate carcinoma even in a resistant situation. It is changed *in vivo* to abiraterone that permanently antagonizes cytochrome P45017, a-hydroxylase/C17,20-lyase (CYP17). CYP17 is required for biosynthesis of androgens and is expressed in testicular, adrenal, and prostatic tumor tissues. Blockade of this receptor prevents further production of testosterone required by prostate cancer cells for duplication (Kluetz *et al.*, 2013).

#### 1.8.3.3 Enzalutamide

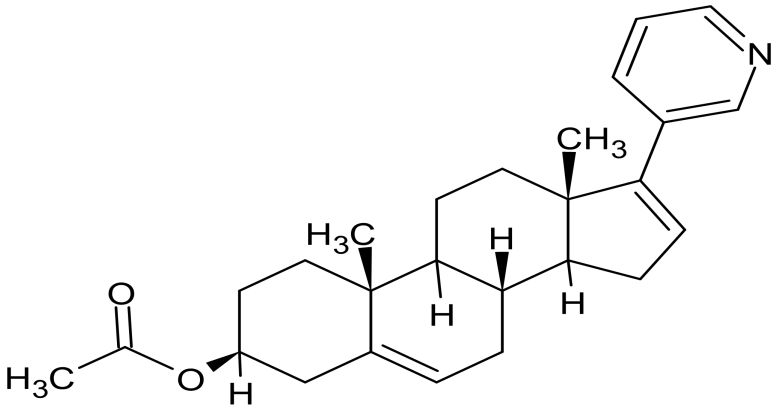
Detailed studies have revealed the receptors coupled to by the compound at three striking phases. It works by inhibiting AR, inhibition of nuclear translocation of activated AR, and by disrupting binding of activated AR with DNA. This prevents the androgen signaling mechanism from working in prostate cancer cells (Teo *et al.*, 2019).

#### 1.8.3.4 Sipuleucel-T

Sipuleucel-T is composed of the PA2024-laden antigen presenting cells (APC). The precise mechanism of action of this preparation remains equivocal, it is made to elicit a systemic immune response against the patient's prostate cancer cells expressing PAP. T-cells bind the processed recombinant antigen to the surface of the APC. Once bound, the T-cell activates circulating T-cell-mediated destruction of tumor cells by immunogenic cell death (Graff and Chamberlain, 2015).

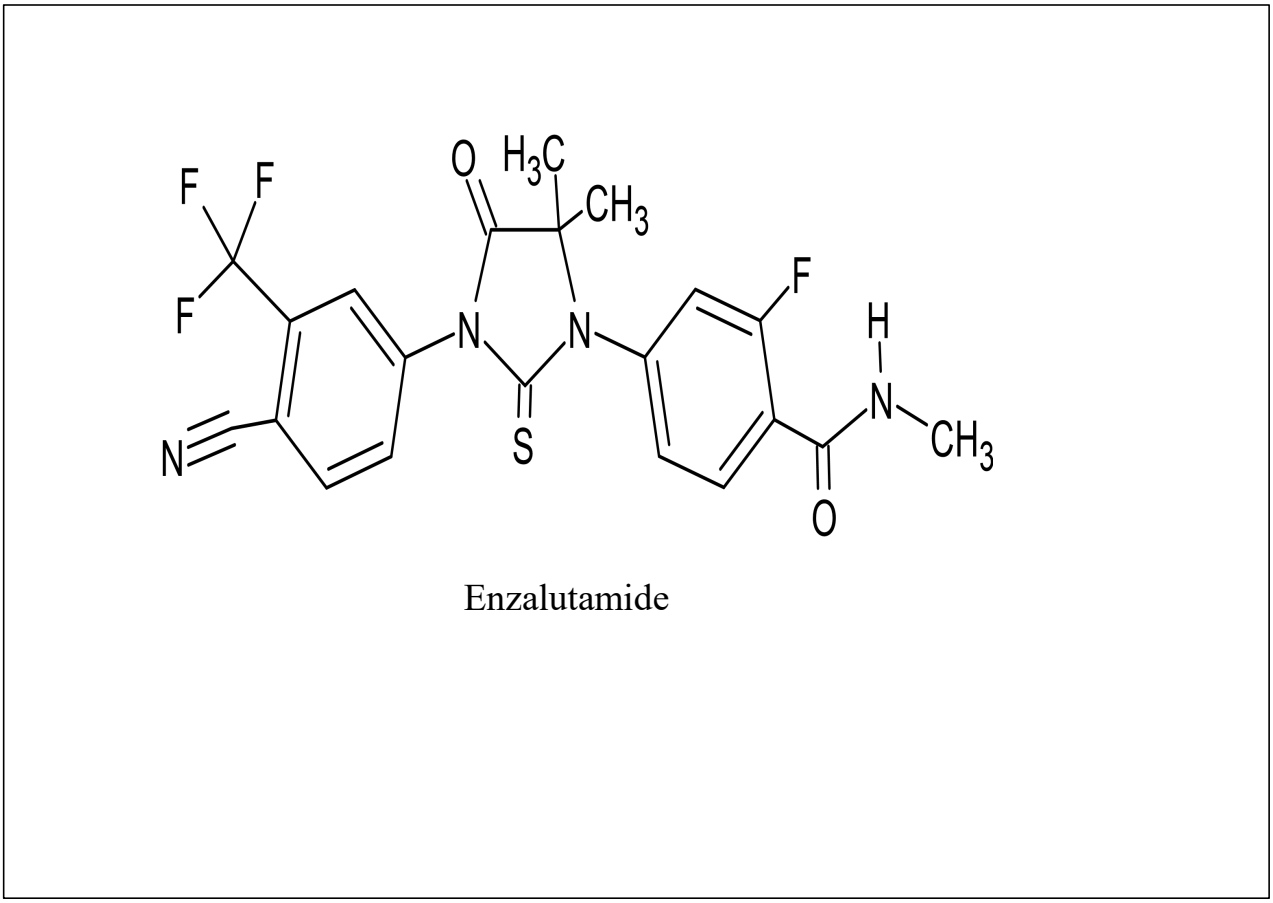


Carbazitaxel



Abiraterone

**Figures 1.11**: Structures of some common drugs used to treat prostate cancer




Enzalutamide

**Figure 1.12**: Structure of enzalutamide used to treat prostate cancer

### 1.8.4 Selected medicinal plants with activities against prostate cancer

#### 1.8.4.1 *Kalanchoe gastonis-bonnieri* Raym. Hamet (Crassulaceae)

The methanolic extract of this plant contains uronic acid compounds that activate apoptosis in cancer cells. These block an essential step in the development of cancer phenotype because

dodging of programmed cell death is a characteristic of cancer cells.. The induction of caspase-8-mediated apoptosis was detected in DU145, LNCaP, and PC-3 cells challenged with the extract (Shamaladevi *et al.*, 2016).

#### 1.8.4.2 *Acacia hydaspica* R.Parker (Fabaceae)

Methanolic extracts of the bark, twigs, and leaves of *A. hydaspica* contain polyphenolic compounds that attenuate expression of anti-apoptotic molecules and suppress the proliferation of PC-3 cells (Afsar *et al.*, 2016).

#### 1.8.4.3 *Trimeria grandifolia* (Hocsht) Warb. (Salicaceae)

An extract obtained from the leaves of the plant has anti-androgenic properties through the inhibition of LNCap in prostate cancer cell. These cancer cells depend on androgenic signals to promote growth (Bobach *et al.*, 2014).

#### 1.8.4.4 *Euphorbia triaculeata* Forssk. (Euphorbiaceae)

This a large family of flowering spurges. A stable food source known as cassava belongs to this family. Extracts of this plant are known to be cytotoxic to PC-3 cells. This effect was elicited through a mechanism that damages the DNA and leading to cellular apoptosis (Wu *et al.*, 2014).

#### 1.8.4.5 *Thevetia peruviana* (Pers.) K.Schum. (Apocynaceae)

Methanolic extract of the aerial parts of the plant contains some polyketides that induced apoptosis in HTB-81 (DU145) prostate cancer cells through the inhibition of cell-proliferation,

motility and increased membrane permeability. The resultant effects of these is DNA fragmentation (Ramos-Silva *et al.*, 2017).

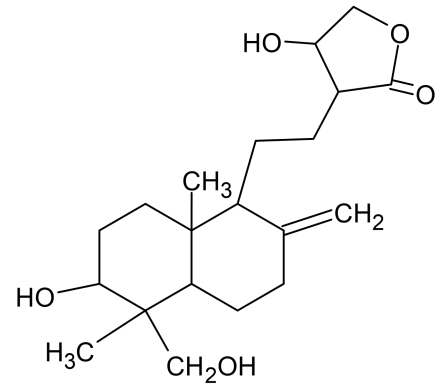
### 1.8.5. Isolated compounds with activity against prostate cancer

1. A diterpenoid lactone called andrographolide is obtained from the plant *Andrographis prague e* (Burm. F.) Wall.(Acanthaceae). It was shown that andrographolide (1–20 M) might block the prague e caused by IL-6 and stimulate STAT3 and the phosphorylation of extracellular signal-regulated kinases (ERK). Meanwhile, it might cause apoptosis in the $PC_3$ and DU145 prostate cancer cell lines and limit cell survival. In a mouse xenograft model with castration-resistant DU145 cell-derived human prostate tumours, andrographolide also inhibited tumour development. These results suggest that andrographolide may be developed as a medicinal drug for the treatment of prostate cancer (Chun *et al.*, 2010).

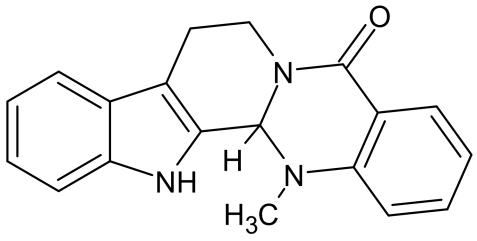
2. The Chinese herbal remedy *Evodia ruticarpa* (A.Juss.) Hook.f. (Rutaceae) contains evodiamine, which has been isolated. According to recent research, evodiamine inhibited the development of LNCaP cells in a dose-dependent manner in prostate cancer cell lines (100 nM–100 M) by causing apoptosis and cell cycle arrest in the G2/M phase (Kan *et al.*, 2004).

3. A new prague e of the cyclobutene type was discovered in *Peperomia tetraphylla* Hook (Piperaceae), this compound inhibited PC-3 cell growth and cancer cells were made to enter the G1 phase arrest and apoptosis (Y. Li *et al.*, 2015).

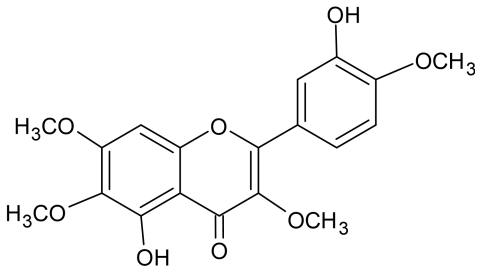
4. A polymethoxy flavone derived from *Vitex rotundifolia* L. (Lamiaceae), is an anti-inflammatory. By upregulating the proapoptotic protein Bax, downregulating the antiapoptotic protein Bcl-2, releasing cytochrome C from mitochondria, and lowering mitochondrial membrane potential in $PC_3$ cells, vitexicarpin (10–50 M) promoted apoptosis (Meng *et al.*, 2012).




Andrographolide




Evodiamine




Vitexicarpin

**Figure 1.13**: Structure of some compounds used to treat prostate cancer

### 1.9 *In vitro* model systems used in prostate tumour studies

*In vitro* models make use of cell cultures to determine the effects of compounds on tumours or cancerous tumours of the prostate. A huge array of cell lines are available for the testing of potential drugs.

#### 1.9.1 DU-145

DU-145 cells were discovered and extracted from a brain metastatic prostate tumour in 1975 (Stone *et al.*, 1978). This cell line is considered one of the gold standards of prostate cancerous tumour cell culture lines. DU-145 cells retain their phenotypic and genotypic properties when injected into mice. They invaded several organs, including the spleen and liver (Mickey *et al.*, 1977; Bastide *et al.*, 2002). The hallmark of cancer therapy is to activate apoptosis to reduce tumour mass. Pro and anti-apoptotic proteins are maintained in a fine balance in normal cells but are usually unbalanced in cancer ailments. DU-145 cells express the pro-apoptotic protein Bax which is an important point for consideration when selecting a cell line for study.

#### 1.9.2 $PC_3$

This cell line was isolated from a metastatic prostate tumour in the vertebra in 1979. This was done to address the concerns of researchers who needed cell lines that are hormone insensitive and express no androgenic receptors. It is similar to DU-145 cells in the aspect of hormone insensitivity. It is a highly aneuploid line with a karyotype modal number of 58. Importantly, this cell line expresses transferrin receptors and also responds to bone marrow transferrin during growth stimulation treatment (Keer *et al.*, 1990; Rossi and Zetter, 1992)

### 1.9.3 LNCaP

The LNCaP cell line was isolated from a human prostate adenocarcinoma that metastasized to the lymph node(Horoszewicz, 1980). This cell line responds to androgen. It also expresses Androgen Receptor (AR) and PSA mRNA/protein.(Veldscholte *et al.*, 1990). This cell line doubling time is every 60–72 hours. Xenografting with this cell line has afforded a 50% success rate (Sobel and Sadar, 2005).

### 1.9.4 C4-2B

The C4-2B cell line was extracted from a mouse vertebra as a result of LNCaP xenografts.(Wu *et al.*, 1994) These cells express AR and PSA mRNA/protein equally. P53 level is subnormal in this cell line and they do not express PTEN (Conley-LaComb *et al.*, 2013).

### 1.9.5 LAPC-4

This cell line was discovered as a result of subcutaneous xenografting experiments in mice (Klein *et al.*, 1997). Explants from prostate cancer patients were observed to maintain growth successfully in mice. LAPC-4 cells are androgen-dependent and are positive for AR/PSA mRNA/protein. They have the modal number 89. The doubling rate was found to be around 72 hour (Neshat *et al.*, 2001).

### 1.9.6 LAPC 9

They were first isolated from a patient's femur (metastatic cells) undergoing androgen ablation therapy. (Craft *et al.*, 1999). It was developed to address the need for cell lines that are responsive to androgens. They are WT AR/PSA sensitized. These cells develop tumours via

subcutaneous administration in normal mice at the dose of 10 cells. Tumour-volume doubling time is about 3 weeks. Removal of androgen usually results in growth arrest (Silvers *et al.*, 2010).

### 1.9.7 VcaP

VcaP expresses AR/PSAmRNA/protein and is androgen sensitive. They also express prostatic acid phosphatase (PAP) and retinoblastoma (Rb) in addition. Doubling time is found to be about 5 to 6 days. These cells proliferate sufficiently in normal mice (Sobel and Sadar, 2005).

### 1.9.8 MDA Pca 2a/2b

These are dual cell lines isolated from the same lesion from an African-American patient. (Navone *et al.*, 1997). They are androgen-sensitive and tumorigenic in mice. The doubling rates of the two cell lines are different. The 2a form doubles in about 82–93 hours while the 2b form doubles in about 42–73 hours. The 2b form proliferates at a faster rate than the 2a form *in vivo*. 2a forms palpable prostatic tumours within 11 weeks post-induction (Alimonti *et al.*, 2010).

### 1.9.9 RWPE-2

This cell line is the genetic product of modified human normal prostate epithelium. Through the art of genetic engineering. The Ki-ras gene and human papillomavirus 18 (HPV) genome was incorporated into the cell line to make it tumorigenic (Bello *et al.*, 1997). They express AR and PSA mRNA/protein and are hormone sensitized. They don't depend on hormonal stimulation to proliferate. HPV DNA dominates samples of cervical, vulvar, penile, and perianal cancers,
which make this cell line a preferred model for examining the role of viral factors in perianal carcinogenesis (Woodworth *et al.*, 1990).

### 1.9.10 PIN

PIN is a premalignant lesion in the development of prostate cancerous tumour (Abate-Shen and Shen, 2000b). PIN cells are a very useful cell line used to study oncogenic mechanisms of premalignant scenario.

### 1.10 *In vivo* model systems for the studies of prostate cancer

The transplant of prostate cancer cells into mice is known as xenograft. Three methods of xenograft are available such as subcutaneous, sub-renal and orthotopic. Subcutaneous xenograft technology gave the first ever transplantable tumour cell line (PC-82) (Hoehn *et al.*, 1980). This method is easy to manipulate and operate. It also requires a lower quantity of tumours for induction. However, only aggressive tumours can grow through this type of method (Wang *et al.*, 2005).

A sub-renal xenograft is the newest discovery in this type of scientific study. With this method, the tumour recovery rate can be as much as 93% (Wang *et al.*, 2005). Irrespective of the fact that it is dependent on the expertise of the scientist, it is still a reliable method of recovering crafted material with the capacity to match treatment to a patient's tumour biology (Lin *et al.*, 2014; Kritsanawong *et al.*, 2016)

The orthotopic xenograft method permits the placement of cancerous tissue of the prostate in the mouse prostate gland. The merit of this technique is the relationship between the deposited tissue and the host gland (An *et al.*, 1998). The take rate is almost 72% (Giavazzi *et al.*, 1986). This technique allows for the study of tumorigenic and metastatic processes.

#### 1.10.1 TRAMP

Rat can frequently develop spontaneous adenocarcinoma naturally. This type of animal was developed in 1995 (Pollard, 1973; Wilson *et al.*, 1981). Further studies revealed that the prostate

glands of rats express a promoter known as probasin (PB) that is responsible for androgen sensitivity and is the basic difference when compared with other models (Rennie *et al.*, 1993). This method is a very useful technique since translation from PIN to full cancer is predictable.

### 1.10.2 LADY

This technique was developed to study the influence of neuroendocrine cells as a driving force on the advancement of prostate cancerous tumour in the absence of androgens (Masumori *et al.*, 2001). Of course, prostate cancer can arise from different cell types, but the nervous system also contributes through the paracrine signals via neuroendocrine cells. This mouse model reveals the progression of PIN to a malignant disease (Kasper *et al.*, 1998; Masumori *et al.*, 2001).

### 1.10.3 Metastatic models

Models for the investigation of cancer of the prostate are available. A typical example is the intratibial injection technique. In this method. Prostate cancer cells are usually suspended in phosphate-buffered saline. This is then injected into the tibias of genetically distorted WT mice. X-rays can be used to monitor changes in bone lesions (Campbell *et al.*, 2012).

### 1.10.4 Cancer cell injection via the tail vein

This method involves the deposition of a bolus dose of cancerous tumour cells in the blood circulation of a mouse. It is easier to use this technique to study different molecules in metaplasia since the relationship between circulating tumour cells and the endothelium is a vital factor (Elkin and Vlodavsky, 2001)

## 1.11 Cervical uteri cancer

### 1.11.1 Epidemiology of cervical cancer

Cervical cancer constitutes twenty five percent cause of cancer related mortality in women globally. According to GLOBOCAN estimates, 604 000 women were diagnosed with cervical cancer of which 56.62 percent were fatal cases due to the disease (Sung *et al.*, 2021). Most geographical regions of the world have experienced a significant reduction in the occurrence and death rate of uterine cervix cancer over the past decades. Morbidity and mortality vary across these regions. The age-standardized incidence rate was found to be 13.1 per 100000 women with variation among nations worldwide (Ferlay *et al.*, 2018). In Sub-Saharan Africa, uterine cervical cancerous tumour is the most reported cause of mortality in females despite interventions such as vaccination, and cancer screening services. However, there is a rise in incidence rate in many regions across Africa such as Eastern, Southern, and Middle Africa (Ferlay *et al.*, 2018).

### 1.11.2 Pathophysiology of uterine cervical cancer

Human Papilloma Virus (HPV) causes cervical cancer by first all infecting the proliferating basal cells of the squamous epithelium. Failure of the immune complex to contain and resolve the infection leads to the accumulation of sufficient genomic instability which result in neoplastic transformation of the epithelial cells. Low-grade Squamous Intraepithelial Lesions (LSIL), the initial infective and potential progressive state develop in the vulnerable transformation zone. Persistent infection of this type may then give way to the development of High Grade Squamous Intraepithelial Lesion (HSIL) within a time frame of two to three years. The development of HSIL causes the expression of viral proteins E6 and E7 that interferes with cell cycle regulator and programmed cell demise machineries. The involvement of E6 and E7 initiates the progression and transformation of a viral infection to carcinogenesis. Subsequently,

genetic alterations resulting in the deletion of tumor suppressor genes and changes in growth modulating factors result in the occurrence of a full blown uterine cervical carcinoma (Steben and Duarte-Franco, 2007).

### 1.11.3 Pathogenesis of uterine cervix cancer

HPV infection has been identified to be involved in the development of cervix uteri cancer in the past decade (Chan and Berek, 2007). A scientist from Germany was the first to elucidate the connection between the HPV and uterine cervix cancer in the early 1980. It was demonstrated that the likelihood of HPV causing cervical cancer and smoking causing lungs cancer was greater in magnitude (Franco, 1995). HPVs are categorized into high-risk and low-risk types. Low-risk HPV types are types 6, 11, 42, 43, and 44. High-risk HPV types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70. Low-risk types are associated with low-grade cervix uteri cellular transformations resulting in infections such as genital warts (Papillomaviruses, 2011). High-risk HPV types are carcinogenic in nature, thus causing the development of high-grade cell abnormalities, cervical cancer and other anogenital cancers (Muñoz, 2003). Type 16 and 18 are the most virulent that are implicated in the cause of over 70% cervical cancer cases worldwide (Steben and Duarte-Franco, 2007). Recurrent microtrauma caused by sexual intercourse to the lining of the cervix allow HPVs to infect basal epithelial cells of inner lining of cervical tissues. Cervical epithelial cells that infected with HPV 16 and 18 DNA possess selective proliferative advantage over cells that contain normal extrachromosomal

viral genomes; this growth advantage promote the over expression of two viral genes E6 and E7. These proteins interact and inactivate the tumour-suppressor gene (p53), and the retinoblastoma tumour-suppressor protein (pRb) respectively. The expression of the E6 and E7 viral genes initiate and sustain the progression of cervical cancers (Papillomaviruses, 2011).

### 1.11.4 Etiology

The general causative factor for the occurrence of cervical cancer is continuous exposure to infection with high-risk oncogenic HPV types (zur Hausen, 2009). Some other well-known risk factors for cervical cancer are the sexual acquisition of HPV, immune dysfunction, exposure to mutagens, genetic and hormonal factors. Other predisposing factors are early commencement of sexual activity, multiple sexual partners, exposure to other sexually transmitted diseases, cigarette smoking, the use of oral contraceptive and human immunodeficiency virus infection. Social risk factors include lack of access to cancer screening facilities and non-compliance with recommendations (Chauhan *et al.*, 2009).

### 1.11.5 Assessment of cervical cancer

United States Preventative Services Task Force (USPTF) recommends Pap smear from the age of 21 years. HPV testing commences at 30 years of age in combination with Pap smear cytology. Screening is recommended per three years for women with normal screening and low risk for cervical cancer. Women beyond 30 years of age, cytology is recommended every five years in addition to HPV testing. It is further recommended that low risk women with consistent screen

can discontinue screening when the clock the age of 65 years. Women who had hysterectomy are exempted from further screening (Farghaly *et al.*, 2006).

### 1.11.6 Management of cervical cancer

Precancerous lesions are managed conservatively for women below the age of 25 years because most findings in this group of women are low risk infections that resolve spontaneously. Lesions with lower risk may be on watchful waiting and assessed regularly. Colposcopy is required to assess continuous lesions suspect to be high risk HPV infection. Very risky lesions are managed according to extent, position, and presentation. Cryotherapy is usually employed to treat early cancerous lesions that are localized in extent and complexity. Loop Electrosurgical Excision Procedure (LEEP) is usually a method of choice to manage or treat extensive lesions or lesions that involve the endocervical canal. LEEP provides better assessment and imaging of the squamocolumnar intersection and afford less bleeding to an outpatient. For a confirmed cancer diagnosis, the usual management strategy is staging to decide on the next step to follow. Staging is based on results from examination, imaging, tissue findings, history and reported signs and symptoms. Grading is done based on the size and depth of cancer and evidence of spread to other organs. Treatment of early-stage cancer includes a complete hysterectomy. Chemotherapy and radiotherapy are initiated after surgical removal of the uterus to impede the progression of cancer (Senol *et al.*, 2016).

### 1.11.7 Drug treatment of cervical cancer

#### 1.11.7.1 Cisplatin

Cisplatin is a chemotherapeutic agent utilized for the treatment of uterine cervix cancer including bladder and ovarian cancers. Once Cisplatin is within the cytosol of the target cell, it is activated. The chloride atoms found on cisplatin molecule are eliminated by water molecules to form an active electrophile that can react with any nucleophile, including the sulfhydryl groups on proteins and nitrogen donor atoms on nucleic acids. Cisplatin exert destruction of DNA in cancer cells through coupling with the N7 reactive receptors on purine bases, thus arresting cell division that leads apoptotic cell death (Reedijk and Lohman, 1985) .

#### 1.11.7.2 Paclitaxel

Paclitaxel was originally known as Taxol, the name was changed to paclitaxel by Bristol-myers when they developed the compound for commercial use. `Paclitaxel, naturally derived diterpene alkaloid was isolated from the bark extract of *Taxus brevifolia* Nutt. (Taxaceae). Paclitaxel is a member of the cytoskeletal drugs that affects tubulin resulting in the abnormality of the mitotic spindle assembly and chromosome segregation that affects cell division. Due to the stabilization of microtubule polymer, paclitaxel arrests cell cycle in the G0/G1 and G2/M phases and induces apoptosis in cancer cells (Zhang *et al.*, 2014).

