UNIVERSITY OF CAPE COAST

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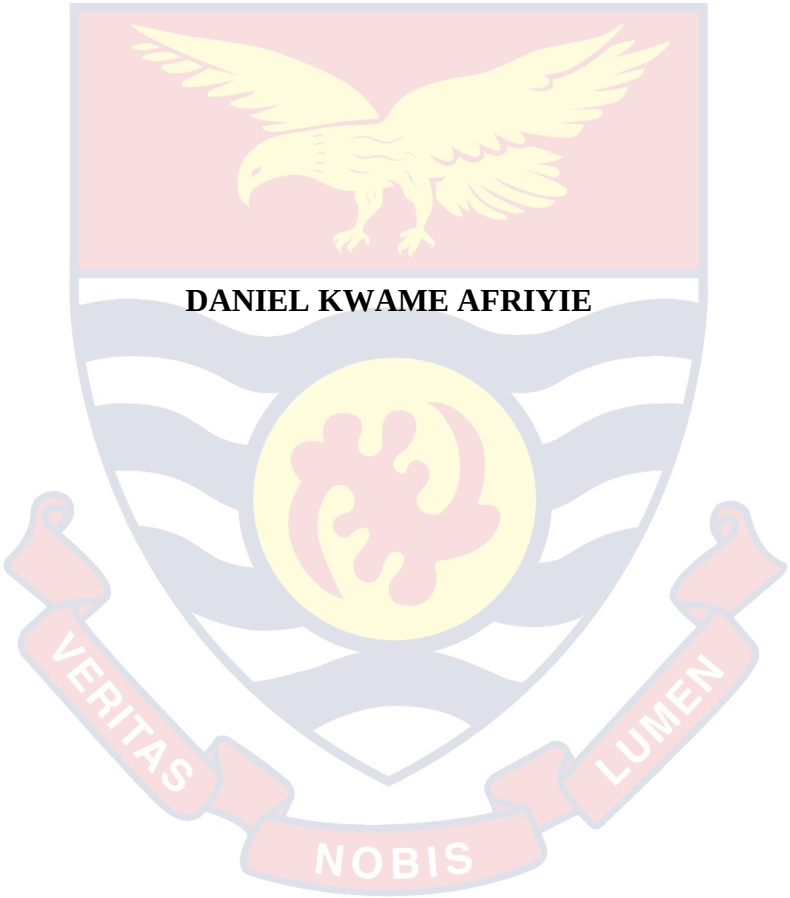

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2020

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BY

DANIEL KWAME AFRIYIE

Thesis submitted to the Department of Biomedical Sciences of the School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Doctor of Philosophy degree in Drug Discovery and Development

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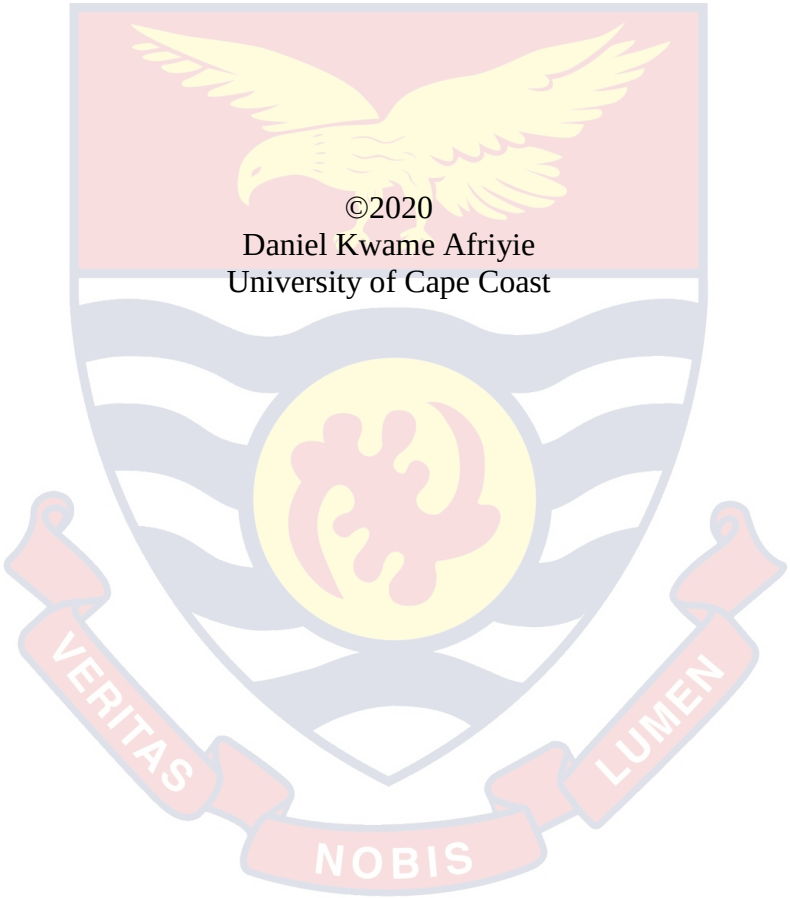

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidates Signature: …… Date: …….....

Name: Daniel Kwame Afriyie

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature: …… Date:…….

Name: Dr. Elvis Ofori Ameyaw

Co- Supervisor's Signature: …… Date: …….

Name: Prof. Regina Appiah-Opong

ABSTRACT

Phytotherapeutic studies on some extracts have revealed promising anti-prostatic growth or anticancer activities. In this study, the pharmacological and toxicological activities of the aqueous stem (CMASE) and sequential stem fractions (SECM) of *Croton membranaceus* were evaluated in; testosterone-induced benign prostatic hyperplasia (BPH) Sprague-Dawley (S-D) male rats and prostate cancer lines (PC3 and LNCaP). Phytochemical screening of CMASE revealed presence of phenols, alkaloids, saponins, terpenoids and tannins. Gas chromatography-mass spectrometry analysis of CMASE showed the presence of n-hexadeconoic acid and 6-octadecanoic acid, known for their anti-androgenic, antioxidant, and 5-alpha reductase inhibitory properties. All extracts (SECM and CMASE) exhibited mild antioxidant potentials, and antiproliferative activities against PC3 and LNCaP cells. Sequential aqueous fraction and CMASE exhibited selective indices for PC3 (SI=75.41, 85.11) and LNCaP (SI=2.2, 2.07) cells, respectively. CMASE induced key apoptotic hallmarks in PC3 cells. Acute (1000, 2500, 5000 mg/kg) and sub-chronic (30, 150 and 300 mg/kg for 90 days) toxicity studies of CMASE in male S-D rats revealed, it was relatively safe, possessed nephroprotective, mild hepatotoxic activity, and had LD_{50} value greater than 5 g/kg. CMASE caused significant ($p<0.001$) reduction in prostatic prostate specific antigens levels, significant ($p<0.01$ and $p<0.05$) reductions in 5-alpha reductase activity, marked reduction in prostatic androgens levels, and size of prostates (between 48.1-70.4%) in BPH-induced rats. Also, CMASE caused marked elevation of prostatic and liver antioxidant enzymes. In conclusion, CMASE was relatively safe, had apoptotic, mild antioxidant potential, anti-prostate growth and anti-cancer activity, and is a 5-alpha reductase inhibitor.

KEYWORDS

Croton membranaceus extracts

Antioxidant and apoptotic potentials

Anticancer activity

Toxicity

Benign prostatic hyperplasia

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DEDICATION

This thesis is dedicated to all scientists pursuing research in drug discovery towards management of benign prostatic hyperplasia and prostate cancer in Ghana.

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LIST OF ABBREVIATIONS

ALT	Alanine amino transferase
ACP	Acid phosphatase
ADT	Androgen deprivation therapy
AFS	Anterior fibromuscular stroma
ALB	Albumin
ALP	Alkaline phosphatase
AQ	Aqueous
AR	Androgen receptor
AST	Aspartate aminotransferase
Bak	BCL-2 homologous antagonist killer
Bax	BCL-2 associated X protein
BHT	Butylated hydroxytoluene
BPH	Benign prostate hyperplasia
BUN	Blood urea nitrogen
CAT	Catalase
Camp	Cyclic adenosine monophosphate
CDER	Center for Drug Evaluation and Research

CK-MB	Creatinine kinase MB
CK-R	Creatinine kinase-R
CMASE	Aqueous stem extract of *C. membranaceus*
COX-2	Cyclooxygenase -2
CSRPM	Center for Scientific Research into Plant Medicine
CZ	Central zone
DAD	Diode array detector
DAQ	Direct aqueous
DHT	Dihydrotestosterone
DLD-1	Colon cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DRE	Digital rectal examination
DU-145	Prostate cancer
EA	Ethyl acetate
EC_{50}	Fifty percent effective concentration

ED	Ejaculatory ducts
EDTA	Ethylene di-amine triacetic acid
EGF	Epidermal growth factor
ETOH	Ethanol
FACS	Flourescence -activated cell sorting
Fas-L	Fas ligand
FBG	Fasting blood glucose
FRAP	Ferric reducing antioxidant power assay
FBS	Fetal bovine serum
GA	Gallic Acid
GAFCO	Ghana Agro Foods Company Limited
GC-MS	Gas chromatography-mass spectrometry analysis
GFR	Glomerular filtration rate
GLOBOCAN	Global Cancer statistics
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione -S-transferase

Hb	Haemoglobin concentration
HCT	Hematocrit
HD	High dose
HDL	High density lipid
HEX	Hexane
HGB	Haemoglobin
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
IGF-1	Insulin-like growth factor 1
IL	Interleukin
Inos	Inducible nitric oxide synthase
IPSS	International prostate symptom score
IR	Infrared spectroscopy
LD	Low dose
LD_{50}	Lethal dose 50%
LDL	Low density lipid
LDH	Lactate dehydrogenase

LUTS	Lower urinary tract symptoms
LYM%	Lymphatic percentage
LYM#	Lymphatic count
MAE	Microwave assisted extraction
MCF-7	Breast cancer cells
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MD	Median dose
MIC	Minimum inhibitory concentrations
Mn	Maganese
mRNA	Messenger Ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NK	Natural killer cells
NMR	Nuclear magnetic resonance
NO	Nitric oxide

NOAEL	No -observed -adverse -effect level
OECD	Organization for Economic and Cooperation and Development
OPA	Ortho-phthaladehyde
PBS	Phosphate buffer saline
PCa	Prostate cancer
PC3	Metastatic prostate cancer cell line
PCV	Packed cell volume
PDE5	Phosphodiesterase-5
PI	Propidium iodide
PI	Prostatic index
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intra-epithelial neoplasia
PLT	Platelet count test
PNT2	Normal prostate cell line
PS	Phosphatidyl serine
PSA	Prostate specific antigen
PZ	Peripheral zone

P53	Tumor suppressor gene
P.o:	*per os*
QC	Qianliening capsules
QE	Quercetin equivalent
QoL	Quality of life
RBC	Red blood cell
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Rosewell park memorial institute
RT	Retention time
SCEM	Sequential stem fractions of *C. membranaceus*
S-D	Sprague-Dawley
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic-pyruvic transaminase
SI	Selective indices
SOD	Superoxide-dismutase
Spk1	Sphignosine phosphokinase 1

Spk 2	Sphignosine phosphokinase 2
TAEC	Trolox antioxidant equivalent capacity
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol
TFC	Total flavonoid content
TLC	Thin layer chromatography
TG	Triglyceride
TGF-α	Tumour growth factor alpha
TGF-β	Tumour growth factor beta
TNF-α	Tumor necrosis factor-α
TPC	Total phenolic content
TRAIL	TNF- related apoptosis -inducing ligand
TRUS	Transrectal ultrasound-guided biopsy
TZ	Transition zone
UTI	Urinary tract infection
UV	Ultraviolet
VLDL	Very low density lipoprotein

WBC	White blood count
WHO	World Health Organisation
WPMY-1	Myofibroblast stromal cell line

CHAPTER ONE

INTRODUCTION

Background Information

Benign prostatic hyperplasia (BPH) is an enlargement of the prostate gland due to progressive hyperplasia (abnormal growth of cells) of glandular (epithelial) and stromal cells (Roehrborn, 2012; Dhirngra and Bhagwat, 2011). Many factors such as ageing, hormonal, dietary, inflammatory mediators, oxidative stress among others have been associated with its development (Minciullo *et al*., 2015).

It is estimated that, 42% of men aged 51-60 years have BPH, compared to over 70% of men 61-70 years and almost 90% of men aged 81 to 90 years (Tanguay *et al*., 2009). In a study among Ghanaian men, the prevalences reported for digital rectal examination (DRE) detected enlarged prostate, prostate serum antigen (PSA) ≥1.5 ng/ml (prostate volume ≥ 30 cm^3), moderate-to-severe lower urinary tract symptoms (LUTS), and an enlarged prostate on DRE plus moderate-to-severe LUTS were 62.3%, 35.3%, 19.9% and 13.3%, respectively (Chokkalingam *et al*., 2012). The symptoms of BPH and its related cancer can cause significant bother and impact on quality of life in patients. Clinical symptoms include urinary hesitancy or straining during initiation of urination.

Mortality rate of prostate cancer in USA stands at 1 out of 34 diagnosed cases, whilst 17.35% of male cancer deaths in Ghana are due to this condition (American Cancer Society, 2012; Wiredu and Armah, 2006).

Conventional drugs such as 5-alpha reductase inhibitors (finasteride and dutasteride) and alpha-1 adrenergic receptor antagonists (tamsulosin and doxazosin) have been used in management of BPH and its related cancers. The 5-alpha reductase inhibitors impede overproduction of dihydrotestosterone in prostate tissues, and consequently hinder proliferation of prostatic cells. Alpha-adrenoceptor antagonists relax the muscles in the bladder neck and prostate, however, there are associated adverse effects with usage of these drugs; impotence, gynaecomastia, harsh myopathy, orthostatic hypotension, dizziness among others (Steineck *et al*., 2002). Besides these drugs also being expensive, some of the adverse effects of conventional therapy include toxicity and inhibition to normal cells growth (Singh *et al*., 2003). Furthermore, the risk and cost of surgical option also discourages its patronage among affected patients. These observations have led to the increasing preference among BPH patients for herbal products. There is also an upsurge in development of phytotherapeutic agents for the management of BPH and its cancers due to the perception that, they are safer, cost-effective and have fewer side effects than their conventional alternatives (Wilt *et al*., 1998; Thompson, 2003).

Croton membranaceus, a plant of the Genus *Euphorbiaceae* has recently been of scientific and medicinal interest in Ghana. *C. membranaceus* roots have a historical and traditional use in the management of prostatic hypertrophy, and it is also used for treatment of secondary infections associated with measles (Bayor *et al*., 2009). Its aqueous root extract has been employed in the treatment and management of prostate enlargement with reports of significant levels of success (Mshana *et al*., 2000). Previous *in vitro* studies revealed that, its ethanolic root

extract was cytotoxic to human prostate and its cancer cells (Bayor *et al*., 2008: Ayim *et al*., 2007).

Recent pharmacological and toxicological studies on the aqueous root extract of *C. membranaceus* revealed its safety in both acute and subchronic toxicity studies, possesses antimicrobial, anticancer, anti-diabetic, prostate targeting activity, anti-antherogenic and anti-ischaemic potentials (Asare *et al*., 2015a; Afriyie *et al*., 2014a; Afriyie *et al*., 2013; Asare *et al*., 2011; Bayor *et al*., 2009). High doses of the aqueous extract have also been shown to possess cytotoxic and genotoxic activity on rat bone marrow (Asare *et al*., 2015b). Furthermore, the aqueous root extract exhibited anti-proliferative activity against BPH-1, and induced apoptosis through mitochondria dependent pathway as one of its mechanisms of action (Afriyie *et al*., 2015). Also, its efficacy in shrinking enlarged prostates in humans and rats has also been established (Asare *et al*., 2015c; Afriyie *et al*., 2014b).

Due to the recent positive pharmacological and toxicological findings on the therapeutic potentials of the root extract of *C. membranaceus* in Ghana, reports suggest there is increasing consumer demand for its root extract for BPH and prostate cancer management. Hence, some traditional medicine practioners, herbal outlets and clinics are adding its stem to the roots to increase its economic yield without consideration to safety of their clients.

There are also anectodal reports on patrons of *C. membranaceus* combining it with conventional or orthodox BPH drugs for management of their prostate health

with the assumption that, the latter being of plant origin is natural, hence very safe without scientific evidence.

However, instances of herb-herb or herb-orthodox drug interaction can usually lead to unpredictable complications (Gurley, 2012). Drug interaction occurs when a drug is administered together with any xenobiotics, altering the therapeutic efficacy of the administered drug with respect to its efficacy or period of action (Rainone *et al*., 2015; Davis *et al*., 2013). Major pathways for drug interactions are via the CYP450 enzymes which have many isoforms. However, the major metabolizing CYP450 isoforms are CYP3A4, CYP2D6 and CYP1A2, and these metabolize between 80% - 85% of administered drugs (Danielson, 2002). Drugs that have the potential to induce CYP enzymes often results in therapeutic failures through rapid excretion of the drugs from the circulation, thus, reducing its plasma concentration and vice visa (Rainone *et al*., 2015).

The promising therapeutic potentials of various plant parts of *Croton membranaceus* and other medicinal plants in Ghana as global agents for the management of benign prostatic hyperplasia and its tumours, have led to concerns over their safety and efficacy. The reason being that, toxicity of medicinal plants has been known to man since their therapeutic effects were discovered. Some plant extracts from different parts of the same plant could be inherently dangerous, containing naturally occurring toxins, which may be cytotoxic or carcinogenic (Humphrey and McKenna, 1997). Toxicity reports on plant extracts is also variable depending on the plant part used, and the solvent employed (Coria-Tellez *et al*., 2016). Possibility of herbal-conventional drugs interactions are well documented,

yet pharmacological evaluation of possible interations of *C. membranaceus* extracts with conventional BPH drugs among patients in Ghana to provide safety guidance are virtually non-existent in spite of the risks of such interactions.

Pharmacological and toxicological data on medicinal plant extracts are currently required as part of the drug evaluation process in many countries to ensure safety, quality and efficacy before marketing. Thus, evaluations of extracts from medicinal plant parts are essential for drug development (Ibarrola *et al*., 2000; Mushtaq *et al*., 2003). However, studies on the safety of the stem extract of *C. membranaceus*, its capability of reducing enlarged prostate, its antiprostate growth or antiprostate cancer mechanisms are unknown. The virtual non availability of in-depth pharmacological and toxicological data on the aqueous stem (CMASE) and other stem (sequential fractions) extracts of *C. membranaceus* (SECM) limits their therapeutic use at national and global markets in an era of evidence-based medicine.

Statement of the Problem

An estimated 75-80 % of the world's population especially from the developing countries depend on plant-derived medicine as the first line of treatment for diseases (Pandey *et al*., 2011; Monteagudo *et al*., 2006). Based on their long history of use, they are generally regarded as safe and are promoted as being "natural' and safer alternatives to conventional medicines. Besides, they are more affordable and easily accessible to users than conventional drugs. However, many medicinal plants used across the world and widely assumed to be safe, have been found to be potentially toxic due to the presence of toxic and potentially lethal constituents such as viscotoxins, saponins, diterpenes, cyanogenetic glycosides and furonocoumarins

(Fennel *et al*., 2004; Pfister *et al*., 2002). Hence, the need to evaluate the safety, quality and efficacy of medicinal plant extracts are currently required as part of the drug development process in many countries to ensure their before clinical use (Morgan *et al*., 2004; Gericke, 1995). Inspite of the recent therapeutic use of the aqueous stem extract of *C. membranaceus* in the management of BPH and prostate cancer, no evidenced-based study has been done on it to ascertain its safety and efficacy.

Hypothesis

1. SECM and CMASE are relatively safe, and have selective cytotoxic effects on normal and cancerous prostate cell lines.
2. SECM and CMASE induces apoptosis as one of its mechanisms of action in BPH and prostate cancer (PCa) models.
3. Administration of sub-chronic rat doses of CMASE to testosterone-induced BPH Sprague-Dawley (S-D) rats will reduce enlarged prostates.

Aim

This study sought to evaluate the pharmacological and toxicological activities of stem extracts of *C. membranaceus* in BPH and prostate cancer models.

Specific objectives

The objectives of the study were to;

1. screen the phytochemical constituents and perform GC-MS analysis of CMASE, and further conduct HPLC and antioxidant analysis on CMASE and SECM.

2. establish the cytotoxic effects and selective indices of CMASE and SECM on prostate cancer cell lines.
3. establish the apoptotic potentials of CMASE on PC3 cell line.
4. describe the toxicity profile of acute and sub-chronic doses of CMASE administered orally to S-D male rats.
5. establish the efficacy and mechanisms of action of CMASE in testosterone induced BPH model.

Significance of the Study

Recent scientific discoveries on the pharmacological potentials of ethanolic and aqueous root extracts of C. membranaceus have generated a lot of interest and demand for it locally and globally. This has led to the inclusion of the stem to root parts by some herbal companies and or practioners to increase its economic yield without any evidence-based studies. The average yield of the stem part of C. membranaceus per plant is estimated at about two and half times in weight compared to the root from the experience of harvesters. Should evidence-based research on the stem extracts proof its safety and efficacy in BPH management, this would justify the use of the stem rather than the roots. Thus, prolong the propagation and economic yield of the plant, as use of its roots terminates further cultivation of the shrub after its initial harvest. Previous studies revealed that, the methanolic root extract of C. membranaceus showed cytotoxic effects on human cancer cells (Bayor et al, 2007). On the other hand, current studies showed that, high doses of the aqueous root extract have cytotoxic activity against BPH-1 cells, and genotoxic effects in rat bone marrow (Asare et al., 2015b). However, there is

virtually no pharmacological and toxicological data on its stem extracts to support their use in management of BPH and its associated cancers. Furthermore, there are reports of co-administration of conventional BPH drugs with stem extracts of C. membranaceus by some patients, thus increasing possible risk of herb-drug interactions among such patients.

Limitations of the study

1. Due to limited resources constraints, this study could not further explore the type of apoptotic pathway(s) through which CMASE exhibited its antiproliferative activity in PC3 cells.

2. Ethical reasons constrained this study to use a small sample size (5) of rats for the *in vivo* experiments. Thus, findings of this study may not be directly extrapolated to humans.

3. Due to limited financial resources, this study could not examine the *in vitro* effects of CMASE and SECM on human BPH-1 cell lines.

CHAPTER TWO

LITERATURE REVIEW

Medicinal potentials of phytochemicals

Phytochemical compounds are primary and secondary plant metabolites such as polyphenols and flavonoids, which play vital roles in the normal development, reproduction and protection of the plants against harsh conditions (Shankar, 2015). Some phytochemicals have been found to possess pharmacological properties such as antioxidants, anti-cancer, anti-inflammatory and analgesics (Pierson *et al*., 2012). For example, secondary metabolites such as brassinosteroids, polyphenols and alkaloids have been documented to modulate cell cycle progression, proliferation and cell apoptosis (HemaIswarya and Doble, 2006).

Saponins which have a foaming characteristic have been found to exhibit anti-cancer activities (Wang *et al*., 2012). Tannins are phenolic compounds with hydroxyls groups are known for their antiviral, antimicrobial, anti-inflammatory properties, inhibits free radicals among other activities which are linked to their therapeutic benefits of medicinal plants in which they are present (Sieniawsk, 2015). Alkaloids are phytochemicals that contain heterocyclic nitrogen atoms, and some alkaloids have anti-cancer properties among other pharmacological activities. For example, alkaloids with anti-cancer properties include dimeric indoles, vincristine, and vinblastine (Jordan and Wilson, 2004; Solowey *et al*., 2014).

Flavonoids are phenolic substances with over 8000 individually known compounds. Studies have revealed that, some of their pharmacological properties include antiviral, antiallergenic, vasodilatory actions and anti-inflammatory actions

(Anand *et al*., 2016; Amin and Buratovich, 2007). Reports from studies on some phytochemicals obtained from ethanolic root extracts of *C. membranaceus* revealed marked cytotoxic activity of crotonmembrafuran, beta-sitosterol-3-D-glucoside and DL-thrietol against human prostate cancer (PC-3) cells (Bayor, 2008).

Relevance of phytochemical analysis of plant extracts

Qualitative preliminary phytochemical analysis of plant extracts helps to detect phytochemical constituents that might be responsible for their pharmacological and toxicological activities. Profiling of medicinal plant extracts is also important to establish the authenticity and quality of the extract. Standard procedures have been used in many studies in order to guarantee that results obtained from such extracts are a true reflection of specified plant (Chatterjee *et al*., 2010). Many solvents of varying polarities are commonly used to extract phytochemicals, and usually the dried powders of plants are used for the extraction of bioactive compounds and elimination of the interference of water at the same time (Altemimi *et al*., 2017). A review of animal and human studies for management of BPH with natural products by Azimi *et al*. (2012) concluded that, there are numerous plants with beneficial influence on BPH management. Furthermore, as their active molecules which have been identified, could be employed as potential lead compounds for developing new, safer and effective medications for BPH management.

Isolation and purification of secondary metabolites in plants

Plants have been a source of a wide array of secondary metabolites with potential pharmacological properties (Russell and Duthie, 2011). The presence of diverse therapeutically active constituents in crude extracts may increase the high possibility of triggering other pathophysiological pathways other than the desired therapeutic outcome. According to El-Shemy *et al*. (2007), because metabolites from medicinal plants originate from natural sources, they may be devoid of the deleterious effects of synthetic drugs. Thus, making medicinal plant extract more preferable due to their natural origin. Large scale cultivation of medicinal plants is a challenge globally, coupled with global climatic challenges. Currently, there is a global shift towards isolation, characterization and the use of pure and or novel plant secondary metabolites which could be safer alternatives to synthetic compounds (Bajpai and Kang, 2011). Analysis of the ethyl acetate extract of *Brassica napus* L., a medicinal plant used for BPH management revealed vigorous activity in declining secretion of prostate specific antigen (PSA) (Yam *et al*., 2008). Five flavonoids; Naringenin, Luteolin, Kaempferol, Kaempferol 3-(3-E-p-coumaroyl-alpha-Lrhamnopyranoside), and Kaempferol 3-(2,3-di-E-p--alpha-Lrhamnopyranoside) were further isolated from the extract, and ten active phytosterol components of the supercritical carbon dioxide fluid extract (SFE-CO (2) of *B. rapa* L. were purified and identified. Linolenic acid and monolinolein were observed to exhibit significant 5 alpha-reductase inhibitory action. Use of crude extracts from plant parts using various solvents and techniques have been employed

in the therapeutic management and treatment of various diseases worldwide for centuries.

According to Wong and Kitts (2006), solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest. A solvent of similar polarity to the solute will properly dissolve the solute. Multiple solvents can be used sequentially in order to limit the amount of analogous compounds in the desired yield (Altemimi *et al*., 2017). Though various solvents have been used for extraction from plants, ethnic communities often use aqueous extract. Hence, therapeutic or active constituents are expected to be present in the aqueous extract. Consequently, in the pharmacological examination of plant extracts for therapeutic efficacy, generally there is need to use polar (hydrophilic) solvents first: methanol, ethanol and pure acetone. To identify and isolate secondary plant metabolites which are non-polar, lipophilic organic solvents such as petroleum ether, chloroform, diethyl ether, ethyl acetate, n-hexane are employed. However, due to high toxicity of organic solvents such as methanol, acetone, chloroform and dichloromethane to cells for example in the liver, alternate safer solvents are often preferred. Gradient solvent system (non-polar to high polar solvent system) provides one of the best elution and separation of various compounds from any plant-based organic extract (Bajpai *et al*., 2016). The polarity, from least polar to most polar, and a few commonly used solvents are as follows: hexane < chloroform <ethyl acetate< acetone < methanol < water (Altemimi *et al*., 2017). Water extracts are the most commonly used, followed by alcoholic extracts due to their low cost, ease of availability and safety. The aqueous-ethanolic extract of Hai Jin Sha

(Lygodii Spora) has been found to possess testosterone 5-alpa-reductase inhibitory action, which the lipophilic (fatty acids) compounds i.e. oleic, linoleic, and palmitic acids were the substantial active principles (Matsuda *et al*., 2001). Microwave-Assisted Extraction (MAE) is also as a technique of interest to researchers to extract bioactive compounds from plants and natural sources by heating the solvent and extracting from plants with a lesser amount of these solvents (Anokwuru *et al*., 2011). This technique reduces losses associated with desired biochemical compounds being extracted (Kingston and Jessie, 1998).

Plants extracts are made up of high amounts of a mixtures of complex phytochemicals which makes their separation difficult, and diverse economical and convenient techniques are mostly employed to isolate and purify these extracts. Fractionation and isolation of biologically active plant secondary metabolites using column-chromatographic techniques is the primary method of compound purification (Zhang *et al*., 2005). Fractions obtained from column-chromatography or sequential fractionation can be further analyzed for partial separation of both organic and inorganic bioactive compounds that may be present using thin-layer chromatography (TLC). Thus, the latter two techniques are useful for isolation and or checking purity of fractions. The number of bands (compounds) that appear on the TLC plate can be further purified or analyzed using high performance liquid chromatography (HPLC).

To elucidate the chemical structure of purified compounds, spectral analyses such as infrared (IR), mass spectrometry, and nuclear magnetic resonance (NMR) could be used based on the nature of the compounds (Popova *et al*., 2009). NMR

spectroscopy has the capacity of providing detailed data normally needed for thorough structure determination of natural compounds. And further determination of its relative stereochemistry as well as discrimination between positional isomers. Researchers often use the spectra produced from passing some of these radiations through the test compound(s) from either three or four regions– ultra violet (UV), infra red (IR), radio frequency, and electron beam for their structural elucidation (Popova *et al*., 2009). Thus, in the search for new pharmacologically active molecules from natural products, there is the need for repeated chromatographic separations of diverse compounds or molecules before determination of the structures of purified constituents.

HPLC Fingerprint analysis

Fingerprint analysis has been accepted by World Health Organization as a methodology for the quality control of herbal samples (WHO, 2000). The concept of biological fingerprinting has been primarily developed for the purposes of the quality control of complex traditional Chinese medicines (Su *et al* 2007; WHO, 2012). It is used to identify closely related plant species, to detect adulterations, to control the extraction process or to study the quality of a finished product. Herbal sample fingerprint can be defined as a set of characteristic chromatographic or spectroscopic signals, whose comparison leads to sample recognition. Traditional chromatographic fingerprint analysis provides the researchers only with qualitative and quantitative information (Lukasz, 2012; Tistaert *et al*., 2011). With HPLC analysis, the presence of the active phytochemical biomarkers with well separated

peaks, in their right ratios, and reasonable retention time have been used as a reliable indicator of the plant's composition (Sasidharan *et al*., 2011).

A HPLC analysis of the aqueous root extract of *C. membranaceus* in a study by Afriyie *et al*. (2014b) revealed four main peaks (active phytochemicals) under the conditions examined. Lambert *et al*. (2005) investigated the ethanolic root bark extract of *C. membranaceus* by reverse-phase HPLC on a (150 x 4.6 mmi.d. octadecylsilyl) silica column using an acetonitrile gradient in water. The results showed it was dominated by one constituent eluted at retention time of 14.3 min. The identity of this major extract constituent determined by an HPLC–SPE–NMR experiment, was scopoletin. Established chemical profile of crude extracts provides guidance for further investigations of active ingredients for their pharmacological or toxicological properties.

Studies have also revealed that, direct correlation exists between presence of phytochemicals such as total phenolic content and their antioxidant activity in plants (Yang *et al*., 2009).