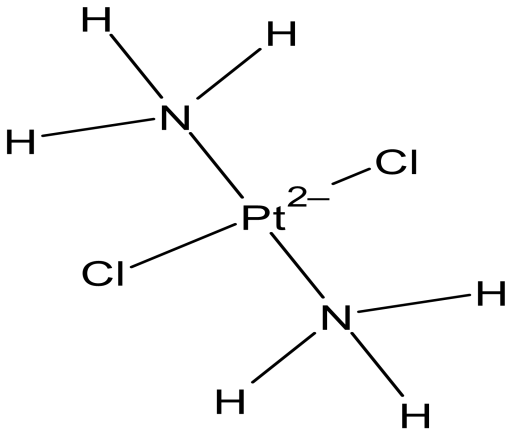
#### 1.11.7.3 Ifosfamide

Ifosfamide is an oxazaphosphorine alkylating agent that is a prodrug. It is metabolized in the liver by CYP450 enzymes to the active form phosphoramide mustard derivatives and acrolein which bind to DNA and inhibit DNA synthesis. These metabolites act by causing cell damage through inter-strand or intra-strand crosslinking, leading to cell death of the affected cancer cells. It is known also that the active metabolites initiate an upregulation of the Reactive Oxygen

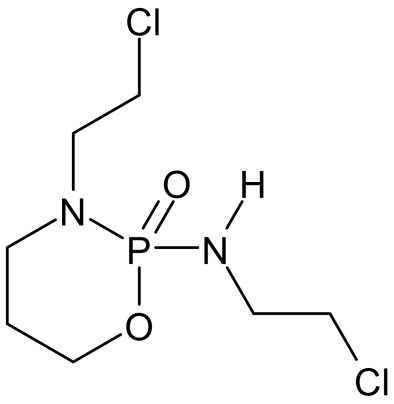
Species (ROS) that consequently result in the irreparable DNA injury and the cessation of protein formation (Gangireddy and Nookala, 2021).

#### 1.11.7.4 Methotrexate

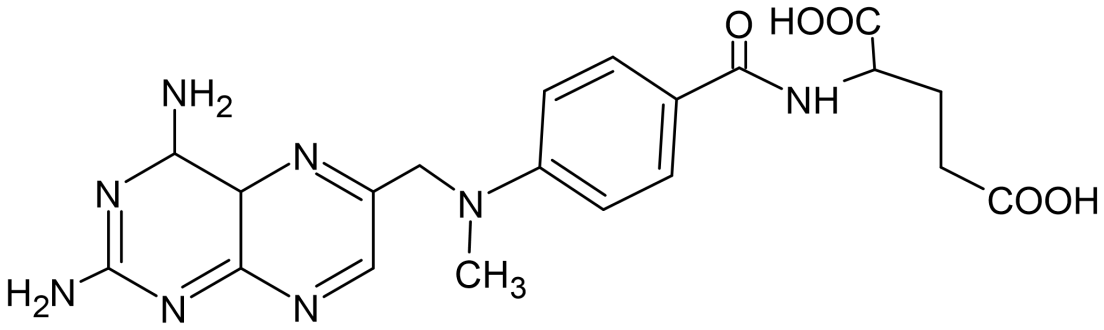
Methotrexate is used in the chemotherapy of common neoplasms and as immunosuppressant in auto-immune diseases. It belongs to anticancer class of drugs known as an antifolate antimetabolite. During the synthesis of nucleotides of RNA and DNA, an important enzyme called dihydrofolate reductase drives the conversion of dihydrofolate into tetrahydrofolate, which is a necessary step in the process. Methotrexate is absorbed to the cell by folate carriers (SLC19A1), which form methotrexate-polyglutamate. The compound and the active metabolite block the enzyme dihydrofolate reductase thus blocking cell synthesis. Methotrexate-polyglutamate also prevents the initial purine synthesis of purine and thymidylate synthase arresting DNA formation (Hannoodee and Mittal, 2022).




Cisplatin




Ifosfamide




Methotrexate

**Figures 1.14**: Structures of some common drugs used to treat uterine cervical cancer

### 1.11.8 Medicinal plants with activity against uterine cervix carcinoma

#### 1.11.8.1 *Cannabis sativa* L. (Cannabaceae)

*Cannabis sativa* has been shown to be effective in treating uterine cervical cancer in studies. Arachidonyl ethanolamide (AEA) from the plant was able to cause uterine cervical cell lines to die through aberrantly produced vanilloid receptor-1, according to the researchers' main results (Contassot *et al.*, 2004).

#### 1.11.8.2 *Abrus precatorius* L. (Fabaceae)

Agglutinin peptide fraction isolated from the seed of *A. precatorius* have demonstrated significant antiproliferative activity against uterine cervix cancer cell lines ( Hela). The proposed mechanism of action is cancer cell apoptosis through the Generation of reactive oxygen species and induction of mitochondrial permeability transition (Wang *et al.*, 2013).

#### 1.11.8.3 *Achyranthes bidentate* Blume. (Amaranthaceae)

The aqueous extract from the root of *A. bidentate* which forms part of a polyherbal preparation for the treatment of uterine cervix neoplasm caused a 52.9% resolution of the cancer ailment in women treated with the formula (de Moura *et al.*, 2002).

#### 1.11.8.4 *Rosa prague e* Mill. (Rosaceae)

The plant is known to contain high level of flavonoids with a significant antioxidant activity with a potential DNA protective effect. Hydro-ethanol extract of the flowers of *R. damascene* was tested against the human uterine cervical cell line (Hela). The result shows that the extract has cytotoxic activity against Hela cell lines (Zamiri-Akhlaghi *et al.*, 2011).

#### 1.11.8.5 *Coryphantha robbinsorum* W.Earle (Cactaceae)

The aqueous extracts of the fruit of *C. robbinsorum* show significant cytotoxic activity against human cervical cancer cells (HeLa). One percent solution preparation of the fruit caused about sixty percent suppression of HeLa cells growth. A five percent solution of the same preparation exterminated almost all the Hela cell in the culture medium within five days with $IC_{50}$ value of 1.8% (Chakraborty, 2019).


**Figure 1.15** *Lonchocarpus griffonianus* in its natural habitat (Urueoffong/Oruko community)

### 1.12. Taxonomic description of *Lonchocarpus griffonianus* (Baill) Dunn

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Tribe : Millettieae

Genus: *Lonchocarpus*

Species: *griffonianus*

### 1.13 The genus *Lonchocarpus*

*Lonchocarpus* comprises of about 150 species. The genus overlaps morphologically with some other millettiod genera. The most recent circumscription of *Lonchocarpus* is based largely on the concepts of Geesink (1984), who recognized *Deguelia* and *Philenoptera* as distinct from *Lonchocarpus* (Ihenyen and Mensah, 2009)

### 1.14 Botanical description of *Lonchocarpus griffonianus*

The plant is usually a tree or shrub of about ten to twenty meters tall with compound leaves having three to five pairs of leaflets of about five to ten centimeter long and two to four

centimeters wide. The lilac to violet flowers have a strong scent. They flowers bloom with the new leaves in the dry season and are up to one and a half inches in length. The stipules are curve and two millimeters long, Grows around water banks from Nigeria to Angola (Ihenyen *et al.*, 2009; Latham, 2017).

### 1.5 Ethno-medicinal uses of *Lonchocarpus griffonianus*

The plant is used by the natives of Akwa Ibom State, Nigeria for the treatment of tumor-related ailments. The leaves are used to treat pharyngopulmonary disorders. The root and stem bark are remedies against stomach aches, infertility, amenorrhea, inflammatory disorders, BPH, and an enlarged scrotum in neonates (Bassey and Effiong, 2011). The young leaves of the plant are usually taken as a purgative.

## 1.6 JUSTIFICATION OF THE STUDY

Conventional treatment options for cancer management are saddled with challenges such as obnoxious side effects (apoptosis, mitotic cell death, emesis, stomatitis, neutropenia, and mucositis) and high financial burden. Other limitations to the use of these options are patient rejection of therapy and inaccessibility. Therefore, there is an urgent need to seek promising and effective alternatives to complement or supplement the current treatment modalities in order to mitigate or obviate the undesirable side effects.

## 1.7 AIM AND OBJECTIVES

The aim of the study is to evaluate the antitumor effects of *Lonchocarpus griffonianus* (LG) as claimed in ethnomedicine.

The specific objectives of the study are as follows:

i. to evaluate the cytotoxic and antiproliferative potentials of LG using simple benchtop assays.
ii. to isolate and characterize bioactive principles from the plant
iii. to evaluate *in vivo* BPH attenuation effects of the plant's extract and the isolated compound (s).
iv. to evaluate the cytotoxic effects of isolated compound(s).

# CHAPTER TWO

## 2.0 MATERIALS AND METHODS

### 2.1 Laboratory Materials and Equipment

Mass spectrometer (JEOL JMS 600H-1 model), Nuclear magnetic resonance spectrometer (Brucker Avance 600 MHz model), Thermoregulated water bath (WNB 22), TLC precoated (Merck), TLC tank, chromatography column, spray gun, oven, centrifuge (CF-30 model), mortar and pestle, TLC silica gel, silica gel (60-120 micron) and electric milling machine (Chris Norris, England)

### 2.2 Chemicals and reagents

The following chemicals were utilized during this work: n-hexane (BDH), dichloromethane (BDH), ethyl-acetate (Lobachem), acetone (BDH), ethanol (BDH), methanol (Lobachem), toluene (BDH), DMSO (Gaylord), tween-80, 10% formal-saline, conc. Sulfuric acid (Pharmatrend), gentian violet, haematoxylin and eosin, Accubind tPSA ELISA kit (USA), Accubind Estradiol ELISA kit (USA), Accubind Testosterone ELISA kit (USA).

### 2.3. Plant collection and identification

The stem bark (LGSB) (5 kg) and root bark (LGRB) (5 kg) of *L. griffonianus* were collected at Oro community in the month of November, 2018, in Urue-offong/Oruko Local Government

Area of Akwa Ibom State, Nigeria. A Taxonomist, Prof. Henry Akinibosun authenticated the collected plant at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State. Voucher specimen was deposited at the herbarium section of the Department with specimen number UBH-L611.

### 2.4 Drying of plant materials

Foreign matters were removed from the LGSB and LGRB collected. The plant material were chopped before drying under the shade for ten days. The dried plant parts were comminuted separately to avoid cross contamination using an electric milling machine (Chris Norris, England)

### 2.5. Extraction

The powdered plant's parts were extracted with methanol (absolute) using a Soxhlet extractor at 67ºC. The respective extracts were concentrated *in vacuo* using rotary evaporator. The concentrated extracts were weighed, kept in labelled bottles, and stored in the refrigerator at 4ºC until required.

### 2.6 Preliminary activity evaluation of LGSB and LGRB crude extracts

#### 2.6.1 Acquisition of experimental animal (*Raniceps raninus* )

Tadpoles of *Raniceps raninus* were scooped from small stagnant water bodies around the Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria.

#### 2.6.2 Cytotoxic activity assay using *Raniceps raninus* model

The cytotoxic effect of LGSB and LGRB extracts was carried out using the following procedure: ten tadpoles were selected through a wide pore pipette and transferred into a 50 mL beaker with 15 mL of the water from the pool the tadpoles were collected from. Distilled water (34 mL) was added to each 50 mL beaker to make up the volume to 49 mL. The final volume was adjusted to 50 mL with 1 mL of 1, 2, 4 and 10 mg/mL of the crude extracts of LGSB and LGRB to make the concentration of 20, 40, 80 and 200 µg/mL correspondingly. Distilled water served as the negative control. The experiment was conducted in triplicate for all the groups. The tadpoles were observed for 24 hours for mortality ( Ayinde and Agbakwuru, 2010).

### 2.6.3 Evaluation of growth-suppression activity using the *Sorghum bicolor* model

The growth-suppression effect of LGSB and LGRB extracts was carried out using the method described by Ayinde and Agbakwuru, (2010). Glass Petri dishes were laid with cotton wool followed by Whatman filter paper number 1. 10 mL of 1, 2, 5, 10, 20, and 30 mg/mL of samples were added to the Petri dishes. Distilled water served as negative control. Twenty viable seeds were spread on each plate and kept in the dark. The length of emerging radicles was measured at 24. 48, 72 and 96 hours. The experiment was conducted in triplicates.

### 2.7 Acute toxicity study

This experiment was conducted in two phases. In phase one, Nine rats weighing between 180 - 200 g were randomly assigned to 3 groups of 3 rats each. Groups 1, 2 and 3 rats were given 10, 100 and 1000 mg/kg body weight of the plant extract respectively orally. The rats were observed keenly for the first two hours, then intermittently for the next four hours, and then over twenty four hours. In the absence of any mortality, phase two of the study was conducted. In phase two,

9 rats with a weight range of 170 g to 200 g were randomly assigned to 3 groups of 3 rats each. Group 1, Group 2 and Group 3 were administered 1600 mg/kg, 2900 mg/kg and 5000 mg/kg body weight of the plant extract orally respectively. The test rats were observed for signs of toxicity and mortality continuously for 30 minutes, then every hour for the first 24 hours and at least once daily. Animals in both phases were kept under the same conditions and observed for signs of toxicity for 14 days post administration of the extract. Focus was placed on signs of toxicity such as piloerection, sensitivity to sound and touch, motility, aggression, the presence of faeces, salivation, urine pattern, convulsions, coma, and death. The number of survivors was recorded after 24 hours and after 14 days, Animals' weights were observed for days 0, 7 and 14 (Gabriel and Idu, 2021).

### 2.8 Partitioning of the LGSB extract

One hundred and thirty grams of the LGSB (most active) was dissolved in water and successively partitioned with Dichloromethane (DCM) until exhausted (300 mLx7) in the separating funnel. The fractions (Aqueous (AQ) and DCM phases) obtained were concentrated under pressure to dryness. The weight of the DCM fraction was 13.8 g (10.5%), while the weight of the aqueous fraction was 105 g (80%)

### 2.9 Biological screening of the aqueous and solvent fractions

#### 2.9.1. Cytotoxic activity assay using *Raniceps raninus* model

The cytotoxic effect of the aqueous and solvent fractions were done using *Raniceps raninus* method described by Ayinde and Agbakwuru, (2010). Ten tadpoles were selected through a wide

pore pipette and transferred into a 50 mL beaker with 15 mL of the water from the pool the tadpoles were collected from. 34 mL of distilled water was added to each 50 mL beaker to make up the volume to 49 mL. The final volume was adjusted to 50 mL with 1 mL of 1, 2, 4 and 10 mg/mL of DCM and AQ fractions of LGSB extract to make the concentration of 20, 40, 80 and 200 µg/mL correspondingly. Distilled water served as negative control for the aqueous fraction while 2.5% DMSO served as the negative control for the DCM fraction. The experiment was conducted in triplicate for all the groups. The tadpoles were observed for 24 hours for mortality.

### 2.9.2 Evaluation of growth-suppression activity using the *Sorghum bicolor* model

The growth-suppression effect of LGSB and LGRB extracts was carried out using the method described by Ayinde and Agbakwuru, (2010). Glass Petri dishes were laid with cotton wool followed by Whatman filter paper number 1. 10 mL of 1, 2, 5, 10, 20, and 30 mg/mL of DCM and AQ fractions were added to the Petri dishes. Distilled water served as negative control for the AQ fraction while 2.5% DMSO served as the negative control for the DCM fraction.. Twenty viable seeds of *S. bicolor* were spread on each plate and kept in the dark. The length of emerging radicles was measured at 24. 48, 72 and 96 hours. The experiment was conducted in triplicates.

### 2.10.1 Vacuum liquid chromatography (VLC) of the bioactive DCM fraction

Ten grams of the DCM fraction was adsorbed and triturated with silica gel (60-120 µm) to achieve a uniform mass and was loaded on a sintered glass funnel (No. 3) previously packed with silica gel for TLC. The sintered glass funnel was attached to a Buckner flask. A vacuum pump was connected to the assembly and the adsorbed fraction was eluted with 300 mL of 100% hexane, then subsequently eluted with an increasing concentration of dichloromethane (25, 50,

75%). The adsorbed fraction was further eluted with 300 mL of 100% dichloromethane followed by 50% ethyl acetate and then 100% ethyl acetate. Finally, 300 mL of 100% methanol was used to elute the adsorbed fraction. The seven fractions obtained were concentrated *in vacuo*.

### 2.10.2 Thin layer chromatography of the VLC fractions of bioactive DCM

The VLC fractions were subjected to thin layer chromatography (silica gel plate, $GF_{254}$) using the solvent system hexane-dichloromethane (1:4). The TLC plates were developed by spraying with concentrated $H_2SO_4$ spray reagent and then heated in the oven at $110^0$C for 5 minutes. The VLC fractions were bulked together based on the TLC profile of the developed plates to obtain three fractions as follows: A (1-3), B (4) and C (5-7). The weights of the bulked VLC fractions obtained were as follows: A = 5.80 g (35.5%), B = 0.49 g (15.7%), C = 2.36 g (39.3%).

### 2.10.3 Vacuum liquid chromatography (VLC) of the AQ fraction

Fifty grams of the AQ fraction was adsorbed and triturated with silica gel (60-120 μm) to achieve a uniform mass and was loaded on a sintered glass funnel (No. 3) previously packed with silica gel for TLC. The sintered glass funnel was attached to a Buckner flask. A vacuum pump was connected to the assembly and the adsorbed fraction was eluted with 500 mL of each solvent combination was used to elute the flask as follows: 100% DCM, 50% DCM-Ethyl acetate, 100% ethyl acetate, 50% ethyl acetate-methanol, 25% ethyl acetate-methanol, 100% methanol, 75% methanol-water, 50% methanol-water, 25% methanol-water and 100% water. The ten fractions (1-10) were concentrated *in vacuo*.

### 2.10.4 Thin layer chromatography of the VLC fractions of AQ

The VLC fractions were subjected to thin layer chromatography (silica gel plate, $GF_{254}$) using the following solvent systems: DCM-dichloromethane (3:2), DCM-methanol(9:1) and DCM-methanol (1:1). The TLC plates were developed by spraying with concentrated $H_2SO_4$ spray reagent and then heated in the oven at $110^0$C for 5 minutes. The VLC fractions were bulked together based on the TLC profile of the developed plates to obtain four fractions as follows: A (1-3), B (4and 5), C (5-9) and D (10). The weights of the bulked VLC fractions obtained were as follows: A = 0.39 g (0.78%), B = 11.63 g (23.26%), C = 25.41 g (50.82%) and D 1.88 g (3.76%)

**2.11.1 Biological evaluation of bulked VLC fractions of the bioactive DCM fraction**

The bulked VLC fractions were subjected to growth-suppression activity evaluation using *S. bicolor* seeds at the concentration of 5 mg/mL as previously described (Ayinde and Agbakwuru, 2010).

**2.11.2 Biological evaluation of bulked VLC fractions of the AQ fraction**

The bulked VLC fractions were subjected to growth-suppression activity evaluation using *S. bicolor* seeds at the concentration of 5 mg/mL as previously described (Ayinde and Agbakwuru, 2010).

**2.13.1 Column Chromatography (GCC) of bioactive VLC fraction "A"**

VLC fraction A was subjected to column chromatography as follows: The column (37 cm x 2 cm) was packed with silica gel (60-120 μm). The sample was completely adsorbed on silica gel and loaded on top of the column. Gradient elution was done using 200 mL at the flow rate of 6.67 mL/min of the solvents system according to Table 2.2:

Table 2.1: Solvent system combination for column chromatography of fraction "A"

| N-hexane (%) | Ethyl-acetate (%) | Volume (mL) | Position | Remark |
|---|---|---|---|---|
| 100 | - | 280 | 1-14 | |
| 99 | 1 | 580 | 15-44 | Band separation |
| 98 | 2 | 360 | 44-61 | Elution of LO1 |
| 97 | 3 | 420 | 61-81 | Elution of LO1 |
| 96 | 4 | 680 | 81-114 | Band separation |

The eluate (20 mL) were collected in clean test-tubes numbered serially (114).

### 2.13.2 TLC analysis and bulking of column chromatography eluates

Eluates from column chromatography obtained were subjected to TLC profiling using analytical Silica gel $GF_{254}$. The solvent system of n-Hexane:Dichloromethane (1:4) was used to develop the plates. Concentrated tetraoxosulphate (IV) acid (spray reagent) was used to spray the plates and thereafter activated them in the oven at 110 degrees Celsius to develop the spots.. Eluates with similar TLC profile were bulked together to give 7 major column fractions as follows: 7-19 (D), 26-34 I, 35-47 (F), 48-64 (G), 68-71 (I), 75-88 (J), 90-114 (K).

### 2.13.3 Column chromatography of the bulked column fraction "G"

Fraction G (2400mg) was subjected to column chromatography for further purification. The fraction was loaded on a gravity column (37 cm x 2 cm) packed with silica gel (60-120 μm).

Gradient elution was done using 200 mL at the flow rate of 4 mL/min of the solvents system according to Table 2.3.

Table 2.2: Solvent system combination for column chromatography

| N-hexane (%) | Ethyl-acetate (%) | Volume (mL) | Position | Remark |
|---|---|---|---|---|
| 100 | - | 360 | 1-19 | Cleaning |
| 99 | 1 | 280 | 19-32 | |
| 98 | 2 | 200 | 32-41 | Elution of LO1 |
| 97 | 3 | 200 | 41-50 | Elution of LO1 |
| 96 | 4 | 220 | 50-60 | |

The eluates (20 mL) were collected in clean test-tubes numbered serially (60).

Column eluates obtained were subjected to TLC profiling using analytical Silica gel ($GF_{254}$): N-Hexane: Dichloromethane (1:4) solvent system was used to developed the plates. Concentrated tetraoxosulphate (IV) acid (spray reagent) was used to spray the plates and thereafter activated them in the oven at 110 degrees Celsius to develop the spots. Fractions 34-47 with similar profile were bulked together to give a pure compound coded LO1 (1920 mg).

### 2.13.4 Column chromatography of the bulked column fraction J

Fraction J (160 mg) was subjected to column chromatography for further purification. The fraction was loaded on a gravity column (400 mm x 15 mm) packed with silica gel (60-120 μm). Gradient elution was done using 200 mL at the flow rate of 5.71 mL/min of the solvents system according to Table 2.4.

Table 2.3: Solvent system combination for column chromatography

| N-hexane (%) | Ethyl-acetate (%) | Volume (mL) | Position | Remark |
|---|---|---|---|---|
| 100 | - | 200 | 1-10 | |
| 99 | 1 | 200 | 10-20 | |
| 98 | 2 | 300 | 20-34 | |
| 97 | 3 | 400 | 34-53 | |
| 96 | 4 | 200 | 53-72 | Elution of LO2 |

The eluates (20 mL) were collected in clean test-tubes numbered serially (72).

Column eluates obtained were subjected to TLC profiling using analytical Silica gel $GF_{254}$.: N-Hexane: Dichloromethane 1:4 solvent system was used to developed the plates. Concentrated tetraoxosulphate (IV) acid (spray reagent) was used to spray the plates and thereafter activated them in oven at 110 degrees Celsius to develop the spots. Fractions 62-66 with similar profile were bulked together to give a pure compound coded LO2 (33 mg).

**2.14 Determination of melting point of the isolated compounds LO1 and LO2**

Kofler hot-stage microscope equipment was used to test the melting point (m.p.). A few quantity of the powder was put in a capillary tube with a thin wall made of glass, and the tube was then inserted through an opening in the device into the side of the heating block. After then, heat was

applied to the compound until it melted. The melting point range of the compound was defined as the range between the temperature at which the solid started to melt and the temperature at which it turned completely to a liquid.

### 2.15 Biological activity evaluation of the isolated compounds

Two compounds were isolated from the bioactive DCM fraction of LGSB extract. They were coded LO1 and LO2 respectively and were further subjected to biological activity evaluations.

#### 2.15.1 Antiproliferative activity evaluation of LO1

LO1 was subjected to growth-suppression activity evaluation using *S. bicolor* seeds at the concentration of 1 mg/mL as previously described (Ayinde and Agbakwuru, 2010).

#### 2.15.2 MTT assay of LO1 and LO2

The cytotoxic analysis was done using a 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The human prostate cancer ($PC_3$) and cervical cancer (HeLa) cell lines were obtained from the molecular bank of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. The cancer cells were placed in 96-well plates at a density of 10,000 cells/well/100µL incubated for 24 h in complete media at 37 °C and 5% $CO_2$ for multiplication. The solutions of the compounds (30 µM) were prepared in sterile DMSO. Doxorubicin (30 µM) was used as the standard drug. The cells were challenged with the compounds in triplicates. The cell cultures were incubated for 48 hours at 37 °C in a humidified atmosphere of 5 % $CO_2$. 200 µL of MTT (0.5mM) dye was added to each well and then

incubated for another 3-4 h. Formazan crystals formed were dissolved in 100 µL of DMSO. The absorbance of the resulting solution was measured at 570 nm (Imade *et al.*, 2021).

**2.16 Spectroscopic analysis of the isolated compounds and structure elucidation**

Compounds LO1 (amorphous white powder) and LO2 (amorphous white powder) were subjected to spectrometry analysis using mass spectrometer (JEOL JMS 600H-1) on electron impact EI+ ionization mode. 1D and 2D Nuclear Magnetic Resonance (NMR) spectrometry on the compounds were done on Avance Neo 600 MHz Spectrometer at frequencies of 600 MHz in $CDCl_3$.