Role of free radicals and antioxidants in disease development and management

Oxidative stress is a condition in which there is imbalance in the rate of release or production of free radicals (reactive oxygen/nitrogen species) in tissues compared to its detoxification, hence resulting in tissue damage (Minciullo *et al*., 2015). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are among free radicals produced by various endogenous systems, when exposed to different physiochemical conditions in pathological conditions. These free radicals have been implicated in the pathogenesis of many diseases including cancer (Singh

et al., 2015). The production of by-products of cell metabolism such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) leads to significant decline in antioxidants defense systems with associated damage to DNA, protein and lipid in cells, and ultimately cell death (Udensi and Paul, 2016). Similarly, Dzialo *et al*. (2016) reported that, excessive production of ROS and RNS could lead to oxidative stress triggering damage in cell structures, including lipids, proteins and DNA, and consequent disorders such as cancer. Lipid peroxidation results in a number of degradation products in biological systems such as malondialdehyde (MDA) which is considered to be an important cause of cell membrane destruction and cell damage (Lizcano *et al*., 2012).

Antioxidants are molecules which delay or inhibit cellular damage mainly through their free radical scavenging property stable through their ability to donate electron(s) to neutralize free radicals, consequently reducing its capacity to damage cells and associated diseases (Halliwell, 1995). Antioxidants can be classified into natural or synthetic antioxidants. In recent times there has been growing interest in the potential health benefits of dietary plant polyphenols as antioxidants due to safety concerns (i.e liver damage, carcinogenicity) associated with synthetic antioxidants such as butylated hydroxytoluene, propyl gallate and terbutylhydroquinine (Altemimi *et al*., 2017). High concentrations of phytochemicals with antioxidant properties such as phenolic compounds; phenols and phenolic acids, flavonoids, carotenoids, anthocyanins, tannins, lignans, tocopherols, as well as vitamins A, C and E are said to accumulate in fruits and

vegetables, and may protect against free radical damage (Suffredini *et al*., 2004; Jakubowski and Bartosz, 1997).

Phenolic compounds and flavonoids have been found to be associated with antioxidative action in biological systems, acting as scavengers of free radicals (Kolli *et al*., 2015). The antioxidant activity of phenolic compounds (aromatic ring, bearing one or more hydroxyl group) is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations (Dzialo *et al*., 2016). Flavonoids are also known as strong metal chelators that inhibit lipid peroxidation (Geetha *et al*., 2004). According to Azimi *et al.* (2012) plants with flavonoids such as naringenin, luteolin, kaempferol, kaempferol 3-(3-E-p-coumaroyl-alpha-Lrhamnopyranoside), kaempferol 3-(2,3-di-E-p--alpha-Lrhamnopyranoside), icaritin, xanthohumol, baicalein, and soybean isoflavone have been found to possess anti-BPH effects, such as 5 alpha-reductase enzyme inhibitory activities.

Plants are rich sources of these secondary metabolites which are generally involved in defense against ultraviolet radiation or aggression by pathogens, are also used in the treatment of various chronic or infectious diseases (Duraipandiyan *et al*., 2006). Recent epidemiological studies and meta-analyses strongly indicates that long term consumption of diets rich in plant polyphenols provides some protection against the development of chronic diseases such as cancers, cardiovascular diseases, diabetes, osteoporosis, neurodegenerative diseases and skin disorders (Dzialo *et al*., 2016; Pandey and Rizvi, 2009).

Studies on plant extracts and some phytochemicals especially phenolic compounds have revealed that beta carotene, ascorbic acid, and many phenolics

play dynamic roles in delaying aging, reducing inflammation, and preventing certain cancers (Duthie *et al*., 1996). With increasing awareness of the possible adverse effects of synthetic preservatives, there has been increased in demand for the use of non toxic, natural preservatives, many of these are likely to have either antioxidant or antimicrobial activities (Negi *et al*., 2005). Recent human and animal studies provide credence to the role of prostatic MDA, antioxidants and defense enzyme levels as markers in the development of BPH (Udensi and Paul, 2016; Kalu *et al*., 2016a).

Assessment of antioxidant activity of compounds

Several methods such as 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, ferric reducing or antioxidant power assay (FRAP assay), and Trolox antioxidant equivalent capacity (TAEC) have been adopted individually or in combination in the determination of antioxidant capacity of pure or phytochemical compounds (Prasad *et al*., 2010). These methods are based on the ability of the antioxidants in scavenging specific radicals by hydrogen atom transfer (i.e DPPH free radical-scavenging assay), by inhibiting lipid peroxidation or chelating metal ions. DPPH is a stable radical that forms a dark purple colour in methanolic solution. The dark purple colouration disappears after the DPPH is reduced by antioxidants through hydrogen transfer to DPPH-H (non-radical form). In the assessment of the antioxidant potentials of the various extracts from the stem of *C. membranaceus* in this study, DPPH free radical scavenging assay was employed due to its exclusive ability to assess the total antioxidant activity. According to Zlotek *et al*. (2015), extraction conditions or procedures could

influence appreciably the stability, efficacy of extracts, and yield of polyphenols. Furthermore, total antioxidant activity has been associated with the plant phenolic compounds as well as other secondary metabolites.

Total antioxidant activities of bioactive compounds have been found to be concentration-dependent (Srikanth *et al.*, 2010). Studies by Blois (1958) have showed an inverse relationship between antioxidant activity of compounds or extracts and their 50 % effective concentration values (EC_{50}). Thus, the higher the antioxidant activity of an extract or compound, the lower the value of EC_{50} and vice-versa. Blois (1958) further classified the antioxidant activity of plants extracts based on their EC_{50} values. The EC_{50} values of extracts less than 50 µg/ml are considered very strong antioxidants, between 50-100 µg/ml are strong antioxidant, from 101-150 µg/ml possess medium antioxidant activity, and values above 150 µg/ml are considered weak antioxidants.

Prostate

The prostate is a small gland about the shape and size of a walnut, that is part of the male reproductive system. It is situated at the neck of the bladder, and surrounds the beginning of the urethra and is anterior (in front of) to the rectum of males. As one ages, the prostate can become large, and enlargement of it is considered a normal phenomenon for most men. Because it surrounds part of the urethra, the enlarged prostate can squeeze and or obstruct it. Thus, interferes with frequency and flow of urine which causes reduction in the quality of life (QoL) in such people. The prostate also helps to make some of the fluid in semen, which carries spermatozoa from the testicles during ejaculation. The prostate produces above 30%

of the non-cellular constituent of semen that provides the prime conditions necessary for the survival and motility of spermatozoa within the reproductive organ of males (Gat *et al*., 2008). The prostate gland can be divided into three to main zones based on the different types of tissue it is composed of (Sperling, 2019; Livermore *et al*., 2016, McNeal, 1981).

The peripheral zone (PZ) contains majority of prostatic glandular tissue and the largest area of this zone is at the back of the prostate gland, closest to the rectal wall. During digital rectal examination (DRE) which is a diagnostic procedure to establish BPH, it is the back surface of the gland that is felt. Examination and study of this zone is important because between 70-80% of prostate cancers originate from it (Sperling, 2019; Livermore *et al*., 2016). The central zone (CZ) is the portion that surrounds the ejaculatory ducts and it claimed that, a very small percentage of prostate cancers originate here (less than 5%), though thought to be more aggressive and more likely to invade the seminal vesicles. The transition zone (TZ) surrounds the urethra as it enters the prostate gland. Though small in young adults, it grows throughout life, eventually taking a bigger percentage of the gland, and is responsible for benign prostatic hyperplasia (BPH, or normal gland enlargement that occurs with aging). It is estimated that 20% of prostate cancers originate in this zone. According to McNeal (1981), the transition, central and peripheral zones contain highly organized glandular epithelium structures separated by a fibromuscular stromal network. The surrounding fibromuscular stromal tissue contains smooth muscle cells, fibroblasts, nerve cells, blood vessels, extracellular matrix and lymphatics (Livermore *et al*., 2016). However, according to Visual

AidMD (2014), the prostate is generally partitioned in two different ways namely zones and lobes; the lateral, the median, and posterior lobes virtually corresponds with TZ, CZ and PZ, respectively.

Minutoli *et al*. (2014) have reported that, proliferation of smooth muscle and epithelial primarily within the prostatic zone results in BPH with its associated lower urinary tract symptoms (LUTS). Figures 1 and 2 depict the various zones of the prostate gland.

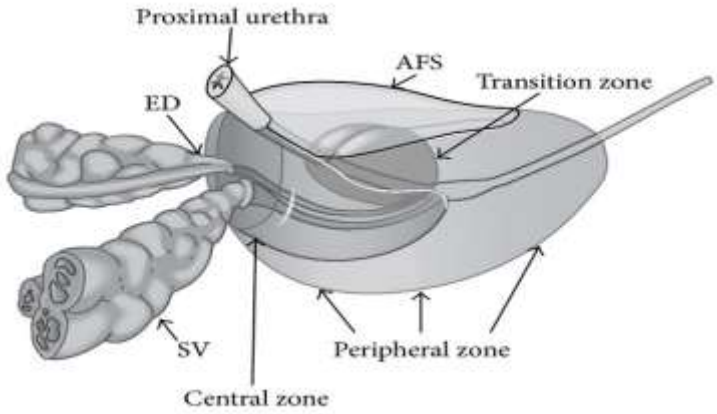


Figure 1: Zonal anatomy of the prostate gland; ejaculatory ducts (ED); seminal vesicles (SV); anterior fibromuscular stroma (AFS).

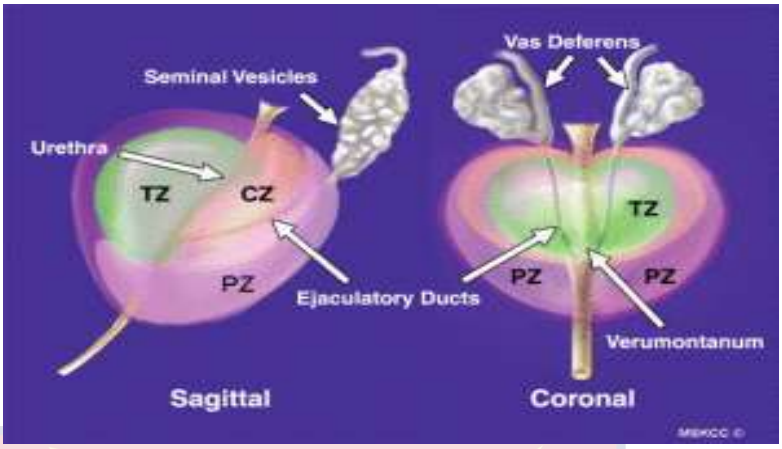


Figure 2: The sagittal view is from the side, with the back of the peripheral zone (PZ) toward the rectal wall. The Coronal view is from the top, showing the left and right sides of the gland. TZ (transition zone) and CZ (central zone)

Source: Image from Geneva Foundation for Medical Education and Research at http://www.gfmer.ch/selected_images.

Benign prostatic hyperplasia (BPH)

Epidemiology

Prostate enlargement presents both health-related and socioeconomic problems for today's society. It is estimated that, 42% of men aged 51-60 years have BPH, compared to over 70% of men 61-70 years, and almost 90% of men aged 81 to 90 years (Tanguay *et al*., 2009). Often men over the age of 40 begin to experience symptoms of BPH, if the prostate enlargement presses on the urethra. The prostate reaches maturity at puberty and essentially maintains its size for several decades, after which growth continues again in majority of the men in midlife and beyond. Since BPH commonly occurs in the transitional zone of the prostate, which surrounds the urethra, this could lead to restriction of urine flow out of the bladder. This condition is known as benign prostatic obstruction. The hyperplasia of the prostate includes the smooth muscle cells and glandular connective tissue. Also, the

smooth muscles in the prostate could be further contracted by the sympathetic nervous system perpetuating any existing obstruction.

Clinically, the palpable enlargement of the prostate can be detected by digital rectal examination or ultrasonographic examination (Roehrborn *et al*., 1997). The correct term for the diagnosis of an enlarged prostate, felt on digital rectal examination is benign prostatic enlargement. Studies report that, only about 30% to 50% of BPH cases with clinical gland enlargement manifest lower urinary tract symptoms (Oesterling, 1995a). It is estimated that, approximately over 50% of men above 80 years of age will experience this condition (Aleksandra *et al*., 2015). A study by Chokkalingam *et al*. (2012) reported among Ghanaian men that, the prevalences for digital rectal examination (DRE) detected enlarged prostate, PSA≥1.5 ng/ml (prostate volume ≥ 30 cm^3), and moderate-to-severe lower urinary tract symptoms [LUTS] (IPSS≥8), were 62.3%, 35.3%, 19.9% and 13.3% respectively. Studies have also revealed that, hypertensive men were at higher risk of developing BPH, and would require medical and surgical intervention than non-hypertensive men (McVary, 2006).

Etiology and Symptoms

According to Aleksandra *et al*. (2015), BPH starts as a simple micronodular hyperlasia which leads to obstruction of the bladder, and consequent development of LUTS. As the prostate organ enlarges (BPH develops), it constricts the urethra which induces several lower urinary tract symptoms. However, histologic diagnosis of BPH may not necessarily lead to clinical symptoms. LUTS associated with BPH are classified as obstructive; hesitancy, weak stream, straining, and prolonged

voiding, as irritative; increased frequency during the day and night, urgency, nocturia, urge incontinence, and voiding small volumes, or post micturition; post void dribble, incomplete emptying of bladder, and painful or bloody urination (Chughtai *et al*., 2011; Wolters *et al*., 2004; Eisenberg *et al*., 1998). These symptoms can cause significant bother and negatively impact on quality of life of affected men.

Many risk factors are associated with BPH development and includes: aging, inflammation, metabolic syndrome, environmental and oxidative stress (Minciullo *et al*., 2015; Roehrborn and McConnell, 2002). Other variables such as lifestyle or dietary risk factors that have been found to be associated with PCa development include; consumption of smoked or overcooked fish in high amounts of long chain polyunsaturated fatty acids, red meat, vitamin D deficiency, smoking, and sexually transmitted diseases (Cunningham and You, 2015).

Role of Hormones

The role of serum sex hormones such testosterone, dihydrotestosterone (DHT) and estrogens binding to their respective receptors in the prostate have been implicated in the development of BPH (Kreig *et al*., 1993; George *et al*., 1991). The conversion of testosterone to dihydrotestosterone by 5 alpha-reductase type 2 found in the nuclear membrane of both the stroma and epithelium of the prostate has been documented as a major pathway for its development (Aleksandra *et al*., 2015; Roehrborn and McConnell, 2002). Three types of 5α-reductase (type 1, 2 and 3) isoenzymes have been identified, and 5α-reductase type 2 is responsible for

inducing the irreversible conversion of testosterone in the prostate to DHT (Stiles and Russell, 2010; Bartsch *et al*., 2002).

Studies have proven that, as men age their testicular functions decreases which results in decline in bioavailable prostatic testosterone levels decline (Alberto *et al*., 2009). The precursor of DHT which is testosterone, is synthesized in the testes and adrenal glands and then converted to DHT, found mainly in prostate, epididymis, hair follicle, and liver tissue. DHT is said to be about ten times potent than testosterone, hence dissociates slowly from nuclear androgen receptors which signals the transcription of growth factors which are mitogenic to the epithelial and stroma cells, resulting in prostatic hyperplasia. Furthermore, binding of DHT to androgen receptors in the prostate leads to the production of proteins such as prostate specific antigen (PSA), and other regulatory proteins that induce prostate cell proliferation, resulting in development of BPH.

Finasteride, a medication for BPH treatment, is a 5α-reductase inhibitor that decreases the DHT concentration in the prostate. It is based on this understanding that, 5-alpha reductase inhibitors are used therapeutically to suppress production of prostatic DHT levels. This results in the reduction of prostate volume and LUTS (Alberto *et al*., 2009). However, other studies have revealed the stimulation of prostatic growth by estrogens (Kalu *et al*., 2016b; Aleksandra *et al*., 2015). Zhou *et al*. (2012) reported conversion of androgens to estrogens also increases with decline in testicular functions in the aged. The resultant increase in plasma estrogen to androgen ratio and levels of circulating constant free estradiol leads to stimulation of the stroma and its excessive proliferation (Kalu *et al*., 2016b; Ho and Habib,

2011). Natural plant extracts from tomato, black raspberry among others, have been observed to be useful for prevention and treatment of BPH through their ability to regulate expressions of DHT, 5α-reductase, androgen receptors, and prostate-specific antigen in the prostate and serum (Lee *et al*., 2014; Kang *et al*., 2007).

Role of aging

Aging in males has been proven in several studies to be a risk factor in the progression of BPH development and incease in its markers. Additionally, tissue remodeling significantly occurs in the basal cells within the transition zone which leads to prostatic enlargement (Kalu *et al*., 2016a; Liu *et al*., 2009; Alba *et al*., 2009; Roehrborn and McConnell, 2002).

Role of inflammation

Inflammation has been found to play a role in the development of BPH or its progression (De Nunzio *et al*., 2013). It is a natural cell defense (immune) mechanism against pathogens (microbial and viral infections), exposure to toxic chemicals, radiation, allergens, diseases, drugs among others (Tarique *et al*., 2016). Inflammation of the prostate may represent a mechanism for hyperplastic changes to occur in the prostate. There is scientific evidence to suggest the presence of acute and chronic inflammation in prostate biopsy and BPH specimens (Di Sliverio *et al*., 2003). Inflammation of the prostate could result in the generation of free radicals such as nitric oxide (NO), hydroxyl, superoxide anion, hydrogen peroxide, oxygen, hypochlorite, peroxynitriteand other oxygen species. Also, inflammatory cytokines

or markers such as C-reactive protein, interleukin-6 (IL-6) and interferon-gamma have been implicated in the induction of hyperplastic changes observed in BPH (Steiner *et al*., 2003). Chronic and acute inflammation through a variety of mechanisms, notably oxidative stress may result in events that can cause proliferation within prostatic tissue (Naber and Weidner, 2000). According to Chughtai *et al.* (2011), tissue damage and oxidative stress may lead to compensatory cellular proliferation with resulting hyperplastic growth. Furthermore, inflammation of prostatic tissues could result in generation of free radicals such as inducible nitric oxide synthase (iNOS) among various species of oxygen free radicals (Hamid *et al*., 2011). INOS is the principal agent for activating reactive nitrogen species that could cause cell damage. These reactions may result in production of arachidonic acid from membranes, and cause an increase in of prostaglandins levels a vital factor associated with proliferation of prostatic cells through conversion by cyclooxygenase (COX) enzymes (Sugar, 2011).

Studies by Mishra *et al*. (2007) found that, 70% of men with urinary retention had pathologic evidence of acute and or chronic inflammation as against 45 % of those without LUTS. Studies have revealed that, interleukins such as IL-17, IL-6, IL-8, IL-1β which are pro-inflammatory, and up regulation of tumor necrosis factor-α (TNF-α) may lead to the progression of BPH and prostatic inflammation (Chughtai *et al*., 2011). Healthy prostates have been found not to express IL-17, whereas prostates with inflammation and BPH do express interleukin-17 and cyclooxygenase 2 (COX-2) (Wang *et al*., 2004; Steiner *et al*., 2003). Studies on COX-2 inhibition in human BPH tissue results in significant increase in prostate

cell apoptotic activity (Di Silverio *et al*., 2003). Nitric oxide (NO) has been found to enhance activity of COX-2, and COX-2 inhibition in human BPH tissue could increase apoptotic activity significantly (Di Sliverio *et al*., 2003). Naber and Weidner (2000) reported that, the prostate tissue is typically protected from oxidative stress by free radical scavengers such as superoxide-dismutase (SOD) and glutathione-*S*-transferase (GST)-P1.

Role of oxidative stress in BPH development

Oxidative stress has been implicated in cellular reactions involved in the development and progression of BPH. Oxidative stress is the situation where there is imbalance in the rate of release or production of free radicals and or reactive oxygen species in tissues compared to its detoxification, hence resulting in tissue damage (Minciullo *et al*., 2015). According to Udensi and Paul (2016), the release of by-products of cell metabolism such as ROS and RNS results in significant decline in antioxidants defense systems resulting in damage to DNA, proteins, lipids in cells, and ultimately cell death. Endogenous antioxidants such as; GST-P1 and SOD have been found as the main scavengers of free radicals in the prostate tissue during oxidative stress (Naber and Weidner, 2000).

Cederbaum (2001), reported that, there is overproduction of ROS the main mediators of oxidative stress and reduction of antioxidant enzymes such as catalase (CAT), GST, glutathione peroxidase (GPx), SOD and glutathione reductase (GR) during oxidative stress. Catalase is a known biomarker of oxidative stress which protects cells against hydrogen peroxide, whilst SOD is a metalloenzyme can convert superoxide (O^{2-}) radicals (anions) during oxidative stress into either

ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Aebi, 1984). The most abundant antioxidant in cells is reduced glutathione (GSH) and it plays a key role in maintenance of intracellular redox balance which protects cells against oxidative stress and induced-cellular injury (Dey and Cederbaum, 2009). Glutathione reductase also known as glutathione-disulfide reductase (GSR) allows the regeneration of glutathione through the conversion of oxidized glutathione to the GSH state (Ramesh *et al*., 2012). On the other hand, GST catalyze the conjugation of electrophilic products of oxidative stress with glutathione (GSH) (Habig and Jakoby, 1981).

Antioxidant activities in cells could be mediated by the prevention of the generation of radicals or scavenging of the formed radicals (Gutierrez and Naarro, 2010). Increased ROS levels leads to lipid peroxidation (leading to generation of malondialdehyde) in the membranes through a chain of self-perpetuating reactions or oxidation of unsaturated fatty acids which causes immediate damage to cell membranes and DNA (Dursun *et al*., 2009; Kumari and Menon, 1987). Glutathione peroxidase's (GPx) primary work is to remove products of lipid hydroperoxidation and hydrogen peroxides from tissue (Zamora *et al*., 2007). Udensi and Paul (2016) reported that, antioxidant levels in prostatic tissues were found to be significantly low. Similar studies on finasteride and kolaviron treated BPH rat models revealed significant decline in prostatic GSH, GPx, SOD and catalase levels with significant elevation in MDA levels compared to model BPH group (Kalu *et al*., 2016a).

Role of apoptosis

Apoptosis or 'programmed cell death' occurs normally during development, aging, and plays a key role as a homeostatic mechanism to maintain cell populations in tissues (Hassan *et al.*, 2014). This phenomenom is generally observed in eukaryotic cells, and may occur as a defense mechanism through immune reactions, when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Programmed cell death can be induced by intrinsic (mitochondria pathway) and extrinsic (death receptor pathway) stimuli such as: hormones (corticosteroids), DNA damage, growth factors, chemotherapeutic agents, and ionizing radiation. For instance, elements such as radiation or drugs used for cancer chemotherapy may result in DNA damage in some cells, which can lead to apoptotic death through a *p53*-dependent pathway (Tumour suppressor p53 activates proapoptotic proteins from Bcl-2 family). Intracellular signals such as DNA damage, cytokine and growth factors deprivation are involved in triggering apoptosis (Zaman *et al*., 2014). Changes such as activation of caspases -2, -8, -9, -10 (initiator caspases) caspase-3, -6, -7 (executioners) results in cleaving of cellular components i.e cytoskeletal and nuclear proteins needed for routine cellular function when apoptosis is signaled (Hassan *et al*., 2014). The executioner caspases cleave the target proteins that results in cell death. Activation of caspase activity characteristically results in shrinkage of apoptotic cells, and changes in plasma membrane leading to signaling of macrophage response.

Intrinsic apoptotic pathway is regulated principally by the Bcl-2 (Beta-cell lymphoma-2) family. During intrinsic apoptosis there is inhibition or down

regulation of anti-apoptotic protiens such as Bcl-2, and upregulation of pro-apoptotic effector protiens associated X protein (Bax) and Bcl-2 homologous (Bak) (Lopez and Tait, 2015; Elkoli *et al*., 2014). Furthermore, it has been observed that, over expression of Bcl-2 proteins occurs in almost half of all human cancers, and these types of tumours are resistant to some anticancer drugs which act via the intrinsic pathway (Yip and Reed, 2008). Studies have revealed that, most of the traditional anticancer drugs are dependent on the Bax/Bcl-2 mechanisms to destroy cancer cells (Yip and Reed, 2008).

The extrinsic pathway uses extracellular or cell death signals (death ligands) such as Fas ligand [Fas-L], tumour necrosis factor [TNF], TNF-related apoptosis-inducing ligand [TRAIL] to bind tumour necrosis factor [TNF] family death receptors and induce apoptosis (Zaman *et al*., 2014). Elkholic and Renault (2014) reported that, cancer cells are more responsive to the extrinsic pathway than to the intrinsic pathway of apoptosis. The reason being, overexpression of Bcl-2 protein in over half of the diverse kinds of tumours seen, resulting in resistance to any intrinsic apoptotic pathway.

Apoptosis is genetically controlled, and defects in this process may lead to the development of several diseases as seen in acute and chronic degenerative diseases, and neoplasms (Hassan *et al*., 2014). Prevention of cancer is one of the main functions of apoptosis, and many anticancer drugs target several stages of the intrinsic and extrinsic pathways which the goal of up regulating proapoptotic molecules, whilst inhibiting of anti-apoptotic Bcl-2 proteins (Villa-Pulgarin *et al*., 2017; Bao *et al*., 2017; Lopez and Tait, 2015; Hassan *et al*., 2014).

Cellular and morphological alterations are established hallmarks of apoptosis, and are characterized by features such as; cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation formation, and are eventually engulfed by macrophages (Bayne *et al*., 2000; Majno and Joris, 1995; Collins *et al*., 1997). Other studies reported that, essentially there is no inflammatory reaction associated neither with the process of apoptosis nor with removal of apoptotic cells (Savill and Fadok, 2000; Kurosaka *et al*., 2003). The reason is that, apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue. They are readily phagocytosed by surrounding cells. Thus, preventing secondary necrosis and the engulfing cells do not produce anti-inflammatory cytokines.

Imbalance in the molecular mechanisms that regulate cell proliferation and apoptosis in stromal and epithelial cells have been implicated in both the development of BPH, and progression of prostate cancer (Kyprianou *et al*., 2000). Growth of prostate cells is governed by a delicate balance between cell proliferation and apoptosis (Kyprianou and Jacobs, 1996). The normal stroma to epithelium ratio of the prostate is 2:1, however in patients with BPH this ratio has been found to be 5:1 (Prabhav and Bairy, 2009). Some studies on extracts from several Croton species used in the management of tumors such *C. zambesicus, C. argyratus,* and *C. pierrei* have been found to induce apoptosis in cells (Sandoval *et al*., 2002; Block *etal*., 2005; Morales *et al*., 2005). A previous study on the aqueous root extract of *C. membranaceus* by Afriyie *et al*., (2015) revealed that, it suppressed the proliferation of BPH-1 cells through apoptosis via mitochondria-dependent pathway. This was characterized by loss of membrane potential, significant nuclear

condensation, DNA fragmentation, and significant upregulation of Bax proteins. Studies have also shown that oxidative stress plays a primary role in the pathophysiology of age-induced apoptosis via accumulated free-radical damage to mitochondrial DNA (Harman, 1992; Ozawa, 1995).

Necrosis which refers to the degradative processes that occur after cell death. It is an uncontrolled and passive process that usually affects large fields of cells. Furthermore, this process is also said to be a toxic process and follows an energy-independent mode of death. Whereas apoptosis is considered a controlled, energy-dependent, and can affect individual or clusters of cells.

Etiology of Prostate cancer (PCa)

Prostate cancer is a state or condition of the prostate, in which the prostate cells differentiate, become malignant and rapidly proliferate to form solid tumours of glandular origin. Figure 3 shows at cellular level the development of prostate hyperplasia and its progression from asymptomatic to metastatic stage. Studies have shown that, 1 out of 6 men are now being diagnosed with prostate cancer, though most often the cases are not clinically relevant (Taichman *et al*., 2007). Incidence of PCa increases as people get older and major risk factors include age, ethnicity, obesity, and positive family history, hypertension, lack of exercise, smoking, persistently elevated testosterone levels (Islami *et al*., 2014; Jha, 2014; Gann, 2002). Development of the PCa is most common at the peripheral zone and is often adenocarcinomas, whereas non-adenocarcinomas like transitional cell carcinoma, small cell carcinoma and sarcoma rarely occur (Nutting *et al*., 1997). Prostate cancer is classified as localized, locally advanced or metastatic. When PCa is

limited to the prostate, it is considered localized and potentially curable compared to metastasize one (i.e of the bone).

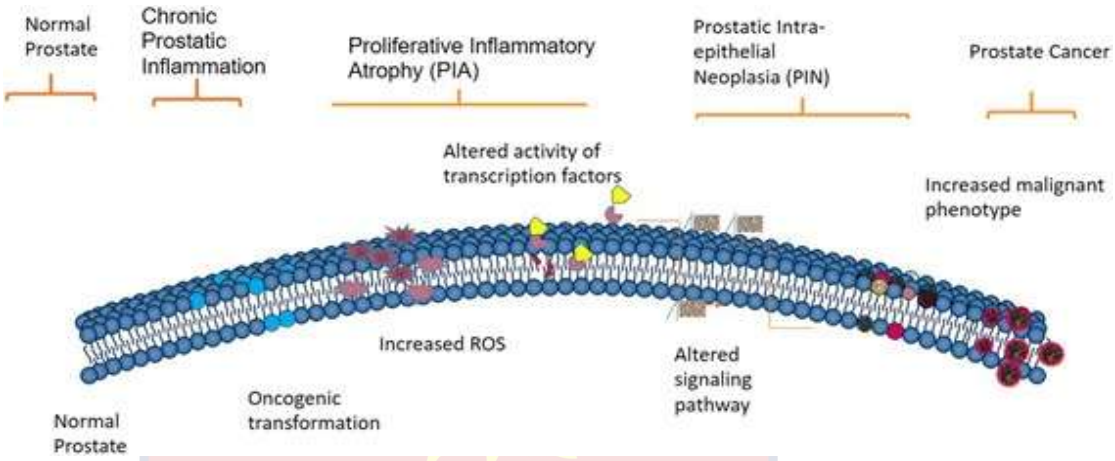


Figure 3: Prostate Carcinogenesis Model: This illustrates what happens at the cellular level as prostate hyperplasia progresses from asymptomatic to metastatic stage.

Source: Udensi (2016).

Like BPH, the etiology and pathogenesis of PCa are poorly understood, however it appears most of known risk factors associated with BPH development such as oxidative stress and genetic predisposition (Alba *et al*., 2009; Gronberg, 2003), diet, aging, hormonal imbalance, apoptosis among others are linked to PCa initiation and progression (Yeboah, 2009). Epidemiological, experimental and clinical studies have showed that markers of oxidative stress are associated with the development and progression of cancer (Reuter *et al*., 2010).

Epidemiology of Prostate cancer

Prostate cancer is the second highest cause of male cancer mortality, after lung cancer in the Western world including Australia and United State of America (Ferlay *et al*., 2013; Smith, 2012). It is the most frequently reported male cancer in the UK and estimated up to 25% of all newly reported male cancer cases in 2012 (Cancer Statistics Registration, 2012). Also, in the USA, it accounts for approximately 29% of newly reported cases of cancer, and 9% of all reported cancer deaths among men in 2012 (American Cancer Society, 2012). In Australia, PCa accounts for approximately 30% of cancers diagnosed each year (Australian Institute of Health and Welfare Cancer Incidence Projections, 2012). Studies have revealed that, descents of black African or Caribbean ethnic origin have 25% lifetime incidence of prostate cancer, and people with a family history have a relative risk of up to 3.5 times the risk of developing the disease (Prostate Cancer-UK, 2017; Johns and Houlston, 2003).

The incidence in West Africa is estimated at 4.7–19.8 per 100,000 men per year (Chu *et al*., 2011). Prostate cancer was found to be the fourth highest, and the most common among cancers in Gambia and Nigeria (Bah *et al*., 2001; Ogunbiyi and Shittu, 1999) respectively. The GLOBOCAN 2002 database estimated that in Ghana, 1-year PCa prevalence is 734; whilst 5-year prevalence is estimated at 2,451; with the number of deaths per year being 758 (Ferlay *et al*., 2002). In Ghana, prostate cancer is reported to be responsible for 17.35% of all male cancer deaths making it second only to liver cancer at the Korle-Bu Teaching Hospital (Wiredu and Armah, 2006). According to Yeboah (2009), prevalence of PCa is about 5% in

men over the ages of 50 years, and about 120 to 150 new cases are seen at the Korle-Bu Teaching Hospital every year, constituting 18% of prostate disorders. A recent study in prostate cancer patients by the National Center for Radiotherapy (Ghana) revealed that, about a third of these patients presented with metastatic disease, suggesting the need for earlier detection and curative therapy (Yamoah *et al*., 2013). Metastasized or extracapsular PCa may invade the bones and lymph nodes and present problems such as discomfort in the pelvic area, vertebral pain, leg weakness from compressed spine and faecal discharge problems (Van der Cruijsen-Koeter *et al*., 2005; Johassan *et al*., 2004).

Symptoms of PCa

Common symptoms experienced in PCa are similar to BPH, and includes urinary incontinence, nocturia, dysuria, haematuria, and difficulty starting and maintaining a constant urinary stream or decreased force of stream, sexual dysfunction; difficulty achieving erection and painful ejaculation, and blood in semen (Chughtai *et al*., 2011; Miller *et al*., 2003). However, most of these common symptoms of PCa are not present during the initial phase in up to two-thirds of the population (Miller *et al*., 2003).

Diagnosis of BPH and PCa

Presence or absence of LUTS in males coupled with the age of occurrence is employed in diagnosis of BPH and PCa (Aleksandra *et al*., 2015; Jha *et al*., 2014; Minutoli *et al*., 2014; Miller *et al*., 2003). On the otherhand, men under the age of 50 presenting with voiding LUTS are unlikely to have BPH, it should be suspected

in middle-aged and elderly men with LUTS. It is recommended that all patients presenting LUTS with suspected BPH should have a urine dipstick and/or culture and sensitivity test to rule out a urinary tract infection UTI or haematuria. It is also worth noting that, one must also exclude a UTI prior to a prostate specific antigen (PSA) test, and not perform the test within a month of a proven UTI. Renal impairment should be suspected if there is a palpable bladder, nocturnal enuresis, recurrent UTI and history of stone disease.

Prostate specific antigen test is useful in the assessment of LUTS by acting as a surrogate marker in BPH diagnosis, and evidence suggests that men with a PSA >1.4 ng/ml be considered at increased risk of developing BPH (Melia, 2005; Bartsch *et al*., 2004). Melia (2005) reported that, urologists do not approve the indiscriminate use of the PSA tests alone in order to minimize unnecessary invasive biopsies and over diagnosis of carcinoma. A study revealed that, up to 50% of patients identified on PSA screening do not have a clinically significant carcinoma. According to Hewitson and Austoker (2005), the presence of BPH symptoms does not predispose a patient to prostate carcinoma; so, PSA test is not essential in men with BPH unless an abnormal prostate is felt on digital rectal examination (DRE) or the patient is specifically concerned about or has a family history of prostate carcinoma.

Examination of patients with suspected BPH begins with abdominal, digital rectal and a focused neurological examination. Whilst abdominal examination is done to assess palpable bladder and other abdominal masses, DRE is done to assess perianal sensation, anal tone, prostate size and for any prostatic irregularity. Digital

rectal examination is a technique by urologists for inspecting the prostate for abnormalities by inserting the finger through the rectum to assess or feel for any hard/irregular areas than expected for a particular age. DRE is one of the reliable methods of diagnosing a prostate cancer, and when prostatic nodule is palpable it is recommended that PSA test be considered along with appropriate referral to a urologist. It is worth noting that, PSA test is not essential in men with BPH unless an abnormal prostate is felt on DRE or the patient is concerned about carcinoma. DRE is also able to detect prostate cancer that has progressed beyond early stage, with a sensitivity of about 60% and 90% specificity (Chodak *et al.*, 1989).

Active surveillance is the recommended option for men with low-risk disease of PCa, whilst detection of metastasis may require the need for radical treatment at a later date. Due to the insidious nature of PCa, many PCa patients with early disease are asymptomatic, and LUTS of locally advanced disease may be difficult to distinguish from those associated with BPH. Prostate magnetic resonance imaging resonance (MRI) aids in viewing the entire prostate with precision. A definitive diagnosis of PCa is made histologically through the use of targeted biopsies.

Currently, the gold standard diagnostic for detection of prostate cancer with prostate biopsy is the transrectal ultrasound-guided (TRUS) technique (Rodríguez-Patrón *et al*., 2006). However, this procedure is associated with significant risks of infection, bleeding, pain and occasionally, acute urinary retention. Furthermore, the Gleason score is used to report the histological grading of prostate biopsies (Gordetsky and Epstein, 2016). The total score may range from six (non-aggressive disease) to 10 (highly aggressive). This grading system is used as an accurate

predictor of disease progression in conjunction with PSA measurement and radiological staging. When diagnosis of renal impairment is confirmed in BPH or PCa, a transabdominal ultrasound scan should be requested to measure the size of the prostate, to assess hydronephrosis and post-void residual. CT scan unlike MRI, uses a combination of x-rays and computer technology to scan images, can identify any abnormally in prostate. Acute urinary retention (inability to pass urine) is often associated with suprapubic pain and a palpable bladder. It is considered as a urological emergency and requires immediate drainage of the bladder.

Conventional management of BPH and PCa

Watchful waiting or active surveillance

Normally the onset of BPH and PCa does not present any harmful symptoms, and is often undetected early by medical professionals. In the treatment of LUTS suggestive of BPH, the goal is to; relieve patients of symptoms, improve quality of life, minimize potential side-effects of recommended medications, prevent progression and possible development of associated complications such as chronic retention, renal impairment, recurrent UTI, bladder stones, haematuria and bladder. Hence, there is increasing advocacy for what is termed "watchful waiting" or the even newer alternative term "active surveillance" for early detection and management of BPH and PCa (Levy and Samraj, 2007; Andren *et al*., 2006; Johansson *et al*., 2004). Wasson *et al*. (1995) reported that, of 276 men with BPH symptoms who were assigned to watchful waiting and received no medications, 24% progressed to require surgery within 3 years. When conservative non-pharmacotherapy options fail, medical therapy may be instituted in primary care.

Management of BPH

Conventional 5α-reductase inhibitors (finasteride and dutasteride) as well as alpha-1 reductase inhibitors (tamsulosin and alfuzosin), have contributed significantly to the management of BPH (Wilt *et al*., 2003). Five-alpha reductase inhibitors are employed therapeutically to suppress production of prostatic DHT levels. This results in the reduction of prostate volume and LUTS (Alberto *et al*., 2009). Studies have found that, usage of 5-alpha reductase inhibitors decreases LUTS scores, decreases prostate volume by 20-30%, increases flow rate and reduce the risk of surgery and acute retention (O'Leary *et al*., 2008; Kaplan, 2005; McConnel *et al*., 1998). Furthermore, men with larger prostate glands, higher IPSS scores, PSA>1.4ng/ml and Qmax<12ml/s were more likely to progress with LUTS, and benefit most from 5-alpha reductase inhibitors.

Alpha adrenoceptor blockers inhibit α1-adrenergic receptors which results in clinically reducing the sympathetic tone, and relaxation of smooth muscles found in the hyperplastic prostate (bladder smooth muscle and prostate urethra), and thus relieve urinary obstruction (Chapple, 2001). Alpha-adrenergic blockers act rapidly (usually within 48 hours) and provide a symptomatic improvement that is immediately noticeable in BPH patients (Patel and Chapple, 2006). A similar study revealed that, these agents alleviate symptoms quicker and more effectively than 5-alpha reductase inhibitors (Trachtenberg, 2005). Related studies have reported improvement in symptom scores of 30-40% with usage of the alpha-antagonists in BPH management, and also there is improvement in Qmax of 15-30% (Samli and Dincel, 2004; Chapple *et al*., 1996).

Side effects associated with usage of alpha-receptors include postural hypotension, tachycardia, dizziness, headache and somnolence or abnormal ejaculation (Lowblaw *et al*., 2014; Steineck *et al*., 2002). However, the use of tamsulosin which has high alpha-1 adrenoceptor affinity or selectivity is often not associated with the latter adverse effects unlike other common non-selective α-blocking agents such as doxazosin, alfuzosin and terazosin (Kaplan, 2005). According to Wilt *et al*. (2003), clinicians currently prescribe combination of alpha-blockers and 5-alpha-reductase inhibitors to achieve better therapeutic outcomes in BPH management.

Furthermore, findings from two large studies revealed that, combination of alpha-blockers and 5-alpha reductase inhibitors resulted in greater symptomatic relief and reduced progression of BPH in patients (Montorsi *et al*., 2010; McConnel *et al*., 2003). Although combination therapy was significantly superior to alpha-blocker monotherapy, it was not superior to use of 5-alpha reductase inhibitor monotherapy at reducing the relative risk of acute retention or BPH-related surgery (Roehrborn *et al*., 2010). Furthermore, combination therapy which is increasingly being used currently are alpha-antagonist with anticholinergics, even though there are concerns that the use of anticholinergics in men with voiding symptoms may result in acute urinary retention.

Another group of pharmacotherapeutic agents currently being employed in the management of LUTS associated with BPH management are known as phosphodiesterase-5 (PDE5) inhibitors; sildenafil, vardenafil, and tadalafil. Studies suggest their possible mechanisms of actions include; relaxing of the smooth

muscle fibers of the bladder and prostate, and reducing the hyperactivity of the autonomic nervous system. Furthermore, it possesses potent anti-inflammatory and anti-proliferative effects (Peixoto and Gomes, 2015). Besides being effective in the management of LUTS in men with BPH, these drugs can improve sexual function, and urine flow rate to comparable levels as the alpha-blocker tamsulosin. However, it has been observed that a third of the BPH patients never respond to medicinal therapy, and thus would need surgery (Bechis *et al*., 2014; Oesterling *et al*., 1995). According to Wasson *et al*. (1995), surgery provides option for improving symptoms and decreasing progression of the disease for BPH patients who develop complications or have inadequately controlled symptoms whilst on medical therapy.

Management of PCa

Active surveillance (watchful waiting), radical prostatectomy (surgery), drugs, radiation therapy or high intensity frequency ultrasound are often used in management of clinically diagnosed localized prostate cancer (Thompsons *et al*., 2003). However, hormonal therapy (androgen-deprivation therapy) and chemotherapy are the main remedies in advanced cancer treatment regimen. Radiation treatment may be combined with androgen-deprivation therapy (ADT), and men with metastatic PCa are managed almost exclusively with ADT and chemotherapy (Loblaw *et al*., 2007). Conventional drug treatment regimens besides being expensive are associated with adverse effects such as urinary and erectile dysfunctional problems, and in some instances may cause toxicity and growth inhibition to normal cells (Singh *et al*., 2006; Steineck *et al*., 2002). Hence, clinicians' ability to risk and stratify patients with clinical PCa, and the stage of the

disease, helps to personalize therapeutic strategies with the aim of minimizing harm to low risk patients and maximizing therapeutic outcomes in high risk patients. Though these therapeutic options are quite effective, development of resistance of drugs to BPH, progression of PCa to metastasis stage, toxicities associated conventional drugs and efficacy-related issues in diverse populations have been of concern to clinicians and scientists.

However, due to the common drug associated side-effects with the conventional BPH and PCa drugs such as; impotence, decreased libido, orthostatic hypotension, fatigue, dizziness among others have underpinned the increased shift towards search and usage of "safer" natural plant extracts for the management of these conditions (Hutchison *et al*., 2007; Madersbacher *et al*., 2005; Buck, 1996). Furthermore, studies have revealed that PCa usually progresses from the androgen-dependent to the androgen-independent stage, thus increasing in metastatic potential, as well as incurable malignancy owing to the ineffectiveness of previously recommended antiandrogen therapy (Jemal *et al*., 2005; Rini *et al*., 2002). Hence, continuous research for efficacious and safer therapeutic strategies using *in vitro* and *in vivo* prostate models has led to the increase in scientific studies on medicinal plant therapies generally claimed to be safer, and cost effective to address these major concerns for the clinical treatment and management of PCa.

Models systems used in BPH and PCa research

The scientific global community in its efforts to understand the molecular pathogenesis of BPH and PCa, its clinical effects, and to develop therapeutic

targets, employs several models including *in vitro and in vivo*. Immortalized cell lines such as normal (prostate) or tumour prostate (PCa) cells have been manipulated under conditions affecting their growth and differentiation to grow and divide indefinitely. These provide useful *in vitro* model systems to study the mechanisms of prostate cell function, regulation, as well as cellular transformation (Cunningham and You, 2015; Mahapokai *et al*., 2000).

In vitro studies provide ease, reliable, affordable screening of several compounds or agents in a relatively shorter period of time, and also permits quantitative analysis. *In vitro* models also offer useful techniques for testing the cytotoxicity of new synthetic or natural compounds or products in established cancer cell lines (Carocho and Ferreira, 2013). According to Cunningham and You (2015), the most useful *in vitro* PCa model is cell line culture, and a vast variety of cell lines are available due to the different origin of PCa in the prostate organ or tissue. Despite the benefits of *in vitro* models in prostate disease studies, they capture only limited aspects of the normal prostate or tumour microenvironment, and also have reduced physiological relevance which may not directly reflect the *in vivo* situation (Katt *et al*., 2016).

Several BPH and PCa *in vivo* models have also been used in the study of the pathogenesis of prostate diseases, depending on the concept of the study. According to Mahapokai *et al*. (2000), the use of spontaneous BPH is rare in other species other than man, though spontaneous animal models have been developed and currently limited to chimpanzees and dogs. However, ethical and financial factors restrict the applicability of these models. BPH rat models are much cheaper and

easier to develop for drug efficacy studies. On the other hand, the use of *in vivo* PCa models takes much longer period of time to induce, and have high failure rate of cancer induction (Evans *et al*., 2016). *In vivo* prostate models usually involve the induction of BPH or PCa in mice or rats, and treatment with a test agent (Mahapokai *et al*., 2000). It is worth noting that, the prostate of rodents consists of three lobes; the ventral, dorsolateral and anterior lobes, which are confined to the urethra. Whereas in male humans, there are four distinct regions of different tissue composition; central, peripheral, transitional zones and the anterior fibromuscular stroma (McNeal, 1980).

Normal prostate (PNT2) cell lines

PNT2 is a normal human prostate immortalized cell line which originated from the normal prostate of a deceased 33-year-old male. It is a plasmid containing a Simian virus genome with a defective replication origin (SV40 ori), and is currently employed in many *in vitro* studies (Cussnot *et al*., 2001). It is thought to have highly differentiated luminal epithelia (Lang *et al*. 2001). PNT2 cell lines may not be wholly normal, but has been used widely as a model of the normal prostate epithelia. These cells are attached cell lines which grow with an epithelial morphology. It has a well differentiated morphology and expresses cytokeratin 8, 18 and 19 members of the keratin family which are responsible for maintaining the epithelial structure (Gonias *et al*., 2001). PNT 2 cell lines are cultured in RPMI 1640 media which are widely used in cancer research. The cell cytotoxic value obtained for a potential anticancer drug or extract for the cell line is employed in the determination of the selectivity indices of the drug or extract vis-a-vis other prostate

cancer cell lines. PNT2 (serum free) is also a related cell line derived from PNT2 and adapted to grow in serum free media.

BPH cell lines

Studies have revealed that, epithelial prostate cells obtained from normal humans and BPH patients that have undergone prostatectomy, and cultured under condusive conditions which promotes its differentiation and growth; are useful *in vitro* models for researchers to study the etiology of BPH, as well as the mechanisms of action of potential novel therapies (Kaseb *et al*., 2007; Webber, 1979). Non-tumorigenic human prostatic epithelium cell lines such as RWPE-1, BPH 1 and pRNS-1-1 are used by researchers to study the contrast of PCa pathogenesis. The most prevalent of this type of cell lines is RWPE-1. RWPE-1 were immortalized with human papilloma virus (HPV) 18, are androgen dependent, and are elongating growth factors (EGF) and tumour growth factor beta (TGF-β) treatment sensitive. These are therefore good models to investigate the molecular mechanisms underlying the proliferation of benign prostatic epithelial cells (Bello *et al*., 1997). Purity of epithelial cell cultures partly depends on the source of prostate tissue, and those obtained from open prostatectomies are said to primarily provide pure epithelial cells with occasional fibroblast colonies in some cultures, and the latter can be removed (Webber, 1979). According to Mahapokai *et al*. (2000), these epithelial cells can be serially passaged at several times under proper culture conditions. Studies have been conducted on the aqueous root extract of *C. membranaceus* by Afriyie *et al*. (2015) to ascertain whether apoptosis was involved in the antiproliferative activity using human BPH-1 cells. Findings confirmed

mitochondria dependent apoptotic pathway as one of its antiproliferative mechanisms of action in BPH.

Due to the essential complex interaction between epithelium and stroma during the normal prostate gland development and function, researchers have extensively studied and also cultured stromal cell lines to facilitate the understanding of BPH development and potential drugs for its management. For instance, WPMY-1 (myofibroblast stromal cell line) which was derived histologically from stromal cells in the peripheral zone of the normal adult prostate is useful for studies on paracrine, and stromal: epithelial interactions. The prostatic stroma which is made up principally of smooth muscle cells is suspected to play an important role in urethral obstruction (tension in the urethral muscle) secondary to BPH. Qianliening capsules (QC), a clinically proven effective traditional Chinese formulation which has been used for decades in the management of BPH in China was found to inhibit the proliferation of bFGF-stimulated WPMY-1 stromal cell lines (Zhong *et al*., 2015). Studies have shown that, epithelial and stroma cell lines when cultured under conducive conditions, are able to maintain their cellular and functional properties of the human prostate; sensitivity to steroid hormones and androgen receptor mRNA expression (Planz *et al*., 1999; Zhang *et al*., 1997). Webber (1979) reported that, epithelial cells obtained from transurethral resection of the prostate usually have many fibroblast colonies as a result of incomplete separation of acini from the stroma.

Scientists have documented a simple rapid and reproducible method for the isolation and culture of epithelial and stromal cells from benign and neoplastic

prostates; because the growth enhancing property of the culture could be achieved in the absence of undefined exogenous factors (Krill *et al*., 1997). Using their technique, they showed that primary cultured epithelial or stroma cells could undergo three to four passages before became senescent. Furthermore, *in vitro* prostate animal cell lines have also been employed in the study of the etiology of BPH. According to Danialpour *et al*. (1994) epithelial cell lines; NRP-152 and NRP-154 have been obtained from Lobund and Wistar rat prostates for studies of normal prostate growth and prostatic carcinogenesis. Isolation of canine prostate epithelial cells from a sexually intact adult beagle dogs have been reported (Lehr *et al*., 1998). Mahapokai *et al*. (2000) reported that, only a limited number of prostate cell lines are known to be non-tumorigenic, and most of the human prostate cell lines currently available are derived from malignancies. Furthermore, PCa cell lines are not suitable for studying normal prostate physiology, and the initiation and development of tumours of the prostate.

Prostate Cancer cell lines

Human prostate cancer cell lines are generally classified into two groups; androgen dependent and non-androgen-independent (Claas and van Steenbrugge, 1983; Horoszewicz, 1980). Majority of human prostate tumours express androgen receptors. And in selection of PCa cell model, it is important to consider factors such as; growth rate, behaviour as a xenograft, response to growth factors (Hanahan and Weinberg, 2011). Especially as a growth factor which is independent through autocrine signalling pathway is considered an important hallmark of cancer. Due to the vast array of prostate cancer cell lines available, this literature would focus on

LNCaP and PC3 cell lines which are the most frequently used prostate cancer cell lines for studying toxicity and anti-prostate cancer drugs (Horoszewicz *et al*., 1983; Horoszewicz *et al*., 1980; Kaighn *et al*., 1979). Prostate cancer cell lines grow in Rosewell Park Memorial Institute media supplemented with 1% penicillin/ streptomycin and 10% foetal bovine serum (FBS) (Alonzeau *et al*., 2013). The PC3 cells isolated from the vertebral metastatic prostate tumour, have a deletion mutation of p53 gene, lacks androgen receptor or PSA mRNA/protein and 5α-reductase enzyme (Carroll, 1993). It does not respond to androgen hormones, glucocorticoids or fibroblast growth factors and possesses highly metastatic ability than DU 145 and LNCaP cells which have low metastatic potentials (Pulukuri *et al*., 2005). High expression of TGF-alpha and EGF-R by PC3 cells accounts for its autonomous growth, a key factor which is speculated to underpin bones being a hospitable metastatic site (Ching *et al*., 1993). Futhermore, PC3 has a doubling time of approximately 33 hours, and highly aneuploidy. PC3 bears some similarity with DU-145 prostate cell lines with respect to them being hormone insensitive, and the absence of AR or PSA mRNA/protein.

Androgen sensitive prostate cancer cell lines LNCaP was derived from a metastatic deposit in a lymph node (Horoszewicz *et al*., 1980). LNCaP cell lines are adherent elongated epithelial cells that grow in either aggregates or as single cells, and are often used in the field of oncology due to the inherent ability to closely mimic human prostate cancer progression (Horoszewicz *et al*., 1983). LNCap cell lines possess a T877A mutated androgen receptor (as a result of a T877A mutation in the AR coding sequence), has a silent mutation on the tumour suppressor gene

p53, and lacks 5α-reductase enzyme (Carroll, 1993; Veldscholte *et al*., 1990). Furthermore, the androgen and estrogen receptors found in this cell line expresses prostate specific antigen, exhibit high affinity to 5α-dihydrotestosterone and estradiol respectively, as well as to a range of steroid compounds (Veldscholte *et al*., 1990). Furthermore, it has a doubling time of between 60-72 h depending on the serum, making it slower in growth compared to PC3, responds to TGF-alpha, EGF, insulin growth factor-1 (IGF-1) and expresses their receptors. PCa cell lines such as LNCaP, PC3 cells, and DU-145 cells were formally considered part of the triad that constituted the gold standard for prostate cancer research (Cunningham and You, 2015).

Need for *in vivo* PCa models is as a result of increase in the understanding that, cancer cell lines in general (including prostate lines) do not and cannot recapitulate the disease processes that occur in living tissues. Mice have been the most favoured models (i.e TRAMP, Pten, LADY) in recapitulating various stages of PCa process compared to rats and canine. Besides, dog models being expensive and having a pet affection, there is inadequate genetic manipulation in the case of rats (Cunningham and You, 2015). Furthermore, a major challenge currently being faced in prostate cancer research is the lack of an animal model which can recapitulate metastasis of prostate cancer.

In vivo models of BPH

Several animal models of rats, mice, chimpanzees and dogs have been developed and used to study the etiology of BPH and the therapeutic effects of potential drug candidates in BPH management (Mahapokai *et al*., 2000). Studies on

experimental rat models have revealed that, the most susceptible rat strains with the capabilities of developing benign and atypical prostatic hyperplasia after exposure to exogenous testosterone in both intact and post castrated males are the Wistar and Sprague-Dawley (Sconik *et al*., 1994). Furthermore, development of BPH in F344 and ACl/Ztm rat strains were found to be difficult compared to the latter strains. In the study of the etiology of BPH in man using animal models, it is worth noting that only the dorsolateral lobe of rodent's prostate is ontogenetically comparable to the human prostate (Nevalainen *et al*., 1991). Unlike the prostate anatomy of rodent models, dogs with BPH have many features similar to man and in both species, the development of BPH occurs spontaneously with advanced age. Studies have also revealed that, prostatic epithelial hyperplasia in both man and the dog are androgen sensitive, undergoes involution with androgen deprivation and resumes epithelial hyperplasia when androgen is replaced (Tutrone *et al*., 1993). However, the enlarged prostate in the dog hardly leads to urethral obstruction (Krawie and Heflin, 1992).

Human BPH cells obtained from primary surgical specimens can be transplanted into athymic rats or mice to create xenograft models of human BPH. These transplants have been found to exhibit histologically typical BPH acini, stroma, epithelial lining and possess the ability to express prostatic biomarkers such as PSA and prostate acid phosphatase (Debiec-Rychter *et al*., 1994; Claus *et al*., 1993). These types of models have contributed immensely to the studying of the etiology, and potential drug efficacy in BPH management (Otto *et al*., 1992). For example, human genes have been transferred into the genetic make-up of mice

(transgenetic mice) in order to elucidate the various stages of BPH development and its management with potential drug candidates. In 1992, a Harvard-developed mouse model for prostate enlargement was established and found to be hormonally sensitive and suitable for investigating BPH and growth-factor-induced epithelial cell hyperplasia (Tutrone *et al*., 1993). The availability of human and animal *in vitro* and *in vivo* models has made it cost-effective for studying toxicity, cytotoxicity and therapeutic potential drug extracts or candidate using reliable and reproducible methods. Thus, enhancing research on potential bioactive compounds in plants as well as synthetic compounds.

Plants as sources of bioactive compounds with anticancer activity

Several chemopreventive agents and radiation employed in the management of cancer have been associated with serious adverse reactions or toxicity (Katherisan *et al*., 2006). Plant-derived products have also been reported to exhibit potent antitumor activity in several rodent and human cancer cell lines (Lin *et al*., 1996). Other studies have revealed that, many naturally occurring substances present in the human diet have been identified as potential chemopreventive agents, and the consumption of relatively large amounts of vegetables and fruits is believed to prevent the development of cancer (Vecchia and Tavani, 1998). Over 50% of all modern drugs in clinical use were derived from natural products, with many of them having the ability to inhibit growth of cancer cells (Madhuri and Pandey, 2009). The isolation of the vinca alkaloids, vinblastine and vincristine from Madagascar periwinkle, *Catharanthus roseus* (Apo-cynaceae) for clinical

management of cancer, and combination with other cancer chemotherapeutic drugs have introduced a new era of use of plant material as anticancer agents (Cragg and Newman, 2005). Futhermore, two plant derived natural products, paclitaxel and camptothecin were estimated to account for nearly one third of the global anticancer drug market in the year 2002 (Oberlines and Kroll, 2004). Within the period of 1940s to the end of 2014, 49% of cancer drugs in clinical use were derived from natural compounds, and Taxol is an example of a very common chemo drug obtained from the bark of the Pacific Yew tree (Taxus) (Newman and Cragg, 2016). A survey revealed that, more than 60% of cancer patients use vitamins or herbs as therapy (Rosangkima *et al.*, 2004).

Medicinal plants also contain several phytochemicals, which have active biological compounds with disease preventive, cytotoxic, strong antioxidant and apoptototic activities among others. These antioxidants may prevent cancer and other diseases by protecting the cells from damage caused by free radicals from the highly reactive oxygen compounds (Madhuri and Padley, 2009). Thus, consuming a diet rich in antioxidants (e.g. fruits and vegetables) is believed to provide phytochemicals that possess cancer protective effects.

Quercetin is a popular phytochemical of interest to researchers. It is a strong anticancer, anti-inflammatory, and antiviral agent found in grapes, onions, apples, red wine, licorice root, and berries (Anand *et al*., 2016). Another phytochemical of clinical importance is resveratrol found in red wine, red grapes, and dark berries, which inhibits cancer by anti-proliferation properties, and metastasis by inducing apoptosis (Lee *et al*., 2015; Donnelly *et al*., 2014). It also acts against several genes

that promote prostate cancer, colorectal, and other cancers, through activation of the tumor suppressor gene p53. This gene regulates cell division by keeping cells from growing and replicating too fast or in an uncontrolled way. It is also vital in determining whether damaged DNA will be repaired or a damaged cell will undergo apoptosis. p53 activates other genes to fix the damaged repairable DNA and if the DNA cannot be repaired it prevents the cell from dividing and signals it to undergo apoptosis. Resveratrol has also been found to inhibit the gene that make aromatase, the enzyme that controls estrogen production, thus inhibiting the growth of estrogen-sensitive cancers (Saluzzo *et al*., 2016).Lycopene found in abundance in watermelon, and tomato (particularly when cooked), has proven scientific data of efficacy against cancers of the pancreas, colon, rectum, esophagus, oral cavity, stomach, lungs, prostate, breast, and cervix (Chottanapund *et al*., 2014). A review article published by the American Institute for Cancer Research tends to suggest that eating tomatoes and other lycopene-containing foods lowers prostate cancer risk (Grossarth-Maticek *et al*., 2001).

A diet rich in selenium is believed to protect against cancers such as those of the stomach, breast, esophagus, lung, tongue, liver, colon, kidneys, bladder, pancreas, prostate, colon, and rectum. Studies by Walfish *et al*. (2007) revealed that, increased selenium intake lowered the incidence of cancers of the lung, colon, and prostate by roughly 50%, and higher levels of selenium tend to lower the incidence of tumor formation, bringing the risk-reduction even higher for some cancers.

Over the past decade, herbal medicines with its phytochemicals have been accepted as alternative medicines in the management of BPH and its related

cancers. Their use has made considerable contribution towards improving and maintaining prostate health nationally and globally.

Medicinal plants used for BPH management and PCa

Phytotherapy is the first-line treatment for mild-to-moderate lower urinary tract symptoms in Germany and Austria, and represents more than 90% of all drugs prescribed for the treatment of BPH (Wilt *et al*., 2000; Buck, 1996). In Italy, phytotherapeutic agents represent nearly 50% of the medications dispensed for treatment of BPH, compared to 5% for alpha 1-adrenoreceptor blockers, and 5% for 5 alpha-reductase inhibitors (Di Silverio *et al*., 1993). In the United States, phytotherapies for BPH are readily available as non-prescription dietary supplements and most of these compounds are unlicensed, often promoted to "maintain a healthy prostate" as natural and harmless (Wilt *et al*., 1998).