**2.17 Anti testosterone-induced BPH evaluation of LGSB extract and LO1 in rats model**

**2.17.1 Experimental animals**

Sixty-four male in-bred Sprague-Dawley rats weighing 264 ± 6.91 g were obtained from the Animal House unit of the Department of Biochemistry, University of Benin, Nigeria. The National Institute of Health (NIH 45) protocols for the use and care of laboratory Animals were strictly followed. The animals were housed under standard environmental factors. They were allowed free access to standard pellets and water *ad libitum*. Ethical approval (EC/FP/022/15) was secured from the Ethical committee, Faculty of Pharmacy, University of Benin, Edo state, Nigeria.

**2.17.2 Experimental design**

Sixty-four male Sprague-Dawley rats were randomly selected into eight (8) groups of eight (8) animals (Table 2.4). Group 1, known as the negative control group, were administered (4

mg/kg/day, s.c.) of testosterone propionate (T) diluted in olive oil daily. Groups 2-4 were administered the extract (100, 200 and 400 mg/kg/day) dissolved in distilled water and T (4 mg/kg/day, s.c.) diluted in olive oil daily. Group 5 was given 5 mg/kg/day of lupeol and T (4 mg/kg/day, s.c.) diluted in olive oil daily. Group 6 was given a combination of the extract (200 mg/kg/day) and finasteride 5 mg/kg/day and T (4 mg/kg/day, s.c.) diluted in olive oil daily. Group 7 was administered 5mg/kg/day of finasteride orally as positive control and T (4 mg/kg/day, s.c.) diluted in olive oil daily. Group 8 served as the standard control and was allowed free access to feed and water. All animals were treated daily for twenty-eight days, and their body weights were recorded once weekly. They animals were fasted overnight on the 28$^{th}$ day, then on the 29$^{th}$ day, the animals were sacrificed under anaesthesia (chloroform gas chamber), and their blood was collected from the abdominal aorta biochemical analysis. The wet weight of the prostate and volume were taken. Prostate index was calculated as a ratio of the prostate weight to the final body weight of the animals (Sasidharan *et al.*, 2022) (Table 2.4).

Table 2.4: Experimental design showing treatment groups and administration

| Group | Treatment |
|---|---|
| Group 1: Negative control | 4 mg/kg/day of Testosterone propionate s.c. + I mL/100g/day of distilled water orally |
| Group 2:100 mg/kg | 4 mg/kg/day of Testosterone propionate s.c. + 100 mg/kg/day of extract orally |
| Group 3: 200 mg/kg | 4 mg/kg/day of Testosterone propionate s.c. + 200 mg/kg/day of extract orally |
| Group 4: 400 mg/kg | 4 mg/kg/day of Testosterone propionate s.c. + 400 mg/kg/day of extract orally |
| Group 5: Lupeol 5 mg/kg | 4 mg/kg/day of Testosterone propionate s.c. + 5 mg/kg/day of lupeol orally |
| Group 6: 200 mg/kg + 5mg/ kg finasteride | 4 mg/kg/day of Testosterone propionate s.c + 200 mg/kg/day of extract and 5 mg/kg of finasteride |
| Group 7: 5 mg/ kg finasteride | 4 mg/kg/day of Testosterone propionate s.c. + 5 mg/kg/day of finasteride orally |
| Group 8: normal control | Received feed and water *ad libitum* |

Initial body weights were recorded and then once weekly until the study elapsed.

### 2.17.3 Measurement of serum testosterone, PSA and estradiol levels

Enzyme-linked immunosorbent assay (ELISA) technique was used to assay the serum concentrations of testosterone, Prostate Specific Antigen (PSA) and estradiol following the instruction of the manufacturer.

#### 2.17.3.1 ELISA test for prostate specific antigen (PSA)

Twenty-five microlitres of test rats' serum were pipetted into the microwells. A hundred microlitres of Total Prostate Specific Antigen (tPSA) enzyme were added to the serum in the microwell and swirled gently for 30 seconds to properly mix the serum and the enzyme. The plate was washed thrice with 350 of wash buffer. One hundred microlitres of the working substrate solution were added to the microwells and incubated at room temperature for 15 minutes. Fifty microlitres of stop solution were added to all the wells and mixed adequately for 20 seconds. The absorbance of the microwells was read at 450 nm using a microplate reader within 30 minutes of the addition of the stop solution.

#### 2.17.3.2 ELISA test for testosterone

Ten microlitres of tests rat serum were pipetted into the microwells. Fifty microlitres of testosterone enzyme reagent were added to the serum in the microwell and swirled gently for 30

seconds to properly mix the serum and the enzyme. Fifty microlitres of testosterone biotin reagent were added to the wells and swirled for mixing. The plate was covered and incubated at room temperature for 60 minutes. After 60 minutes, the enzyme reagent was discarded and blotted dry with blotting paper. The plate was washed thrice with 350 of wash buffer. One hundred microlitres of the working solution were added to the microwells and incubated at room temperature for 15 minutes. Fifty microlitres of stop solution were added to all the wells and mixed adequately for 20 seconds. The absorbance of the microwells was read at 450 nm using a microplate reader with 30 minutes of the addition of the stop solution.

#### 2.17.3.3 ELISA test for estradiol

Twenty-five microlitres of test rats' serum were pipetted into the microwells. Fifty microlitres of estradiol biotin reagent was added to the serum in the microwell and swirled gently for 30 seconds to properly mix the serum and the enzyme. Fifty microlitres of estradiol enzyme reagent was added to the wells and swirled for mixing. The plate was covered and incubated at room temperature for 60 minutes. After 60 minutes, the enzyme reagent was discarded and blotted dry with blotting paper. The plate was washed thrice with 350 µL of wash buffer. One hundred microlitres of the substrate solution were added to the microwells and incubated at room temperature for 20 minutes. Fifty microlitres of stop solution were added to all the wells and mixed adequately for 20 seconds. The absorbance of the microwells was read at 450 nm using a microplate reader within 15 minutes of the addition of the stop solution

### 2.17.4 Histological examination of the animal's prostate tissues

The harvested prostate glands were fixed in 10% forma-saline. They were embedded in paraffin wax blocks, and sliced into 4 $\mu$m thick sections. The cut sections were transferred to glass slides and stained with routine haematoxylin and eosin stains. The stained sections were observed under a light microscope at magnifications of ×40 and ×100 (with oil immersion). Morphological and toxicological observations were recorded. Photomicrographs of the observed sections were taken through the use of Amscope (Fan *et al.*, 2022).

**2.18 Statistical analysis**

Values are expressed as mean ± SEM. Differences between groups were analyzed using analysis of variance (ANOVA) followed by the Dunnett's post hoc test at a 95% confidence interval using Graphpad prism version 6.01.

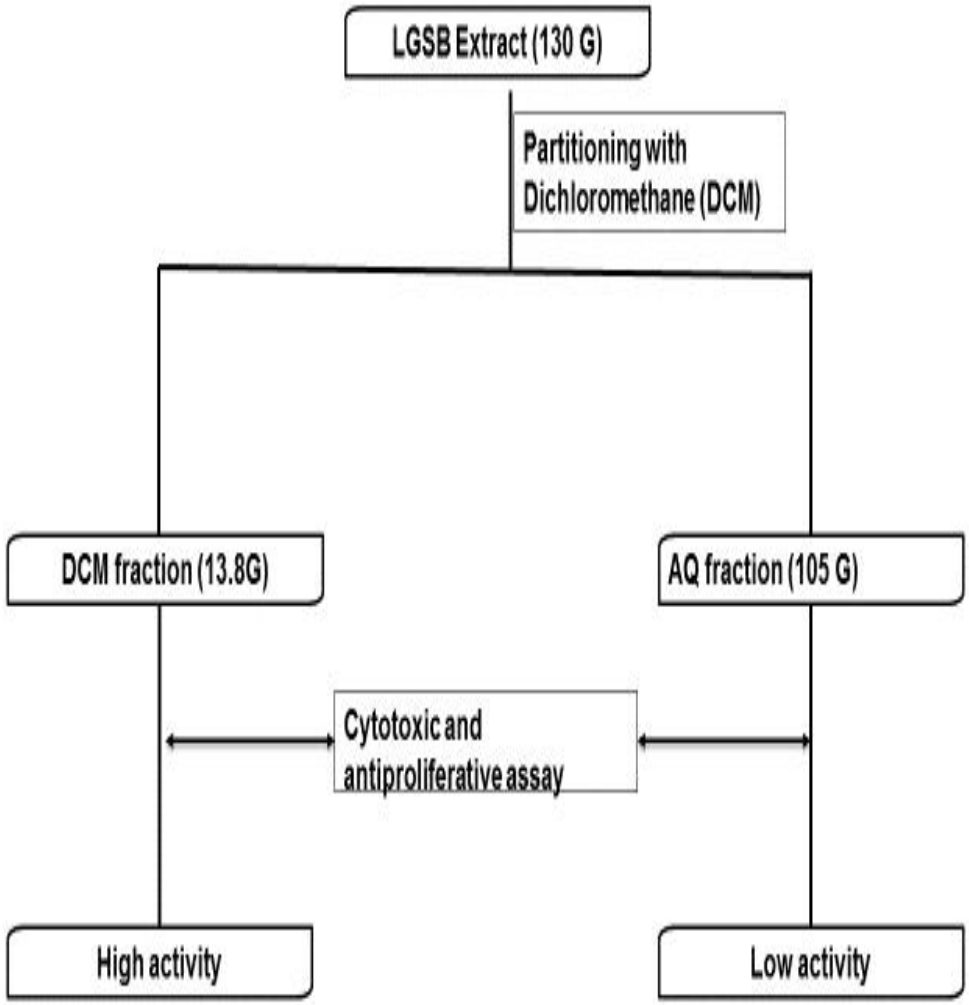



**Figure 2.1**: Schematic representation of the partitioning of the LGSB extract and activity evaluation of the solvent fractions.

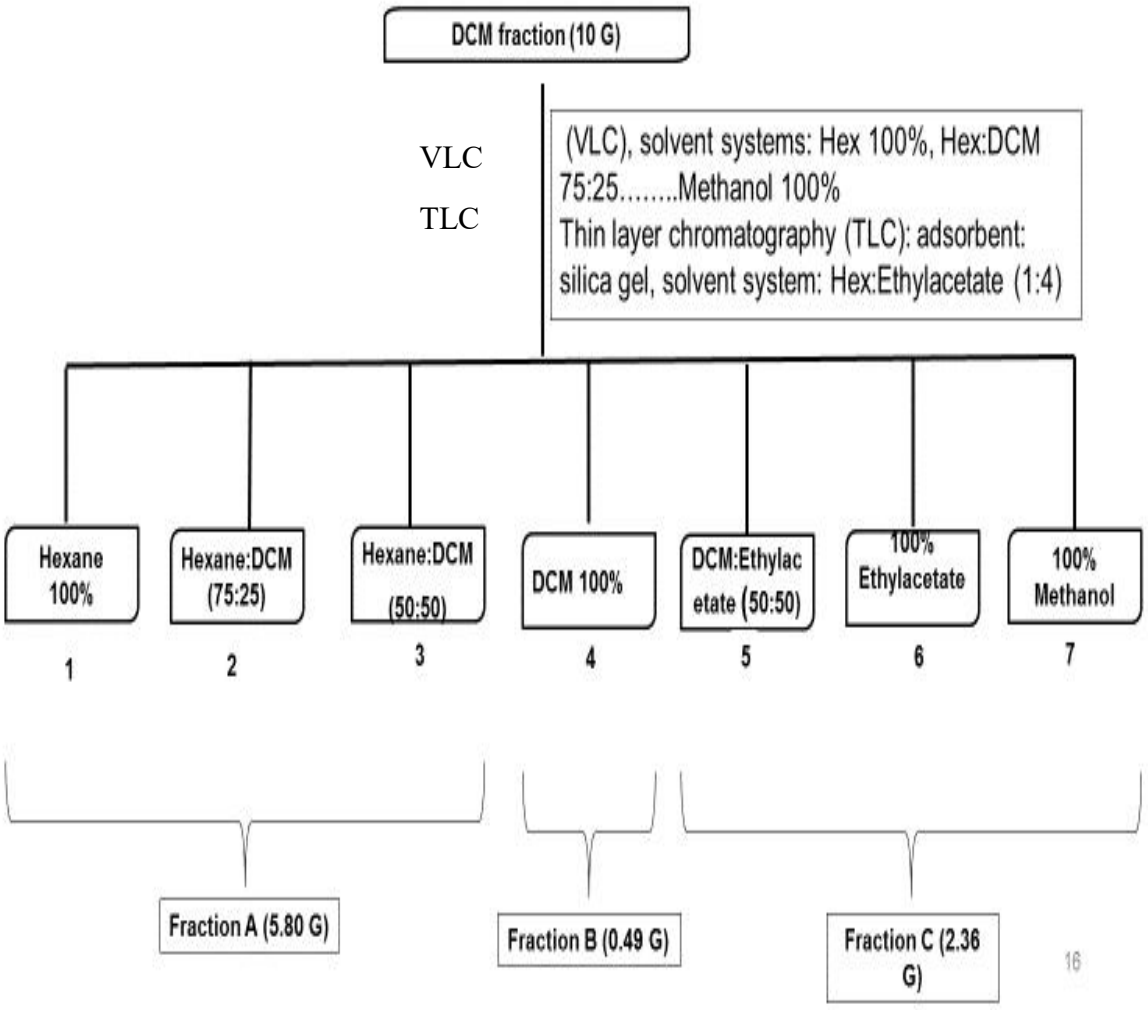



**Figure 2.2**: Schematic representation of VLC of the bioactive DCM fraction.

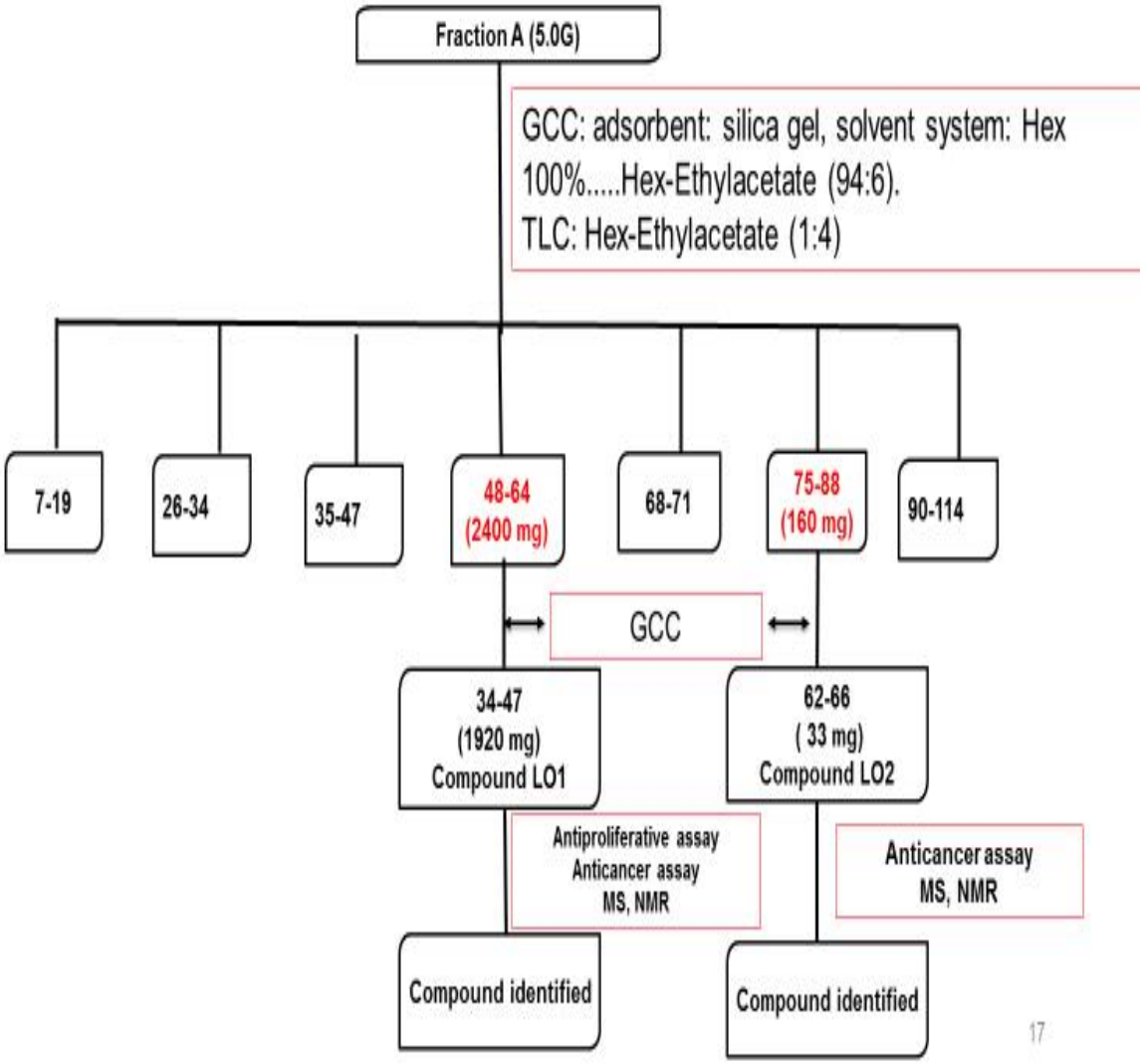



**Figure 2.3**: Schematic representation of GCC of the bioactive VLC bulked fraction "A"

# CHAPTER THREE

## 3.0 RESULTS

### 3.1 Yield of extracts and solvent fractions

The stem bark of *L. griffonianus* (2.0kg) gave 166 g (8.30%) of the methanol extract. The methanol extract (130 g) following partitioning, gave 13.8 g (10.5%) of the DCM fraction and 105 g (80%) of the AQ fraction. The root of the plant (2.0 kg) gave 98 g (4.90%) of the crude extract (Table 3.1).

Table 3.1: Percentage yield of extracts and solvent fractions

| **Extract and fraction** | **Weight in grams (g)** | **Percentage yield (%)** |
|---|---|---|
| LGSB extract | 166 | 8.30 |
| LGSB DCM fraction | 13.8 | 10.5 |
| LGSB AQ fraction | 105 | 80 |
| LGRB extract | 98 | 4.90 |

### 3.2 Preliminary biological evaluation

#### 3.2.1 Result of cytotoxic effect of LGSB on *Raniceps raninus*

The result of the cytotoxic analysis done on the stem bark of *L. griffonianus* using *R. raninus* is shown in figure 3.1. Results showed that the methanol extracts of stem bark possess dose-dependent cytotoxic effect. The concentration of 20 µg/mL of the methanol extract of the stem bark caused a statistically significant 73.33±8.82% mortality in the tadpoles. 40, 80 and 200 mg/mL caused 80.00±5.77, 100.00±0.00 and 100.00±0.00% respectively. Results were significant at $P<0.0001$.

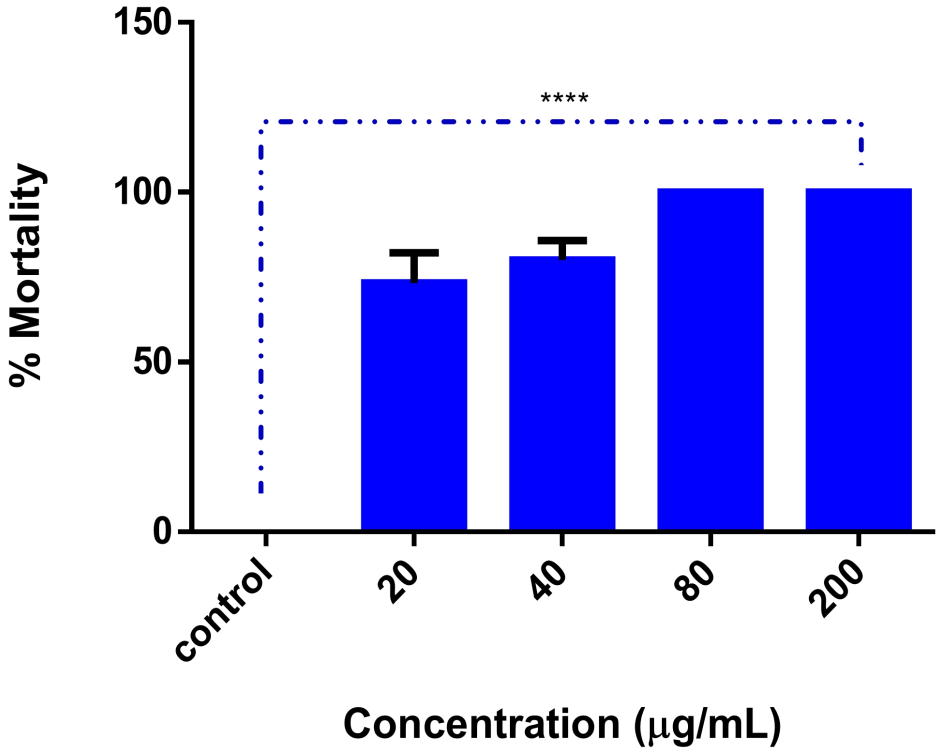



**Figure 3.1** Cytotoxic screening of LGSB on *R. raninus* tadpole. Each bar represents mean ± SEM. Bars with **** represent significant results at $p<0.0001$. n=10

### 3.2.2 Result of cytotoxic effect of LGRB on *Raniceps raninus*

The result of the cytotoxic analysis done on the root bark of *L. griffonianus* using *R. raninus* is shown in figure 3.2. Results showed that the methanol extracts of root bark possess dose-dependent cytotoxic effect. The concentration of 40 µg/mL of the methanol extract of the root bark caused a statistically significant 30.00±5.77% mortality in the tadpoles at $P<0.001$. 80 and 200 mg/mL caused 63.30 ± 3.33 and 93.30 ± 3.33% respectively. 80 and 200 µg/mL results were significant at $P<0.0001$.

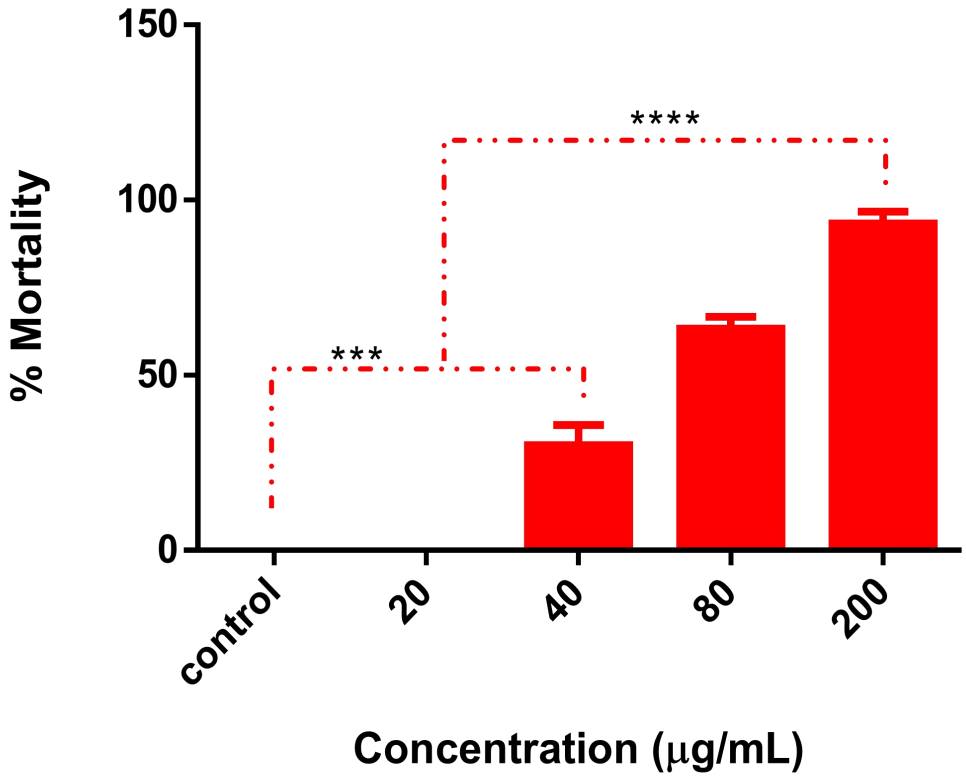
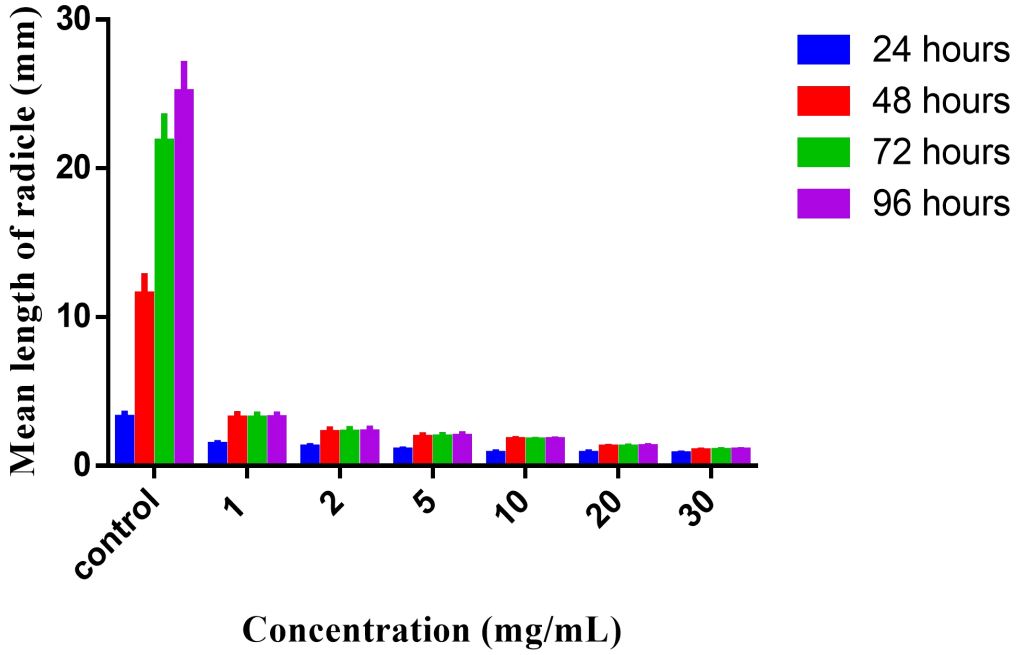



**Figure 3.2** Cytotoxic screening of LGRB on *R. raninus* tadpole. Each bar represents mean±SEM. Bars with *** represent significant results at $p<0.001$ and **** represent significant results at $p<0.0001$. n=10

### 3.2.3 Result of growth inhibition effect of LGSB extract

The growth inhibition evaluation result of the stem bark extract of *L. griffonianus* tested with *S. bicolor* seed radicles for ninety-six hours showed a dose-dependent inhibition of the radicle length by all the concentrations (1-30 mg/mL). 1 mg/mL concentration within 24 hours caused a statistically significant ($p<0.0001$) 1.40 ± 0.12 mm (56.25% suppression) growth inhibition of the emerging radicle, which increased to 3.20 ± 0.27 mm (87.2% suppression) by 96 hours. Equally, 30 mg/mL of the extract caused 0.75 ± 0.06 mm (76.56% suppression) inhibition of the mean radicle length of guinea corn seeds at 24 hours and 1.00 ± 0.05 mm (96% suppression) at 96 hours. Results were statistically significant at all concentrations at $p<0.0001$. The result is shown in Figure 3.3.