Phytotherapeutic evaluation and clinical studies on the root extract of *Urtica dioica* (Stinging nettle), extract of European mistletoe (*Viscum album*) oil of *Cucurbita pepo* (Pumpkin seed) and *Secale cereale* (Rye grass pollen) have revealed promising natural anti-prostatic growth and anti-prostate cancer activities (Tsai *et al*., 2006; Grossarth-Marticek *et al*., 2001; Nickel *et al*., 2008). Epigallacotechin 3 gallate, an anticancer agent isolated from green tea is used for the management of prostate cancer (Raza and John, 2005). Studies have shown that, the hot aqueous extracts of *Hypoxis hemerocallidea* (African potato) is useful in the management of BPH and prostate adenoma, and beta-sitosterol has been found to be one of the phytochemicals implicated for its therapeutic effect in these conditions (Singh, 1999; Hutchings *et al*., 1996). *Hypoxis rooperi* and *Secale cereal* (Rye

grass pollen) have demonstrated preliminary evidence of improving symptom score and urological symptoms in BPH (Wilt *et al*., 2000). Pygeum bark extract has been used in Europe since the mid-1960s to treat men suffering from benign prostatic hyperplasia (Isaac, 1990). The liphophilic extract of *Pygeum africana* have been used commercially in Europe in the management of BPH and prostatitis (Plosker and Brogden, 1996; Andro and Riffaud, 1995). Additionally, the presence of beta-sitosterols in the extract (known to possess anti-inflammatory activities) was found to inhibit inflammation and enlargement of the prostate. Furthermore, short term clinical studies (< 6 months) have confirmed its efficacy in relieving symptoms of BPH. Saw palmetto (*Serenoa serrulata*) have been used for decades in relieving lower urinary tract symptoms associated with BPH and prostate adenoma (Wilt *et al*., 1998). Studies have revealed that, some constituents of this plant inhibit 5-alpha reductase, and dihydrotestosterone from binding to androgen receptors in the prostate gland (Plosker and Brogen, 1996). Paclitaxel and docetaxel which are widely used anti-prostate cancer drugs, were isolated from Pacific yew tree and *Taxus brevifolia*, respectively (Cragg, 1998).

In Ghana, aqueous root extract of *Croton membranaceus* have been used for management of BPH and related prostate cancer for several decades (Bayor *et al*., 2009; Mshana *et al*., 2000). Currently, the root, stem and leaf extracts from *C. membranaceus, Hibiscus sabdariffa* and *Annona muricata* are available on the Ghanaian market in capsules or syrups formulations as non-prescription drugs for management of BPH and its related cancer. Although, there are claims of symptomatic relief of LUTS with use of these formulations in Ghana, there remain

virtually no scientific data on the toxicity, efficacy and mechanism(s) of action of the aqueous stem extract of *C. membranaceus.*

Relevance of toxicological studies

Toxicological studies on medicinal plant extracts

Toxicity test examines toxic effects of a chemical when it is absorbed into the body via the mouth, skin, nose, lungs, among other routes (OECD, 1987; 1998). Pharmacological and toxicological evaluations of medicinal plant extracts or potential drug molecules are essential for drug development (Mushtaq *et al*., 2003; Ibarrola *et al*., 2000). These are done to establish safety levels and to assist in determining whether a new drug should be adopted for clinical use or not. Toxicity studies need to be done even if the plant has a long history of ethnomedicinal use without any documented toxicological effect(s) especially if the plant is meant for either long term treatment, chronic conditions or as food supplements. Toxicological evaluation of medicinal plants has often been neglected based on the belief that their sources are natural and therefore are not toxic. Furthermore, owing to the fact that they have been part of our diet and their apparent uneventful use usually is considered a testimony of its safety. However, the fact remains that, many of these medicinal plants and their extracts are administered in most disease conditions over long period of time without proper dosage monitoring and consideration of possible toxic consequences from such prolonged usage (Ogbonnia *et al*., 2010). According to Sofowora *et al.* (1993), researchers have been aware of the potential toxicity of these plants and their extracts, inspite of their immense

potential therapeutic effects. It has been reported that, some plant extracts could be inherently dangerous as a result of naturally occurring toxins, which may be cytotoxic or carcinogenic (Humphrey and McKenna, 1997). Fennel *et al*. (2004) found that, many African plants used medicinally and widely assumed to be safe, are potentially toxic due to the presence of constituents such as aristolochic acids, pyrrolizidine alkaloids, benzophenanthrine alkaloids, lectins, viscotoxins, saponins, diterpenes, cyanogenetic glycosides and furonocoumarins. Traditional practitioners often do not have the expertise to detect or monitor delayed effects such as carcinogenicity or mutagenicity, and adverse effects that could arise from long-term use of even food supplements and nutraceuticals (Ernst, 1998).

Even though several studies have been done in screening medicinal plants for efficacy based on traditional claims, less emphasis has been placed on the issue of safety. Chanabra *et al*. (2003) reported that, plants that contain compounds with known mutagenic or carcinogenic activities must also be subjected to special toxicity studies. Thus, the need to carry out short term and/or long-term toxicological studies on medicinal plants need not be overemphasized.

Information from safety studies in animals is used to characterize target organ toxicity, relationship between dose or exposure/response, potential reversibility of any observed effects, and for monitoring adverse effects in clinical studies. According to Remirez (2006), toxicological studies on potential drug targets or plant extracts should include tests such as acute, subchronic, and special toxicological studies such as genotoxicity, carcinogenicity, and reproductive toxicity that are impossible to detect clinically.

Acute toxicity studies

The examination of possible or potential adverse effects produced by a test substance when it is administered in one or more doses during a period not exceeding 24 hours is classified as acute toxicity testing (Adeyimi, 2010; CDER, 1996; OECD, 1987). Acute toxicity studies in animals are usually required to be performed on any potential drug canditate intended for human use and the information obtained from these studies is useful, in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, occasionally in revealing delayed toxicity. Findings from such studies may aid in the selection of starting doses for phase 1 human studies, and provide information relevant to acute overdosing in humans. The aim(s) of acute studies can usually be achieved in rodents, using small groups of animals (for instance, three to five rodents per sex per dose) (CDER, 1996). Furthermore, in instances which non-rodent species are appropriate for investigation, the use of fewer animals may be considered. The test compound or crude extract is often administered to animals in varying high doses to groups once or same dose given in divided doses over a period of time within 24 hours to identify doses causing no adverse effect and doses causing major or life-threatening toxicity.

A key toxicological index employed for the assessment of the safety of drugs is lethal dose 50% (LD_{50}), and it is the most common test done in acute toxicity studies and varies depending on the route of administration. Oral LD_{50} value is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of the animals when administered via the oral route (Deora *et al*.,

2010). It is expressed in terms of weight of test substance per unit weight of test animal (g/kg). The lower the LD_{50} value, the more toxic the chemical or crude extract and vice versa. According to Deora *et al*. (2010) values of LD_{50} may be affected by factors such as species, age, sex, and amount of food, route of exposure and temperature or humidity of the environment. Based on Hodge and Sterner scale (CCOHS, 2005), a test drug administered orally is considered extremely toxic at LD_{50} value between 1-50 mg kg^{-1} as highly toxic, moderately toxic at 50-500 mg kg^{-1}, slightly toxic at 500-5000 mg kg-^{1,} practically non-toxic at 5000-15,000 mg kg^{-1} and relatively harmless at 15,000 mg kg^{-1} or more. Three alternative methods have been currently adopted to replace the classical LD_{50} method; the fixed dose procedure, acute toxic class, and Up and Down procedures (Deora *et al*., 2010; Asiedu-Gyekye *et al*., 2005). Several animals such as mice, rats, rabbits, guinea-pigs, cats, dogs, fish, monkeys and birds have been used for LD_{50} studies of chemicals.

Lethal dose (LD_{50}) values of various species of Croton have been estimated currently by the fixed dose method. The essential oil from *Croton cajucara* bark was investigated for acute toxicity in mice and LD_{50} values of 9.3 g/kg and 680 mg/kg were estimated by the oral route and intraperitoneal route, respectively (Hiruma-Lima *et al*., 1999). The LD_{50} values of 273.86 mg/kg and above 3 g/kg were estimated for the ethanolic root extract of *Croton zambesicus* and the essential oil from *Croton zehtneri* leaf, respectively, using rat models (Hiruma-Lima *et al*., 2008; Okokon *et al*., 2004). However, the LD_{50} value of the ethanolic leaf extract of *Croton zambesicus* was estimated at 1400 ± 148 mg/kg in mice (Okokon *et al*.,

2006). Acute toxicity studies by Asare *et al*. (2011) showed that, ingestion of the aqueous-ethanolic root extract of *C. membranaceus* does not produce general acute toxicity in Sprague-Dawley rats and the LD_{50} value was found to be greater than 3 g/kg. Similar acute toxicity studies on the ethanolic root extract of *C. membranaceus* by Sarkodie *et al*. (2014) in S-D rats showed oral LD_{50} value is greater than 5000 mg/kg. A preliminary study by Appiah (2011) on the aqueous extracts of the root, stem and leaf of *C. membranaceus* in male S-D rats also revealed that, the LD_{50} values were greater than 5000 mg/kg. Thus, based on Hodge and Sterner scale, LD_{50} values of ingested aqueous and ethanolic root extracts of *C. membranaceus* were virtually greater than 5 g/kg, and may be classified practically non-toxic. According to Asiedu-Gyekye *et al*. (2005), LD_{50} value obtained in a study may be used to estimate the starting dose of a substance, because under normal circumstances, animals will tolerate about a quarter of the LD_{50} dose of a given extract or compound.

Repeated Toxicity Study (Sub-chronic toxicity study)

Repeated or sub-chronic toxicity studies are designed to reveal toxic or adverse effects associated with repeated doses of a chemical over part of an average lifespan of experimental animals. Standard sub-chronic toxicity studies with rodents are generally conducted up to 90 days (OECD, 1998), and in some studies it ranges between 30-90 days (Adeyimi *et al*., 2010; Hamid *et al*., 2005, Rasekh *et al*., 2005). Research have revealed that repeated toxicity studies even up to 90 days usually may not determine the carcinogenic potential of a test substance because of the long latency period for development (OECD, 1998). And extrapolation of the results of

such studies to human is valid to a limited degree. The objective of these toxicological studies includes, estimation of appropriate doses of the test substance for future chronic toxicity studies, indication of organs adversely affected by test substance, information on possibility of product accumulation, and determining the no observable adverse effect level (NOAELs) for some toxicology endpoints. NOAEL doses obtained depend on how closely the test dosages are spaced, the type or species, and or number of animals assigned for the study. Repeated toxicity studies also allow future long-term toxicity studies in rodents and non-rodents to be designed with special emphasis on identified target organs.

The real usefulness of these studies is their ability to provide information on health hazards that are likely to arise from repeated exposure to a test substance or medicinal plant extract. Sub-chronic studies are usually conducted in two species of rodent and non-rodent (rabbit or dog) by the route of intended exposure. However, the preferred species is the young healthy adult (8-9 weeks) rat (OECD, 1998), although other rodent species such as the mouse, could be used. At least three doses are employed in standard sub-chronic toxicological studies; a high dose that produces toxicity but does not cause more than 10 percent fatalities, a low dose that produces no apparent toxic effects, and an intermediate dose (OECD, 1998). The three dose levels- low, medium and high are normally based on the LD_{50} value and, unless otherwise stated, it is recommended that the therapeutic dose (or multiples of it) be used as the low dose and one-third of the lethal dose LD_{50} as the high dose (Asiedu-Gyekye *et al*., 2005). The highest dose level is chosen with the aim of inducing toxicity but not death or severe suffering, whiles decreasing sequence of

dose levels should be selected with a view to demonstrating any dosage related response. In such studies, the lowest dose level selected should give no toxic effect or no-observed-adverse-effect level (NOAEL) (Redbook, 2000).

Clinical toxicological signs

The Organization for Economic Cooperation and Development (1998) recommends routine cage-side observations are made on all test animals during the experimental period of administration of test substance or medicinal plant extract in pre-clinical toxicological studies for general signs of toxicity, morbidity and mortality until sacrificed. Furthermore, these observations should include but are not limited to, changes in skin, eyes, fur, mucous membranes, occurrence of secretions and excretions. It also involves observing for autonomic activity such as lacrimation, piloerection, pupil size, respiratory patterns (such as noisy breathing). Also, observations for toxic symptoms such as weakness, aggressiveness, food refusal, loss of weight, diarrhoea, discharges from the eyes and ears are necessary during acute and subchronic toxicity studies. The experimental animals are to be critically observed during the first six hours for signs of toxicity (OECD, 2001; WHO, 2000). However, the nature of the toxic reactions, the time of onset and length of recovery period or period of reversibility from the effect of test substance or extract underpins the duration and frequency of observation for clinical signs (Asiedu-Gyekye *et al.*, 2005). The OECD (1998) however recommends that, general clinical observations be made at least once or twice a day, preferably at the same time each day, taking into consideration the peak period of anticipated effects after dosing. Acute toxicity study of the aqueous root extract of *Croton*

membranaceus with doses of 1500 mg/kg and 3000 mg/kg in Sprague-Dawley rats, did not elicit any clinical signs of toxicity 14 days after oral administration (Asare *et al.*, 2011). Neither did sub-chronic oral administration of 30, 150 and 300 mg/kg of the aqueous root extract of *Croton membranaceus* for 90 day elicit any clinical signs of toxicity in S-D rats during the experimental period (Afriyie *et al*., 2013).

Auletta (1995) recommends that, it is critical for objective evaluation of the effect of a compound on test animals to include changes in general behaviours, body weight, internal organ weight since such changes are often the first signs of toxicity. Weights of test animals during such toxicity studies should be determined at least once a week. Alteration (such as a significant decrease) in general body weight and internal organ weight are often the first signs of toxicity (Auletta, 1995). The rationale is that, since the experimental animals often used in toxicological studies are young adults in the early phase of their growth, a decrease in the rate of gain relative to control animals is a very sensitive indicator of systemic toxicity. According to Teo *et al.* (2002) body weight changes in experimental animals are indicators of toxicity, it is recommended that, animals that survive should not lose more than 10% of their initial body weight. And OECD (1998) recommends that, food or water consumption (if the test substance is administered in the drinking water) by test animals be monitored (or estimated) every week during sub-chronic toxicity study. Findings from acute and sub-chronic toxicity studies on the aqueous and ethanolic extract of *C. membranaceus,* respectively in S-D rats did not significantly affect their food and water consumptions, neither did it adversely impact the body and organ weights of the rats (Asare *et al*., 2011; Afriyie *et al*.,

2013). In a similar sub-chronic toxicity study using ethanolic crude root extract of *C. zambesicus* on rats, considerable increase in body weights in both extract treated and control groups were observed (Okokon *et al.*, 2010). They further inferred that, the extract does not interfere with growth processes in the rats, and may have promoted growth by stimulating the synthesis of body proteins.

Assessment of haematological parameters during toxicity studies

One of the most sensitive targets of toxic compounds is the haematopoietic system, and it is an important index of physiological status in man and animals (Adeneye *et al.*, 2006). Analysis of haematological data from test animals or humans are vital for diagnosis, prognosis and effective therapy of diseases, thus it is an important physiological index (Baker, 1985). The, assay and interpretation of haematological parameters in test animals at various time intervals during the course of a study is a common practice in toxicological studies (Frith *et al.*, 1980). Another study reported that, analysis of blood parameters is relevant in toxicological risk evaluation as changes in the haematological system have higher predictive value (91%) for toxicity in humans in assays involving rodents and non-rodents (Olson *et al.*, 2000). Parameters normally assessed include, haematocrit (HCT), haemoglobin concentration (HGB), erythrocyte count, total and differential leukocyte count, platelet count (PLT#) and a measure of clotting potential such as clotting time, prothrombin time, or thromboplastin time. According to OECD (1998), assessment of the effect of test compounds or plant extracts on these parameters in experimental animals are done at the commencement of the study, then either at monthly intervals or midway through the test period and finally at the

end of the test period (OECD, 1998). Usually, the mean values and standard deviations of the test compound groups are calculated for each parameter after the study period, and statistical comparisons of group mean values among test groups and are controls done (Mckie and Parkar, 2006).

Haemoglobin, haematocrit and other red blood cell indices such as mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) are also helpful in differential diagnosis of anaemia (Gregg and Voigt, 2000; Coles, 1986). With respect to causative factors attributed to causing anaemia, Ihedioha and Chineme (2004) reported that it could be attributed to one or a combination of these; excessive blood loss (haemorrhage), excessive red blood cell destruction (haemolysis), and decreased erythrocyte production. Furthermore, significant reduction in packed cell volume and haemoglobin in treated groups in toxicity studies, tends to suggest possible signs of anaemia (Anaigu *et al*., 2005).

A rise in white blood cell (leukocytosis) count in rats is a normal reaction to foreign substances which alters their normal physiological processes (Adebayo *et al.*, 2010). However, significant reduction in the count of white blood cells could be suggestive of suppression of leucocytosis (Okokon *et al*., 2004). Rise in white blood cell count in rats is a normal reaction to foreign substances which alters their normal physiological processes (Adebayo *et al.*, 2010). However, significant reduction in the count of white blood cells could be suggestive of suppression of leucopenia (Okokon *et al*., 2004).

Sub-acute toxicity study on the effects of ethanolic leaf extract of *Croton zambesicus* on haematological parameters of rats employing doses from 100-400 mg/kg for 21 days, revealed reductions in packed cell volume (PCV), haemoglobin concentration (HGB), mean corpuscular haemoglobin (MCH) and white blood count (WBC), in a dose-dependent fashion (Okokon *et al.*, 2004). Thus, the extract was suspected to possess the potential of suppressing erythropioesis and causing anemia. However, significant elevations of mean cell volume in treated groups were observed compared to control. The significant reduction in erythrocyte counts and haemoglobin concentration were attributed to failure to supply the blood circulation with cells from haemohepatic tissues, due to the role the liver plays in the regeneration of erythrocyte (Adebayo *et al.*, 2010). Further analysis of the haematological parameters revealed dose-dependent reduction in the total white blood cells and this was attributed to suppression of their production in the bone marrow by extract, resulting in leucopenia (Okokon *et al.*, 2004). The acute toxicity study of the ethanolic root extract of *Croton membranaceus* on haematological indices using S-D rats did not have significant changes on RBC count, MCV, MCH, MCHC in the treated groups, after 48 hours and 14 days were not statistically significant (Asare *et al.*, 2011). After a 90-day sub chronic administration of the aqueous root extract of *C. membranaceus*, the absence of deleterious effects on the white blood cells (WBC), red blood cells (RBC), HGB, HCT, mean cell volume (MCV), MCH, and platelets (PLT) in the rats largely suggested no adverse effect of extract on haematological parameters (Afriyie *et al.*, 2013). Sub-acute administration of ethanolic crude root extract of *C. zambesicus* with doses ranging

between 27-81 mg/kg to rats for 21 days, caused dose-dependent increase in RBC, PCV, HGB, WBC, bleeding time and clotting (Okokon *et al.*, 2010). Significant increases were observed in the highest group (81 mg/kg) for RBC and PCV when compared to the control. It was also observed that the extract caused dose-dependent rise in the level of WBCs, with significant ($p<0.05$) increase in the high dose group (Okokon *et al.*, 2010).

Biochemical indices examination in toxicity studies

Clinical chemistry parameters in toxicity studies

Clinical chemistry examinations are required to be performed on the serum obtained from blood of test animals at the start, then either at monthly intervals or midway through the test and finally at the end of the test period (OECD, 1998). Furthermore, a standardized list of clinical biochemistry and urinalysis parameters are to be monitored during the course of toxicity studies to assess the potential target organ of toxicity of medicinal plant extracts, pharmaceuticals, direct and indirect food additives, and industrial chemicals.

Kidney function indices

Serum urea, creatinine, potassium, calcium and chloride levels are often used as markers of kidney functions. Plasma concentration of creatinine is a better index of measuring glomerular filtration rate (GFR), as it is relatively constant under normal circumstances unless GFR changes (Whitby *et al.*, 1989). Furthermore, increased creatinine levels in the blood is indicative of abnormal kidney function as a result of decreased excretion of creatinine in the urine (Sireeratawong *et al.*, 2008b; Gad,

1994). Plasma urea concentration however, is less reliable than creatinine as an index of glomerular filtration rate by virtue of the fact that, about 40 % diffuse back into the renal tubular cell (Mckie and Parkar, 2006; Tilkian *et al.*, 1979). Obidah *et al.* (2009) were of the view that, insignificant rise in serum urea and creatinine is suggestive of normal functional kidney of the rats. Administration of ethanolic crude root extract of *C. zambesicus* (27-81 mg/kg) to rats in a sub-chronic toxicity study revealed no significant changes in mean serum concentrations of urea, creatinine, sodium, potassium and chloride ions in the test group compared to that of control (Okokon *et al.*, 2010). Thus, it was therefore inferred that, the extract was non-nephrotoxic. Urinalysis done during acute toxicity study of the aqueous root extract of *Croton membranaceus* in S-D rats at 0 h, 48 h, and the 14th day by Asare *et al.*, (2011) revealed that: the pH, protein, glucose, ketone bodies, bilirubin, occult blood and urobilinogen were negative. Findings of the latter study concluded that, the renal system assessed by urea and creatinine levels, were normal post extract administration during the study. Urinalysis data from sub-chronic toxicity on the aqueous root extract of *C. membranaceus* did not reveal any noticeable differences in urobilinogen, glucose, bilirubin, specific gravity, blood (RBC), nitrite, and leukocytes between control and treated groups (Afriyie *et al.*, 2013). Further examination of renal function indices; urea, creatinine, and electrolytes suggested the absence of nephrotoxicity.

Liver function indices

The liver is the primary target organ for toxic effects of xenobiotics, and detoxification of toxic agents occurs mainly in the liver (Balistreri and Shaw, 1987).

According to OECD (1998), the levels of some serum parameters or enzymes such as albumin, total protein, bilirubin, alkaline phosphatase (ALP), acid phosphatase (ACP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and cholesterol could be used as indices of toxicity for xenobiotics. Severe hepatic injury due to the metabolism of toxic phytochemicals and failure of the metabolic products to be eliminated by the liver, could be associated with marked distortion of serum enzyme activities, or serum biomarkers (Geidam *et al.*, 2004). Alanine amino transferase (ALT) is a hepatospecific enzyme principally found in the cytoplasm and is a specific marker of hepatic injury (Tilikian *et al.*, 1979). This enzyme (ALT) has its highest concentration in the liver, whilst kidney and skeletal muscles having lesser activities of the enzyme. Studies have revealed that, about 80% of AST is present in the mitochondria whereas ALT is a purely cytosolic enzyme (Al-Mamary *et al.*, 2002).

Furthermore, AST levels appear higher in a number of tissues such as liver, kidneys, heart and pancreas, and it is released slowly compared to ALT. Similar studies have revealed that, high levels of AST and ALT are usually present within hepatocytes, and plasma levels rise as hepatocytes membrane integrity is disturbed during hepatocellular cell injury. Whitby *et al.* (1989) reported that, ALT measurements are more liver specific than AST, and its activity is usually greater than AST activity at early or acute hepatocellular disease. However, they reported that, AST tend to be released more than ALT in chronic liver diseases such as cirrhosis. Other similar studies suggest that, since the liver and the heart release AST and ALT, and elevation of both enzymes in the blood could indicate

possibility of liver or heart damage (Crook, 2006; Wasan *et al.*, 2001). Acute toxicity study on the aqueous root extract of *C. membranaceus* on S-D rats, did not reveal any significant elevation in liver function enzyme levels for AST and ALT in the test groups compared to control group (Asare *et al.*, 2011). Findings from the sub-chronic toxicity effect of ethanolic crude root extract of *C. zambesicus* in rats however, showed a significant increase in ALT, AST and ALP levels (Okokon *et al.*, 2010). Results from the latter study was indicative of possible hepatic injury (biochemical or pathological), though this was not confirmed during the histological examination of the tissues. On the contrary, results from the 90 day sub-chronic toxicity study on the aqueous root extract of *C. membranaceus* revealed, serum levels of ALT and AST were reduced by more than 50% at the end of the study period suggesting it does not cause hepatocellular injury nor does it disturb hepatocytes membrane integrity (Afriyie *et al*., 2013).

Serum lipid indices

Total cholesterol constitutes low density lipid (LDL) cholesterol, high density lipid (HDL) cholesterol, and very low-density lipid (VLDL) cholesterol (Birtcher and Ballanchyne, 2004). Several studies from animals, laboratory investigations, and epidemiology have showed that elevated LDL cholesterol is a major cause of cardiovascular disease (Cleeman *et al.*, 2001). Edijala *et al*. (2005) revealed that, reduction of serum total cholesterol and low-density lipoprotein is a primary factor for prevention of cardiovascular disease. High HDL level suggests reduced risk of cardiovascular disease as HDL transports cholesterol away from the peripheral tissues to the liver, thus prevents the formation of atherosclerosis (Wilson *et al*.,

1988). Triglycerides are the most common lipids and higher than normal range levels suggest higher risk of atherosclerosis, heart disease or stroke (Austin, 1989; Wilhelmsen *et al*., 1973). Triglyceride rich proteins are degraded to very low density lipoprotein (VLDL), commonly called remnant lipoproteins. In clinical practice, VLDL cholesterol is the most readily available measure of atherogenic remnant lipoproteins (Birtcher and Ballanchyne, 2004).

A 30-day sub-chronic toxicity study of *Stachytarpheta augustifolia* using Wistar rats, resulted in significant increase in HDL-cholesterols and reduction in LDL-cholesterol levels in all treated animals (Ogbonnia *et al.*, 2009). This indicated that, the extract could reduce cardiovascular risk factors which contribute to deaths in diabetic subjects. The observed decrease in the total cholesterol and triglycerides suggested the presence of hypolipidemic agents in the extract. In a related study by Ojokuku *et al.* (2011) on *Croton pendilflorus* oil, results showed significant reduction in total cholesterol, LDL, HDL/LDL ratio but a non-significant elevation in triglycerides. Thus, suggesting that, the oil had hypocholesterolemic and cardiac protective activity. Makinen *et al*. (2008) reported that, low HDL and high triglycerides levels was associated with low serum testosterone concentrations observed in aging men. Findings from a sub chronic toxicity study on the aqueous root extract of *C. membranaceus* revealed, non-significant changes in serum total cholesterol, LDL and HDL, however, there were reduction in TG and VLDL levels suggesting it had cardioprotective potentials (Afriyie *et al*., 2013).

Serum protein indices

Most plasma proteins are synthesized and degraded in the liver, and significant reduction in the blood could suggests either impaired hepatocellular production and or increased catabolism in various physiological or pathological processes (Hutadilok-Towatana *et al.*, 2010; Gatsing *et al.*, 2005). Albumin is the main plasma protein which binds water, cations such as Ca^{2+}, Na^{+} and K^{+}, fatty acids, hormones, bilirubin and drugs. Its main function is to regulate the colloidal osmotic pressure of blood, transport both endogenous and exogenous substances, and serves as protein reserve.

Hypoalbuminemia is a liver disorder thought to be a consequence of decreased hepatic synthesis of albumin (Burtis and Ashwood, 1994). Albumin levels are generally rapidly reduced in clinical conditions such as congestive heart failure, chronic liver diseases such as hepatitis, liver cirrhosis, and nephritis. It plays an important role in fat metabolism by binding fatty acids, keeps them in a soluble form in the plasma, and this is one reason why hyperlipemia occurs in clinical situations of hypoalbuminemia (Busher, 1990). In the normal individuals, the liver increases albumin synthesis in response to the increased availability of amino acids provided by the portal blood following each protein-containing meal (Busher, 1990). The only clinical situation that causes an elevation in serum albumin is acute dehydration.

The globulin fraction includes hundreds of serum proteins including carrier proteins, enzymes, complement, and immunoglobulins. Most of these are synthesized in the liver, although the immunoglobulins are synthesized by plasma

cells (Busher, 1990). Also, malnutrition and congenital immune deficiency could cause a decrease in total globulins due to decreased synthesis, and nephrotic syndrome which can cause a decrease due to protein loss through the kidney. In a 35-day sub-chronic toxicity study of hydroethanolic extract of *Chromola enaodorata* to Wistar rats, it was observed that, there was a significant increase ($p \leq 0.05$) in the plasma protein level in all the treated animals suggesting that there was no sign of impaired renal function as reported in a similar study by Kachmar and Grant (1982). Findings from both acute and subchronic toxicity studies of crude aqueous leaf extract of *Albizia chevalieri* by Saidu *et al.* (2007) in rats showed that, total serum protein profiles were not significantly different between the animals on different doses of the extract (150-3000 mg/kg) for both tests. They concluded that, the synthetic function of the liver of the animals exposed to oral and subchronic doses was not affected.

Administration of aqueous root extract of *C. membranaceus* on rats in an acute toxicity study showed that, albumin levels were not affected at the end of the study at all dose levels (Asare *et al.*, 2011). In a sub-chronic toxicity study of the ethanolic crude root extract of *C. zambesicus* to rats, albumin levels in all dose groups were not affected by the extract as there was no statistical differences between the mean level of albumin in the extract group and that of the control group (Okokon *et al.*, 2010). Progressive elevation in the level of the total protein and cholesterol in the extract treated rats increased in a dose dependent manner when compared to the control group. Examination of histopathological changes in the

organs of experimental animals remains a cornerstone in safety evaluation of test drugs or plant extracts for possible organ toxicity (Greaves 2007; Gad, 1994).

Histopathological indices

According to Gad (1994), histopathological data on gross pathology observations, organ weights and macroscopic pathology are considered the single most significant data derived out of a repeat dose toxicity study. Alteration (gain or reduction) in body weight and internal organ weights are simple and sensitive indices of toxicity after exposure to toxic substances (Tan *et al.*, 2008; Raza *et al.*, 2002). According to Chukwunonso and Irene (2006), an increase in relative organ weight could be attributed to induction of xenobiotic enzymes resulting in increased proteins synthesis. According to Bailey *et al.* (2004), significant difference in organ weights between treated and control group experimental animals may occur in the absence of any morphological changes.

Acute and sub-chronic toxicological studies on the aqueous leaf extract of *Byrsocarpus coccineus*, revealed no reductions in body weight gain (Adeyimi *et al*., 2010). Furthermore, gross examination of the organs in which changes in weight were observed, did not reveal any obvious abnormalities. Thus, findings suggested safety of prolonged oral administration of the aqueous leaf extract of *B. coccineus* on body and organ. Sub-chronic administration of the aqueous root extract of *C. membranceus* after 90 days of administration revealed that, there was absence of significant differences in the mean relative organ weights in the treated animals compared to the controls (Afriyie *et al*., 2013). The slight differences were attributed to individual variation in size of internal organs (Bailey *et al.*, 2004).

Evaluation of pathological alterations induced in laboratory animals by novel drugs represents the cornerstone of their safety assessment, before they can be first tried in patients (Greaves, 2007). According to OECD (1998), full histopathology is recommended on the preserved organs and tissues in at least all animals in control and high dose groups in toxicological studies. Furthermore, examination should be extended to animals of all other dosage groups, if treatment-related changes are observed in the high dose group. Histological investigations on the liver of Swiss albino mice to assess the effects of administering the leaf extract of *C. zambesicus* (5 mg and 10 mg/kg) for five consecutive days revealed that, no evidence of degenerative changes, necrosis or cyto-architectural distortions of the hepatic parenchyma in the treated groups (Ofusori *et al.*, 2008). Studies suggest that, *Croton zambesicus* consumption has no deleterious effects on the liver of Swiss albino mice up to 10mg/kg. A related study using the ethanolic root extract of *C. zambesicus* employing doses (27-81 mg/kg) for 21 days in rats, did not induce any pathological lesions in the kidney, heart, brain, testes, spleen and ovary during histological examination (Okokon *et al.*, 2010). Macroscopical analysis of organs and tissues harvested after acute toxicity study of the ethanolic root extract of *C. membranaceus* in S-D rats revealed that, they were normal (Asare *et al.*, 2011). According to Afriyie *et al*. (2013), microscopic examination of tissues of the kidney, heart and liver of S-D rats after sub-chronic administration of the aqueous root extract did not reveal any degenerative alterations, necrosis, lesions, inflammation or any abnormality as seen in similar toxicity studies. These findings on the heart, liver and kidney collaborated with results obtained from the clinical

chemistry markers for these organs. Hence, suggesting that the aqueous root extract of *C. membranaceus* was cardioprotective, not nephrotoxic or hepatotoxic during the experimental period.