**Figures 3.3:** Growth inhibitory effect of the crude extract of LGSB on *S. bicolor* radicle. Each bar represents mean ± SEM. All data are significant at $p<0.0001$. n=20

### 3.2.4 Result of growth inhibition effect of LGRB extract

The growth inhibition evaluation result of the root bark extract of *L. griffonianus* tested with *S. bicolor* seed radicles for ninety-six hours showed a dose-dependent inhibition of the radicle length by all the concentrations (1-30 mg/mL). 1 mg/mL concentration gave 0.79 ± 40.09 mm (33.61% suppression) growth inhibition of the mean guinea corn radicle length within 24 hours, which increased to 3.88 ± 0.36 mm (76.34% suppression) by 96 hours. The mean radicle length of guinea corn seeds was suppressed by 0.63 ± 0.07 mm (47.06% suppression) at 24 h when challenged with 30 mg/mL of LGRB extract. The inhibition increased to 1.08 ± 0.06 mm (93.41% suppression) by 96 hours. Results were statistically significant at all concentrations at $p<0.0001$. The result is shown in Figure 3.4.

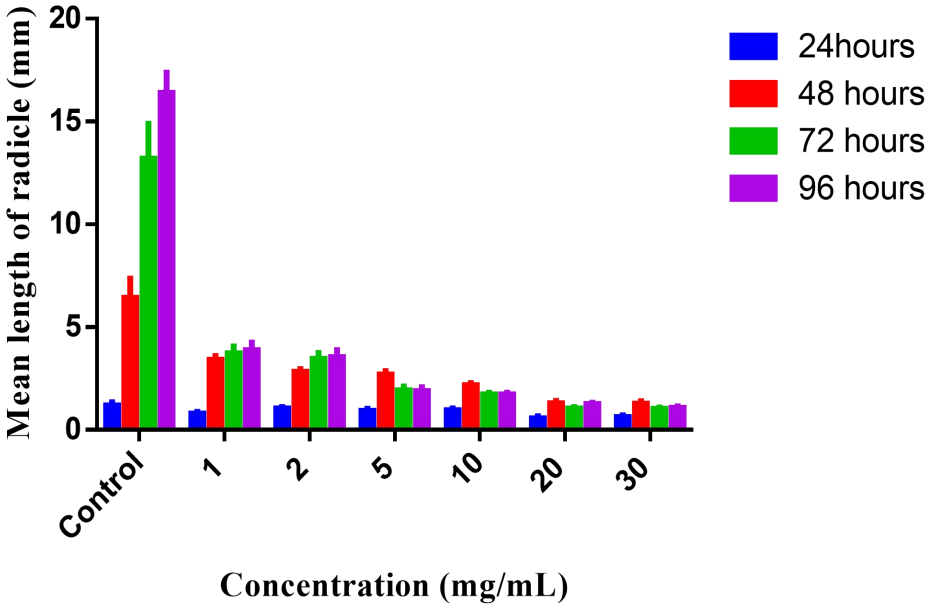



**Figure 3.4:** Growth inhibitory effect of the crude extract of LGRB on *S. bicolor* radicle. Each bar represents mean ± SEM. All data are significant at $p<0.0001$. n=20

### 3.3 Acute toxicity study

The administration of 10, 100 and 1000 mg/kg dose of the extract in phase 1 of the acute toxicity study did not cause any mortality to the experimental animals. Equally, 1600, 2900 and 5000 mg/kg dose of the stem bark extract in phase 2 of the study did not cause any mortality to the experimental animals. The animals were stable and expressed no signs of toxicity. This indicated that the $LD_{50}$ of the extract is greater than 5000 mg/kg dose. The result is shown in Table 3.2.

Table 3.2: Result of oral acute toxicity of LGSB extract

| Group | Number of rat | Dose (mg/kg) | Mortality (%) |
|---|---|---|---|
| Phase 1 | | | |
| 1 | 3 | 10 | 0 |
| 2 | 3 | 100 | 0 |
| 3 | 3 | 1000 | 0 |
| Phase 11 | | | |
| 1 | 3 | 1600 | 0 |
| 2 | 3 | 2900 | 0 |
| 3 | 3 | 5000 | 0 |

$LD_{50}$ > 5000 mg/kg

### 3.4.1 Cytotoxic effect of DCM fraction of methanol extract of LGSB

The cytotoxic result of the dichloromethane (DCM) fraction derived from the solvent partition of the crude methanol extract of the stem bark of *L. griffonianus* is shown in Figure 3.5.

Results showed that the DCM fraction of the extracts of the stem bark possess dose-dependent cytotoxic effect. The concentration of 20 µg/mL of the DCM fraction caused a statistically significant 73.30 ± 6.67% mortality in the tadpoles. 40, 80 and 200 mg/mL caused 86.70 ± 6.67, 100.00 ± 0.00 and 100.00 ± 0.00% respectively. Results were significant at $P<0.0001$.

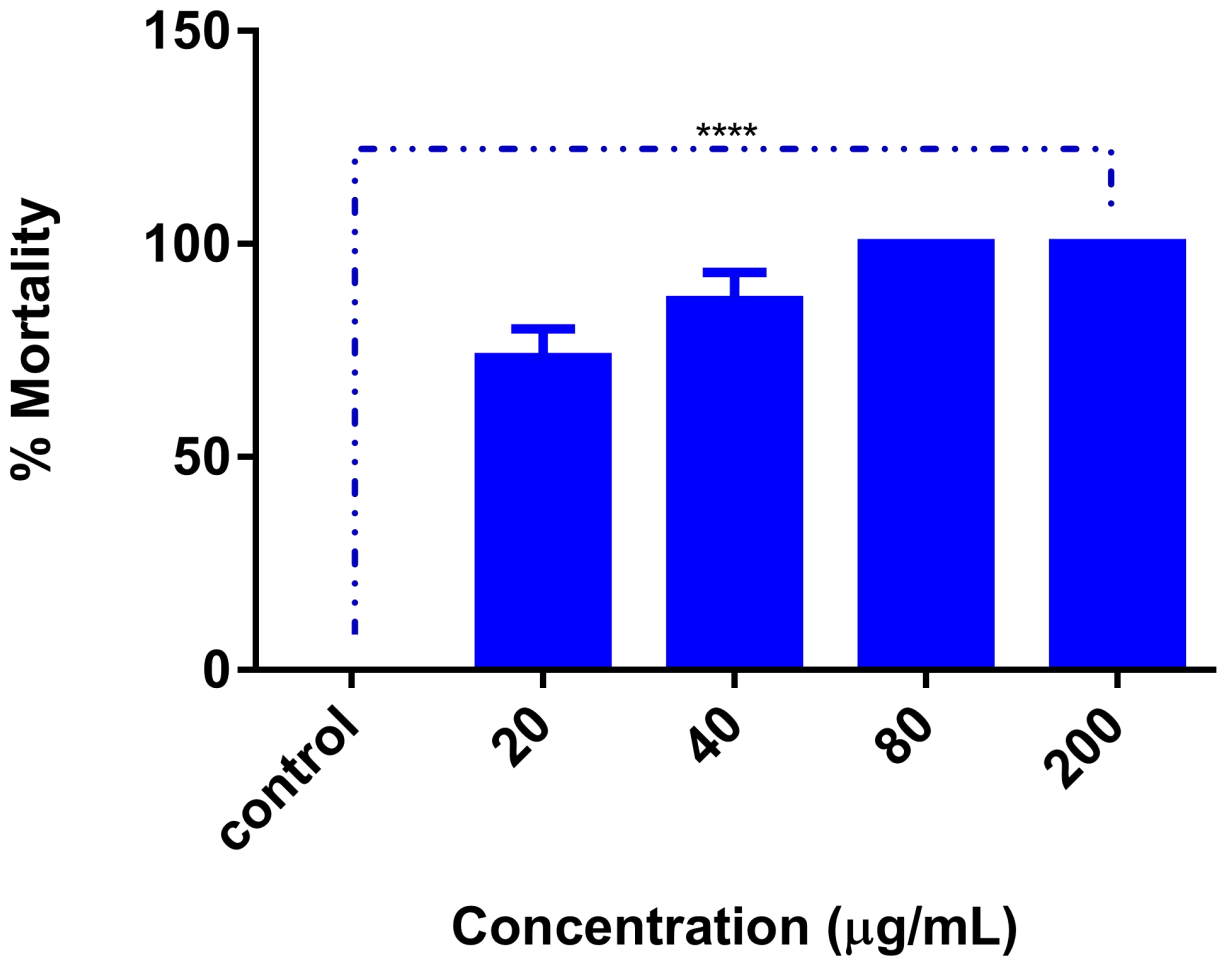



**Figure 3.5:** Cytotoxic effect of the dichloromethane (DCM) fraction of methanol extract of LGSB on *R. raninus* tadpole. Each bar represents mean±SEM. Bars with superscript **** represent significant results at $p<0.0001$ n=10

### 3.4.2 Cytotoxic effect of AQ fractions of methanol extract of LGSB

The cytotoxic result of the aqueous fraction (AQ) derived from the solvent partition of the crude methanol extract of the stem bark of *L. griffonianus* is shown in Figure 3.6.

Results showed that the AQ fraction of the crude extract of the stem bark lack cytotoxic effect at the tested doses. The concentration of 20 and 40 µg/mL of the AQ fraction did not cause any mortality in the tadpoles. 80 and 200 mg/mL caused 3.33 ± 3.33 and 6.67 ± 3.33 % respectively. Results were not significant at $P<0.05$.

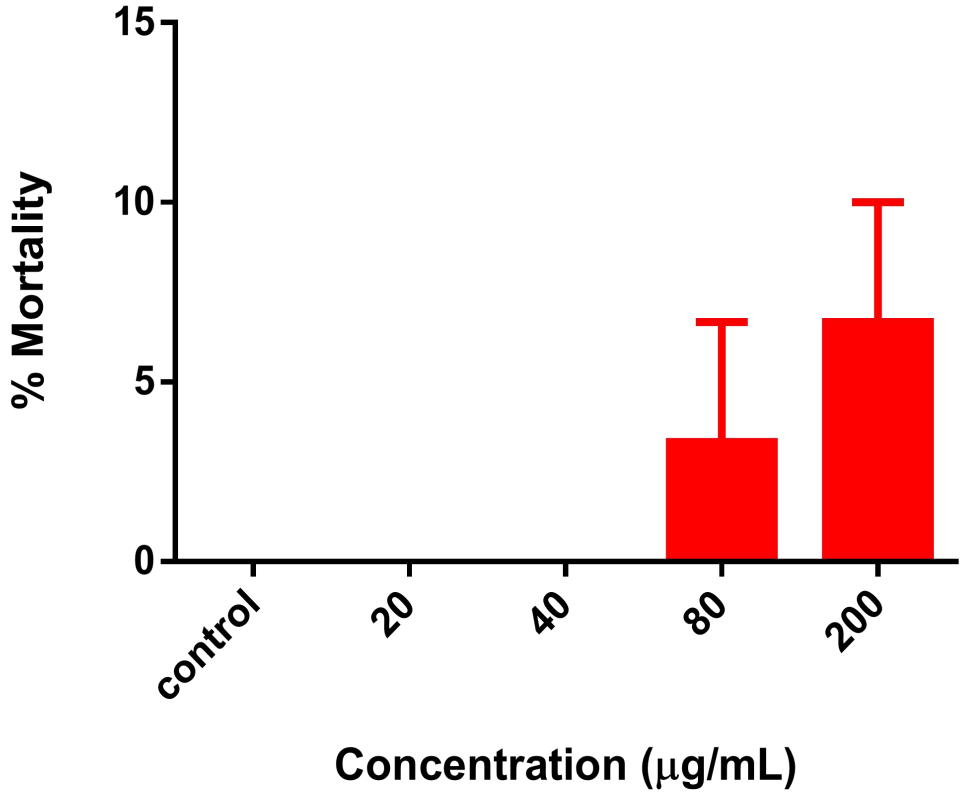



**Figure** 3.**6:** Result of **c**ytotoxic effect of the aqueous fraction (AQ) of methanol extract of LGSB on *R. raninus* tadpole. Each bar represents mean±SEM. Data were not significant at $p<0.05$. n=10

### 3.4.3 Growth inhibition effect of DCM fraction of methanol extract of LGSB

The result of growth inhibition effect of the DCM fractions of the crude methanol extract of LGSB is shown in Figures 3.7. DCM showed a dose-dependent inhibition of the *Sorghum bicolor* seed radicle. 1 mg/mL concentration gave a statistically significant ($P<0.0001$) growth inhibition effect of 2.05 ± 0.48 mm (77.80% suppression) by 48 hours and 5.95 ± 0.66 mm (43.33% suppression) by 96 hours. 30 mg/mL exerted a significant ($P<0.0001$) growth suppression of 1.27 ± 0.19 mm (77.39% suppression) within the time frame of 48 hours which increased to 1.91 ± 0.27 mm (81.81% suppression) by 96 hours. Which were significant when compared to the control.

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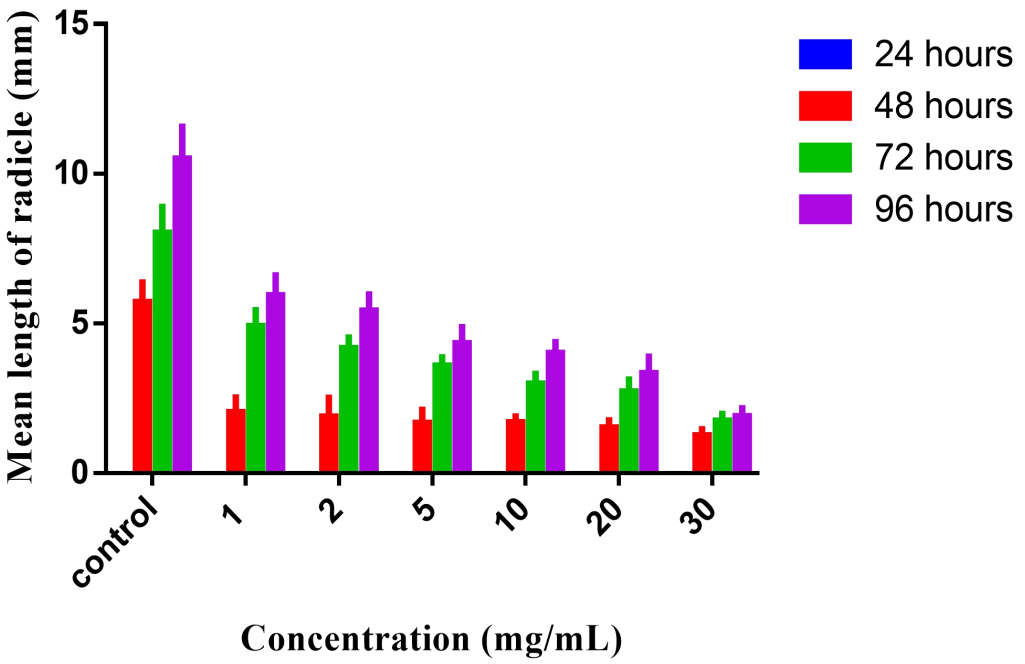



**Figure 3.7:** Growth inhibition effects of the DCM fraction on *S. bicolor* radicle. Each bar represents mean±SEM. All data are significant at $p<0.0001$. n=20

### 3.4.4 Growth inhibitory effect of the AQ fraction of methanol extract of LGSB

The result of growth inhibition effect of AQ fractions of the crude methanol extract of LGSB is shown in Figure 3.8. AQ fraction was lethal to the radicles. The mean radicle length of guinea corn seed challenged with 1 mg/mL of the AQ at 24 hours was 36.71% suppression, while at 96 hours, it increased to 58.86% suppression. 30 mg/mL exerted a mean radicle length suppression of 82.89% suppression at 24 hours, while at 96 hours, the mean radicle length suppression was 94.4%. These results were statistically significant at $P<0.0001$

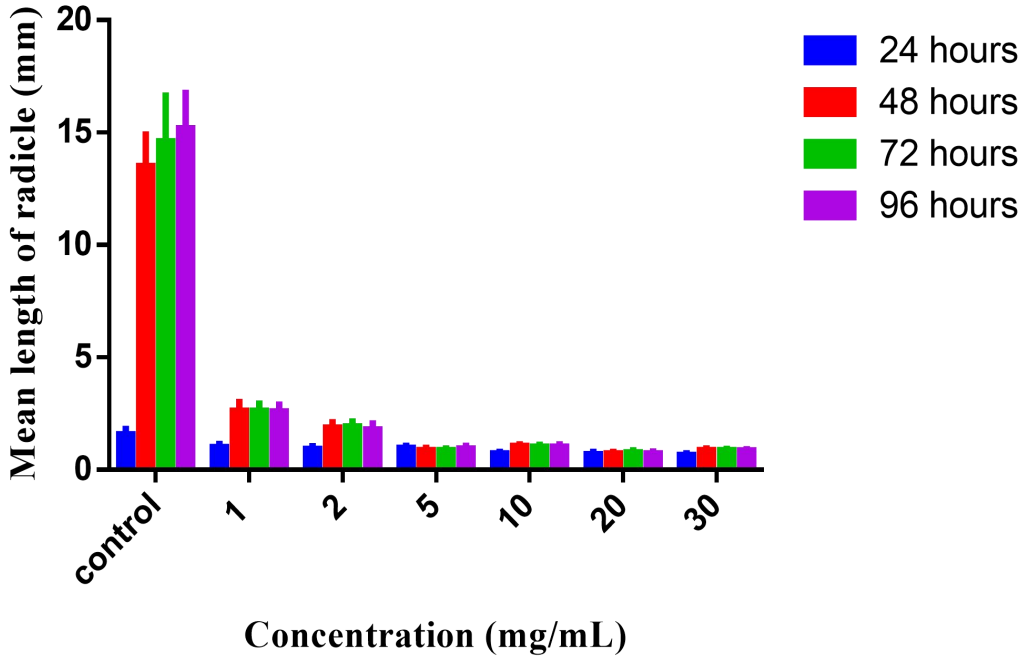



**Figure 3.8:** Growth inhibitory effects of the Aqueous fraction (AQ) on *S. bicolor* radicle. Each bar represents mean ± SEM. All data are significant at $p<0.0001$. n=20

### 3.4.5 Growth inhibition effects of LGSB extract VLC bulked fractions on S*orghum bicolor* radicles

Fraction "A" that was obtained by bulking VLC fractions 1-3 together exerted growth inhibition effect on *Sorghum bicolor* radicles. The result is shown in Figure 3.9. The mean radicle length of guinea corn seed challenged with 5 mg/mL of the fraction "A" at 24 hours was 0.09 ± 0.09 mm (100.00% suppression), which translated to 7.67 ± 0.32 mm (56.66% suppression) by 96 hours. These results were statistically significant at $P<0.0001$

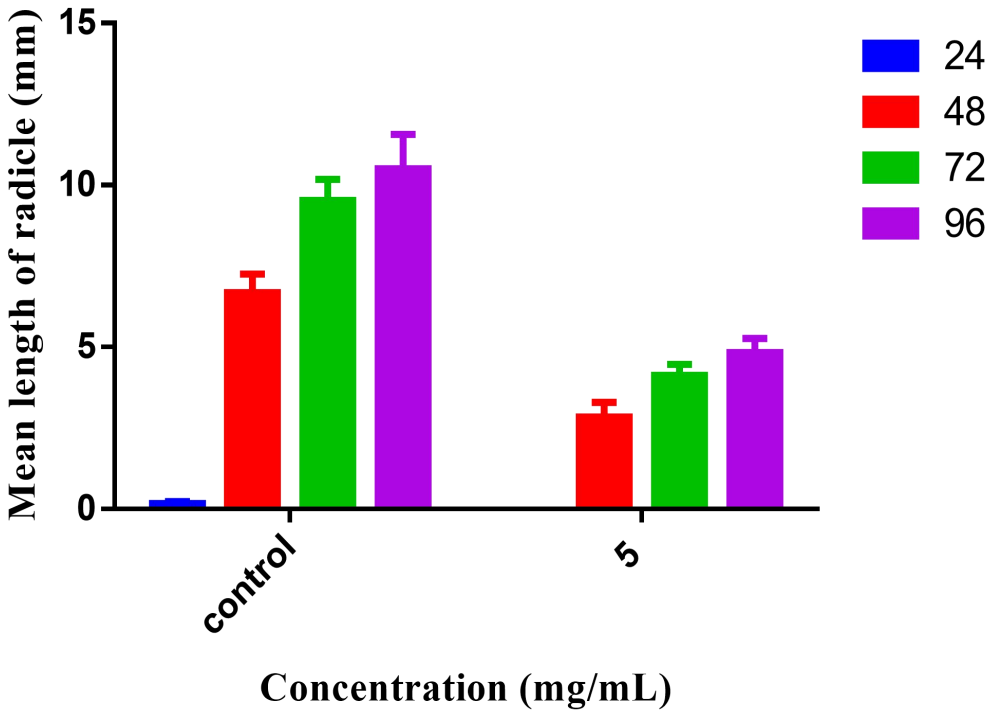



**Figure 3.9:** Growth inhibitory effects of fraction "A" on *S. bicolor* radicle. Each bar represents mean ± SEM. All data are significant at $p<0.0001$. n=20

### 3.5 Spectroscopic analysis of compound LO1

#### 3.5.1 1H-NMR [$CDCl_3$ 600 MHz]

1D NMR analysis of compound LO1 analysis resulted in the generation of the following spectra data used for characterization and identification of the compound.

Comparison with the reported literature values showed similar signals to the seven methyl protons cited in the literate (Shwe *et al*., 2019) (Table 3.3) : δ 0.74, δ 0.76, δ 0.80, δ 0.92, δ 0.94, δ 1.01 and δ 1.66. A sextet of a single proton occurring at δ 2.36 corresponds to 19 β-H proton and is a characteristic signal of lupeol. The olefinic protons located at C29 is a distinguishing feature of the lupane-type triterpenoid and was exhibited at chemical shifts δ 4.55 and δ 4.67. The proton attach to C3 proton occurred as doublet at δ 3.18

Table 3.3 1H NMR (400 MHz) chemical shifts pattern of LO1

| Position | 1H NMR reported in literature | 1H NMR experimental | Nature of proton |
|---|---|---|---|
| 1 | 0.90 (m) | 0.88 (m) | CH2 |
| 2 | 1.52 (m) | 1.48 (m) | CH2 |
| 3 | 3.2 (dd) | 3.18 (dd) | CH |
| 5 | 0.67 (d) | 0.67 (d) | CH |
| 6 | 1.37 (m) | 1.38 (m) | CH2 |
| 11 | 1.2 (m) | 1.2 (m) | CH2 |
| 15 | 1.05 (m) | 1.06 (m) | CH2 |
| 19 | 2.4 (m) | 2.36 (m) | CH |
| 21 | 1.36 (m) | 1.32 (m) | CH2 |
| 22 | 1.37 (m) | 1.3 (m) | CH2 |
| 23 | 0.90 (s) | 0.94 (s) | $CH_3$ |
| 24 | 0.76 (s) | 0.74 (s) | $CH_3$ |
| 25 | 0.83 (s) | 0.80 (s) | $CH_3$ |
| 26 | 1.01 (s) | 1.07 (s) | $CH_3$ |
| 27 | 0.94 (s) | 0.92 (s) | $CH_3$ |
| 28 | 0.79 (s) | 0.76 (s) | $CH_3$ |
| 30 | 1.67 (s) | 1.66 (s) | $CH_3$ |

### 3.5.2: Correlated-spectroscopy (COSY)

The COSY spectrum of LO1 exhibited cross peaks between proton attached to C19 appearing at δ 2.36 and one Sp3 methylene proton attached to C21 occurring at δ 1.32 and another Sp3 methine proton signal at C18 appearing at δ 1.65. There is another coupling between oxygenated methine proton on C30 with signal at δ 3.16 and Sp3 methylene proton attached to C2 occurring at δ 1.50.

### 3.5.3: Mass spectrometry (MS)

The fragmentation pattern of compound LO1 is as shown in figure . The molecular ion peak is seen at m/z 426.6.3 which corresponds to the molecular of formula of $C_{30}H_{50}O$. The loss of water molecule resulted in the formation of the peak at m/z 408.3. Demethylation of the methyl ($CH_3$) functional group at C8 result in the formation of a ionic compound with m/z 393.3. Further cleavage of an alkane ($C_2H_4$) led to the daughter peak observed at m/z 365.3. the loss of $C_{13}H_{20}$ due to Mc Lafferty rearrangement resulted in the formation of a stable peak at 189.3. This pattern reflects the basic fragmentation features of a lupane-type triterpenoid molecule.

### 3.5.4: Melting point of LO1

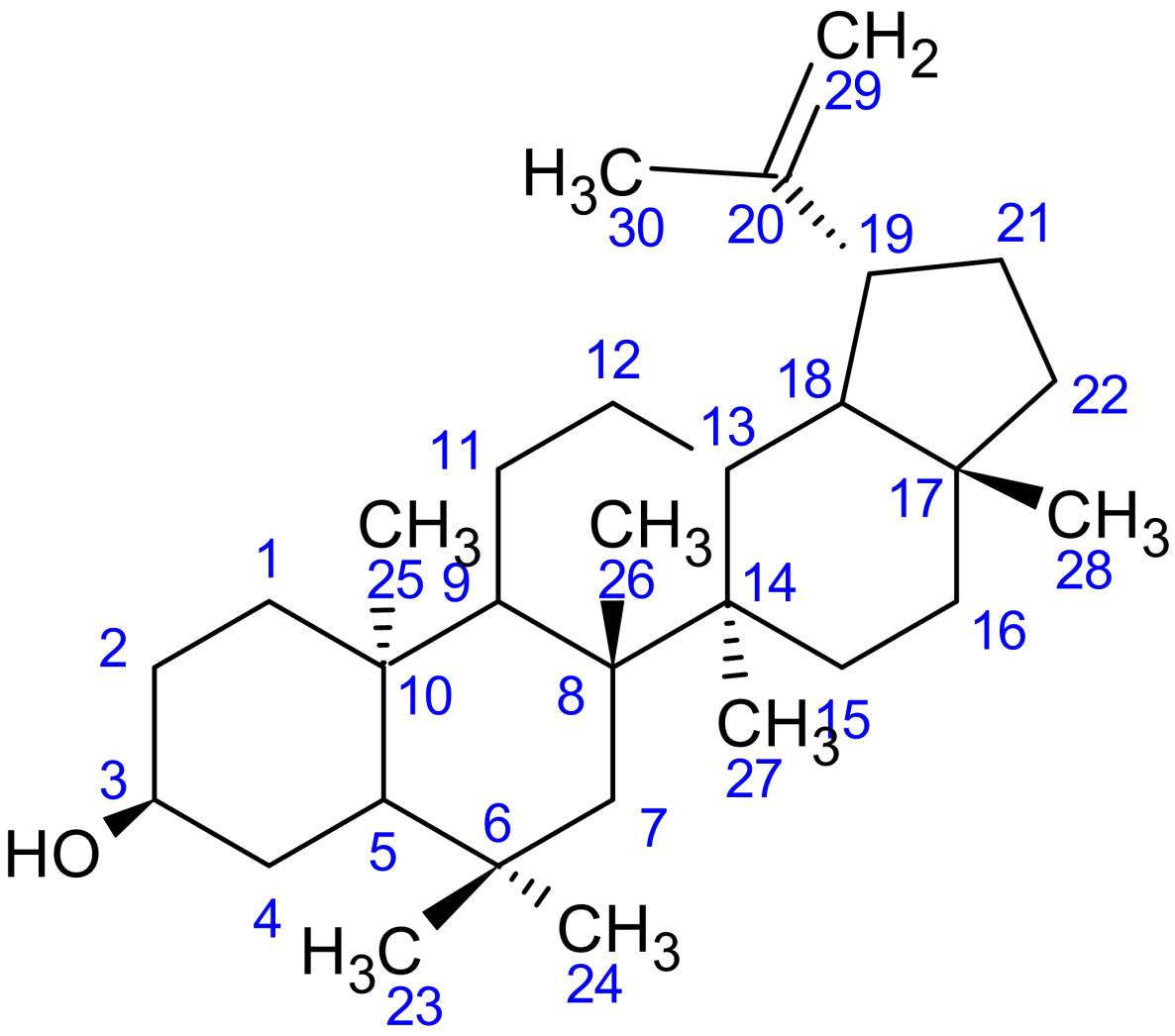
The melting point of LO1 was found to be within the range of 214-215ºC. This further confirms the identity of the isolated compound.

Table 3.4: Melting point of LO1

| S/N | Compound | Mp (ºC) |
|---|---|---|
| 1 | LO1 | 214-215 |

### 3.5.5: The name and structure of LO1

Comparison of the experimental spectroscopic data generated for LO1 and the data reported in the literature (Shwe *et al*., 2019) afforded the identification of the isolated compound to be 3β-lup-20(29)-en-3-ol (lupeol) (Figure 3.10).




3β-lup-20(29)-en-3-ol (Lupeol)

**Figure 3.10**: The structure and name of LO1

### 3.5.6 Growth inhibition effects of LO1 on *Sorghum bicolor* radicles

The isolated compound coded LO1 exerted growth inhibition effect on *Sorghum bicolor* radicles as shown in Figure 3.11. 1 mg/mL of the compound coded LO1 completely suppressed (00.00 mm) the growth of radicles at 24 hours. At 96 hours, the mean length of the emerging radicle translated to 7.67 ± 0.32 mm (56.66% suppression) by 96 hours. These results were statistically significant at $P<0.0001$

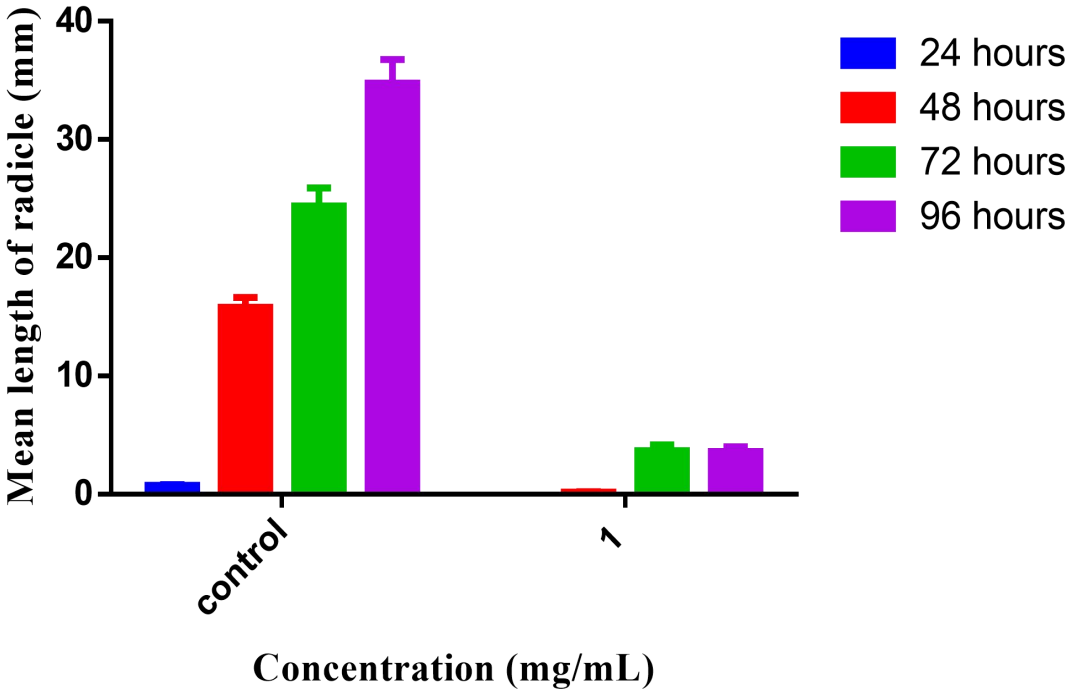



**Figure 3.11:** Growth inhibition effect of LO1 on *Sorghum bicolor* radicle. Each bar represents mean ± SEM. Data are significant at $p<0.0001$. n=20

### 3.6 Spectroscopic analysis of compound LO2

#### 3.6.1 1H-NMR [$CDCl_3$ 600 MHz]

1H NMR analysis of compound LO2 (white amorphous powder) resulted in the generation of the following spectra data used for characterization and identification of the compound.

Comparison with the reported literature values (Cayme and Ragasa, 2004) showed similar signals coinciding to the presence of double angular methyl protons attached to C18 and C19 appearing at δ 0.64 and δ 1.23 respectively. Branching methyl protons at C21 was seen at δ 0.99. Similarly, the signature methyl protons of the isopropyl group at C26 and C27 appeared at δ 0.78 as overlapping 6H duplet. Methine proton that is highly deshielded due to the attachment of electronegative oxygen atom at C3 appeared at δ 3.57 as a single proton multiplet, The overlapping triplet signal occurring at δ 5.20 is indicative of olefinic protons at C6 (Table 3.5).

Table 3.5 1H NMR (400 MHz) chemical shifts pattern of LO2

| Position | 1H NMR reported in literature | 1H NMR experimental | Nature of carbon |
|---|---|---|---|
| 1 | 1.08 | 1.10 | $CH_2$ |
| 2 | 1.49 | 1.49 | $CH_2$ |
| 3 | 3.53 | 3.57 | CH |
| 4 | 2.24 | 1.98 | $CH_2$ |
| 6 | 5.35 d | 5.20 | $CH_2$ |
| 7 | 1.56 | 1.51 | $CH_2$ |
| 8 | 1.46 | 1.39 | CH |
| 9 | 0.94 | 0.95 | CH |
| 11 | 1.45 | 1.50 | $CH_2$ |
| 12 | 1.15 | 1.12 | $CH_2$ |
| 14 | 1.03 | 1.98 | CH |
| 15 | 1.05 | 1.15 | $CH_2$ |
| 16 | 1.24 | 1.24 | $CH_2$ |
| 17 | 1.13 | 1.15 | CH |
| 18 | 0.68 s Me | 0.64 Me | CH3 |
| 19 | 1.10 s Me | 1.23 Me | CH |
| 20 | 1.36 | 1.25 | CH |
| 21 | 0.94 s Me | 0.99 Me | $CH_2$ |
| 22 | 1.32 | 1.30 | $CH_2$ |
| 23 | 1.15 | 1,25 | $CH_3$ |
| 24 | 0.93 | 0.94 | $CH_3$ |
| 25 | 1.65 | 1.63 | $CH_3$ |
| 26 | 0.82 d Me | 0.83t Me | $CH_3$ |
| 27 | 0.83 d Me | 0.87 d Me | $CH_3$ |
| 28 | 1.22 | 1.24 | $CH_3$ |
| 29 | 0.85 t Me | 0.82 Me | $CH_3$ |

### 3.6.2: Correlated-spectroscopy (COSY)

The COSY spectrum of LO2 revealed a cross peak between the methylene proton at δ 1.10, (H-1) and the methylene proton at δ 1.49, (H-2). The methylene protons at δ 1.49 (H-2) and δ 1.98 (H-4) exhibited cross peak association with the carbinol proton of δ 3.57 (H-3). The olefinic proton singlet at δ 5.20 (H-6) displayed cross peaks with the methylene proton at δ 1.51 (H-7). The methine proton at δ 1.39 (H-8) and the methylene proton at δ 1.51 (H-7) were correlated. The cross-peaks connection between the methylene proton at δ 1.50 (H-11) and the methylene proton at δ 1.12, (H-12) were observed.

### 3.6.3: Mass spectroscopy (MS)

The mass spectrum of LO2 showed peaks at m/z ratio of 414.3 which corresponds to the molecular formula $C_{29}H_{50}O$. Peak at m/z 396 is as a result of the loss of water molecule [$H_2O$] from the molecular ion. Further dealkylation [$CH_3$] gave the peak at m/z 381. Peak at m/z 273 is as a result of fragmentation of the side chain [$C_{10}H_{21}$] at C17-C20. The dehydration (-$H_2O$) of the daughter fragment at m/z 273 gave the peak at m/z 255.

### 3.6.4: Melting point of LO2

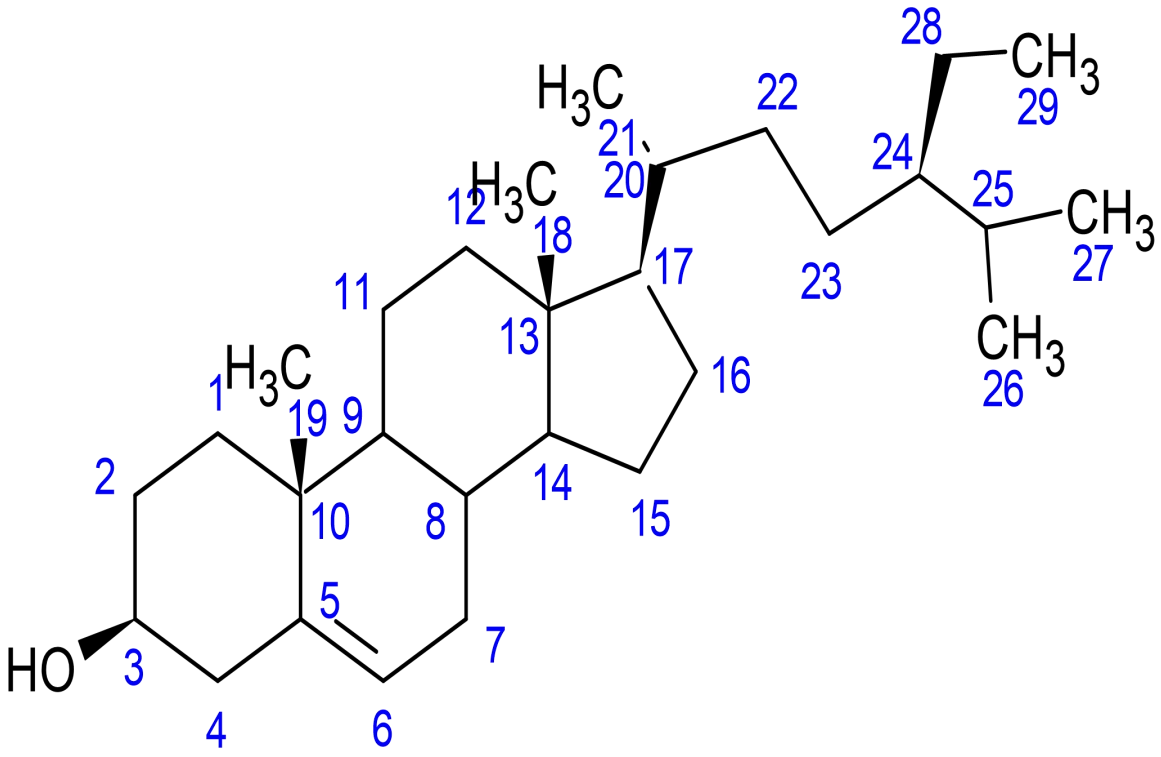
The melting point of LO2 was found to be within the range of 133-135ºC. This further confirms the identity of the isolated compound.

Table 3.6: Melting point of LO2

| S/N | Compound | Mp (ºC) |
|---|---|---|
| 1 | LO2 | 133-135 |

### 3.6.5: The name and structure of LO2

Comparison of the experimental spectroscopic data generated for LO2 and the data reported in the literature afforded the identification of the isolated compound to be stigmast-5-en-3β-ol (β-sitosterol).




Stigmast-5-en-3β-ol (β-sitosterol)

**Figure 3.12**: The structure and name of LO2

### 3.7 MTT assay result of the isolated compounds (Lupeol and β-sitosterol)

Cytotoxic activity of lupeol and β-sitosterol against human prostate cancer cell lines ($PC_3$) and uterine cervical cancer cell lines (Hela) is as shown in Table 3.6. Lupeol exhibited greater cytotoxic activity than that of beta-sitosterol. The isolated compounds were observed to be less toxic to the normal human body cells (BJ)

Table 3.7**:** Cytotoxic effect of compounds isolated from LGSB against human cancer cell lines

| Compound (30 μM) | Percentage inhibition (%) | | |
|---|---|---|---|
| | $PC_3$ | Hela | BJ |
| Lupeol | 40.80 | 36.60 | 14.10 |
| β-sitosterol | 23.60 | 25.10 | 42.70 |
| Doxorubicin | 89.91 | 90.60 | 89.61 |

$PC_3$ (prostate cancer cells) Hela (uterine cervical cancer cells) BJ (normal cells)

### 3.8.1 Effect of LGSB extract and lupeol on the weight of prostate gland of rat

The crude extract exerted a dose dependent reduction in the prostate weights of the experimental animals. 100 and 400 mg/kg body weight of the extract caused 58.33% (325.00 ± 22.50) and 69.87% (235.00 ± 32.30) reduction in the enlargement of the prostate gland of rats. The isolated compound lupeol at 5 mg/kg body weight caused a 63.08% (288.00 ± 36.50) reduction of the testosterone induced enlargement of the prostate gland of experimental animals (Figure 3.13).

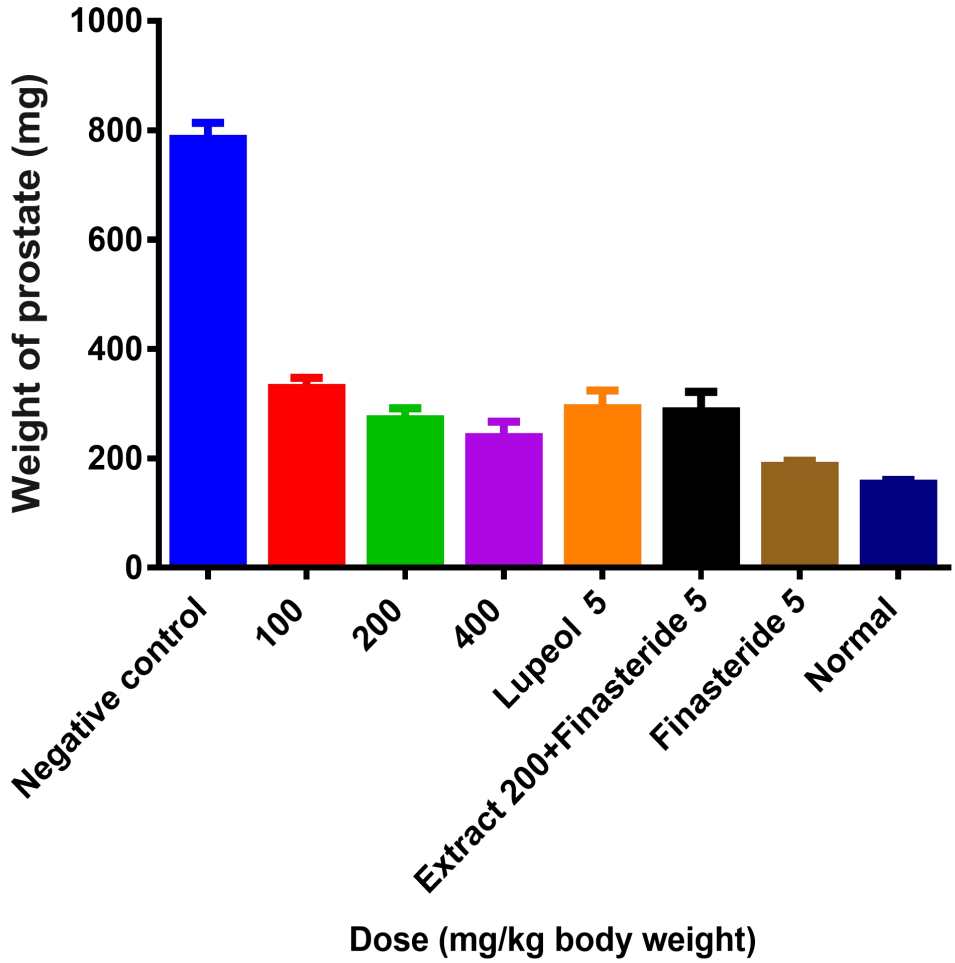

1000
800
600
400
200
0
Weight of prostate (mg)
Negative control
100
200
400
Lupeol 5
Extract 200+Finasteride 5
Finasteride 5
Normal
Dose (mg/kg body weight)

**Figure 3.13**: Effect of LGSB extract and compound lupeol on weight of rat prostate
The values are expressed as mean±SEM. Data are significant at $p<0.05$. n=5

### 3.8.2 Effect of LGSB extract and lupeol on the volume of rat prostate

The crude extract exerted a dose dependent reduction in the prostate volume of the experimental animals. 100 and 400 mg/kg body weight of the extract caused 51.23% (0.80 ± 0.06) and 59.76% (0.66 ± 0.06) reduction in the prostate volume of rats. Lupeol at 5 mg/kg body weight caused a 59.76% (0.66 ± 0.06) reduction of the testosterone induced increment of the prostate volume of experimental animals (Figure 3.14).

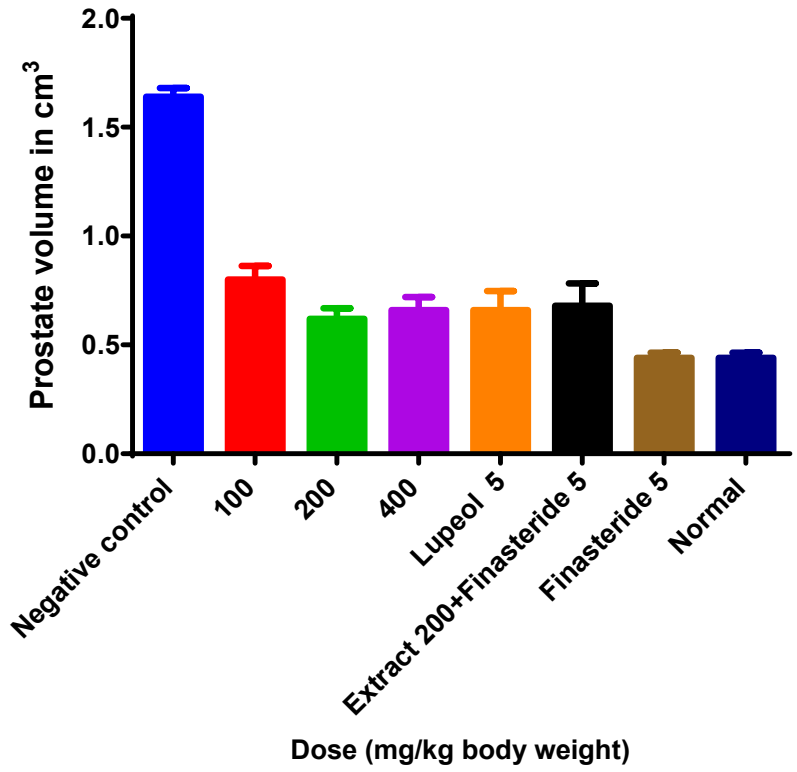



**Figure 3.14:** Effect of LGSB extract and lupeol on the volume of rat prostate. The values are expressed as mean ± SEM. Data are significant at $p<0.0001$. n=5

### 3.8.3 Effect of LGSB extract and lupeol on the prostate index (PI) of rat

The crude extract exerted a dose dependent reduction in the prostate index of the experimental animals. 100 and 400 mg/kg body weight of the extract caused 45.28% (1.39 ± 0.13) and 50.79% (1.25 ± 0.14) reduction in the prostate index of rats. Lupeol at 5 mg/kg body weight caused a 43.70% (1.43 ± 0.09) reduction of the prostate index of experimental animals (Figure 3.15).

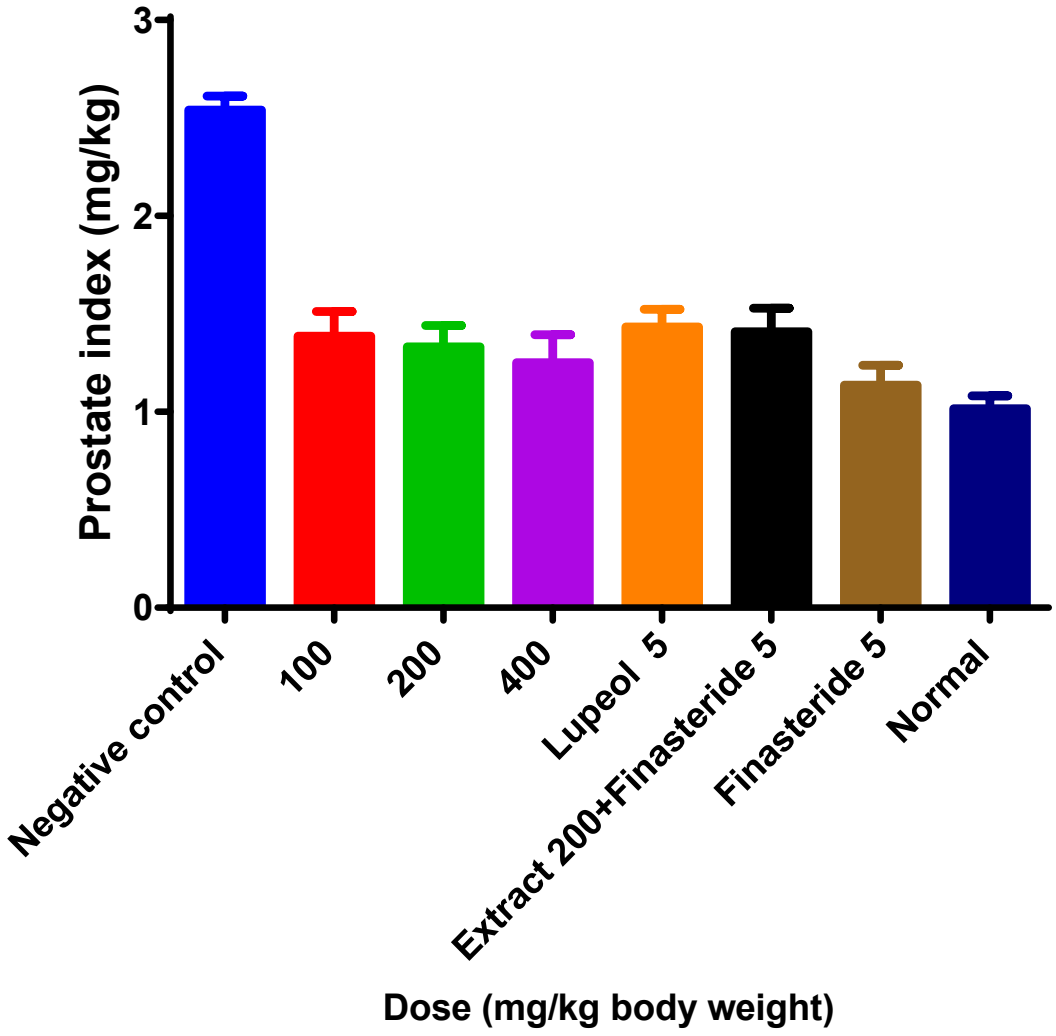



**Figure 3.15**: Effect of LGSB extract and lupeol on prostate index of rat. The values are expressed as mean ± SEM. Data are significant at $p<0.0001$. n=5

### 3.8.4 Effect of LGSB extract and lupeol on PSA of rat

PSA is an important marker that is used to assay the state of health of prostate gland. The crude extract caused a dose dependent reduction on the level of serum PSA of the test animals (Figure 3.16). The crude extract exerted a dose dependent reduction in the PSA of the experimental animals. 100 and 400 mg/kg body weight of the extract caused 48.19% (1.29 ± 0.07) and 70.28% (0.74 ± 0.18) reduction in the PSA of rats. Lupeol at 5 mg/kg body weight caused a 69.88% (0.75 ± 0.09) reduction of the PSA of experimental animals.