Croton (The Genus)

The genus *Croton* belongs to the family of Euphorbiaceae, that has about 1300 species of trees, shrubs, and herbs and are widely distributed throughout tropical and subtropical regions of the world (Xu *et al*., 2018). Furthermore, it is reported to be present in the Americas with about 65 species occurring in Africa and about 125 in Madagascar. It has numerous varieties and it is widely cultivated as houseplants for their brilliant, glossy and multicoloured multifoliage (Bayor, 2007). The name Croton is derived from the Greek word Kroton which means "ticks" because of the seed's resemblance to ticks. The genus Croton is pantropical, with the best-known being *Croton tiglium*, a tree or shrub native of Southeast Asia from which croton oil is extracted from its seeds. The genus Croton is also well known for its diterpenoid content, and a lot of different types of diterpenes such as; phorbol esters, clerodane, labdane, kaurane, trachylobane, primarane among others have been isolated from this genus (Block *et al*., 2002). Similar studies have also revealed that the genus Croton is one of the richest sources of alkaloids such as aporphine, proaporphine, morphinandienone skeletons, and in addition flavonoids, lignans, phenols and diterpenes with the clerodane skeleton (Ahmat *et al.*, 2007; Amaral and Barnes, 1998; Pieters *et al*., 1993). However, fewer flavonoides have been reported from this genus (Zou *et al*., 2010). Figure 4 shows the percentages of compounds and type of diterpenoids isolated from *Croton* Species.

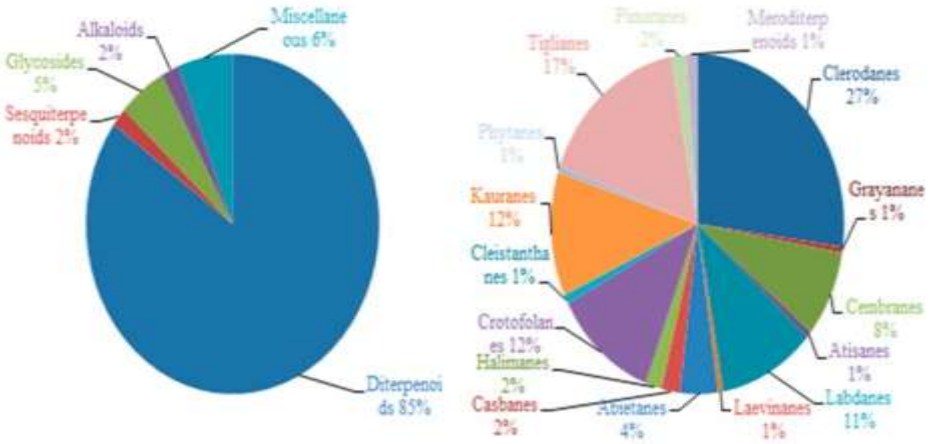


Figure 4: Pie charts showing percentages of compounds (left) and the percentages ofthe type of diterpenoids (right) isolated from *Croton* Species. (Adapted from Xu *et al.*, 2018).

Croton membranaceus

Croton membranaceus grows wildly in West African countries such as Cote d'Ivoire, Ghana, Nigeria, and is often found near big rivers (Aboagye, 1997). It has also been found to grow in moist bush vegetation and savanna, at low altitudes. In Ghana, it is found mainly in the Krobo-Gyakiti area near the Volta where the locals refer to it as Bokum. According to Abbiw *et al*. (2002), it has a limited area of distribution in Ghana, apparently uncommon but has been cultivated in the Aburi Botanical Gardens and at the Centre for Scientific Research in Plant Medicine. The species *Croton membranaceus*, however, has very distinguishing features. The plant is a monoecious herb which grows up to 1-2 m high, and its branches are slender, slightly angular and stellate-pubescent (Schmelzer, 2007; Baker and Wright, 1913). Its leaves are ovate and acutely acuminate, rounded and not glandular at the base. The leaves have entire margins and are covered with stellate hairs on both surfaces. The slender, stellate-pubescent petioles may attain a length of 7cm and generally have reddish-brown tinge. The flowers are monoecious, with racemes axillary and

terminal of about 5cm long, with male flowers at the end and female flowers at base. The female flowers are very small and petals may be rudimentary or completely absent (Baker and Wright, 1913). Fruit is ellipsoid, slightly 3-lobed capsule of 5mm in diameter, and 3-seeded. *Croton membranaceus* has a strong characteristic taste of ginger and also bears a characteristic pleasant odour in all parts, including the roots (Mshana *et al*., 2000). Its fragrance may be useful for purposes of identifying the plant in an event of doubt (Aboagye, 1997).

Figure 5: Picture showing the aerial view (left), stem and roots (right) of *C. membranaceus*
Source: Centre for Plant Medicine Research Library (Mampong-Akwapem, Ghana).

Organoleptic characteristics and phytochemical properties of *Croton membranaceus*

Powdered root (coarse powder) of *Croton membranaceus* has the following organoleptic characteristics such as large amounts of fibres and fragments, strawlike to very pale yellow in colour; possesses indistinguishably characteristic fragrance and almost tasteless at first but develops a slightly bitter and biting taste with astringent properties when chewed (Bayor, 2007).

Aboagye *et al*. (2000) found that, the root bark contains scopoletin and julocrotine (a glutarimide alkaliod) and also contains calcium oxalate crystals. Scopoletin, appears to have some structural resemblance with synthetic alpha-blocker terazosin, which is used for relaxing smooth muscles around the urethra in BPH management (Lambert *et al*., 2005). Phytochemical tests by Bayor (2009) on *C. membranaceus* root showed the presence of reducing sugars, saponins, alkaliods and true tannins.

Bayor *et al*. (2007) separated the ethyl acetate fraction obtained from the methanolic extract by column chromatography, and isolated six compounds; a novel furano-clerodanediterpenoid [12-oxo-15,16-epoxy- 3,13(16), 14-clerodatrien-17,18-dioic acid dimethyl ester] also known as "crotomembranafuran", a glutarimide alkaloid [julocrotine], p-sitosterol, p-sitosterol-3-D-glucoside, a labdane diterpenoid [labda- 8(17), 13E-dien-6a, 15-di-O-glucopyranoside] commonly called gomojoside H and DL-butane-1,2,3,4-tetraol (DL-threitol). Further analysis revealed the presence of phytosterols, glutarimide alkaloids, labdane and clerodane kind of diterpenoids in the root extract of *C. membranaceus*. Sarkodie *et al*. (2014b) recently isolated N [N-(2-methylbutanoyl) glutaminoyl] -2-phenylethylamine from *C. membranaceus*. Phytochemical analysis on the methanolic stem extract by Appiah (2011) resulted in the isolation of Larixol, phytosterols (beta–sitosterol, stigmasterol and campesterol) and a fatty acid, whilst chromatographic separation of the leaf extract resulted in the identification of only phytosterols.

Isolation of cis-terpine and N[N-(2-methyl butanoyl) glutaminoyl]-2-phenylethylamide from the roots of *C. membranaceus* was undertaken by Aboagye

(1997). However, the latter isolate is reported to be a likely product of the chemical reaction between julocrotine and ammonia (Aboagye,1997).

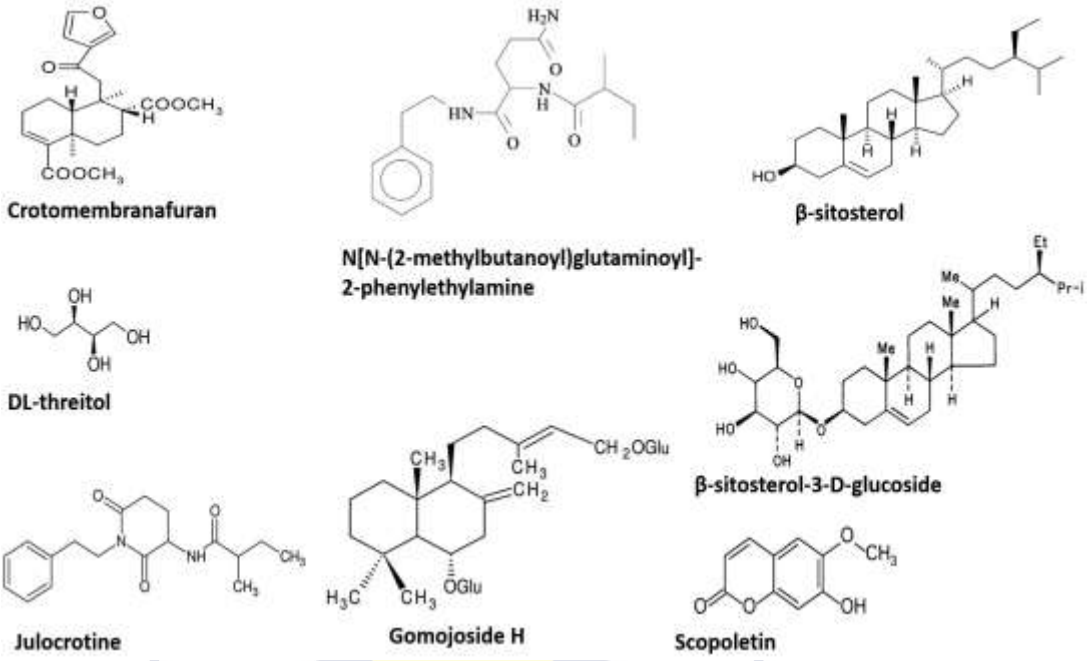


Figure 6: Bioactive isolates of the root extract of *C. membranceus* and their structures (Maroyi, 2018).

Trace elements and natural radionuclides in parts of *C.membranaceus*

Appiah (2011) reported that, high levels of manganese (Mn) have been found to be accumulated in the root, stem and leaf extracts of *C. membranaceus* in weights of 339 ± 4 mg, 252 ± 3 mg and 701 ± 5 mg respectively. Further analysis revealed that, the quantity of Mn in the daily dose of *C. membranceus* in root preparation was 132.5% of the recommended dietary allowance, whilst quantity of copper, chromium, iodine, iron and zinc were below 20% (Food and Nutrition Board, 2001). Tettey-Larbi *et al*. (2013) study on the root of *C. membranaceus* found that, the activity concentration of natural radionuclides 238U and 232Th were 36.1± 2.8 and 65.5 ± 2.4Bq/kg, respectively (compared to the average national values of sampled medicinal plants 31.78 ± 2.80 Bq/kg [238U] and 56.16 ± 2.32 Bq/kg

[232Th]), and 40K was 808.8 ± 12.9 Bq/kg [839.80 ± 11.86 Bq/kg]) whilst the average annual committed effective doses (mSv a^{-1}) was 0.013 ± 0.002. Findings from the latter study showed that, the annual committed effective dose to any individual's organ or tissue in the population group due to ingestion of *C. membranaceus* is far below the average radiation dose of 0.3 $mSva^{-1}$ received per head worldwide due to ingestion of natural radionuclide. Hence, the radiological risk associated with intake of *C. membranaceus* was viewed as in significant, and safe.

Pharmacological and Toxicological Review on *C. membranaceus*

The last decade has seen tremendous steady scientific work after an initial study that sought to determine whether the plant extract was a 5-alpha reductase inhibitor (Aboagye, 1997). Another study has revealed that extracts from parts of *C. membranaceus* possessed antimicrobial activities. The methanolic root extract of *C. membranaceus* was found by Bayor *et al*. (2009) to exhibit significant ($p<0.01$) antibacterial and antifungal activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and fungi; *Aspergillus niger* and *Candida albicans* using agar and disc diffusion techiques. The minimum inhibitory concentrations (MIC) was found to range between 0.53-1.43 mg/ml. Further antibacterial assay on the six isolated compounds from its ethyl acetate fraction revealed that, only gomoside H exhibited significant antibacterial activity against the aforementioned bacteria (MIC < 10 mg/ml) which was comparable to gentamicin (Bayor *et al.* 2007). The MICs for the rest of the isolates were greater than 200mg/ml. These findings in part supported the use of formulations of the root

extract of *C. membranaceus* for management of secondary bacterial infection in measles in Ghana (Mshana *et al*., 2000).

Afriyie and Asare (2016) studied the antibacterial activity of the aqueous stem and leaf extracts of *C. membranaceus* against pure cultures of *Salmonella typhi*, *Klebseilla pneumoniae*, *Staphlococcus aureus* and *Pseudomonas aeroginosa* using disc and well diffusion techniques. DMSO and levofloxacin (5µg) were used as negative and positive controls. Using the disc diffusion method, the various concentrations of stem and leaf extracts ranging between (5-100µg) did not show any activity against any of the bacteria, though levofloxacin produced zones of inhibition ranging between 20 -32mm. Further assay using the well diffusion technique revealed weak antibacterial activity against *S. aureus and K. pneumoniae*. The zones of inhibitions obtained for the *S. aureus* assay were 9 ± 1 and 12 ± 0.5 mm for 5 mg/ml and 10 mg/ml of the leaf extracts respectively, and inhibitions of 9 and 14 ± 2 mm were recorded for 5 and 10 mg/ml of the stem extracts, respectively (against Levofloxacin with zone of inhibition of 27 mm). Only the aqueous leaf extract showed some activity with inhibition zones of 10 ±2 mm and 12 ± 1 mm with 5 and 10 mg/ml, respectively against *K. pneumonia* (whilst that of the positive control was 20 mm). The rest of the organisms did not show any susceptibility to the extracts.

Studies on the methanolic (50%) root extract of *C. membranaceus* against *S. aureus, Bacillus subtillis, P. aeruginosa, E. coli and Salmonella typhi* by Gbedema *et al*. (2010) using agar diffusion methods confirmed its antimicrobial activity with MIC ranging from 13 mg/ml to beyond 20 mg/ml. Significant ($p<0.05$) potentiation

of antibacterial activity of amoxicillin against *S. aureus* was observed in the presence of the extract. Maroyi (2018) is of the view that, the presence of phytochemical compounds such as tannins, flavonoids, alkaliods, and terpeniods in the extract could be responsible for its antimicrobial activities.

Previous acute and sub chronic toxicity studies on the root extracts of *C. membranaceus* have confirmed its safety, anti-antherogenic potential and anti-ischaemic potentials (Afriyie *et al*., 2013; Asare *et al*., 2011). A similar study by Sarkodie *et al*. (2014a) in S-D male rats found that, the oral LD_{50} value is greater than 5000 mg/kg. Preliminary acute toxicity studies by Appiah (2011) on the freezed dried extract of the root, stem and leaf of *C. membranaceus* in male S-D rats showed that, the LD_{50} value was greater than 5000 mg/kg. However, it worth stating that, with exception of the study by Asare *et al*. (2011) which further examined heamatological and biochemical parameters, the other acute toxicity studies on the stem or root extracts of *C. membranaceus* focused on the observance of only clinical signs of toxicity. Oral administration of the aqueous root extract of *C. membranaceus* to Sprague-Dawley rats at doses of 30, 150 and 300 mg/kg body weight for 13 weeks revealed no deaths, nor visible signs of toxicity or changes in heamatological indices or animal behaviour (Afriyie *et al*., 2013).

Using spectrophotometric 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the ethanolic root extract of *C. membranaceus* exhibited concentration dependent scavenging activity with IC_{50} value of 100 mg/L compared to IC_{50} value of 3.4 mg/L for gallic acid (positive control). This observation suggests that some

phytochemicals in the ethanolic root extract of *C. membranaceus* possessed antioxidant properties (Sarkodie *et al*., 2014a).

In vitro assays using root extract of *C. membranaceus* on human cancer lines DLD-1 (colon), MCF-7 (breast) and M14 (melanoma) revealed cytotoxic and growth inhibitory activity of its methanolic root extract, with IC_{50} values of 16, 17.4 and 33.5 µg/ml, respectively (Bayor *et al*., 2007). Further bioassay guided fractionation of the methanolic extract showed that, its cytotoxic activity was due to bioactive compounds which mainly resided in the ethyl acetate fraction. Furthermore, three bioactive compounds obtained from the ethyl acetate fractions namely; crotomembranafuran, p-sitosterol-3-D-glucoside and DL-threitol and showed inhibitory activity against human PC-3 cells, with IC_{50} values of 4.1, 9.7 and 6.6 µg/ml, respectively. These observations may lend credence to the use of the root extract of *C. membranaceus* for treatment of cancers.

Cellular studies using bone marrow techniques demonstrated the potential of the aqueous root extract of *C. membranaceus* being cytotoxic and genotoxic (Asare *et al*., 2015b). Further study undertaken at the cellular level demonstrated that *C. membranaceus* possessed anti-proliferative on human benign hyperplasic cells [BPH-1] (Afriyie *et al*., 2015). Although the mechanisms of action of the plant extracts remained elusive, a recent study strongly suggested that, at the molecular level the aqueous root extract of *C. membranaceus* has apoptotic potentials through the mitochondria dependent pathway which involves Bcl-2 and Bax (Afriyie *et al*., 2015).

The aqueous root extract of *C. memebranaceus* have also found to be prostate targeting at the organ level during the sub-chronic toxicity study, though it did not reduce the prostate of the normal S-D rats significantly (Afriyie *et al*., 2014a). Antihyperglycaemic activity of root extracts of *C. membranaceus* has been reported in streptozotocin-induced diabetic rats (Sarkodie *et al*., 2014). Another related study demonstrated that, the aqueous root extract had some hypolipidaemic and hypoglycaemic potentials using spontaneous hypertensive rats and db/db mice models (Asare *et al*., 2015a). Using testosterone-induced castrated S-D rat models, oral doses of the aqueous root extract of *C. membranaceus* resulted in significant shrinkage of the prostate similar to the finasteride treatment group. Futhermore, there was significant prostatic and seminal vesicle index reduction (Afriyie *et al*., 2014b).

The first human observational studies to attest the efficacy of the capsulated ethanolic root extract of *C. membranaceus* revealed overwhelming evidence in improving the quality of life of BPH patients (Asare *et al*., 2015c). Through the use of the International Prostate Symptoms Score (IPSS), Prostate Specific Antigen (PSA), CT scan and other biomedical methods, it was found; it improved the quality of life (QoL), increased high density lipoprotein (HDL) and apo lipoprotein A-1 significantly, reduced PSA by 40.8%, and prostate volume by 46.6% within three months (Asare *et al*., 2015c). The HDL/apo lipoprotein A-1 reduction gives credence to the involvement of metabolic syndrome in the development of LUTS associated with BPH. The totality of the observational study agreed with the anecdotal evidence of the past four decades. Furthermore, unlike finasteride and

other allopathic medicines, *C. membranaceus* did not affect sexual quality or erectile function.

Calcium and magnesium imbalance have been shown to be an etiologic factor in the development of PCa. Calcium channels when largely influxed by calcium, reduces magnesium inflow and instructs the mitochondria to initiate cell proliferation. When magnesium is predominant, anti-proliferation occurs, thereby inducing apoptosis. The reversal of the Ca/Mg imbalance was demonstrated for the first time in about 80% of the patients with BPH who underwent phytotherapeutic treatment with the capsulated ethanolic root extract of *C. membranaceus* (Asare *et al*., 2017a). The latter observation agrees with findings from previous studies that demonstrated the antiproliferative ability of the aqueous root extract of *C. membranaceus*.

A recent study by Asare *et al*. (2018) revealed for the first time that, a lipid associated antioxidant, arylesterase, was responsible for the increased HDL and apo A-1 in previously observed patients after three (3) months treatment in BPH at the Ghana Police Hospital. Another related study demonstrated for the first time that, the tumour suppressor lipid ceramide was implicated in the apoptotic pathway of the ethanolic root extract of *C. membranaceus*, with the modulation of Sphingosine phosphokinase 1 and 2 (Spk1 and 2) and more importantly its ratio (Asare *et al*., 2017b).

Anecdotal reports from the Urology Department (Korle Bu Teaching Hospital, Ghana) and patients also suggest better treatment outcome for patients using combination therapy of finasteride and *C. membranaceus*. These recent numerous

promising scientific findings on extracts of *C. membranaceus* have led to the surge in the demand and use of this medical plant among BPH and PCa patients. This has resulted in the addition of the stem parts to its root by some manufacturers of this herbal product to increase its economic yield. However, there is not much scientific evidence-based data to support this practice.

CHAPTER THREE

MATERIALS AND METHODS

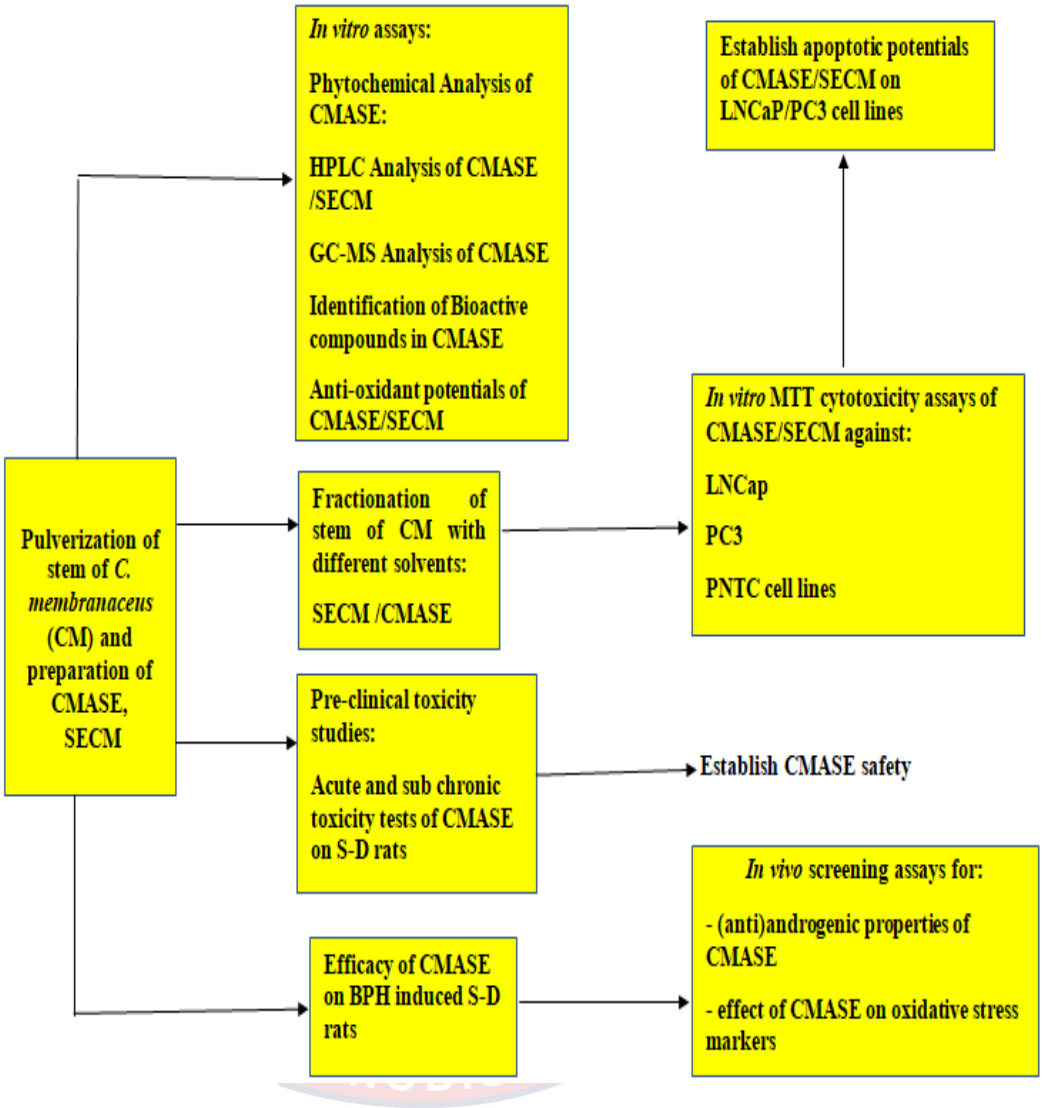


Figure 7: Flow chart from pulverization of stem of *C. membranaceus* (CM), phytochemical screening of the sequential stem fractions (SECM) and the direct aqueous stem extract (CMASE) of *C. membranaceus*, and their *in vitro* and *in vivo* safety, efficacy and mechanisms of actions assays.

Plant material identification and collection

Whole stems of *C. membranaceus* were collected in the morning (between 9-10 am) from Mampong-Akwapem (GPS: E2-0698-1121) in the Eastern Region of Ghana in December 2018. It was authenticated by taxonomist at the Centre for Plant Medicine Research (CPMR), Mampong-Akwapem. A voucher specimen of the plant (UCCG-DCOP-007) was deposited at the Herbarium of the University of Ghana.

Ethical clearance

The ethical clearance for this study protocol conducted in line with the requirements of the University of Cape Coast Ethics committee. All animal experimentations were conducted at the Noguchi Memorial Institute for Medical Research (Department of Animal Experimentation), Legon, Accra in line with the requirements and approval of University of Ghana Institutional Animal Care and Use Committee. Ethical clearance number issued was UG-IACUC 003/18-19.

Plant extraction

Direct aqueous extract of *C. membranaceus* (CMASE)

The modified validated procedure by Afriyie *et al*. (2013) was used to prepare the extract. Briefly, the stem parts of *C. membranaceus* was thoroughly washed, air-dried for three weeks, machined crushed into powder, packed in labeled zip lock bags, and stored at room temperature (25±2 ^{o}C). Then 1 kg of powdered plant part was macerated for 24 hours with four litres (4L) of distilled water, and boiled for 1 hour at 100 ^{o}C in a water bath. The extract was filtered using sterile gauze and then

refrigerated. Three litres (3L) of distilled water was again added to the sediments, cold macerated for another 24 hours, and the previous process (boiling and filtering) was repeated to obtain the second aqueous extract and refrigerated. The latter procedure was repeated to obtain the third extract. The extracts were pooled, and freeze dried. Total extract was determined, before it was stored in a labeled and sealed container in a refrigerator between 2-8 ^{o}C until used. The choice of the aqueous stem extraction of *C. membranaceus* (CMASE) was based on folkloric preparation. This extract (CMASE) obtained directly from the pulverized stem of *C. membranaceus* in this study was labelled 'direct aqueous extract of *C. membranaceus*' (DAQ).

Sequential extraction for hexane, ethyl acetate, ethanol and aqueous fractions from pulverized stem of *C. membranaceus* (SECM)

The pulverized stem of *C. membranaceus* was cold macerated and sequentially extracted using hexane, ethyl acetate, ethanol, and lastly using hot water extraction for the last residue as described by Appiah-Opong *et al*. (2016a). Hundred grams (100 g) of pulverized *Croton membranaceus* was weighed on an analytical balance (Mettler Toledo XP105, USA) and transferred into an Erlenmeyer flask. Five hundred milliliters (500 ml) of hexane was then added to it, and the content was placed on an electric shaker (Yamato Shaker, SA-31, Japan), for 24 h at 300 rpm. The content was filtered using Whatman Filter paper, Grade 91, diam. 150 mm. The procedure was repeated twice (and the residue air dried for further extraction). The filtrate of each of solvent obtained was pooled, subjected to a rotary evaporator (BuchiRotavapor, pump VAC V-500, Germany) to obtain hexane extract. Ethyl

acetate and ethanolic extracts were sequentially obtained using the air-dried residue of *C. membranaceus* from previous procedure. Aqueous extraction was carried out using hot extraction method. Five hundred milliliters (500 ml) of distilled water was added to the air-dried residue obtained from the ethanolic extraction. The content was heated in UGO Basile Julabo GmbH Water Bath, Germany, at 80^{o}C for 1 h. The content was filtered using Whatman Filter paper, Grade 91, diam. 150 mm. The procedure was repeated. Filtrate obtained was pooled and freezed dried using LTE scientific 18 kg ice capacity Lyotrap freeze dryer, United Kingdom) to obtain the sequential aqueous stem extract of *C. membranaceus* (AQ).

Phytochemical analysis of CMASE

Qualitative preliminary phytochemical screening

Phytochemical screening of freezed dried CMASE was performed for to determine the presence of secondary metabolites using standard methods reported in literature (Harborne, 2005; Trease and Evans, 2002; Sofowora, 1993). Alkaloids in extract was screened for using Mayer's and Dragendorff reagents, saponins with froth and emulsification tests, flavonoids with the Schinoda Test, cardiac glycosides with Keller-Kiliani and Kedde tests, tannins with ferric chloride reagent and anthraquinones with ammonia test.

Test for saponins

A volume of 5 ml of distilled water was added to 500 mg of CMASE in a test tube. The content of the test-tube was shaken vigourously and observed for a stable

persistent froth. Three (3) drops of olive oil was added to the frothing and again shaken vigourously, after which it was observed for the formation of an emulsion.

Test for tannins

An amount of 500 mg of the CMASE was weighed and added to 10 ml of water in a test tube. It was vortexed, then heated over burner to boil and then filtered. Few drops (100 µl) of 0.1 % ferric chloride was then added to filterate and observed for brownish green or a blue-black colouration which indicated the presence of tannins.

Test for alkaloids

An amount of 500 mg of CMASE was weighed and transferred into a test-tube. Subsequently, 10 ml of acidified alcohol was added, vortexed, boiled and filtered. A volume of 5 ml of the filtrate was added to 2 ml of dilute ammonia, after which 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid, and divided into two portions to confirm the presence of tannins with two different reagents. Mayer's reagent was added to one portion and the formation of a cream precipitate (with Mayer's reagent) was regarded as positive for the presence of alkaloids. Whilst the addition of Draggendorff's reagent to the other potion resulted in reddish brown precipitate, was an indication of presence of alkaliods.

Test for terpenoids

To 500 mg of the CMASE was added 2 ml of chloroform. Then 3 ml of concentrated H_2S0_4 was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpeniods.

Tests for flavonoids

An amount of 500 mg of CMASE was heated with 10 ml ethyl acetate over a steam bath (40 – 50 ^{o}C) for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids. Curcumin was used as positive control.

Test for reducing sugars

Five millilitres (5 ml) of Benedict's solution was added to aqueous solution of CMASE (5ml of water was added to 500 mg of CMASE, shaken well to mix in a test tube). The resulting solution was boiled for 2 min, and allowed to cool. A brick red precipitate of the solution, copper (I) oxide forms confirms the presence of a reducing sugar.

Test for anthraquinones

An amount of 500 mg of the CMASE was dissolved in 5 ml of water. The solution was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube, and 1 ml of dilute ammonia was added. The resulting solution was observed for colour (pink, red or violet coloured precipitate) changes to confirms the presence of anthraquinones.

Test for Steriods

To 500 mg of CMASE, 2 ml of chloroform was used to dissolve the extract in a test-tube. Two millilitres of acetic anhydride and 2 drops of concentrated sulphuric

acid were added to the mixture. Formation of bluish green coloured solution is an indication of the presence of phytosterols.

High performance liquid chromatography analysis

Sample preparation for HPLC fingerprint analysis

Five milligrams (5 mg) of each sample (SECM, and CMASE) was weighed and dissolved in 1 ml of (50 % methanol) in a 2 ml micro tube. The content was placed in an ultrasonic basin for 5 min and vortexed. The mixture was centrifuged at a speed of 12,000 rpm for 10 min, and the supernatant transferred into HPLC vials for analysis.