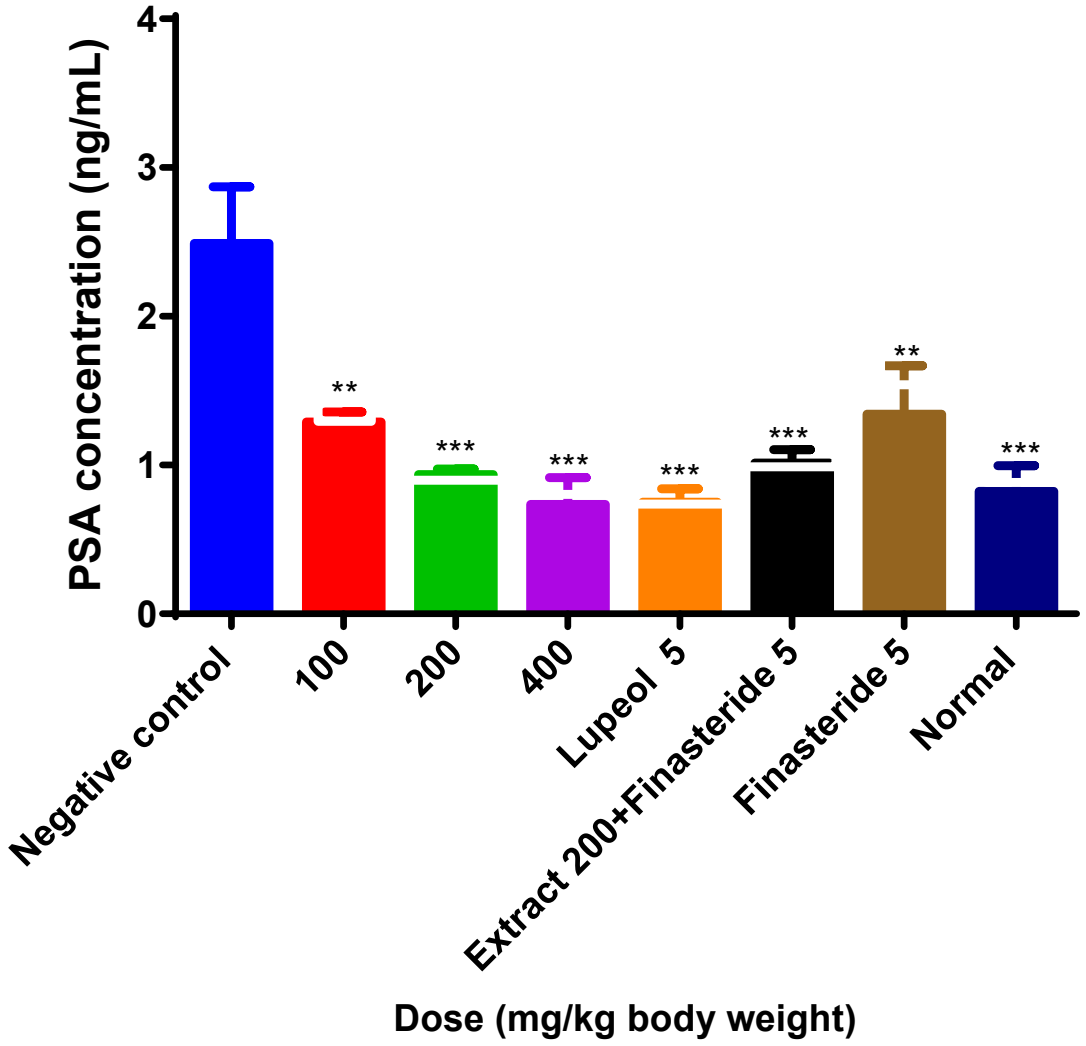



**Figure 3.16:** Effect of LGSB extract and lupeol on PSA of rat. The values are expressed as mean ± SEM. Data with ** are significant at $p<0.01$ while values with superscripts *** are significant at $p<0.001$. n=5

### 3.8.5 Effect of LGSB extract and lupeol on testosterone level of rat

The crude extract caused a reduction in the circulating serum concentration of testosterone when compared to the negative control. Lupeol was found to exert the highest effect when compared to other treatment groups (Figure 3.17). The crude extract exerted a reduction in the circulating serum of the experimental animals. 100 and 400 mg/kg body weight of the extract caused 79.26% (0.28 ± 0.06) and 62.96% (0.50 ± 0.13) reduction in the serum concentration of rats. Lupeol at 5 mg/kg body weight caused a 88.67% (0.18 ± 0.04) reduction of the serum concentration of experimental animals.

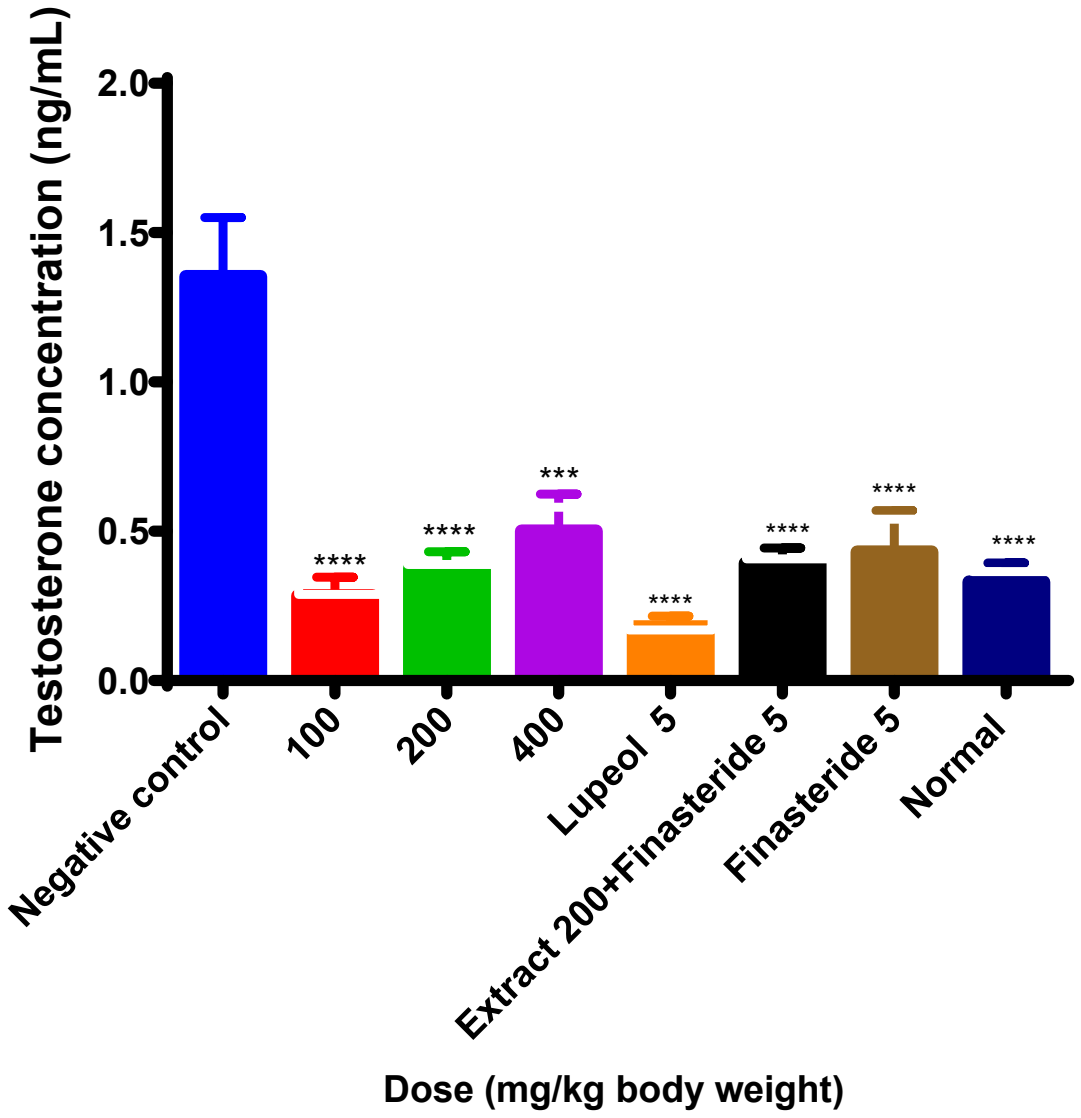



**Figure 3.17:** Effect of LGSB extract and lupeol on testosterone level of rat. The values are expressed as mean ± SEM. Data with *** are significant at $p<0.001$ while values with superscripts **** are significant at $p<0.0001$. n=5

### 3.8.6 Effect of LGSB extract and lupeol on testosterone/ estradiol ($T/E_2$) ratio of rat

Crude extract caused various levels of reduction in the T/E2 ratio with peak effects seen in lupeol (Figure 3.18). The crude extract exerted a reduction in the $T/E_2$ ratio of the experimental animals. 100 and 400 mg/kg body weight of the extract caused 92.50% (0.01 ± 0.00) and 75.00% (0.02 ± 0.01) reduction in the serum concentration of rats. Lupeol at 5 mg/kg body weight caused a 96.25% (0.003 ± 0.00) reduction of the $T/E_2$ ratio of the experimental animals.

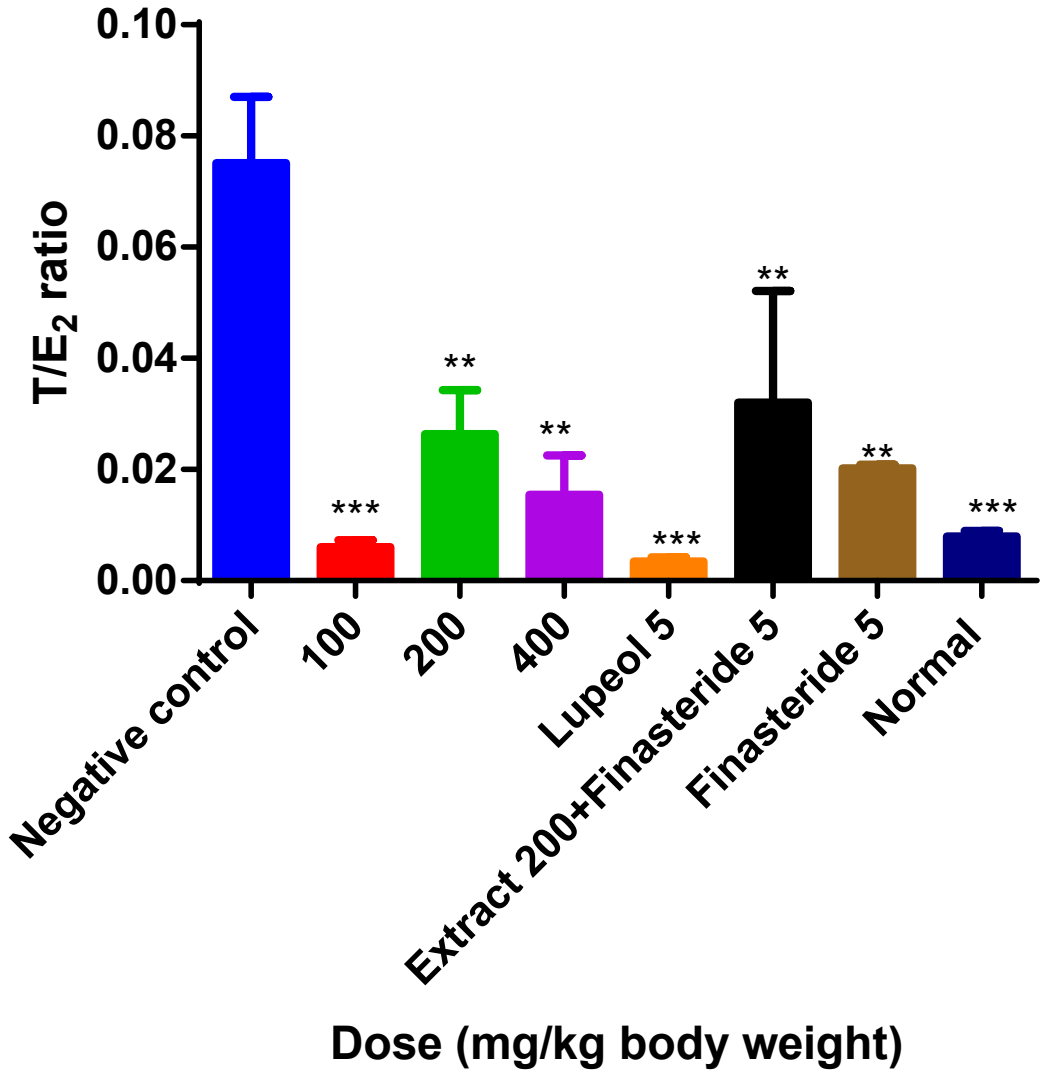



**Figure 3.18:** Effect of LGSB extract and lupeol on $T/E_2$ ratio of rat. The values are expressed as mean ± SEM. Data with ** are significant at $p<0.01$ while values with superscripts *** are significant at $p<0.001$. n=5

### 3.9 Histopathology analysis result

Histopathology result of the prostate tissues reveal morphological features consistent with a challenged prostate and that of a resolving or suppressed insult to the prostate gland of experimental animals.

### 3.9.1 Histopathology result of the normal group

Histopathological presentation of the normal control group has revealed a normal prostate gland histoarchitecture. Acini were seen to be regular. A simple columnar epithelium was distributed in a single layer. The stroma was not thickened and enlarged. Result is evidenced in Figure 3.19.

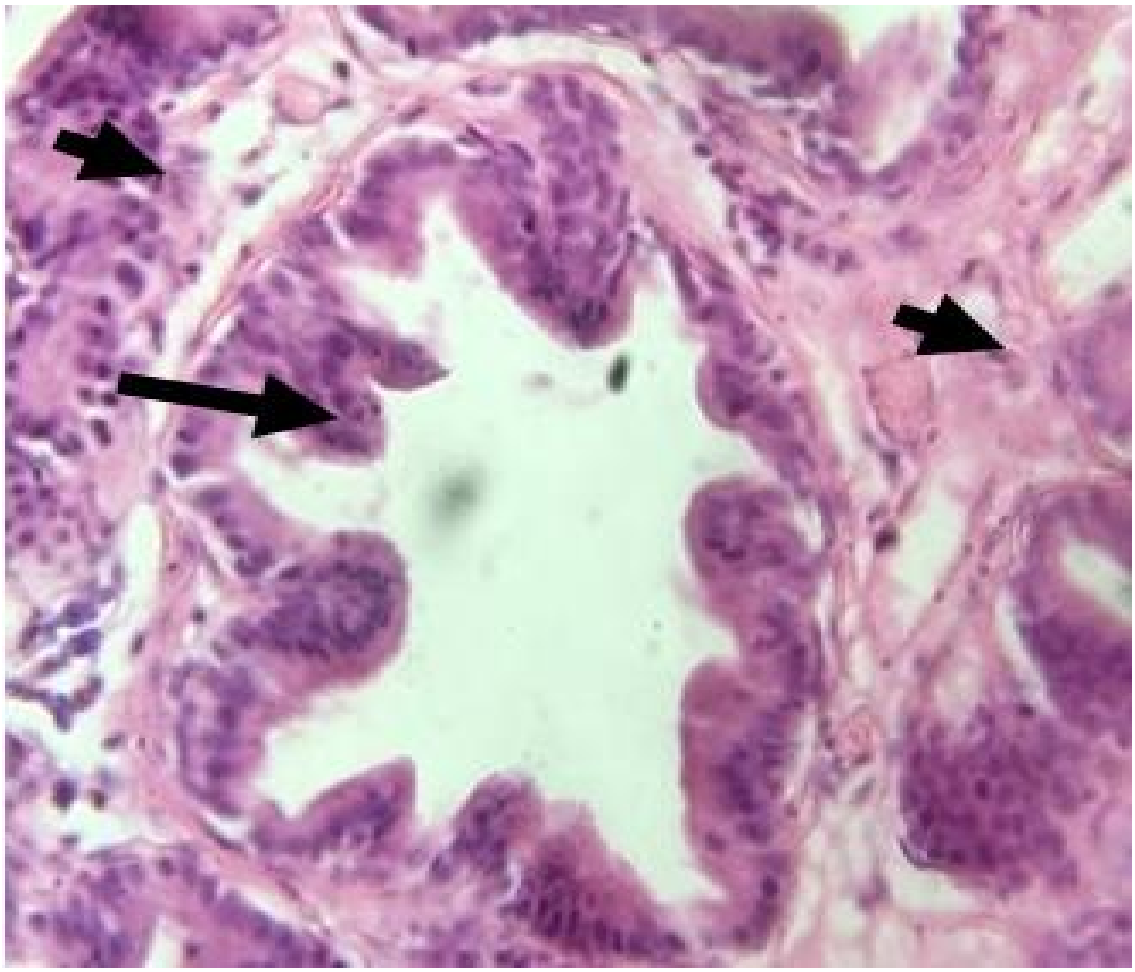


**Figure 3.19**: Photomicrograph of prostatic sections from the control group showing normal prostate gland histoarchitecture with regular size acini (long arrow). The lumen of the acini contains prostatic secretions. Simple columnar epithelium distributed in a single layer lines the acini. There is also fine stroma and a continuous basal layer composed of collagen fibers and fibroblasts (short arrow). X400

### 3.9.2 Histopathology result of the 100 mg/kg group

Histopathological presentation of the 100 mg/kg dose of the extract treatment group showed a mild reduction in hyperplasia and hypertrophic activity. The stroma was slightly thickened and a basal layer composed of collagen fibers. See Figure 3.20.

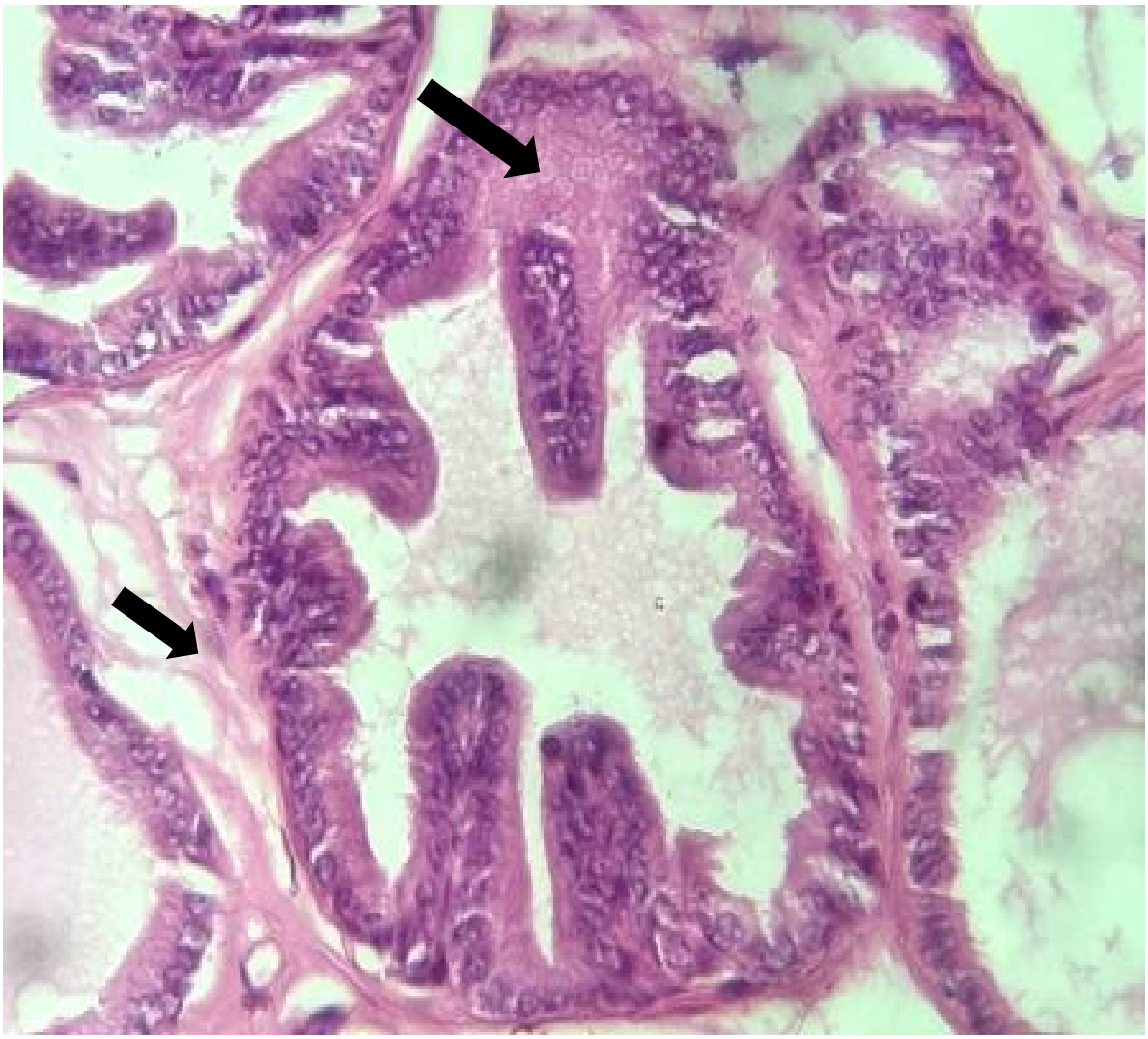


**Figure 3.20**: Photomicrograph of prostatic sections from 100 mg/kg dose of the extract treatment group showed a mild reduction in hyperplasia and hypertrophy (long arrow) with slightly thickened stroma and a continuous basal layer composed of collagen fibers (short arrow).

### 3.9.3 Histopathology result of the 200 mg/kg group

Histopathological presentation of the 200 mg/kg dose of the extract treatment group showed a mild hyperplastic and hypertrophic activity within the prostate. See Figure 3.21.

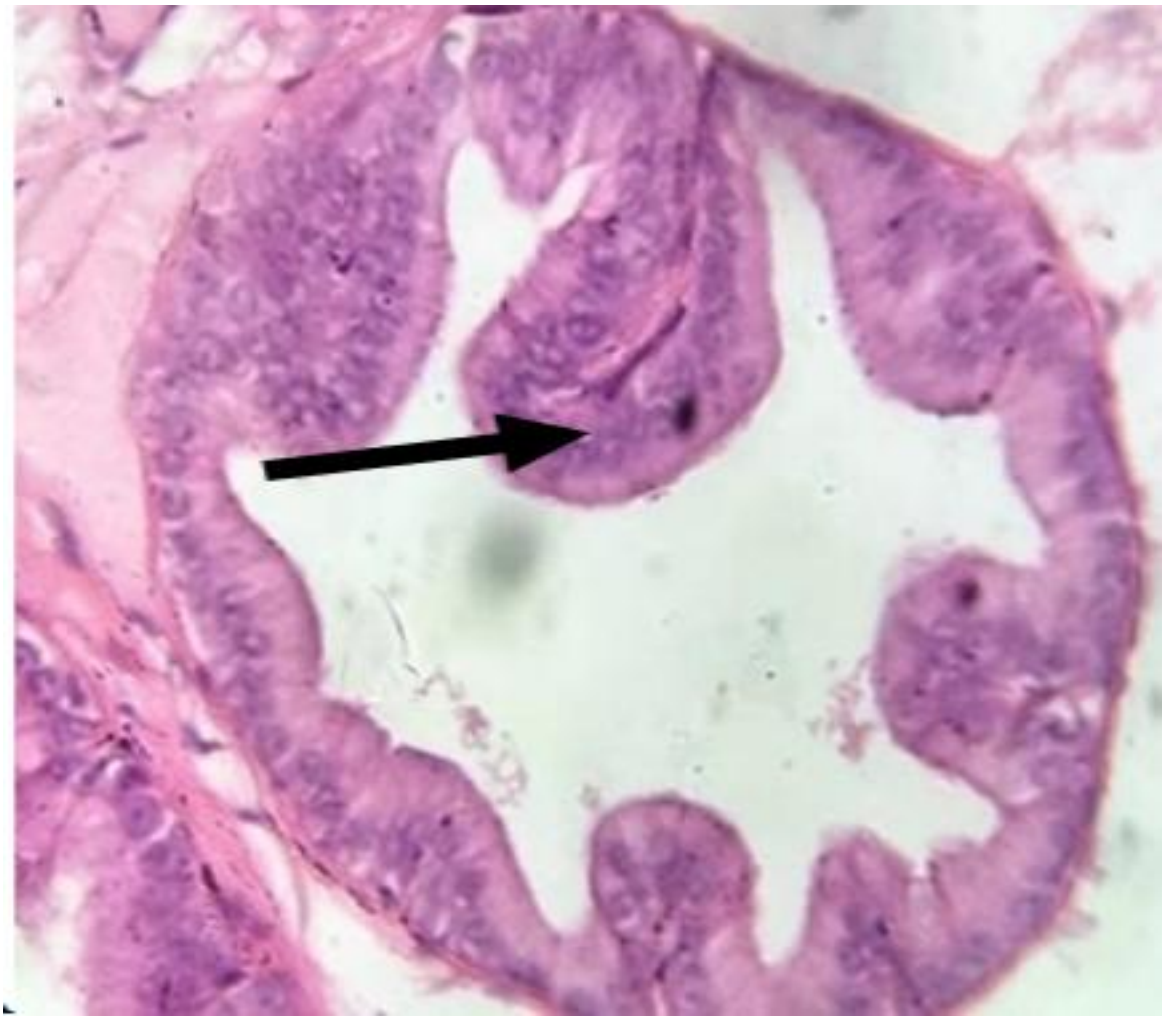


**Figure 3.21**: Photomicrograph of prostatic sections of the 200 mg/kg dose treatment group showed a mild squamous metaplasia of the prostate gland seen in prostate sections, with

squamous differentiation of the prostatic epithelium forming keratin filaments in the lumen (long arrow).

### 3.9.4 Histopathology result of the 400 mg/kg group

Histopathological presentation of the 400 mg/kg dose of the extract treatment group showed a significant reduction in hyperplastic and hypertrophic activity within the prostate. See Figure 3.22.

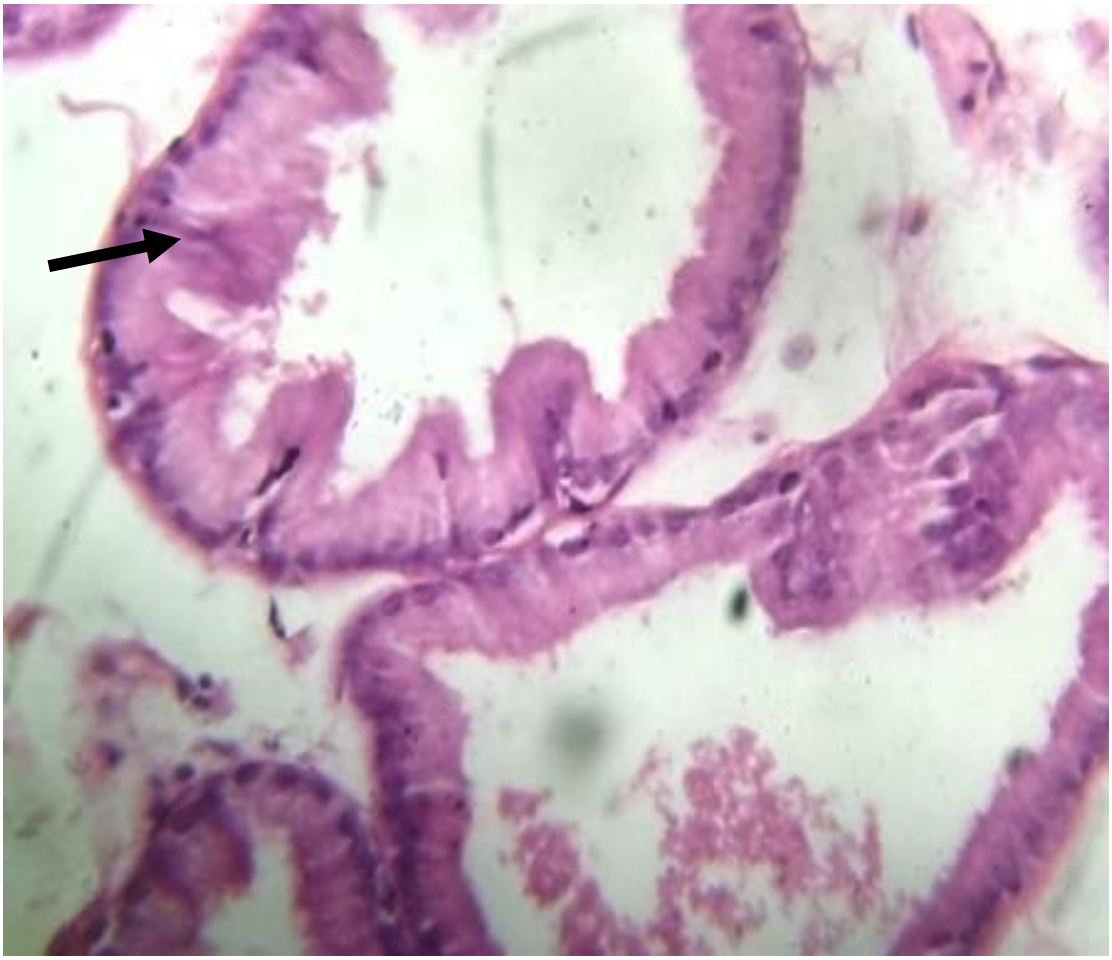


**Figure 3.22**: Photomicrograph of prostate sections from 400 mg/kg dose of the extract treatment group showed marked reduction in hyperplasia and hypertrophy with squamous differentiation of the prostatic epithelium forming keratin filaments in the lumen (long arrow).

### 3.9.5 Histopathology result of lupeol (5 mg/kg) group

Histopathological presentation of the lupeol group revealed mild hyperplasia and squamous activity. There was no infiltration of the lumen by papillary out growth. This indicates minimal effects of testosterone on the prostate gland. See Figure 3.23.

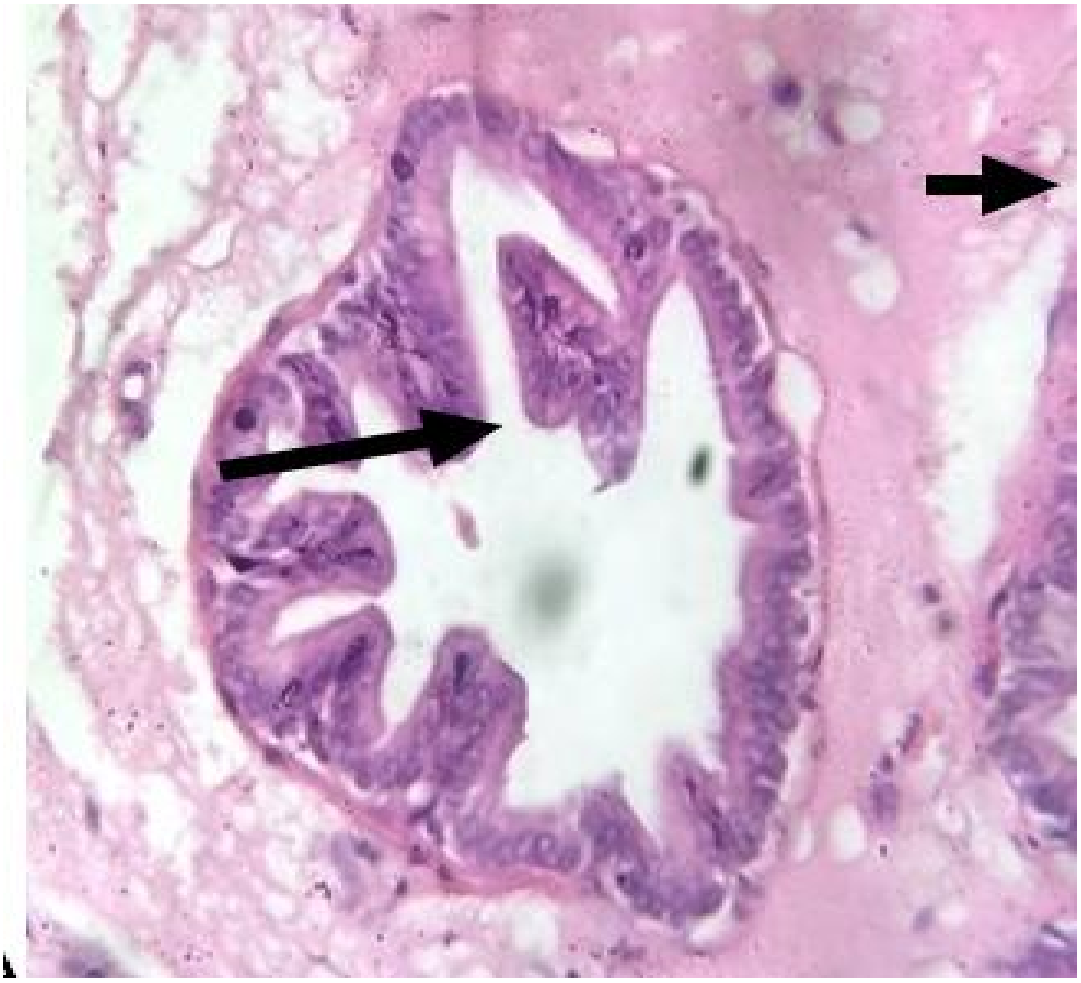


**Figure 3.23**: Photomicrograph of prostatic sections from the lupeol (5 mg/kg) treatment group shows marked reduction in hyperplasia and hypertrophy (long arrow) with fine stroma and a continuous basal layer composed by collagen fibers and fibroblasts (short arrow).

### 3.9.6 Histopathology result of the extract (200 mg/kg) + finasteride (5 mg/kg) group

Histopathological presentation of the combo group revealed non-synergistic activity between co-administration of the extract and finasteride.

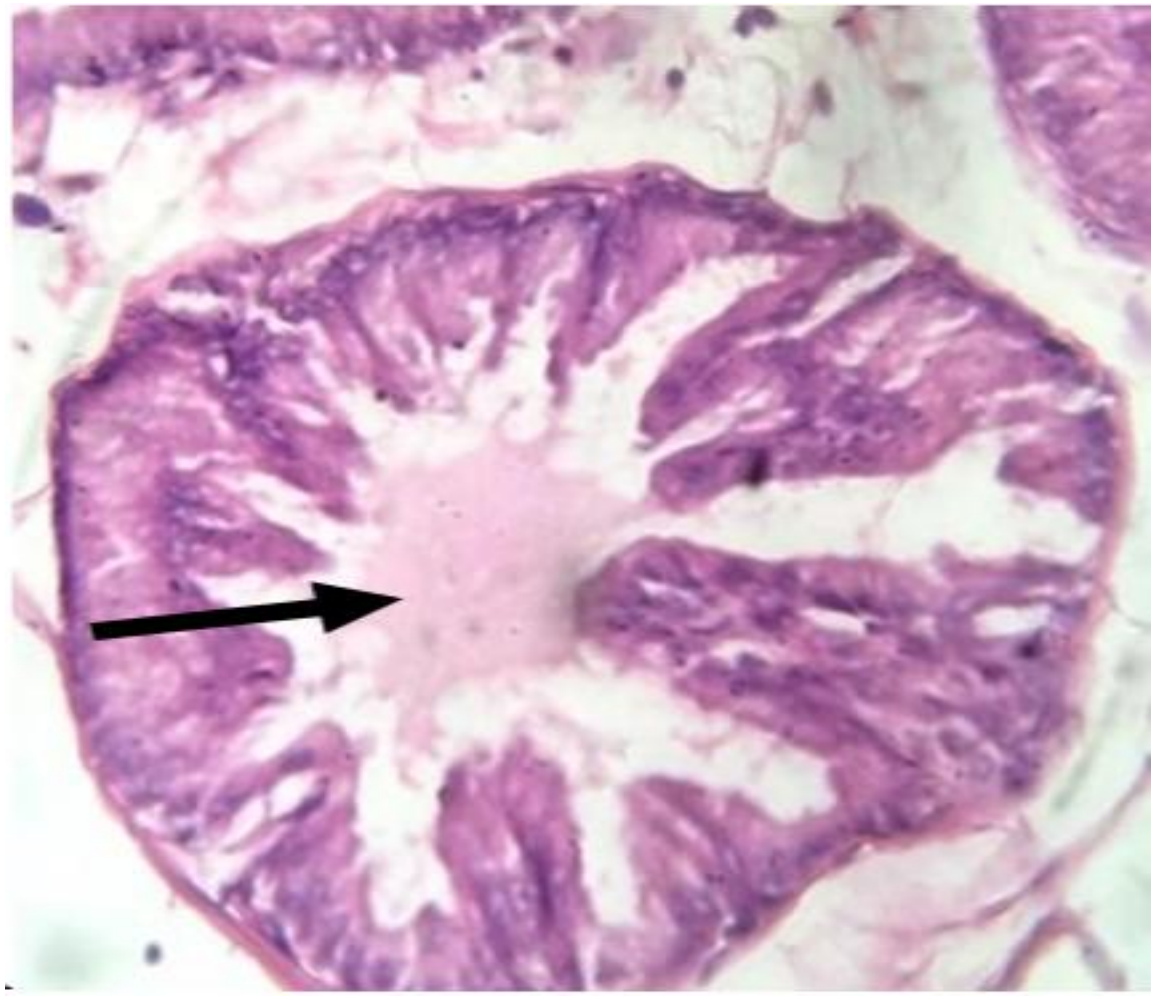


**Figure 3.24**: Photomicrograph of the prostatic sections of the extract (200 mg/kg) + finasteride (5 mg/kg) group show an irregular acinar shape . Squamous metaplasia of the prostate gland is seen. There is an inflammatory infiltrate composed of mononuclear cells (long arrow) and Fibrosis in the interstitium.

### 3.9.7 Histopathology result of finasteride at 5 mg/kg group

Histopathological presentation of the finasteride group (5 mg/kg) showed a significant reduction in hyperplastic and hypertrophic activity within the prostate, see Figure 3.25.

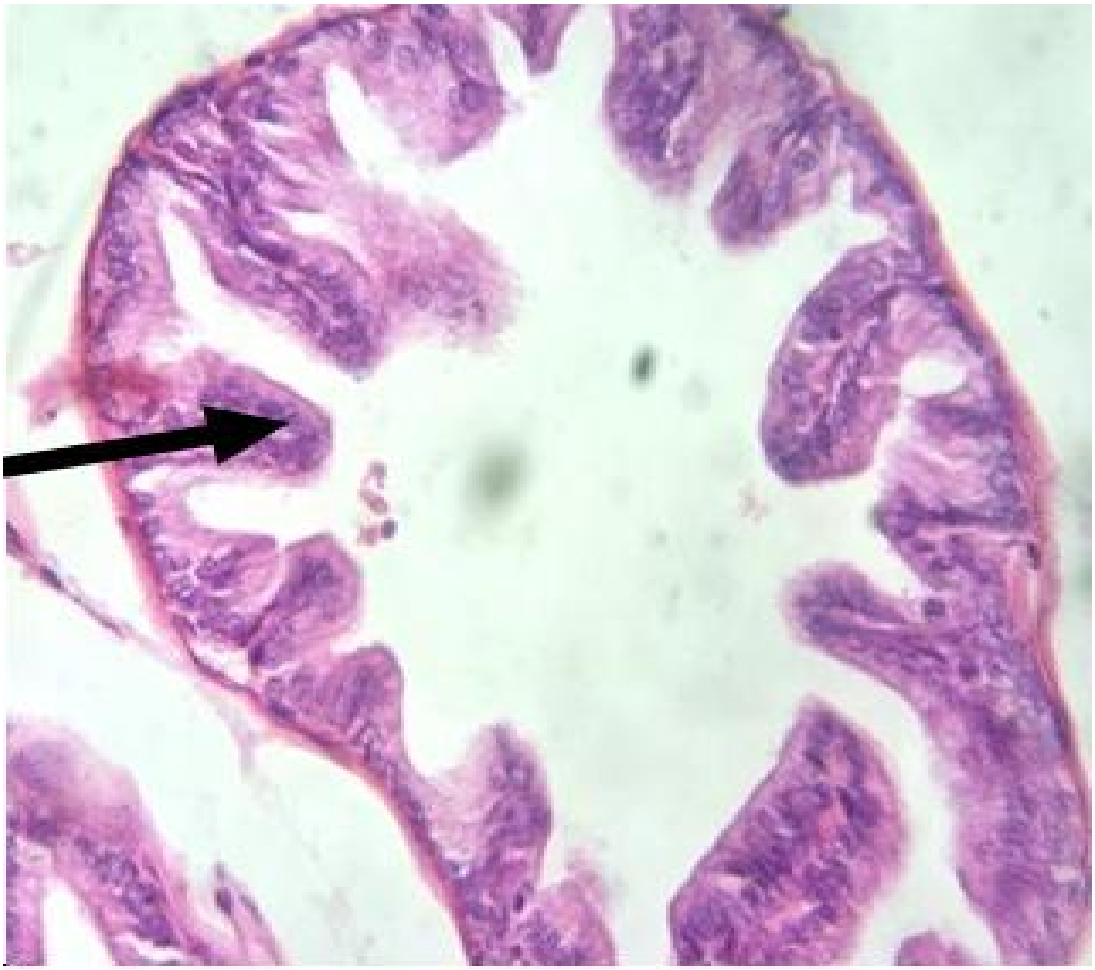


**Figure 3.25**: Photomicrograph of prostatic sections of the positive control group revealed mild Squamous metaplasia of the prostate with squamous differentiation of the prostatic epithelium forming keratin filaments in the lumen (long arrow).

### 3.9.8 Histopathology result of the BPH (negative control) group

Histopathological presentation of the BPH group shows a massive inflammatory events with significant signs of prostate enlargement. There was hyperplasia of the epithelium and signs of congestion of the lumen. See figure 3.26.

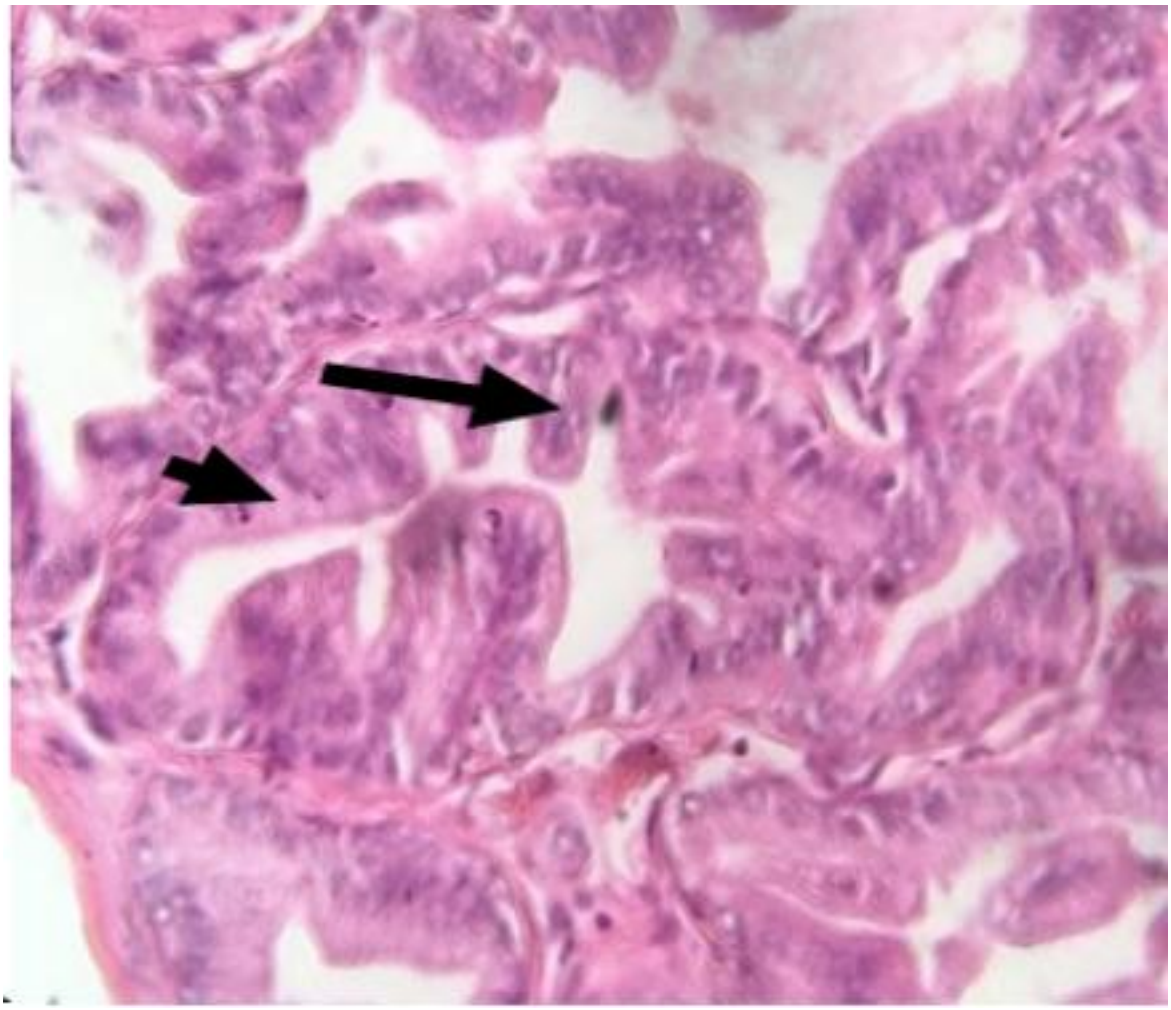


**Figure 3.26:** Photomicrograph of prostatic sections from the untreated negative control group shows irregular acinar shape with papillary projection into the lumen (long arrow), and foci of piling-up hyperplastic nodules are evident. Thickened prostate glandular epithelium was also seen. The epithelium is highly cylindrical, multi layered, and shows irregularly aligned round/ovoid nuclei (short arrow).

# CHAPTER FOUR

## 4.0 DISCUSSION

The results of the study showed that both extracts displayed cytotoxic and growth inhibition effects in a dose-dependent manner with LGSB showing higher activity than LGRB. This indicated that the bioactive compounds responsible for the observed activity could be sequestrated more in the stem bark than in the root bark. It could also be possible that there may be the occurrence of an entirely different molecule with the biological activities in the stem bark that may be lacking in the root bark. (Figures 3.1-3.4)

The acute toxicity result has shown that the extract could be safe and non-toxic to the test animals. At 5000 mg/kg, the was no mortality or signs of toxicity in the test animals. This shows that the extract could be safe for human consumption. However, sub-chronic toxicity evaluation is required to examine the effect of the extract usage in the biological system over an extended period (Table 3.2)

The Dichloromethane fraction exhibited significant cytotoxic activity. The concentration of 20 µg/mL of the DCM fraction caused a statistically significant 73.30 ± 6.67% mortality in the tadpoles. 40, 80 and 200 mg/mL caused 86.70 ± 6.67, 100.00 ± 0.00 and 100.00 ± 0.00% respectively. There was a loss of activity by the aqueous fraction. This was an indication that the components responsible for the cytotoxic effects could be sequestrated in the DCM fraction

rather than the AQ fraction, meaning that they are lipophilic in nature. The lipophilicity of these compounds could make it easier for them to cross the cell membrane of the tadpoles to exert the cytotoxic effect (Figure 3.5).

The DCM fraction dose-dependently inhibited the emerging radicles of *Sorghum bicolor*. The significance of this result is that the DCM fraction may contain bioactive components capable of inhibiting tumour growth in a human system. It also means that it could be useful for the treatment of tumour related ailments. The AQ fraction was lethal to the emerging radicles. The lethality of the AQ fraction at the concentration employed for the study could be due to the solubility of the growth-inhibitory components in the aqueous medium which makes them bioavailable for uptake by the root system of *Sorghum bicolor.*( Figure 3.6)

Natural products have, over time, proven to be a rich source of anticancer agents with lower toxic potentials and wider acceptability. Against this backdrop, the extract of *L. griffonianus* was tested for cytotoxicity and growth inhibition effects, cognizance of the fact that it is used to treat tumour-related ailments. The ability of plant extract to suppress or stimulate the growth of plant organs and animals has been shown to correlate positively with antitumor, herbicidal, plant growth stimulant or inhibitor activity. The traditional use of the plant to treat tumours is shown in the ability of the plant's extract to suppress the growth of emerging *Sorghum bicolor seeds'* radicle length and cytotoxicity to *Raniceps raninus* tadpoles. This effect may be due to the ability of the secondary bioactive metabolites such as triterpenoids present in the plant's part to interfere with DNA division, thus arresting cell proliferation. Some antitumor agents act through such mechanisms of action against multiplying cancer and tumour cells. The observed activity seen in the study seems to follows the chemosystematics arrangement of species in the Fabaceae

plant family (Blatt *et al.*, 2002). Lonchocarpin and Derricin isolated from *Lonchocarpus sericeus* (Fabaceae) have demonstrated cytotoxicity against leukemic cell lines(Cunha *et al.*, 2003)]. Chalcone 2′,4′-dihydroxy-5′-prenylchalcone isolated from *Lonchocarpus cultratus* (Fabaceae) exerted a growth-suppression effect against human breast (Hs578T), prostate ($PC_3$), and liver (Hep G2) cancer cell lines (Da Silva Landim *et al.*, 2021). Chalcones are flavonoids that lacks the heterocyclic c-ring. They are known to exhibit cytotoxic effect both *in vitro* and *in vivo*. Compounds in the triterpenoid class have been reported in the literature to possess cytotoxic effect. This has further substantiated the fact that *L. griffonianus* exhibited the observed activity.