Chromatographic system and conditions for HPLC fingerprinting of CMASE and SECM

The HPLC analysis was carried out using an Agilent 1100 system (Santa Clara, CA, USA), composed of quaternary pump, autosampler, diode array detector (DAD), and HP ChemStation Software. Chromatographic separation was carried out on a Tskgel ODS C18 (250 x 4.6 mm i.d., 5 µm particle size) analytical column maintained at 30^{o}C. The injection volume was 20 µL and the eluents, water in 0.1 % phosphoric acid (A) and methanol (B) as mobile phase at a flow rate of 1 mL/min. The linear gradient program used was set as follows: 0–10 mins, 10–30 % B; 10–15 min, 30–50% B; 15–25 min, 70–90% B; 25–35 min, 90–90% B; 35–38 min, 90–10 % B; 38–40 min, 10–10% B. UV detection was performed at 280 nm.

Microwave-assisted extraction of CMASE

The freezed sample of CMASE was allowed to stand at room temperature for 5 days after removal from the refrigerator. Microwaved assisted extraction (MAE) using methanol as a solvent was performed using closed vessel system with pressure (CEM, Explorer SP 12 S class. Matthews, NC USA) following the method of Xiao *et al*. (2008). An amount of three grams (3g) of the CMASE was weighed and placed in 100 ml Polytetrafluoroethylene (PTFE) extraction vessel with 20 ml methanol solvent. Number of extraction cycles (3 times), methanol concentration (60–100%, v/v), temperature (30^{o}C), irradiation time (30 min) and pressure (10 bars), microwave power (100 W). After extraction, the vessel was allowed to cool at room temperature before opening. The extract obtained from MAE was filtered through Whatman filter paper (No. 2). Extract was concentrated using a Rotary evaporator and kept in the refrigerated for further use. Each sample was loaded in GC-MS compatible vials and analyzed. The remaining freezed-dried extract was placed in airtight container and refrigerated for future use.

Gas chromatography–mass spectrometry (GC-MS) analysis of CMASE

The microwaved assisted extract of CMASE was reconstituted in Methanol/Dichloromethane (MeOH/DCM 1:1) and loaded unto GC-MS compatible vials and analysed. GC–MS analysis of the reconstituted CMASE was performed using Perkin Emler Gas Chromatography (Clarus 580) equipped with MSD mass spectrometer (Clarus SQ8S) instrument with built–in auto sampler employing the following conditions; Column: Elite-5MS (30 m x 0.25 mm id x 0.25 µm). The oven temperature was programmed from 37 to 320°C at a rate of 18-25^{o}C/min and

held for 0.5 min and 1.85 min at 18°C and 320°C, respectively. The injector temperature was set at 250°C and MS Ion Source temperature was 280°C, with full scan and solvent delay of 0–2.30 min. MS Scan Range was 35 – 500 (m/z) in 0.10 sec. One microlitre of the samples was injected in Helium carrier gas at split flow of 20 mL/min. The total running time was completed within 25 min. Chromatograph obtained from gas chromatography was analysed in mass spectrometry to further obtain the mass of all sub fractions of CMASE. The identification of the phytochemical components (sub fractions) was attained through retention time and mass spectrometry by comparing the mass spectra of the unknown stored in Wiley 9 GC-MS library.

***In vitro* antioxidants assay**

Free radical scavenging assay

A modified Blois (1958) 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH) assay was used. DPPH is a stable nitrogen-centered free radical, has a deep violet colour in methanol solution which turns colourless or pale yellow (diphenylpicrylhydrazine) upon reduction by either the donation of a hydrogen atom or an electron (when neutralized) by an antioxidant. Briefly, 20 mg of the various fractions (hexane, ethyl acetate, ethanolic, sequential aqueous (AQ), direct aqueous/DAQ) were dissolved in 1.0 ml of ethanol to obtain stock solutions of 20 mg/ml. Two-fold serial dilutions of the stock were made to obtain concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.015625 mg/ml. Two-fold serial dilutions of the positive control, butylated hydroxyl toluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA) [1 mg/ml in absolute methanol], were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625,

0.03125, and 0.015625 mg/ml. A volume of 100 μL each of the fractions and BHT dilutions were pipetted in triplicates and transferred into 96-well plates. Another volume of 100 μL of 0.5mM DPPH (in methanol) solution (Steinheim, Germany) was added to each of the wells to obtain a total volume of 200 μL. Ethanol and water were used as blank. The 96-well plate was gently shaken to mix the content. The plates were then covered with an aluminium foil and incubated in the dark at room temperature (27^{o}C) for 20 min. Absorbance was read at a wavelength of 517 nm using Tecan Infinite M200 microplate reader (Austria). The percent antioxidant activity of each extract and BHT were calculated from the following formula:

% Antioxidant activity = $[(A_0 - A_1)/A_0 \times 100]$

The scavenging activity is expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of DPPH solution void of the extracts. A_0 is the absorbance of negative control (methanol) and A_1 is the absorbance of test sample with DPPH. Triplicate experiments were performed for each sample/fraction. The EC_{50} value, which is the concentration of the extracts that can cause 50% free radical scavenging activity, were determined from a plot of extract concentrationtested versus percent antioxidant activity.

Assay for total phenolic content

The assay used to estimate total phenols in the fractions was a modification of one reported by Marinova *et al*. (2005). Stock solutions of the fractions (hexane, ethyl acetate, ethanol, AQ, and DAQ) were prepared by dissolving 5 mg of each sample in 1.0 ml of their respective solvents (ethanol for hexane, ethylacetate and ethanolic fraction, whilst distilled water was used for the aqueous fractions). Two-

fold serial dilutions of this stock were made to obtain concentrations of 5.0, 2.5, and 1.25 mg/ml for each fraction. The standard (1 mg/ml) was prepared by dissolving 1.0 mg of gallic acid (generously provided by the Department of Nutrition and Food Science, University of Ghana) in 100 μL of absolute ethanol, and topped up to 1000 μL with 900 μL of distilled water. Two-fold serial dilutions were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 mg/ml. Ten microliters (10 μL) of each of the sample dilutions (concentrations) and the standard was pipetted in triplicates into 24-well plates already containing 790 μL distilled water. A volume of 50 μL of Folin-Ciocalteau reagent (Buchs, Switzerland) was then added to each well and incubated for 8 min. A volume of 150 μL of sodium bicarbonate solution (0.2 g/ml) was then added (to obtain a total volume of 1000 μL). The plates were incubated at room temperature (27^{o}C) for 120 min and the absorbance read at a wavelength of 750 nm using a microplate reader (Tecan Infinite M200, Austria). The phenolic concentration of the extracts were evaluated from a gallic acid calibration curve. Total phenolic content in the fractions of the extract were expressed as Gallic Acid Equivalent (GAE) in grams per 100 g dry weight of sample.

Assay for total flavonoids content

The total flavanoid assays of the stem crude fractions of *C. membranaceus* and CMASE (DAQ) were determined using modified Ordonez *et al.* (2006) procedure. Five milligrams (5 mg) of the fractions (hexane, ethyl acetate, ethanolic, AQ, DAQ) were dissolved in 1 ml of their respective solvents to obtain stock solution of 5 mg/ml. Two-fold serial dilutions of the various fractions of the extract (5, 2.5 and

1.25 mg/ml) were prepared from their stock concentrations of 5 mg/ml. Using Quercetin (Buchs, Switzerland) as standard (1 mg/ml stock methanolic stock solution) serial dilutions were prepared to obtain 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.0015625, 0.000781, 0.000391 and 0.000195 mg/ml for calibration. Then 100 µl of each of the sample dilutions and the standard were pipetted separately into a 96-well plate. Furthermore, 100 µl of 2% aluminium chloride solution (2 mg/100 ml methanol) was added to each concentration in the 96-well plate and incubated for 20 min at room temperature. After the incubation period, the absorbance was determined at 415 nm using a microplate reader (Tecan Infinite M200, Austria). The experiment was carried out in triplicates. The calibration curve was plotted from the various concentrations of quercetin against absorbance.

$$\% \text{ Inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

The quercetin equivalence in each of the fractions were extrapolated from the curve. Total flavonoid content (TFC) was expressed as milligrams of quercetin equivalent (QE) per 100 grams of dry pulverized stem of *C. membranaceus* (mg QE/ 100 g sample).

***In vitro* assay on cell lines**

Determination SECM and CMASE effects on PC3, LNCaP and PNT2 cells

Cell lines

Mammalian cell lines; Lymph Node Carcinoma of the Prostate [(LNCaP), Androgen - sensitive human prostate cancer cells], Prostate Cancer Cell line [(PC3), androgen independenthuman prostate cancer cells] and Human prostate normal,

immortalized with SV40 [(PNT2), human normal prostate cells] were obtained the RIKEN BioResource Center Cell Bank (Nagasaki, Japan).

Preparation of solution of SECM/CMASE

To enhance complete dissolution of the various non-aqueous stem extracts of *C. membranaceus* (SECM), DMSO (100 %) was used as solvent to prepare a stock solution of 100 mg/ml. The stock solution of CMASE was prepared with distilled water and filter-sterilized. Serial dilutions of SECM/CMASE was further made with Rosewell Park Memorial Institute (RPMI) 1640 meduim Basic (with L-Glutamine) in 10% FBS to obtain the required working concentrations.

Cell cultures used for cytotoxicity experiments

The LNCaP, PC3 and PNT2 prostate cell lines were cultured in a T75 culture flask containing RPMI medium. Culture media were supplemented with 10 % Foetal Bovine Serum (FBS) and 0.1 % penicillin-streptomycin-L-Glutamine (PSG). Cultured cells were maintained in a humidified incubator (Panasonic CO_2 Incubator MCO-18 AC, China) at 37°C in the presence of 5 % CO_2.

Cell Recovery

Cells in vials were picked from storage (-80ºC), thawed and resuspended in 10 mL of RPMI culture medium. Culture media were supplemented with 10% Foetal Bovine Serum (FBS) and 0.1% penicillin-streptomycin-L-Glutamine (PSG). The cells were spun at 1000 rpm for 5 min in a table top centrifuge (Tomy LC200, Japan) and resulting supernatant were discarded. Cell pellets were resuspended in 1 mL of culture media and pipetted into a 25 cm^3 culture flask containing 5 mL of

complete culture medium and incubated in a humidified incubator at 37^{o}C in the presence of 5 % CO_2 until they reach confluence.

Passaging of Cells

Cultured cells were daily examined under inverted microscope (Zeiss, Germany) for confluence and health monitoring. Upon reaching 80-100% confluence, cells were sub-cultured (passaged) for two consecutive weeks before they were used for various assays. The spent medium was pipetted off and the cells attached to the surface of the culture plate were washed twice with 5 mL of phosphate buffer saline (PBS) to get rid of dead cells, and to stabilize the pH of the cells. The cells were treated with trypsin (0.5 mL for 25 cm^3 culture flask) and incubated for 3-5 min to detach the cells. Detached cells were resuspended in fresh complete RPMI culture medium (5 mL for 25 cm^3 flasks) and transferred (pipetted) into centrifuge tubes. The cells were then spun at 1000 rpm (Tomy LC200 centrifuge, Japan) for 5 min. The supernatant was discarded and the pellets were initially resuspended in 1 mL of culture media and topped up to 10 mL with fresh culture media. A volume of 10 µL of cells was resuspended in between 20 µL of trypan blue and loaded onto a Haemacytometer to count/estimate cell concentration as shown in the formula below:

$$\text{Concentration of cells} = \frac{\text{Total of cells in 4 Quadrants}}{4} \times 10^4 \times \text{dilution factor} \quad (6)$$

NB: Dilution factor is dependent on degree of trypan blue dilution

Tetrazolium Based Colorimetric (MTT) assay (Cell viability assay)

Cytotoxic effect of the extracts [SECM/CMASE] on PC3, LNCaP and PNT2 cell lines were assessed using tetrazolium-based colorimetric (MTT) assay as described by Appiah-Opong *et al*. (2016a). Briefly, 100 µL of cells were seeded at 1×10^4 cells per well (1×10^5 cells/mL) into 96-well plates and incubated overnight in a humidified incubator (Panasonic, Japan) at 37ºC in the presence of 5 % CO_2. The cells were then treated with 0-1000 µg/ml extracts (SECM and CMASE) in triplicates and incubated for 72 h. After incubation, the cells were treated with 20 µL of 2.5 mg/mL MTT solution (in Phosphate Buffered Saline (PBS)) and re-incubated for 4 h. Subsequently, cells in each well were treated with 150 µL of acidified isopropanol (containing 1 % Triton-X and 1.7% (*v/v*) aqueous HCl in isopropanol) to stop the reaction and the plates were incubated in the dark overnight at room temperature (26^oC) to dissolve any formazan crystals formed. The optical densities of wells were then read using a spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria) at 570 nm. Curcumin was used as a positive control compound. The experiments were performed in triplicates.

Percentage cell survival/viability was evaluated using the formula shown below:

$$\% \text{ Cell viability} = \frac{\text{Average absorbance of test experiment}}{\text{Average absorbance control}} \times 100\%.$$

A graph of % cell viability versus concentration of extracts were plotted and used to determine the IC_{50} (concentration at which extracts inhibits 50% of cell growth) values of each extract.

Safety of extracts were elucidated by determining the selectivity indices (SI) as shown by the formula:

$$SI = \frac{CC_{50} \text{ of extracts on normal cells (PNT2)}}{IC_{50} \text{ of extracts on cancer cells}}$$

Extracts with selectivity indices ≥ 2 are considered as safe or not toxic (Basida *et al*., 2009).

Determination of apoptotic potentials of CMASE on PC-3 cell lines

Cell morphology examination and Immunofluorescent Assay (Hoechst staining)

The morphology of cultured PC-3 cells was examined before treatment with various concentrations (0, 17.5, 35 and 70 µg/ml) of CMASE as described by Uto *et al*. (2013). The nuclei integrity of cells (a marker for apoptosis) was elucidated upon exposure to extract using Hoechst DNA-staining dye method as described by Tuffour *et al*. (2018). Briefly, PC-3 cells were seeded in 6 ml petri dishes at a density of 1 x 10^6 cells/ ml culture media (with 10 % FBS) and incubated in a CO_2 incubator at 37^{o}C overnight. The cells were treated with 600 uL of different concentrations of CMASE (0, 17.5, 35, 70 µg/mL) and a positive control (5 µg/mL curcumin) for 24 hr. The cells were collected into a 15 mL centrifuge tube, centrifuged at 1000 rpm for 5 min (Tomy LC-200, Japan) and the supernatant discarded. The cells were then re-suspended in 1ml of cold PBS, centrifuged at 1000 rpm for 5 min and the supernatant discarded. The cell pellets were then treated with 200 µL of 1% glutaraldehyde, incubated at room temperature for 30 min and the supernatant removed. Subsequently, cells were suspended in 50 µL PBS and 8 µL of Hoechst 33258 solution added, mixed gently and loaded on a microscope

slide with cover slip. The slides were mounted and examined on a fluorescent microscope (Olympus, Japan) equipped with a camera to assess the extent of nucleic condensation. Hoechst stain exhibits high fluorescence on binding to double-stranded DNA in cells. The nuclear morphology (shape and chromatin) of cells were observed under a fluorescence phase-contrast microscope (X40) to determine the degree of cell fragmentation and blebs, using Hoechst 33258 staining. Intact PC-3 cells were considered viable whilst the presence of fragments (apoptotic cells) were indicative of apoptosis. The experiments were performed in triplicates. Photomicrographs of PC-3 cells were obtained at a magnification of X40 using the microscope.

DNA fragmentation and agarose gel electrophoresis

DNA fragmentation effect of CMASE on PC cells was elucidated using the method described by Appiah-Opong *et al*. (2016b). Briefly, 6 ml of cells ($1x10^6$ cell/mL) of PC 3 was seeded into Petri dishes to adhere overnight. The cells were treated with 600 uL of various concentrations of CMASE (0 µg/mL, 17.5 µg/mL, 35µg/mL, 70 µg/mL) and a positive control drug (5 µg/mL curcumin) for 24 h. Cells were then harvested using a scraper and centrifuged at 1000 rpm for 5 min Supernatant were discarded and the cell pellets were resuspended in 1 ml of cold PBS, centrifuged at 1200 rpm for 5 min at 4^oC and supernatant discarded. The cells were treated with 30 µL of lysis buffer and 10 µL of 1mg/mL RNase A solution, and incubated at 50^oC for 30 min in a water bath. A volume of 80 µL of Proteinase K was added and re-incubated at 50^oC overnight. DNA samples from each treatment was run on a 2 % agarose gel and stained with ethidium bromide. The

DNA band patterns were visualized with a UV transilluminator (Vilberlourmat, USA).

Measurement of apoptosis by flow cytometry

Annexin V-FITC/PI staining method is frequently employed in the identification of the early phase and late stages of apoptosis. Flow cytometer was employed to measure apoptotic effect as described by Xing *et al*. (2011) with slight modification. PC3 cells were seeded in 6 ml petri dishes (designated for negative control, three extract concentrations, and positive control) at a density of 1 x 10^6 cells/ ml culture media (with 10% FBS). They were cultured for 24 h to attach to the dish and treated with 0, 17.5, 35, 70 µg/mLof CMASE, and 5 µg/mL of curcumin, was used as positive control for 24 h. RPMI culture medium was used as the negative control. The cells were then harvested and treated with equal volumes of reagent containing Annexin V-FITC and propidium iodide, incubated for 20 min in the dark at room temperature and analyzed immediately after incubation with fluorescence-activated cell sorting (FACS) caliber (Becton Dickinson, CA, USA). In early apoptosis, phosphatidyl serine (PS) residues, which is positioned inside the plasma membrane, become exposed to the cells outer surface. Annexin V-FITC is human vascular anticoagulant which has strong affinity for phosphatidyl serine residues exposed on cell surfaces from their origin in the inner plasma membrane during the early phase of apoptosis. Propidium iodide (PI), a viability dye is used with Annexin V-FITC to enable identification of apoptotic cells from necrotic cells. PI binds to DNA, but can only enter cells that have compromised membrane (Live cells with intact cell membrane will not stained with PI). At late stage of apoptosis

they bind annexin V-FITC and stain brightly with PI. The experiments were performed in triplicates.

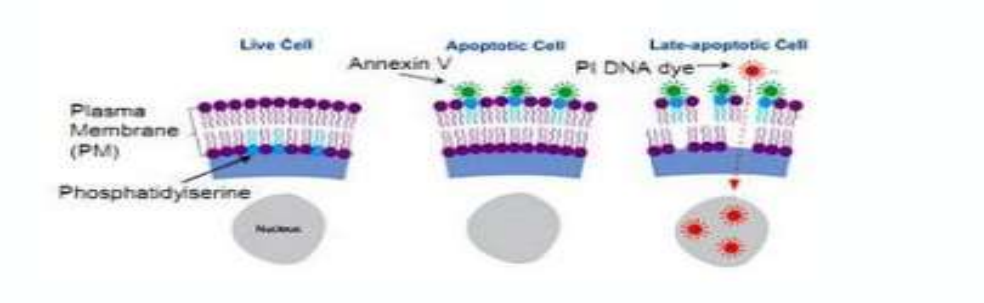


Figure 8: A diagram showing Annexin V FITC/PI staining during apoptosis.

Source: https://www.sinobiological.com/flow-cytometry-fcm-facs-application-apoptosis.html/2 /07/19.

***In vivo* toxicological examinations**

Experimental Animals

Male Sprague-Dawley (S-D) rats weighing between 100-200 g were purchased from Centre for Scientific Research into Plant Medicine and Noguchi Memorial Institute for Medical Research (Animal Experimentation Unit). The animals were made to acclimatize for a week to laboratory conditions in metabolic cages at controlled room temperature of 25-27^{o}C for 12 h light-darkness cycle. The rats were fed standard pellet formulation from AGRICARE, Ghana and water *ad libitum*. Before treatment, the animals were fasted overnight with free access to water.

Acute Toxicity Test [CMASE]

Four groups of Sprague-Dawley male rats including the control group weighing between 110-140 g were used for determination of acute toxicity. Five (5) S–D rats

were randomly selected to constitute each group. Rats in each group were identified by markings with a yellow stain on the head, tail, back, right fore leg and left hind leg for identification during drug administration and observation during study period. Single oral doses of 1500, 3000 and 5000 mg/kg b.wt extract was orally administered (by gavage) once-off to three groups (low dose [LD], median dose [MD], and high dose [HD]) of rats. The control group was given distilled water by oral gavage. Clinical signs of toxidromes such as changes in skin, fur, eyes, movement, mucous membrane as well as presence of mortality among rats were observed after 0.5, 1, 3, and 6 h on the day of administration, and daily till the 14th day. Body weights were measured before administration of extract on the 1st day of administration and on the 14th day. Heamatological, clinical chemistry parameters and histopathological examinations were determined on the 15th day after the rats had been euthanized and sacrificed.

Sub-chronic Toxicity Test [CMASE]

The protocol for this study followed the Organization of European Cooperation and Development Guidelines for testing Chemical (OECD, 1998) using a total of 20 healthy S-D male rats. These animals weighing between 110-140 g were randomly distributed into four groups of 5 rats. Three of the groups were orally administered with single doses of 30, 150 and 300 mg/kg body weight of the CMASE by gavage to the low [LD], median [MD] and high dose [HD] groups respectively, for 90 days. The control group received distilled water equivalent to the volume of extract given to the high dose group. Clinical observations were carried throughout the period, and blood was collected from the tail veins of the rats on the 30th and 60th day. On

the 91st day, blood was obtained by cardiac puncture after the animals had been euthanised with ether. Heamatological, biochemical and histopathological assessment were conducted at the end of the test period. The heart, liver, prostate and kidney tissues were harvested, examined and weighed to obtain organ to body weight ratios.

Heamatological Assessment

Blood was collected into EDTA (ethylenediamine-tetracetate)-2K tubes by cardiac puncture and analyzed for the determination of haematological parameters such as red blood cell counts (RBC), haemoglobin levels (HGB), haematocrit (HCT), packed cell volume (PCV), white blood cell counts (WBC), platelets counts (PCT), mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCHC) using a Sysmex-KX-2IN (Japan) heamatology auto analyzer (Baker *et al*., 1985).

Biochemical Assessment

Blood samples collected into plain test tubes (without anticoagulant) was processed for serum by allowing it to clot and centrifuged for 5 min at 3000 rpm (HUMAX-K, HUMAN-Germany), and stored at -20^{o} C until use. The serum obtained was analyzed for blood chemistry parameters: total protein, albumin, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferases (AST), total cholesterol, urea, lactate dehydrogenase (LDH), creatine kinase-R (CK-R) and creatine kinase-MB (CK-MB), lipids, creatinine,

urea, among others using Selectra Junior Autoanalyzer (Vital Scientific Bv, Version 04, Netherlands).

Antihyperglycaemic activity of CMASE in S-D rats

At the beginning (day 0) of the subchronic toxicity study, the tails of the S-D male rats in the treated and control groups were cleaned with 70 % ethanol and allowed to dry. Baseline level of fasting blood glucose (FBG) of the rats in the various groups were determined using ONE TOUCH Select Plus Flex (Lifescan Johnson and Johnson, China; CE 0344) glucometer, and ONE TOUCH Select Plus test strips (Lifescan, UK, Lot 4505378) with blood obtained by tail vein bleeding. This procedure was repeated on the 30^{th}, 60^{th}, and the 91^{st} day after the rats were fasted overnight, and mean FBG levels for the respective groups determined.

Liver specimen preparation

Harvested livers from the various rat groups were dissected into two (2) parts. Then one portion from each rat in the four groups were placed in a sterile labelled zip envelope, sealed and stored at -80^{o}C until the antioxidant enzymes levels were determined. The other portion of respective organ were placed in labelled specimen containers with some formalin for histopathological examination.

Tissues preparation for oxidative stress and prostatic markers determination

A volume 5ml TNGbuffer (50 mM Tris pH 7.4, 0.1M NaCl, 10% glycerol) was added to each frozen tissue (liver) in a mortar (on ice pack) and homogenized (with a pestle). The homogenized tissue was centrifuged at 4000 xg for 20 min in a refrigerated centrifuge. The supernatant was pipetted into eppendorf tube, labeled

and preserved (on ice packs) for various antioxidant enzyme assays. This procedure was also done for all the prostate organs from each rat in the BPH efficacy study.

In vivo antioxidant assays on liver and serum samples

Reduced Glutathione (GSH) content determination

GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. The glutathione level was estimated using the method described by (Alam *et al*., 2013) with slight changes. The sample (50 µl) was aliquoted into 96 well plate. Sodium phosphate (0.1M, 50 µl) was then added to each of the samples followed by the addition of ortho-phthalaldehyde (OPA) (10 mg/ml, 10 µl) to start the reaction. The mixture was incubated for 15 mins at room temperature. Afterwards, the fluorescence absorbance was measured at 460 nm (emission) and 340 (excitation) against blank using a microplate reader. The absorbance values were compared with a standard GSH curve.

Catalase level determination

Catalase is an important antioxidant enzyme responsible for the degradation of the reactive oxygen species, and hydrogen peroxide. Catalase catalyses the decomposition of hydrogen peroxide into water and oxygen. The catalase activity was determined using the method by Hadwan (2016) with slight modifications. Briefly, the samples (50 µl) were transferred into 1.5 ml eppendorf tubes (each sample/tube) and its control. Freshly prepared hydrogen peroxide (65 mM, 500 µl) was then added. Distilled water was added to the sample controls in place of

hydrogen peroxide. The mixture was vortexed and incubated for 3 min at 37°C. Afterwards, dichromate and acetic acid solution (50 µl of 5% aqueous solution of potassium dichromate with 150 µl of glacial acetic acid, 1 ml) was added and incubated at 100°C for 10 min. After cooling with tap water, it was centrifuged to remove precipitated protein (2500 g for 5 min). The absorbance were recorded at 570 nm against the reagent blank.

The catalase activity was determined using this formula: $\frac{2.303}{T} \times \log \frac{S^o}{S - M} \times \frac{V_t}{V_S}$

Determination of lipid peroxidation

Measuring the end products of lipid peroxidation is one of the most widely accepted assays to check oxidative damage. The lipid peroxidation assay was conducted using the method described by Ohkawa *et al*. (1979) with slight changes in volumes of reagents and samples. Lipid peroxidation refers to the oxidative degradation of lipids and during this process free radicals take electrons from the lipids (in the cell membrane) resulting in cell damage. This forms reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4- HNE) as bi-products. Briefly, the samples (20 µl) were transferred into eppendorf tubes. The following reagents were added: 20 µl of 8.1 % (w/v) sodium dodecyl sulphate, 150 µl of 20 % acetic acid and 150 µl of 8% (w/v) Tris butyrate acetate. Distilled water was added to make a total volume of 400 µl. The mixture was incubated in a water bath at 95°C for 60 min. After cooling with water, the volume was topped up to 500 µl with distilled water. Butanol: pyridine mixture (15:1, 500 µl) was added and vortexed thoroughly. The mixture was centrifuge 3000 rpm for 10 min. The absorbance of the upper layer was read at 532 nm against the appropriate blank

without sample. The levels of lipid peroxide were expressed in moles of thiobarbituric acid reactive substances (TBARS)/mg protein with an extinction coefficient of 1.56×10^5 MLcm^{-1}.

Superoxide dismutase determination

Superoxide dismutase is an antioxidant enzyme that catalyzes the dismutation of the superoxide anion into hydrogen peroxide and oxygen. The SOD activity was determined using a method described by Alam *et al*. (2013) with slight modifications. Samples (70 µl) were aliquoted into 96 well plate. Tris-HCl buffer (75 mM, 200 µl, pH 8.2 containing 30 mM EDTA) was then added, after which pyrogallol (2 mM, 30 µl) was added. The absorbance was measured at time intervals of 0, 3 and 5 min at 420 nm. The enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD was expressed in units/mg protein.

$$\%\text{Inhibition of pyrogallol autoxidation} = \frac{\text{Change in absorbance of test sample}}{\text{Change in absorbance of control sample}} * 100\%$$

$$\text{SOD Activity (U/ml)} = \frac{\%\text{ Inhibition of pyrogallol autoxidation}}{50\ \%}$$

Histopathological analysis for toxicity studies

At the end of the experiment, the animals were anesthetized with ether and sacrificed. The heart, liver, prostate and kidney tissues were harvested, examined and weighed to obtain organ to body weight ratios. These organs were then fixed with 10% buffered formaldehyde solution and processed for histopathological studies. Paraffin-embedded sectioned tissues (5 µm) of these organs were stained

with hematoxylin and eosin (H&E). The slides were evaluated microscopically for histological changes under a light Olympus CX23 microscope (model CX23LEDRFSI, China).

Efficacy of CMASE in BPH Sprague-Dawley rat models

The efficacy assay of CMASE in testosterone-induced hyperplasic prostate in male S-D rats was conducted in line with the Organization of Economic Co-operation and Development (2009) guidelines for testing Chemicals (Hershberger bioassay in rats) and summarized in table 1.

Table 1: Study design for efficacy of CMASE in rats in BPH studies.

Groups (n=5)	**Treatment for 28 days**
Negative control group	Distilled water.
Pathological-[Model]	Testosterone 5 mg/kg s.c
Pathological–CMASE (LD)	Testosterone 5 mg/kg s.c+ 30 mg/ kg
Pathological-CMASE (MD)	Testosterone 5mg/kg s.c + 150 mg/kg
Pathological–CMASE (HD)	Testosterone 5 mg/kg s.c + 300 mg/kg
Pathological-Positive control	Testosterone 5 mg/kg s.c + Finasteride 5mg/kg

Key: LD= low dose group, MD= median dose group, HD= high dose group

Thirty (30) adult male S-D rats weighing 180–240 g were randomly divided into 6 groups (of 5 rats each). Group 1 served as the naïve control and received distilled water *p.o*. Groups 2–6 were anesthetized with ketamine (100 mg/kg, *i.p.*) and castrated aseptically to bilaterally remove testes. These groups of rats (2-6) were then injected with 5 mg/kg b.wt. testosterone propionate (Laborate Pharmaceuticals, India, ETTMI-001) subcutaneously for 28 days. Group 2 (model group) did not receive any further treatment. Group 6 were given simultaneously 0.5 mg/kg b.wt.

finasteride p.o. throughout the experimental period. Groups 3, 4 and 5 received [30 mg/kg b.wt-(LD), 150 mg/kg b.wt –(MD) and 300 mg/kg b.wt (HD)] CMASE, respectively for 28 days p.o. At the end of the study, the rats were euthanised with ether, and blood obtained by cardiac puncture. The rats were then sacrificed, and all liver and prostate organs harvested. Harvested livers and prostates from each rat in the various rat groups were dissected into two (2) parts after they have been weighed. Then one portion from each rat in the six groups were placed in a sterile labelled zip envelope, sealed and stored at -80^{o}C. Tissue preparation techniques on harvested prostate organs to obtain samples for analysis on CMASE possible anti-BPH mechanisms of action, followed previously mentioned procedures (Page 110). Other portions of the prostates were preserved for histopathological examination.

Prostatic weights and index determination

The prostates were carefully dissected, freed from connective tissues and weighed appropriately to obtain wet weights, organ to body weight ratios and the prostatic index (PI) of the various groups of rats were determined.

Percentage of inhibition of increase in prostate weight

Percentage of inhibition of increase in prostate weight (% PW) was calculated:

$$\% \text{ PW: } 100 - \frac{\{\text{PW of treated group} - \text{PW of negative control}\}}{\{\text{PW of model group} - \text{PW of negative control}\}} \times 100$$

(Veeresh *et al*., 2010).