The use of simple benchtop assays like the ones employed in the present study has been successfully used to screen plants with possible antitumor and anticancer activity. For instance, previous work by Owolabi and Ayinde (2022) using this simple but sensitive technique has afforded the isolation of anticancer metabolite catechin from *Musanga cecropioides* (Urticaceae).

The isolated compound LO1 has a melting point (m.p.) of 133–135 °C. The m.p. range of 1–2 °C showed that the isolated compound was pure and was comparable to the one reported in the literature for β-sitosterol (Ododo *et al*., 2016).

The m.p. of the isolated compound LO2 was found to be 214-215ºC. The variation was in the range of 1–2 °C which indicated that the compound was pure. The result also corresponds with the value reported in the literature (Agarwal and Rangari, 2003).

Literature comparison of spectral properties of compounds LO1 and LO2 afforded complete elucidation of the compounds. Mass spectrum (MS) fragmentation pattern, 1D and 2D NMR chemical shifts were compared with the relevant literature information (Adzu *et al.*, 2015; Jenecius *et al.*, 2012). LO1 was found to be lupeol and LO2 found to be β-sitosterol. Analysis of the MS fragmentation pattern of LO1 is as follows: The mass spectrum of LO1 showed the parent ion [M+] at m/z ratio of 426.3 g/mol, which corresponds to the molecular formula of $C_{30}H_{50}O$, The fragmentation pattern of compound LO1 is as shown in figure . The molecular ion peak is seen at m/z 426.6.3 which corresponds to the molecular of formula of $C_{30}H_{50}O$. The loss of water molecule resulted in the formation of the peak at m/z 408.3. Demethylation of the methyl ($CH_3$) functional group at C8 result in the formation of a ionic compound with m/z 393.3. Further cleavage of an alkane ($C_2H_4$) led to the daughter peak observed at m/z 365.3. the loss of $C_{13}H_{20}$ due to Mc Lafferty rearrangement resulted in the formation of a stable peak at 189.3. This was found to coincide with the fragmentation pattern reported in the literature for lupeol (Ngenge *et al*., 2022). In addition, the fragmentation pattern similarity to the one reported in the literature suggests that LO1 is lupeol (Figure 4.1).

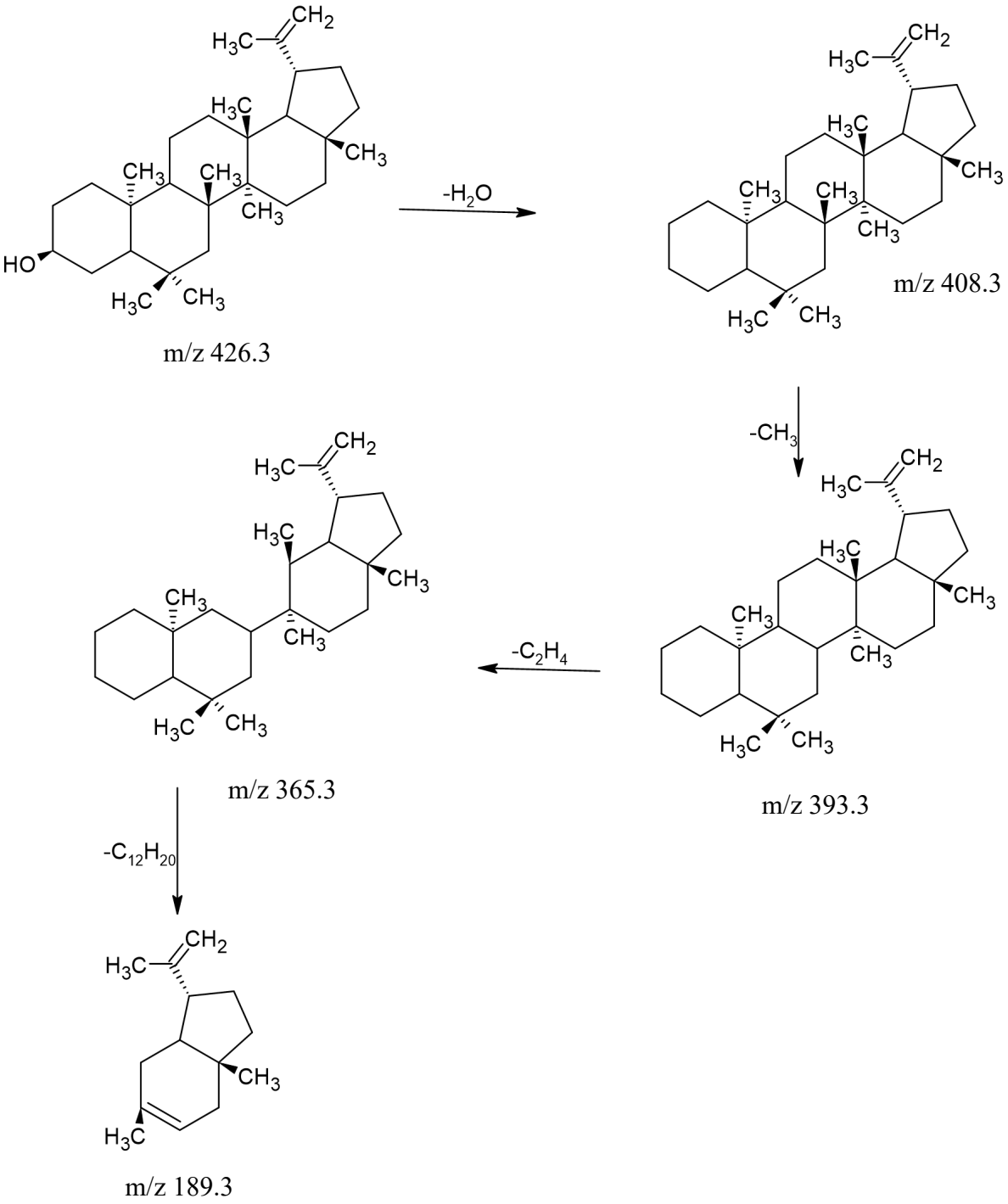



Figure 4.1: The fragmentation pattern of lupeol

1H NMR data of LO1 comparison with the reported literature values showed similar signals to the seven methyl protons cited in the literate (Shwe et al., 2019) (Table 3.3) : δ 0.74, δ 0.76, δ 0.80, δ 0.92, δ 0.94, δ 1.01 and δ 1.66. A sextet of a single proton occurring at δ 2.36 corresponds to 19 β-H proton and is a characteristic signal of lupeol. The olefinic protons located at C29 is a distinguishing feature of the lupane-type triterpenoid and was exhibited at chemical shifts δ 4.55 and δ 4.67. The proton attach to C3 proton occurred as doublet at δ 3.18

The COSY spectrum of LO1 exhibited cross peaks between proton attached to C19 appearing at δ 2.36 and one Sp3 methylene proton attached to C21 occurring at δ 1.32 and another Sp3 methine proton signal at C18 appearing at δ 1.65. There is another coupling between oxygenated methine proton on C30 with signal at δ 3.16 and Sp3 methylene proton attached to C2 occurring at δ 1.50.

Data comparison of MS, 1D NMR and 2D spectral properties of compound coded LO1 suggested its identity as lupeol.

The mass spectrum of LO2 showed peaks at m/z ratio of 414.3 which corresponds to the molecular formula $C_{29}H_{50}O$. Peak at m/z 396 is as a result of the loss of water molecule [$H_2O$] from the molecular ion. Further dealkylation [$CH_3$] gave the peak at m/z 381. Peak at m/z 273 is as a result of fragmentation of the side chain [$C_{10}H_{21}$] at C17-C20. The dehydration of the daughter fragment at m/z 273 gave the peak at m/z 255 (Figure 4.2).

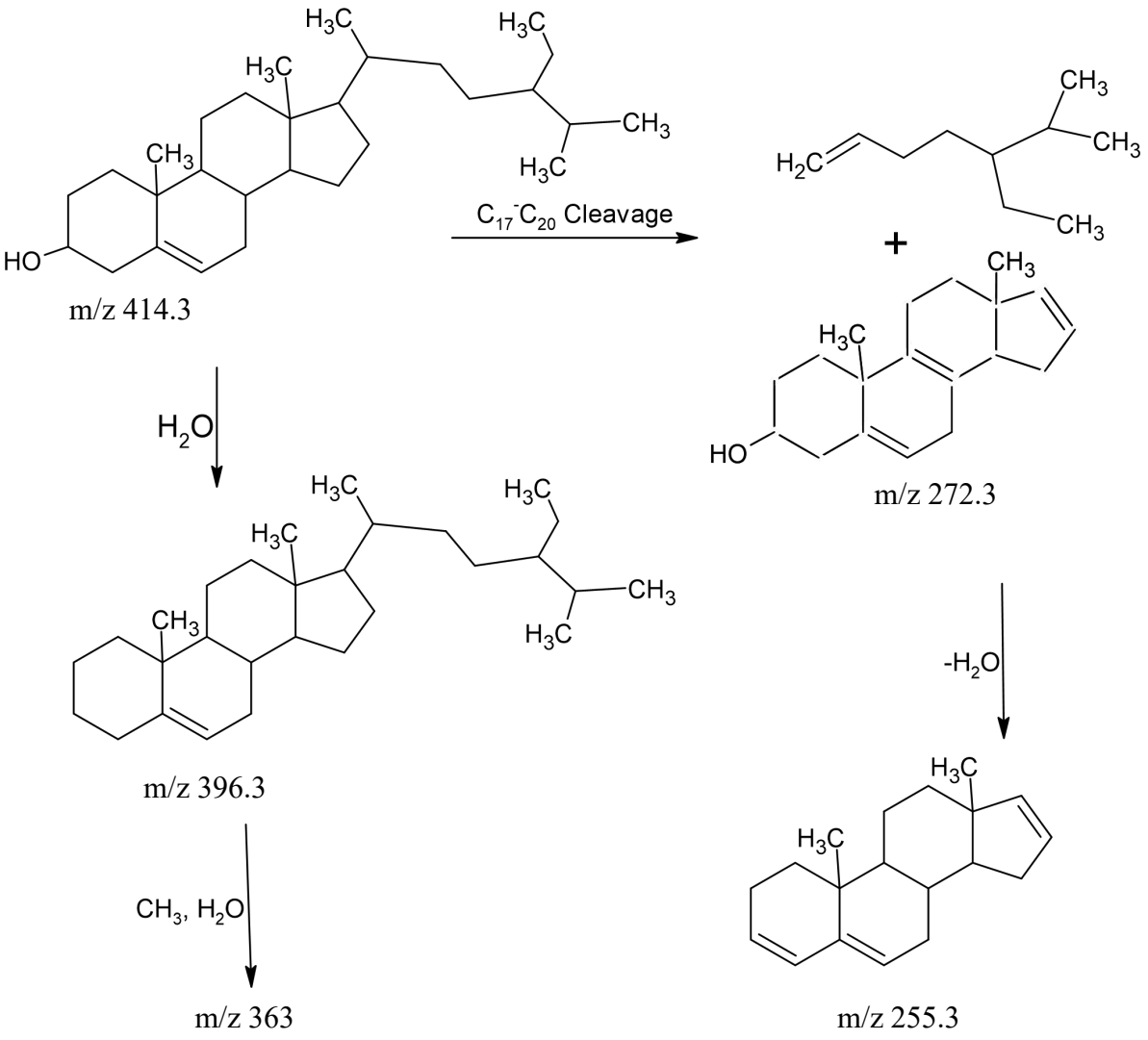



Figure 4.2: The fragmentation pattern of β-sitosterol

1H NMR values for LO2 comparison with the reported literature values (Cayme and Ragasa, 2004) showed similar signals coinciding to the presence of double angular methyl protons attached to C18 and C19 appearing at δ 0.64 and δ 1.23 respectively. Branching methyl protons at C21 was seen at δ 0.99. Similarly, the signature methyl protons of the isopropyl group at C26 and C27 appeared at δ 0.78 as overlapping 6H duplet because they are in the same electronic environment. Methine proton that is highly deshielded due to the attachment of electronegative oxygen atom at C3 appeared at δ 3.57 as a single proton multiplet, The overlapping triplet signal occurring at δ 5.20 is indicative of olefinic protons at C6 (Table 3.5).

The COSY spectrum of LO2 revealed a cross peak between the methylene proton at δ 1.10, (H-1) and the methylene proton at δ 1.49, (H-2). The methylene protons at δ 1.49 (H-2) and δ 1.98 (H-4) exhibited cross peak association with the carbinol proton of δ 3.57 (H-3). The olefinic proton singlet at δ 5.20 (H-6) displayed cross peaks with the methylene proton at δ 1.51 (H-7). The methine proton at δ 1.39 (H-8) and the methylene proton at δ 1.51 (H-7) were correlated. The cross-peaks connection between the methylene proton at δ 1.50 (H-11) and the methylene proton at δ 1.12, (H-12) were observed. The signals compared very well with the literature values for β-sitosterol. Hence, compound LO2 was deduced to be β-sitosterol.

This is the first time lupeol and beta-sitosterol have been isolated from *L. griffonianus*. Lupeol is described as a pentacyclic triterpenoid that belongs to a class of compounds now referred to as phytosterols. The chemical formula of lupeol is $C_{30}H_{50}O$ and its melting point is 215–216 °C as reported in the literature. The molecular weight of lupeol is 426.7174 (g/mol.). The presence of

seven methyl singlet and an olefinic function in the 1H NMR spectrum is characteristic. Lupeol exhibits a parent ion peak at m/z 409 [M+H—18] (Sharma et al. 2020)

This class of compound is unique due to the vast array of pharmacological activities exhibited by plant species expressing them (Das *et al.*, 2021). Triterpenes are terpenes biosynthesized from natural biological systems like plants, animals and fungi. This class of biologically active compounds is highly diversified structurally and is often referred to as triterpenoids (El-Askary *et al.*, 2012; Kim *et al.*, 2013; Rhourri-Frih *et al.*, 2013; Silchenko *et al.*, 2013). Structurally, they are made up of a system of 30 carbons or less which are made up of isoprene (5-carbon unit from acetate) building blocks arising from squalene. They may be divided into linear, two, tricyclic, tetracyclic and pentacyclic types. Other representatives originating from squalene are intermediates [dammarane, lanostane, oleane (oleanolic acid), lupane (lupeol), ursane (ursolic acid)] or triterpenoid sapogenins, for example, cycloartane, friedelane, filicane and cucurbitane triterpenoids. These classes of compounds exhibit anti-cancer properties, anti-inflammatory, anti-bacterial, anti-oxidative, anti-viral, antilipidemic and cytotoxic properties (Chudzik *et al.*, 2015).

B-sitosterol is another type of phytosterol described chemically as (3β)-stigmast-5-en-3-ol, 22:23-dihydro stigmasterol, α-dihydrofucosterol, cinchol, cupreol, rhamnol, quebrachol, and sitosterin. B-sitosterol has a molecular formula of $C_{29}H_{50}O$ with the melting point of 139–142 ◦C. It has the molecular weight of 414.71 g/mol, and is unsaturated with one double bond in the sterol ring structure. It is structurally similar to cholesterol with the addition of an ethyl substituent at the 24-position (Gupta 2020). Based on the number of water molecules added, it is found in three different forms such as anhydrous, hemihydrate and monohydrate (Bin Sayeed *et*

*al.*, 2016) It has a structure similar to that of lupeol, thus making it an important member of the triterpenoid class. Just like lupeol, β-sitosterol is a phytosterol that is biologically active occurring in plants having a chemical structure mimicking that of cholesterol. They form part of our nutrition such as legumes, nuts and oil from olive including rice bran, wheat germ, peanuts, corn oils, and soybeans.. Several sterols form part of human nutrition but beta-sitosterols constitute about 65% of herbal food for humans (Babu and Jayaraman, 2020). The compound is known to display several biological activities such as antioxidant, antimicrobial, angiogenic, antioxidant, immunomodulatory, antidiabetic, anti-inflammatory, anticancer, and antinociceptive (Saleem *et al.*, 2005).

Prostate weight, volume and prostate index were observed to follow a dose dependent reduction pattern. The crude extract at 100, 200 and 400 mg/kg doses exhibited a dose-dependent decrease ($p<0.0001$) in the physical parameters which amounted to 70.86, 78.15 and 83.44% inhibition of prostate weight increase respectively. Lupeol caused 70.86% inhibition same as a 100 mg/kg dose. The standard drug, finasteride, caused 90.07% inhibition of prostate gland enlargement. The combination of 5 mg/kg of finasteride and 200 mg/kg did not exert synergistic activity. These parameters are used to examine the state of health of the prostate gland. An increase in the levels of these parameters reflect prostatic enlargement. Therefore, the reduction of these indices by the extract and lupeol when the prostate glands of the experimental animals were challenged with testosterone showed that the plant can be used to manage BPH in humans (Figures 3.13-3.15). In a similar study, the prostate index of rats was seen to decrease along the concentration

line of the test doses of the administered extract, showing clearly that there was anti-BPH activity (Sasidharan *et al.*, 2022).

The extract reduced the level of testosterone when equated to the negative control group. The normal group and the treatment groups displayed almost the same level of testosterone which confirms the non-enlargement of the prostate as seen in the prostate index result. Lupeol isolated from *L. griffonianus* that is one of the major components contained in the stem bark ameliorated the effect of testosterone induced BPH on the test rats. Equally the level of PSA in the serum of the experimental animals reduced significantly in a dose dependent manner. These results reflect the anti-BPH effect of the extract, which supported the fact that the plant could be used to treat BPH cases in humans. These effects agree with previous literature reports on the plant with anti-BPH effect such as the activity of the stem of extract of *Epilobium angustifolium* L. (Onagraceae) against BPH (Deng *et al.*, 2019). According to Deng *et al*, treatment groups showed a significant reduction in PSA and testosterone circulating levels.. PSA is the marker protein that is expressed during prostate enlargement and is used to monitor prostate health. BPH aetiology has androgens as the key factor in the development of the disease. An increase in the level of circulating testosterone and Dihydrotestosterone (DHT) has been reported to initiate and drive the progression of hyperplasia in the prostate. DHT is known to have the potency of testosterone raised to the ninth power. Both testosterone and DHT can independently activate androgenic receptors at the level of the prostate. First-line treatment targets the inhibition of the seroconversion of testosterone to the more potent metabolite known as DHT by 5-α-reductase enzymes to halt the progression of BPH (Figures 3.16 and 3.17)

The testosterone-estradiol ratio is usually maintained in a fine balance in normal subjects. During BPH, there is an offset of this normal homeostasis leading to an increase of this ratio. The treatment groups significantly reduced the testosterone-to-estradiol ratio with peak effect seen in the lupeol group (Figure 3.18). Therefore, the extract's ability to reduce testosterone, PSA and $T/E_2$ ratio level is a direct display of its efficacy in treating BPH.

The histopathological presentation seen in the prostatic tissues of the negative control group is consistent with the benign prostate hyperplastic enlargement of the rats' prostate glands. Features like irregularly shaped acini were observed. There was a papillary projection into the lumen of the prostatic duct, and foci of piling-up of hyperplastic nodules were evident. The prostate glandular epithelium was thickened. The epithelium was seen to be highly cylindrical and multi-layered and showed irregularly aligned round/ovoid nuclei. The group administered 100-400 mg/kg of the crude extract showed a dose-dependent reduction in the epithelium thickness lining the prostatic lumen. Mild metaplasia showed a markedly reduced effect of testosterone on the prostate gland. Finasteride and lupeol at the administered dose suppressed hypertrophy of the animal's prostate. The co-administration of 200 mg/kg of extract and 5 mg/kg of finasteride have no synergistic effect with an irregular acinar shape with squamous metaplasia of the prostate gland seen. There is an inflammatory infiltrate composed of mononuclear cells and fibrosis in the interstitium. These histological presentations indicated that the extract and lupeol were able to ameliorate the effect of high circulation of testosterone in the blood of the test animals (Figures 3.19-3.26).

Cytotoxicity measures the level of toxicity of compounds on different cells that involve the use of several cellular models. Such analyses are usually based on cell cultures along with the compound of interest and measurement of indices associated with cellular growth such as mitochondrial activity and the ability to undergo cell cycling. Several stains are available to quantify these effects such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (determination of the oxido-reductive activity of mitochondria), which is the capacity to reduce the stain by living cells. MTT assay have been used to evaluate compounds from the triterpenoid class with significant success (Chudzik *et al.*, 2015). Lupeol, a representative member of the lupane type pentacyclic triterpenoid, has been extensively studied for its toxic effect on cells using the MTT assay against several cell lines such as hepatic cell carcinoma (HepG2 and SMMC7721) and colorectal carcinoma (DLDS1, HTC116 and RKO). The result indicated strong activity through inhibition of cellular life, upregulation of apoptosis and the stimulation of NK cells (He *et al.*, 2011; Liu *et al.*, 2013; Tarapore *et al.*, 2013). $PC_3$ cancer cell lines were discovered in 1979 to boost the few available cell lines then. It was isolated from a tumour that metastasize to the vertebra. The cell lines are hormone sensitized and lack androgenic receptors or prostate-specific antigen mRNA/protein. It possesses a karyotype number of 58 and a cycling time of 33 hours. It is well endowed with a transferrin receptor that responds to transferrin sourced from bone marrow. The result of the analysis showed that lupeol suppressed prostate cancer cell growth by 40.8% at 30μM concentration. Several lupeol-expressing plant species have been reported to exhibit anticancer properties such as lupeol isolated from *Chrysanthemum indicum* L. (Asteraceae), which displayed a potent cytotoxic effect against human colon adenocarcinoma (SW620) cell lines with an $IC_{50}$ value of 1.99 μmol/L

(Pathom, 2017). Pathom (2017) reported that *Ficus racemose* Willd. (Moraceae) with an extract with high lupeol content had a cytotoxicity effect that was directly proportional to the amount of lupeol expressed in the plant. Previous reports on *Ficus* species have also shown a strong correlation between the content of lupeol and biological activities (Bopage *et al.*, 2018; Hanafi *et al.*, 2017). Because of the observed cell toxicity effect of lupeol on various cancer cells, it was recommended as a therapeutic and chemopreventive agent (Baltina *et al.*, 2003; Ahmed *et al.*, 2010; e Silva *et al.*, 2012)

Beta-sitosterol isolated from *L. griffonianus* in this research elicited a 25.1% inhibition at 30µM concentration against Hela cells. This is consistent with a 24-hour study that used beta-sitosterol made from Pinellas tuber that showed a 40% reduction in the proliferative activity of Hela cells at a dose of 20 mM. Another study reported by Alvarez-Sala *et al*, (2019), indicated a lower anti-proliferative activity of 20.5% inhibition at the concentration of 13 mM within 24 hours period The reported activity was ascribed to the reduction of the expression of proliferating cell nuclear antigen (PCNA), due to the inhibition of DNA synthesis in this type of cells (Alvarez-Sala *et al.*, 2019). The result indicated that the isolated phytosterols are less toxic on the BJ (normal human cell) cell lines than the standard anticancer drug used as positive control. It further confirms the fact that natural products could be relatively safer. A previous report by Rebeca *et al* (1998) showed that β-sitosterol exerted a 24% suppression of growth on LNCaP human prostate cancer cells at 16 µM concentration. In a related study by Reddy *et al* (2022), it was reported that β-sitosterol has an anticancer effect against prostate cancer cells and can be used to treat cancer with lower side effects (Reddy *et al.*, 2022). B-sitosterol has anticancer effects against breast,

prostate, colon, stomach and ovarian cancer. Studies have shown that interfere with multiple cell signaling pathways, including cell cycle, apoptosis, proliferation, survival, invasion, angiogenesis, metastasis and inflammation. Most of the studies are incomplete partly due to the fact that β-sitosterol is relatively less potent (Rashed 2020). This compound is structurally similar to cholesterol and has huge impact on human physiology. It is a well-known natural sterol with reported potential therapeutic mode of applications in cancer.

A tissue biopsy taken for diagnostic purposes after Henrietta Lacks died in 1951 from an aggressive adenocarcinoma of the uterine cervix produced a significant cell line at Dr. George Gey's tissue culture lab at Johns Hopkins in Baltimore, Maryland (Lucey *et al.*, 2009). The cancer cells now are called Hela cells and are being used all over the World by researchers to evaluate anticancer principles for cervical drug development. Lupeol isolated from *L. griffonianus* at 30 µM concentration inhibited Hela cell growth by 35.6%. This result agrees with the work of Prasad *et al* (2018), where they were able to demonstrate the effect of lupeol on Hela cancer cells at 100µM. According to the study, 100µM produced a 71% inhibition of Hela cells. Previous report indicated that Lupeol treatment of HeLa cells induced S-phase cell cycle arrest which may be the mechanism for decreased cell growth. The effect of lupeol was observed to decrease the expression of Cyclin E and Cyclin at 24 hours period (Prasad *et al.*, 2018).

**CONCLUSION**

The results of the study demonstrated that the crude extract and lupeol isolated from the extract attenuated rat prostate enlargement. Two bioactive phytosterols (lupeol and β-sitosterol) were isolated and characterized with cytotoxic activity against human prostate and uterine cervical

cancer cell lines. Cytotoxic and growth inhibition effects were also observed in the extract of the plant. The ethnomedicinal usage of *L. griffonianus* for the treatment of tumour (BPH) was validated by this study.

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