Histopathological Analysis for BPH

The harvested prostate organs were examined macroscopically. They were then fixed with 10% buffered formaldehyde solution. The samples were the dehydrated

(processed) in graded absolute ethanol. They were then cleared with xylene and infiltrated with paraffin wax. Paraffin beeswax tissue blocks were prepared and employed in portioning the samples into a thickness of 5 µm. Paraffin-embedded sectioned tissues of the prostate organs were stained with hematoxylin and eosin (H&E). The slides were evaluated microscopically for histopathological changes in the epithelium, stromal and acini under a light Olympus CX23 microscope (model CX23LEDRFSI, China).

In vivo screening assays for (anti)androgenic properties of CMASE

Detection of prostatic testosterone and dihydrotestosterone

Prostatic testosterone, dihydrotestosterone (DHT) levels were determined using MyBioSource (San Diego, CA, USA) rat ELISA kits (Rat Testosterone Kit, Catalog Number MBS775388, Lot Number 201902 and Rat Dihydrotestosterone, Catalog Number MBS774535) following the manufacturer's instruction. Standard wells and sample wells were set up and labelled. A volume of 50 µl of standard was added to the standard wells. Next, a volume of 10 µl of the samples were added to the sample wells after which 40 µl of the diluent was added. A volume of 100 µl of HRP-conjugate reagent was added to each well after which the plate was sealed and shaken, then incubated at 37°C for 60 min. The wells were then washed 5 times with 350 µl each with the washing solution after which 50 µl of Chromogen Solution A was added, followed by addition of 50 µl of Chromogen solution B. The plate was then incubated at 37°C for 15 min in the dark, then 50 µl of the Stop solution was added to each well and then shaken gently. The absorbance was taken at 450 nm.

Prostate specific antigen (PSA) determination

Prostate specific antigen levels were determined with MyBioSource (San Diego, CA, USA) rat ELISA kits (Rat Total Prostate Specific AntigenKit, Catalog Number MBS9395952, Lot Number 201902) following the manufacturer's instruction. Standard wells and sample wells were set up and labelled in duplicates. A volume of 50 µl of standard was added to the standard wells. Next, a volume of 50 µl of the prostatic samples were added to the sample wells. A volume of 100 µl of HRP-conjugate reagent was added to each well after which the plate was sealed and shaken, then incubated at 37°C for 60 min. The wells were then washed 4 times with 350 µl each washing solution after which 50 µl of Chromogen solution A was added followed by 50 µl of Chromogen solution B. The plate was then incubated at 37°C for 15 min in the dark, then 50 µl of the Stop solution was added to each well and then shaken gently. The absorbance was taken at 450 nm.

Detection of prostatic 5-alpha reductase expression levels

Expressions of 5-alpha reductase levels within each rat group were determined using prostatic samples (supernants) obtained from the aforementioned tissue preparation procedures, following MyBioSource (San Diego, CA, USA) rat ELISA kits instructions (Rat Steriod 5-Alpha-Reductase 2 Kit [SRD5A2], Catalog Number MBS1600578, Lot number 201902002). A volume of 50 µl of standard was added to the standard well. A volume of 40 µl of sample was added to each sample well, after which 10 µl of anti-SRD5A2 antibody was added. A volume of 50 µl of streptavidin-HRP was then added to the samples and controls but not the blank. The plate was sealed and shaken to mix, covered and incubated for 60 min at 37°C. The

sealer was removed and the plate washed five times with 350 µl of wash buffer for at least 30 sec. After blot drying the plate on paper towels, a volume of 50 µl of substrate solution A was added to each well followed by 50 µl of substrate solution B. The plate was then incubated for 10 min at 37°C in the dark. A volume of 50 µl Stop Solution was added to each well and the optical density at 450 nm was taken immediately.

Examination of liver and prostate antioxidant markers

Preserved samples of the liver and prostate at -80^{o}C in a refrigerator after the rats were sacrificed were used for the determination of antioxidants assays. They were individually homogenized on ice (with a mortar and pestle) and centrifuged at 4000 xg for 20 min following similar procedures stated in the sub-chronic section. The supernants were analysed for levels of antioxidant enzymes; superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) from rats in each group following previously mentioned methods.

Statistical analysis and data evaluation

Statistical analysis of the data was done using Graph-Pad Prism Software version 5.0 for windows (GraphPad Software Inc. San Diego California, USA). All results were expressed as mean ± standard error of mean (S.E.M). The results were analyzed using one-way ANOVA to determine the level of significance, followed by Tukey's *post hoc* multiple comparison analysis to establish significance between the treated and control groups. *P* values less than 0.05 were considered statistically significant.

CHAPTER FOUR

RESULTS

Preliminary qualitative phytochemical screening of aqueous stem extract of *C. membranaceus* (CMASE) indicated the presence of phenols, alkaloids, tannins, saponins and terpeniods. Whilst, reducing sugars, glycosides, phytosterols, and flavonoids were absent (Table 2).

Table 2: Results on qualitative phytochemical screening of the aqueous extract of CMASE

Phytochemical	CMASE
Reducing sugars	-
Alkaloids	+
Tannins	+
Saponins	+
Phytosterols	-
Anthraquinones	-
Phenols	+
Flavonoids	-
Terpenoids	+

Keys: Present phytochemicals are denoted by (+), and absent phytochemicals are denoted by (-).

Following sequential extraction of the pulverized stem of *C. membranaceus*, the dried crude extracts obtained were weighed. The percentage yield of the fractions were calculated and presented in Table 3. The aqueous fraction (AQ) had the highest yield, whilst the lowest yield was obtained from the ethanolic fraction. The

overall yield from 100 g of the pulverized stem was 3.14 % (w/w), and that of the direct aqueous extract from 1000 g was 6 % (w/w).

Table 3: Extracts yields from the sequential fractionation of pulverized stem of *C. membranaceus* (100g) with various solvents.

Type of the fractions	**Percentage (%) yield of 100 g of *C. membranaceus***
Hexane	0.69
Ethyl acetate	0.92
Ethanolic	0.33
Aqueous	1.20

HPLC analysis of SECM and CMASE

The resulting chromatograms from the high performance liquid chromatography analysis of the fractionated extracts of the stem of *C. membranaceus* (SECM) and CMASE were recorded and presented in the Figures (9-13). Similar compounds were seen both the DAQ and AQ fractions with varying concentrations.

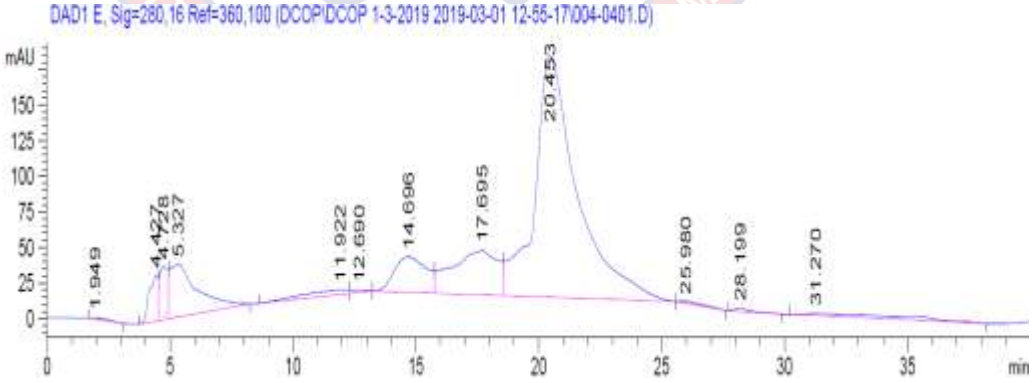


Figure 9: Chromatogram of aqueous stem fraction of *C. membranaceus* (AQ).

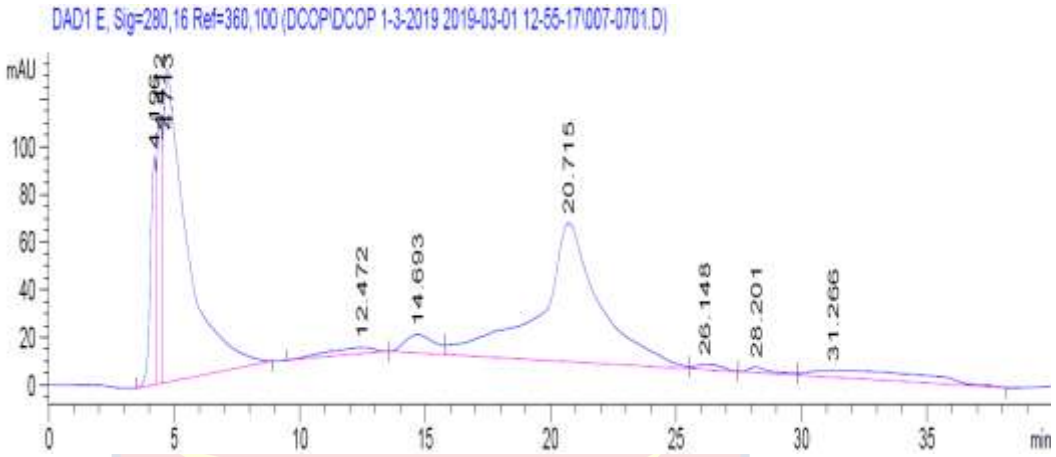


Figure 10: Chromatogram of direct aqueous stem extract of *C. membranaceus* (DAQ).

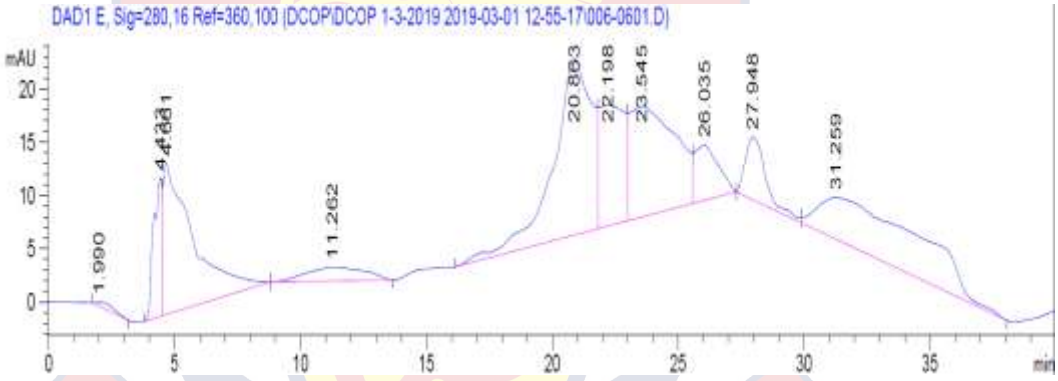


Figure 11: Chromatogram of ethanolic stem fraction of *C. membranaceus*

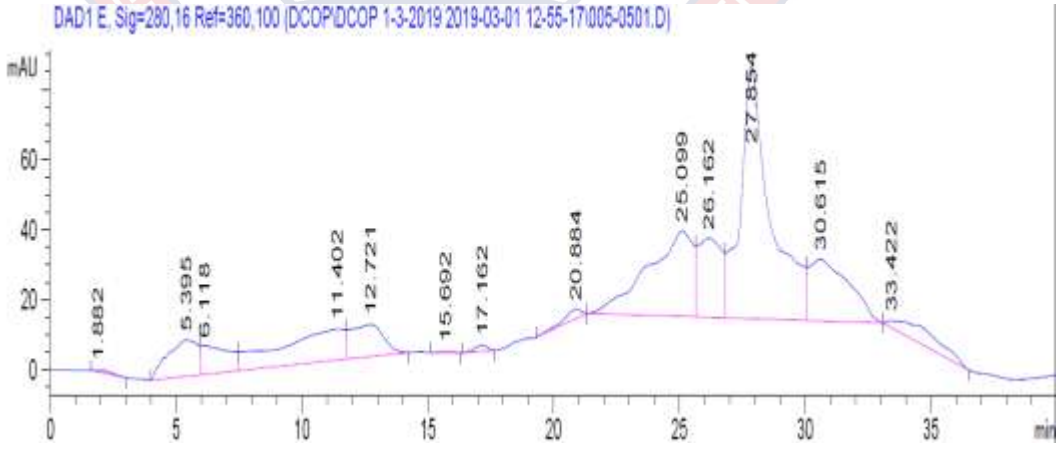


Figure 12: Chromatogram of ethyl acetate stem fraction of *C. membranaceus*

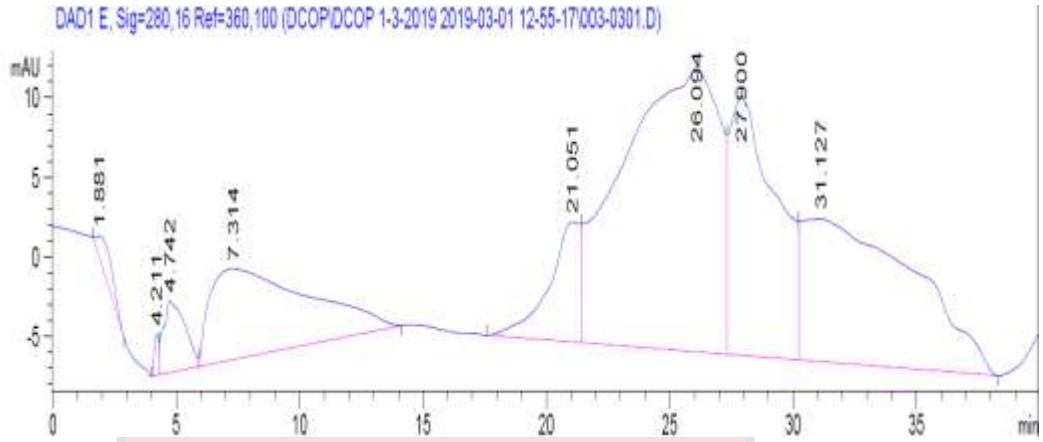


Figure 13: Chromatogram of hexane from the stem extract of *C. membranaceus*

GC-MS Analysis the CMASE dissolved in dichloromethane

The GC-MS chromatograph results on CMASE revealed the presence of 15 main phytochemical constituents which were detected with their retention time (RT), peak area, concentration (peak area %), and names of compounds are presented in Figure 14 and in Table 4. The nine major bioactive phytochemicals and their mass spectra; molecular formula, molecular weight are presented in Figures 15 and 16.

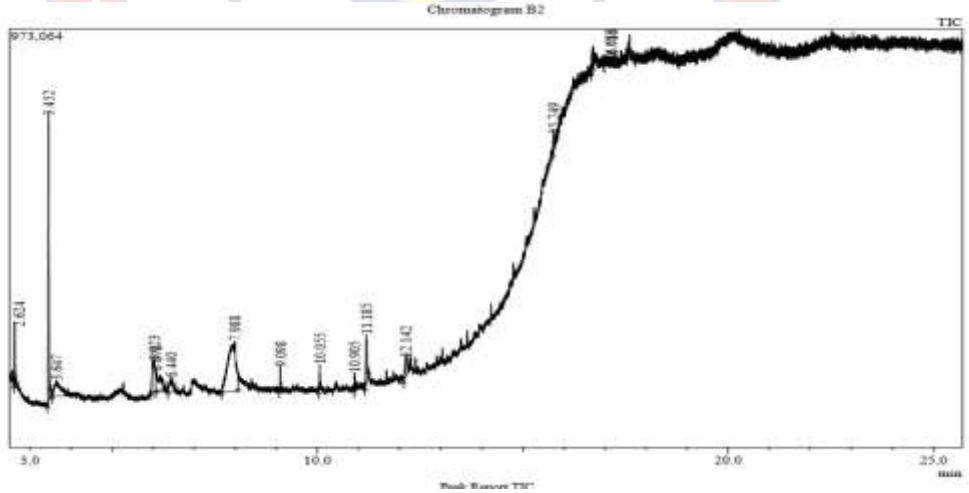
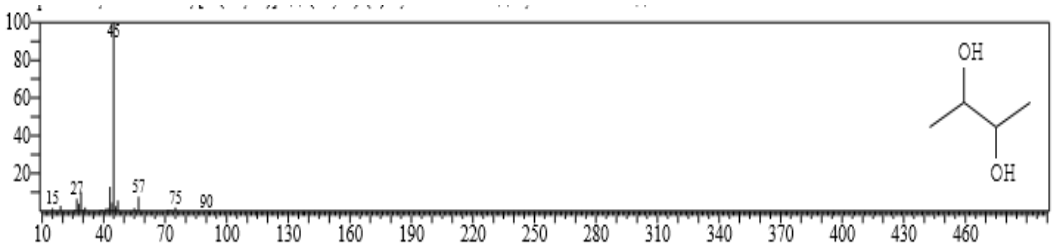


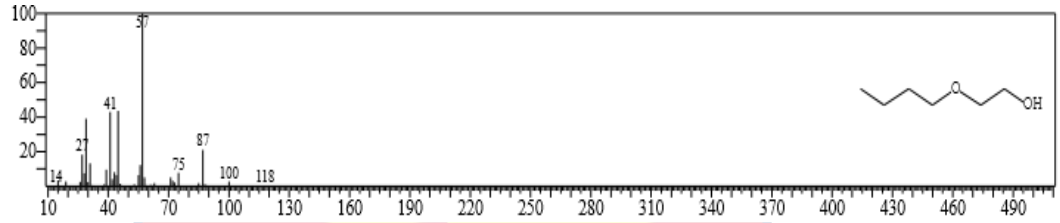
Figure 14: A GC-MS chromatograph of microwave assisted methanolic extract of CMASE in methanol-dichloromethane solvent.

Table 4: Results of GC-MS chromatograph analysis showing constituents of CMASE in methanol-dichloromethane solution.

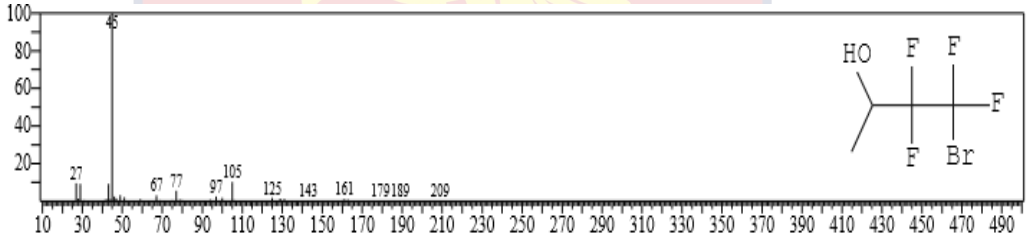
Peak #	R Time	Area	Area %	Height	Heigh t%	Name of compound
1	2.624	17668	3.69	140835	9.10	2,3-Butanediol,[S-(R*,R*)]-
2	3.352	707389	14.78	668060	43.17	Ethanol, 2-butoxy-
3	3.647	391518	8.18	36510	2.36	2-Butanol,4-bromo-3,3,4,4-tetrafluoro
4	6.023	698125	14.59	72167	4.66	Ethanol,2-(2-butoxyethoxy)-
5	6.078	313118	6.54	44110	2.85	Bacchotricumeatin c
6	6.440	155411	3.25	29863	1.93	N-(1-[1-(4-Amino-furan-3-yl)-1H-[1,2,3]tri
7	7.988	15595219	32.58	118072	7.63	1-[-]-4-Hydroxy-1-methyproline
8	9.098	54921	1.15	54200	3.50	Cyclooctasiloxane,hexadecamethyl
9	10.055	75201	1.57	60016	3.88	Cyclohexasiloxane,dodecamethyl-
10	10.905	43888	0.92	38327	2.48	Octasiloxane,1,3,3,5,5,7,7,11,11,13,13,1
11	11.185	24469	5.11	114758	7.42	n-Hexadecanoic acid
12	12.142	190970	3.99	47406	3.06	6-Octadecanoic acid, (Z)-
13	15.749	64263	1.34	42430	2.74	Tetracomesamethyl-cyclododecasiloxane
14	16.685	57243	1.20	39132	2.53	[1,1'-Bicyclohexyl]-4-carboxylic acid, 4'-prop
15	16.716	53273	1.11	41512	2.68	6,6-Diethylhoctadecane
		478676	**100.0**	**1547398**	**100.00**	



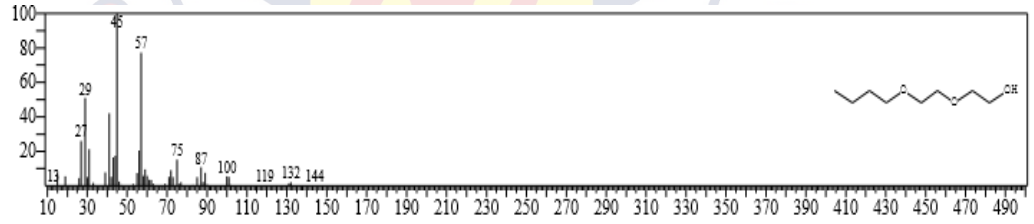
2,3-Butanediol, Formula:C4H10O2 CAS:19132-06-0 MolWeight:90



2-butoxy- Ethanol, Formula:C6H14O2 CAS:111-76-2 MolWeight:118

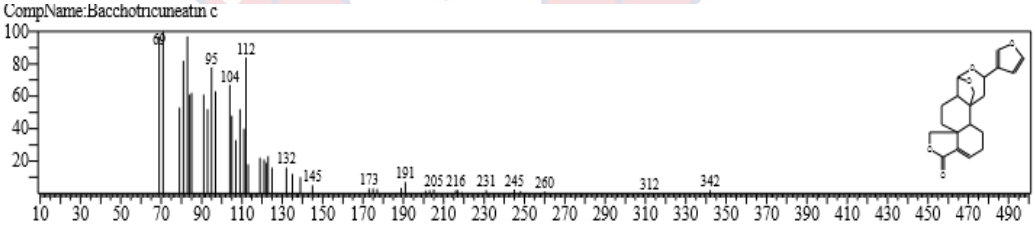


4-bromo-3,3,4,4-tetrafluoro-2-Butanol, Formula:C4H5BrF4O CAS:74646-39-2 MolWeight:224



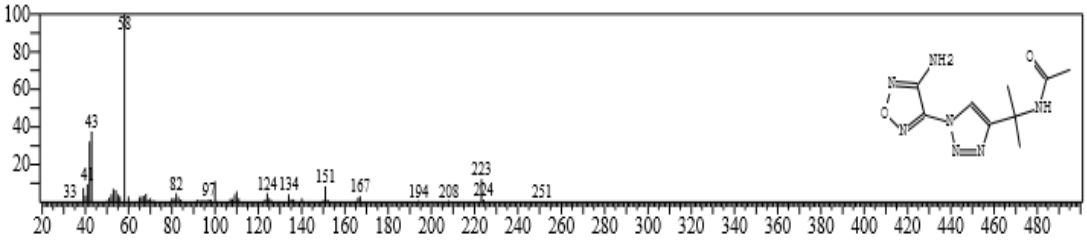
2-(2-butoxyethoxy)-Ethanol,Formula:C8H18O3 CAS:112-34-5 MolWeight:162

CompName:Bacchotricuneatin c



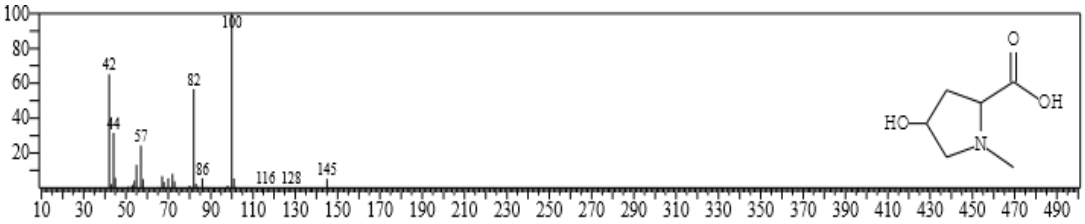
Bacchotricuneatin c Formula:C20H22O5 CAS:66563-30-2 MolWeight:342 RetIndex:2486

Figure 15: Mass spectra of identified major bioactive compounds of CMASE (in methonol-dichloromethane solution)

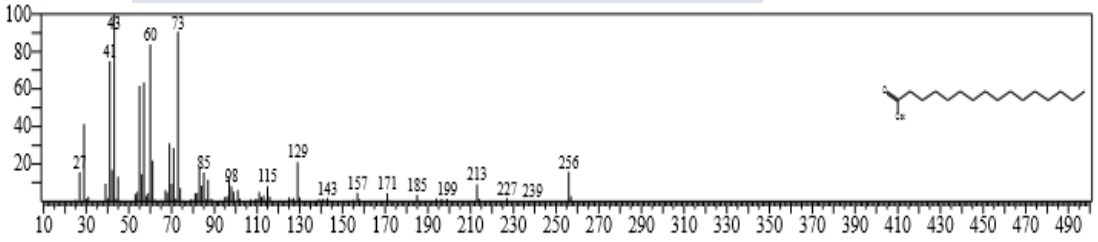


N-(1-[1-(4-Amino-furazan-3-yl)-1H-[1,2,3]triazol-4-yl]-1-methyl-ethyl)-acetamide

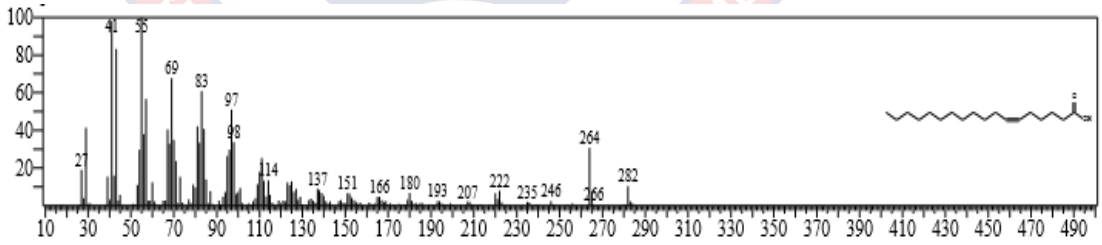
Formula:C9H13N7O2 CAS:0-00-0 MolWeight:251 RetIndex:0



l-[-]-4-Hydroxy-1-methylproline Formula:C6H11NO3 CAS:0-00-0 MolWeight:145 RetIndex:1318



n-Hexadecanoic acidFormula:C16H32O2 CAS:57-10-3 MolWeight:256 RetIndex:1968



6-Octadecenoic acid, Formula:C18H34O2 CAS:593-39-5 MolWeight:282 RetIndex:2175

Figure 16: Mass spectra of identified major bioactive compounds of CMASE (MEOH/DCM)

Antioxidant activity of sequential fractions and direct aqueous extract of the stem of *C. membranaceus*

The effective concentration values from the DPPH scavenging activities of the sequential stem fractions and direct aqueous extract of *C. membranaceus* are presented in Table 5.

Table 5: Summary of EC_{50} values of the fractions and direct aqueous extract of the pulverized stem of *C. membranaceus* (CS).

Test fractions/compound	**EC_{50}(mg/ml)**	**p -value**
BHT	0.02 ± 0.001	
Aqueous	2.03 ± 0.011***	
Direct aqueous (DAQ)	3.44 ± 0.001***	
Ethanolic	0.35 ± 0.001***a	<0.001
Ethylacetate	1.41± 0.011***b	
Hexane	8.57 ± 0.001***	

Keys: BHT: -2, 6-di-*tert*-butyl-4-methylphenol (1 mg/ml). Values represent mean ± SEM from triplicate experiments. '***'p<0.001 BHT is compared to fractions/DAQ from stem of *C. membranaceus*. Concentrations of CS extracts were 10 mg/ml. 'a' p<0.001 was considered significant compared to rest of CS extracts. 'b' p<0.001 was considered significant compared to rest of CS fractions except ETOH fraction. EC_{50} of ETOH>ETAC>AQ>DAQ>HEX.

The DPPH radical scavenging activities of the sequential fractions and direct aqueous extract of the pulverized stem of *C. membranaceus* revealed that, all extracts possessed antioxidant activity. Furthermore, they exhibited antioxidant activity in a concentration dependent manner. The ethanolic fraction of the stem of *C. membranaceus* possessed the highest antioxidant activity (EC_{50}= 0.35± 0.001 mg/ml) when compared to the rest of the fractions. The fraction with the least

antioxidant activity was the hexane extract (8.57 ± 0.001 mg/ml). The EC_{50} value of the ethanolic fraction was significantly ($p<0.001$) lower than the rest of the sequential fractions or direct aqueous extract. Based on EC_{50} values obtained, the order of increasing antioxidant activities of SECM and CMASE (DAQ) in scavenging 50 % of the DPPH free radicals was; hexane, DAQ, sequential aqueous fraction (AQ), ethyl acetate and ethanol. There was significant ($p<0.001$) differences in the EC_{50} values obtained between all the various fractions except between aqueous fraction and direct aqueous extract. Furthermore, the concentration-response curves of DPPH radical scavenging activities (presented as percentage DPPH inhibitions against the log concentrations) of the BHT, the sequential fractions and direct aqueousextract from the stem of *C. membranaceus* are presented in Figure 17 and Figure 18 (B-F).

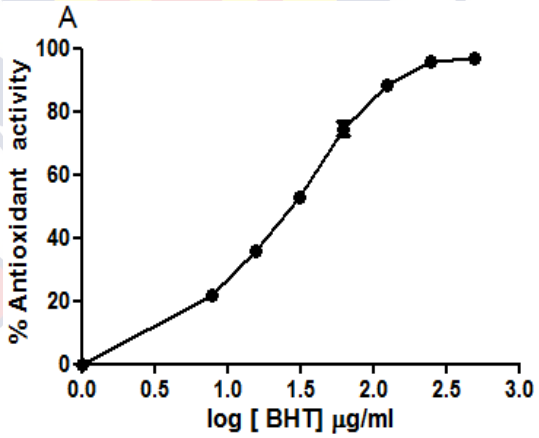


Figure 17: Antioxidant activity of butylated hydroxy toluene (BHT). This figure shows the log concentration dependent curve of BHT. As the concentration increases, so does its antioxidant potential. One millgram per milliliter of BHT was tested as described in the methods.

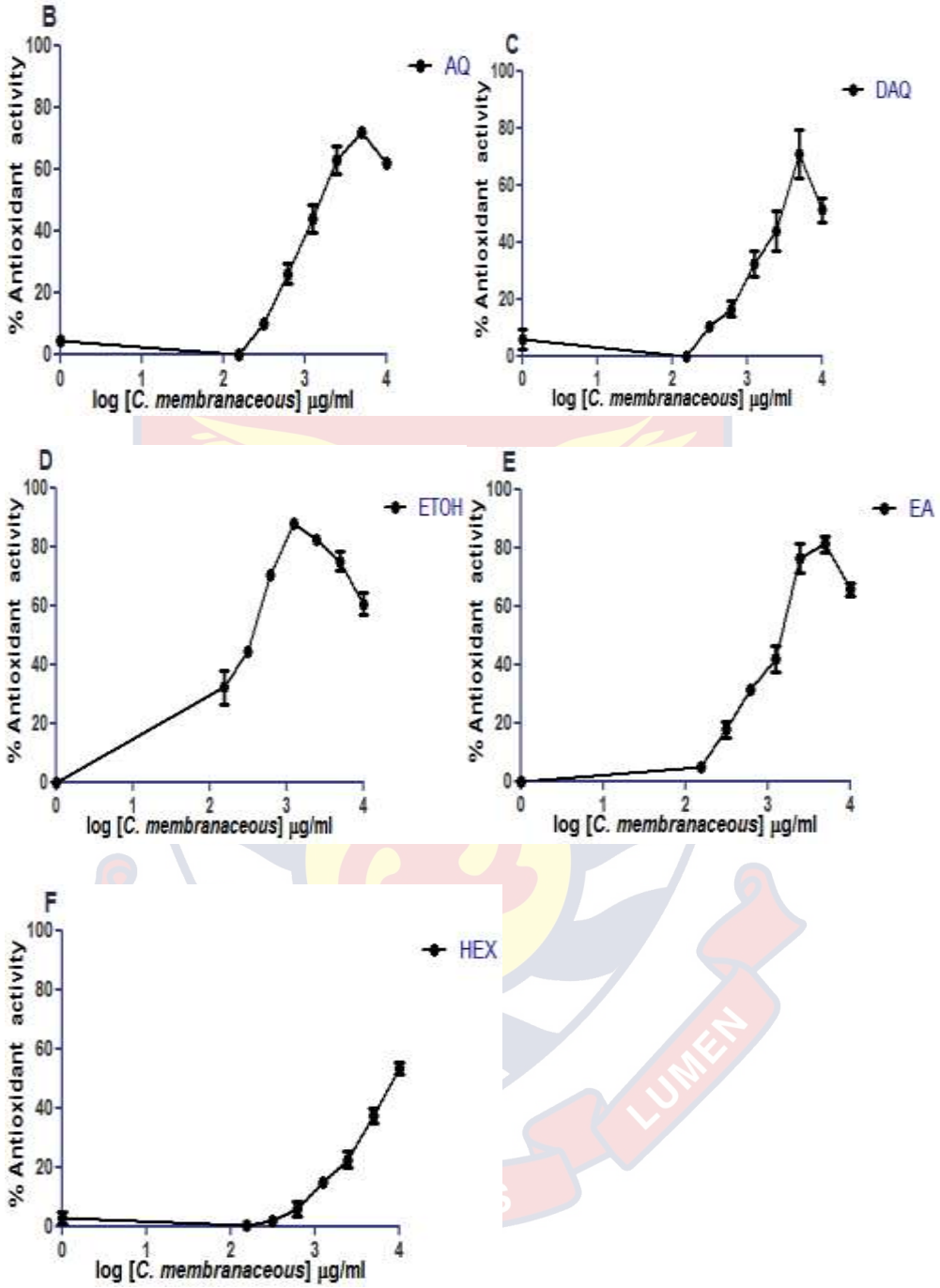


Figure 18: Antioxidant activities of aqueous fraction (B), direct aqueous extract (C), ethanolic (D), ethyl acetate (E) and hexane fractions (F) from the stem of *C. membranaceus.* These figures show the log concentration dependent curve of extracts. As the concentration increases, so does its antioxidant potential. Five millgram per milliliter of each fraction/extract was tested as described in the methods.

Total phenolic content of SECM and CMASE

The absorbance series of concentrations of gallic acid was plotted against their concentration to yield a linear calibration curve of gallic acid as shown in Figure 19 below. The total phenolic content for 2.5 mg/ml concentration of the sequential fractions and direct aqueous extract (CMASE) of the pulverized stem of *C. membranaceus* were extrapolated from the calibration curve for gallic acid standard ($y= 0.384x +0.052$, $R^2 =0.998$).

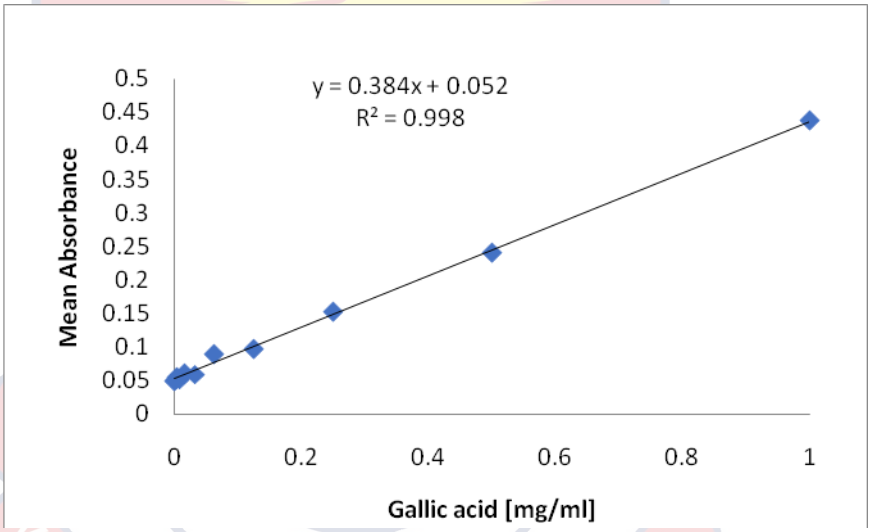


Figure 19: Gallic acid calibration curve.

From Table 6, all sequential fractions and direct aqueous fractions from the pulverized stem of *C. membranaceus* possessed some amount of phenols. The total phenolic contents were expressed as gram equivalent gallic acid per 100 gra of dry stem weight of *C. membranaceus*. The ethanolic extract possessed a significantly higher amount of phenols ($p<0.001$) when compared to the rest of the fractions. The aqueous and direct aqueous possessed a significantly higher amount of phenols

(p<0.01) when compared to the ethyl acetate fraction, and possessed significantly (p<0.001) higher amount of phenols than hexane. There was no significant difference in phenolic contents between the aqueous and direct aqueous fractions. The latter observation was also observed between the ethylacetate and hexane fractions.

Table 6: Total phenolic content of SECM and DAQ (CMASE)

Fractions of CMASE	**TPC [g/100g GAE]**
Aqueous (AQ)	9.72 ±0.58**[b]
Direct Aqueous (DAQ)	9.85± 1.23**[b]
Ethanol (ETOH)	17.46 ± 1.91***[a]
Ethyl acetate (EA)	4.63 ± 0.74
Hexane (Hex)	3.16± 0.16

"[a]" p<0.001 was considered significant compared to rest of fractions. "[b]" <0.01 was considered significantly compared to EA fraction, and significant (p<0.001) compared to hexane. Phenolic contents in ETOH>DAQ>AQ>EA>HEX.

Total flavonoid content in SECM and CMASE

The content of flavonoids was expressed in terms of quercetin equivalents using the regression equation of the calibration curve obtained from quercetin (y = 0.034x + 0.015, R^2 = 0.990) and presented in Figure 20. Results obtained from the flavonoid content assay revealed that, there were no flavonoids in the aqueous and direct aqueous fractions of *C. membranaceus*. The flavonoid content in the rest of fractions in increasing order ranged from 25.25 ± 7.68 to 549.99 ± 64.00 mg QE/100 g sample for; hexane, ethanol and ethyl acetate. Ethyl acetate fractions significantly possessed the highest total flavonoid content (549.99 ± 64.00 TFC/100

g) when compared with ethanolic ($p<0.01$) and hexane ($p<0.001$) fractions respectively.

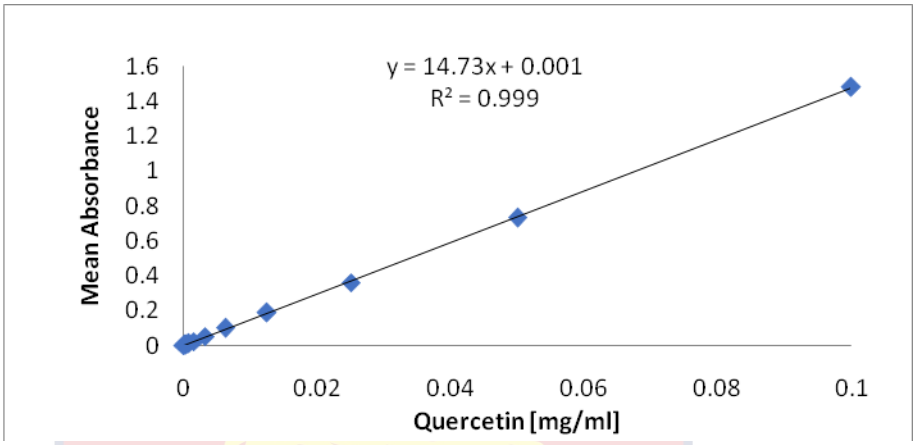


Figure 20: Quercetin acid calibration curve

Furthermore, the ethanolic fraction of flavonoids possessed significantly ($p<0.01$) higher content compared to the hexane fractions. The detailed results on the flavonoid content assay on SECM and CMASE are presented in Table 7.

Table 7: Total flavonoids content (TFC) in the fractions/ aqueous extract of *C. membranaceus*

Fractions of CMASE	**TFC (mg QE/100g)**
AQ	NIL
DAQ	NIL
ETOH	186.92 ± 25.22**[b]
EA	549.99 ± 64.00
Hex	25.25 ± 7.68***[a]

Key: The total flavonoids content (TFC) was expressed as mean ± S.E.M in terms of milligram of quercetin equivalents per 100 grams of dry weight of plant (mg TFC/100g). "[a]" $p<0.001$ and "[b]" $p<0.01$ were considered significant compared to the EA.

In vitro cell viability

Cytotoxic effects of SECM and CMASE on PC3 cells

According to Hostanska *et al*. (2007), cell cultures with more than 90% viable cells after treatment period are considered unaffected, those with 80-90% viable cells were modestly affected, and cultures with <80% viable cells were considered to be susceptible to the cytotoxic effects of the test compound or extract.

Cytotoxicity results obtained from the MMT assay using various concentrations (0, 62.5, 125, 500 and 1000 µg) of SECM and CMASE after 72 h of treatment is presented in Figure 21. All the various concentrations of CMASE and SECM showed antiproliferative effects against PC3 cells with cell viability less than 80 %. The strongest cell growth inhibitions were exhibited by both the aqueous (AQ) and direct aqueous (DAQ/CMASE) extracts with median inhibitory concentrations (IC_{50}) values of 13.26 ± 1.30 and 11.75 ± 1.15 µg/ml, respectively. The weakest cytotoxic effect was exhibited by the ethyl acetate fraction with mean IC_{50} value of 68.3 ± 1.15 µg/ml. The antiproliferative activity of the fractionated ethanolic and hexane extracts were virtually the same (about 55.0 µg/ml). The IC_{50} values obtained from log-concentration curve plots for all SECM and CMASE extracts are presented in Table 8. Although there was a significant difference ($p<0.05$) between the SECM and CMASE compared to the control (curcumin), there was no significant differences in the IC_{50} values of the SECM and CMASE obtained. The cytotoxic profile (cell viability against log concentrations of extracts) and the positive control (curcumin) on PC3 cells are presented in Figure 22 (A-F).

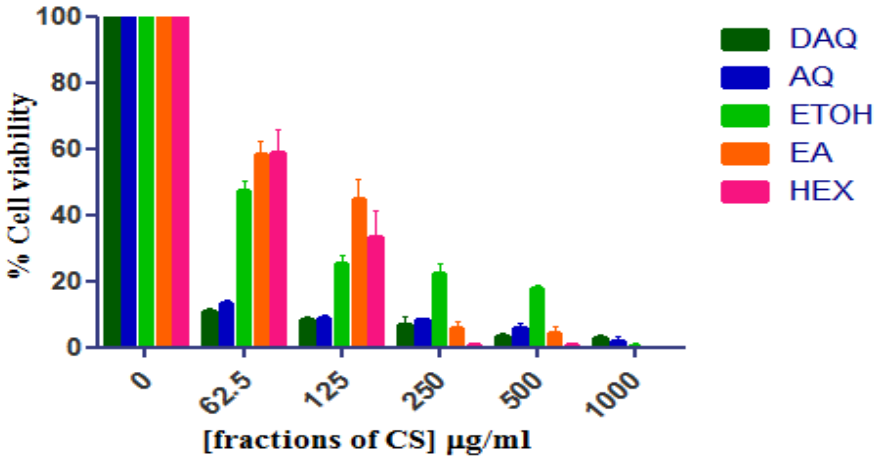


Figure 21: Effect of stem extracts of *C. membranaceus* (SECM and CMASE [DAQ]) on viability of PC3 cells. All fractions inhibit proliferation of PNT2 cells. Data are reported as means ± SEM from at least three independent experiments.

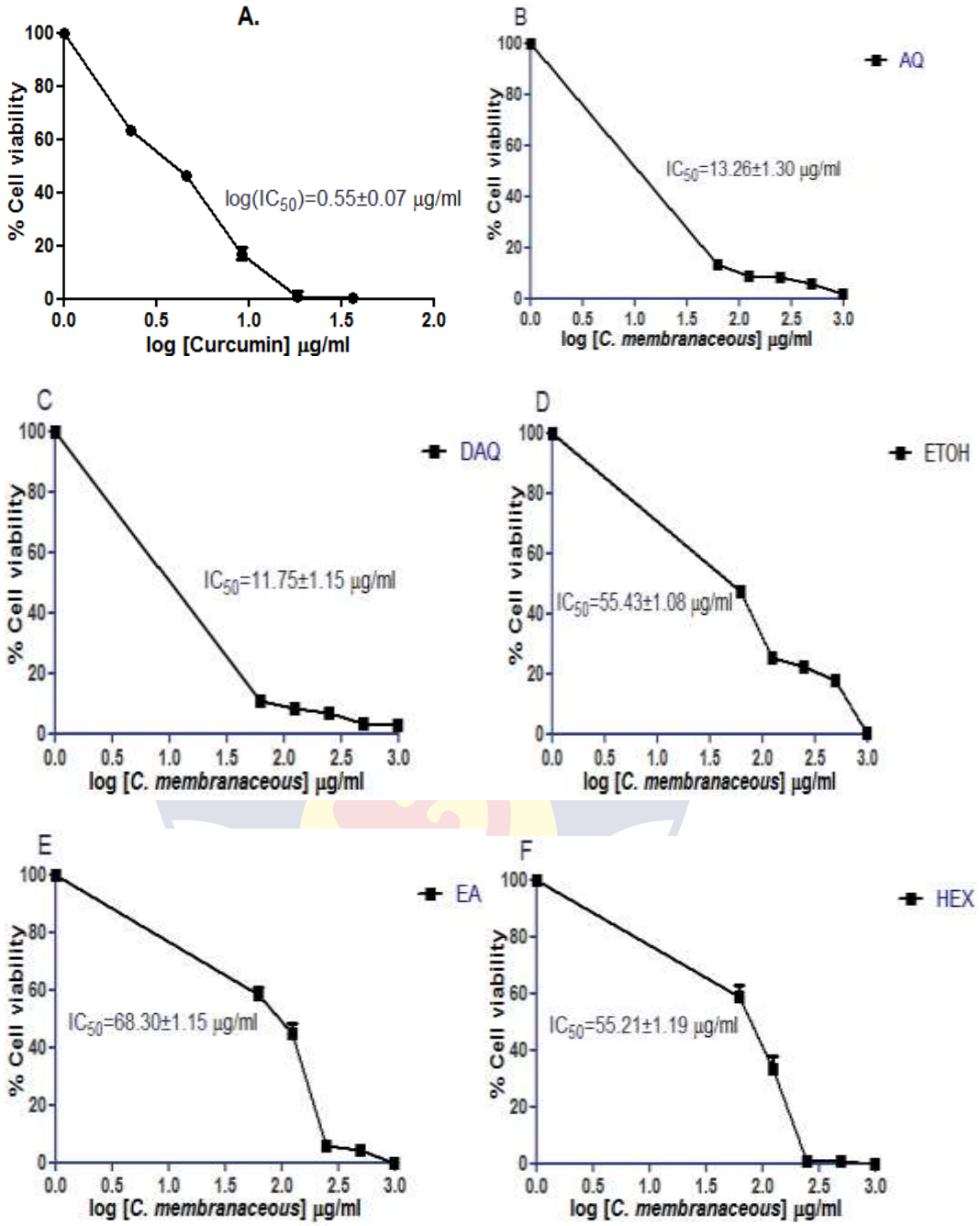


Figure 22: Antiproliferative activities of curcumin (A), AQ(B), DAQ(C), ethanol (D), ethyl acetate (EA) and hexane (F) fractions of CM against PC-3 cell lines. All extracts inhibit proliferation of PC3 cells. Results are representative of three independent experiments. IC_{50} values of fractions obtained indicated in their respective graphs.

Cytotoxic effects of SECM and CMASE on PNT2 cells

Cytotoxic effects of the various concentrations of SECM and CMASE on PNT2 cells after 72 h is presented in figure 23. Generally, there was cell viability of >80 % for both aqueous extracts (AQ and CMASE) after 72 h with mean cytotoxic concentration >1000µg/ml. Some of the extracts showed a concentration dependent inhibition of PNT2 cell growth, with the ethyl acetate fraction exhibiting the highest cytotoxicity with CC_{50} value of 87.9±1.15 µg/ml. Cytotoxic concentration values of ethanol and rest of fractions were 558.0±1.08, and >1000 µg/ml µg/ml respectively. The detailed cytotoxic concentrations (CC_{50} µg/ml) values of the various extracts (SECM and CMASE) against PNT2 cells are presented in Table 8. Figure 24 (A-F) shows the antiproliferative activities (cell viability against log concentrations of extracts) on PNT2 cells.

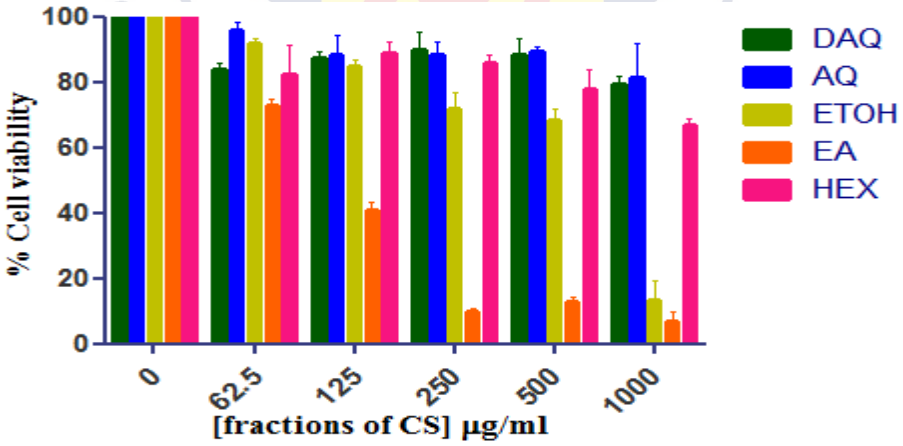


Figure 23: Effect of SECM and CMASE (DAQ) on the viability of PNT2 cells. All fractions inhibit proliferation of PNT2 cells. Data are reported as means ± SEM from at least three independent experiments.

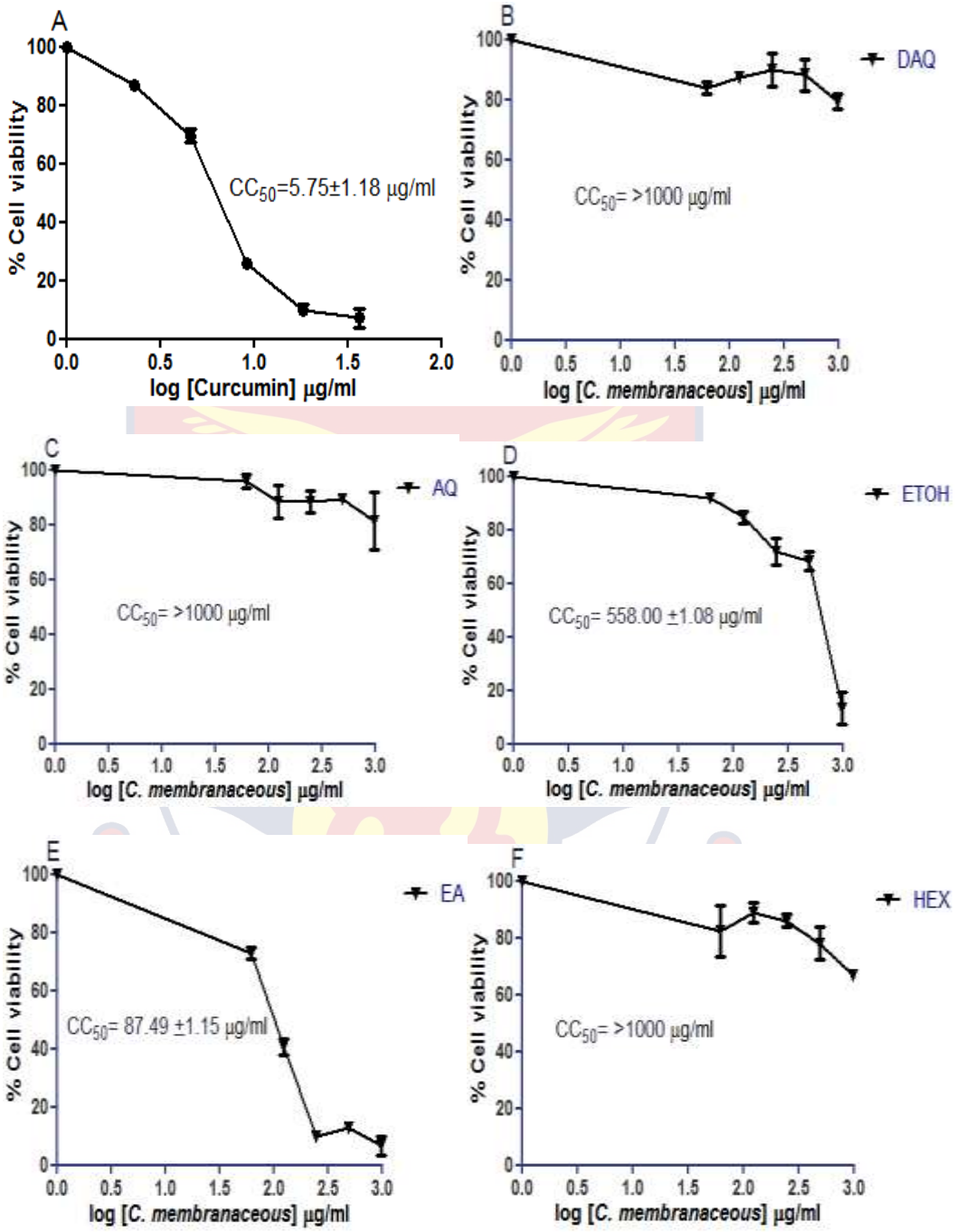


Figure 24: Antiproliferative activities of curcumin (A), AQ (B), DAQ(C), ethanol (D), ethyl acetate (E) and hexane (F) fractions on PNT2 cell line. All fractions inhibit proliferation of PNT2 cells. CC_{50} values of fractions obtained indicated in their respective graphs.

Cytotoxic effects of SECM and CMASE on LNCaP cells

Effect of various concentrations of SECM and CMASE on cell viabilities of LNCaP cells after 72 h is presented in Figure 25. The IC_{50} values obtained for the various stem extracts of *C. membranaceus* (SECM and CMASE) on LNCaP cells after 72 h is presented in Table 8. It was observed that, initial lowest concentrations for CMASE, fractionated aqueous (AQ) and hexane extracts induced minimal proliferations. However, there was a general concentration dependent decrease in cell viability with increasing concentration of the various extracts (CMASE and SECM). The hexane and ethyl acetate solvent extracts exhibited the strongest cytotoxic activities with IC_{50} values of 144.3±1.44 and 226.2±1.31 µg/ml respectively. This was followed by ethanol, fractioned aqueous (AQ) and CMASE in decreasing order of cytotoxicity with IC_{50} values of 366.3±1.33, 482.9±1.38 and 453.3±1.32 µg/ml respectively. Figure 26 (A-F) shows the antiproliferative activities (cell viability against log concentrations of extracts) on LNCaP cells.

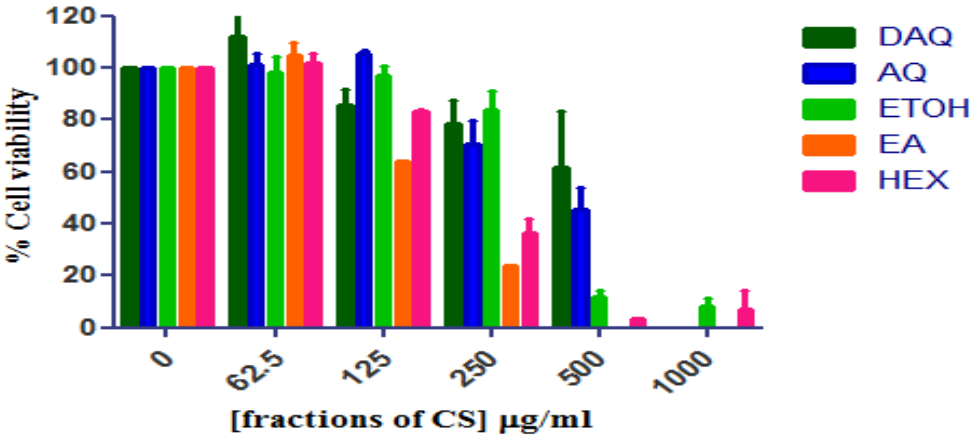


Figure 25: Effect of extracts of SECM) and CMASE (DAQ) on the viability of LNCaP cells. All fractions inhibit proliferation of LNCaP cells, though initial concentrations appeared to induce proliferation. Data are reported as means ± SEM from at least three independent experiments.

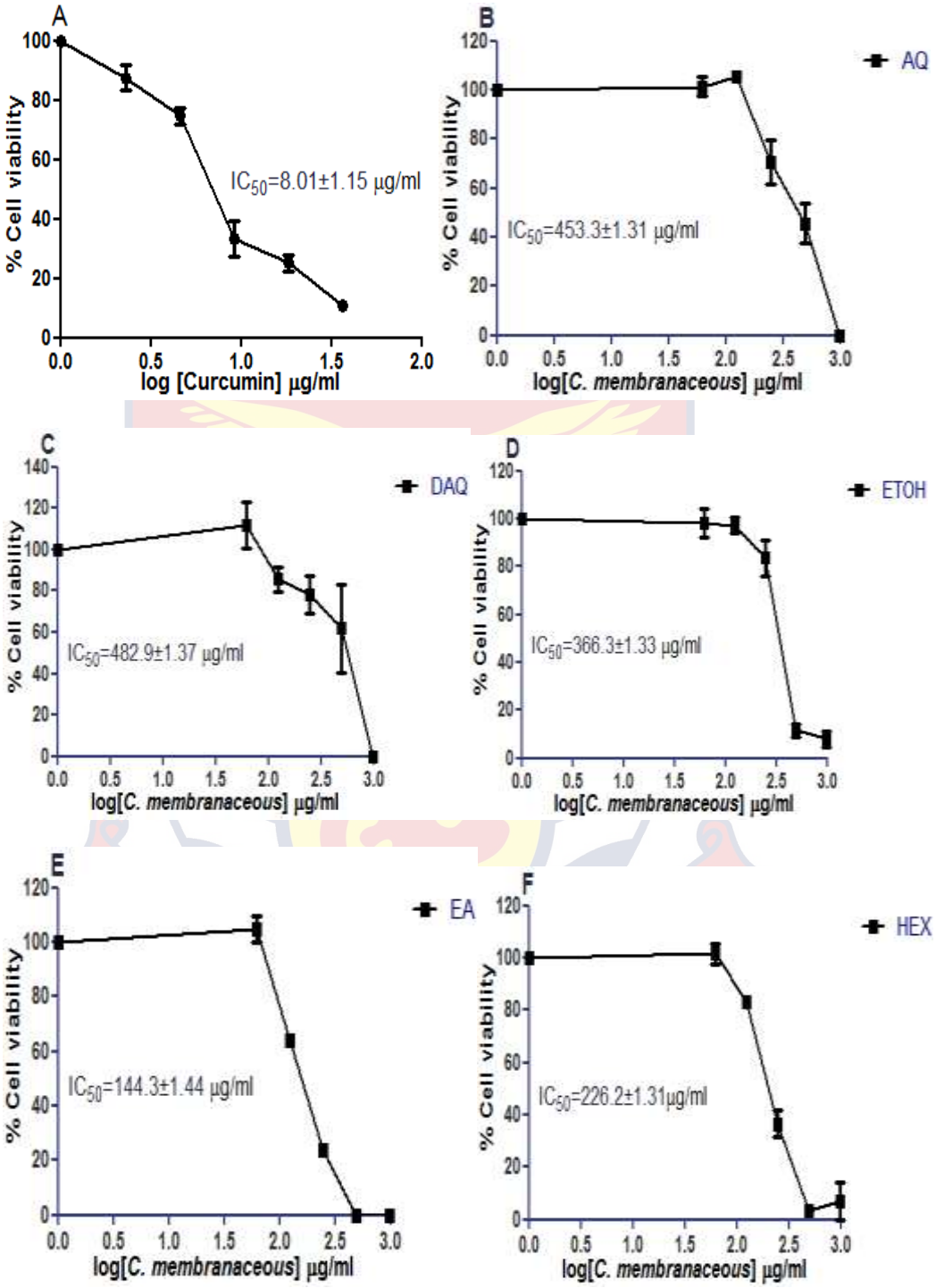


Figure 26: Antiproliferative activities of curcumin (A), AQ(B), DAQ(C), ethanol (D), ethyl acetate (E) and hexane (F) fractions on LNCaP cells. All fractions inhibit proliferation of PNT2 cells. IC_{50} values of fractions obtained indicated in their respective graphs.

Selective indices of SECM and CMASE for PC3 and LNCaP

Table 8 presents the detailed data on the mean IC_{50} and CC_{50} values obtained for the various stem extracts of *C. membranaceus* and that of the positive control (curcumin) from the various prostate cell lines. The IC_{50} values of extracts from the cancerous cells (PC3 and LNCaP) and 50% cytotoxic concentration (CC_{50}) of normal cells (PNT2) obtained, were used to determine the selectivity indices. The results showed that, CMASE and the fractionated aqueous extract of *C. membranaceus* (AQ) showed the best selective indices (SIs) for all the cell lines. The mean SIs values of AQ and DAQ against PC3 being 85.11 and 75.41 respectively, whilst that against LNCaP cells were 2.2 and 2.07, respectively. The hexane extract also showed a good selective index against PC3 and LNCaP cells with values 18.11 and 4.42. The ethanolic fraction showed good selective index for PC3 (10.07) but not in the case of LNCaP (1.52) cells. However, the ethylacetate fractions did not exhibit good selective anticancer indices for PC3 and LNCaP cells.

Generally, all plant extracts analysed except ethyl acetate showed good selective indices against PC3 cells with high values ranging between 10.07-85.11. However, the SIs obtained for the extracts against LNCaP cells were lower when compared respectively against SI values against PC3 cells. Furthermore, only HEX, AQ and DAQ showed good SIs indices of 4.42, 2.2 and 2.02 respectively against LNCaP, whilst the other fractions SIs against LNCaP were below 2.

Table 8: Selective indices of stem fractions of *C. membranaceus* and curcumin

Compound/ Plant extract		PC3 (IC_{50} µg/ml)	Selective index (CC_{50}/ IC_{50})	LNCaP (IC_{50} µg/ml)	Selective index	PNT2 (CC_{50} µg/ml)
Curcumin		3.58 ± 1.18	1.61	8.01±1.15	0.72	5.75 ± 1.20
	HEX	55.21 ± 1.19	18.11	226.2±1.31	4.42	>1000
	EA	68.30 ± 1.15	1.28	144.3±1.44	0.61	87.49 ± 1.15
SECM	ETOH	55.43 ± 1.08	10.07	366.3±1.33	1.52	558.0 ± 1.08
	AQ	13.26 ±1.30	75.41	453.3±1.32	2.2	>1000
CMASE	D AQ	11.75 ± 1.15	85.11	482.9±1.38	2.07	>1000

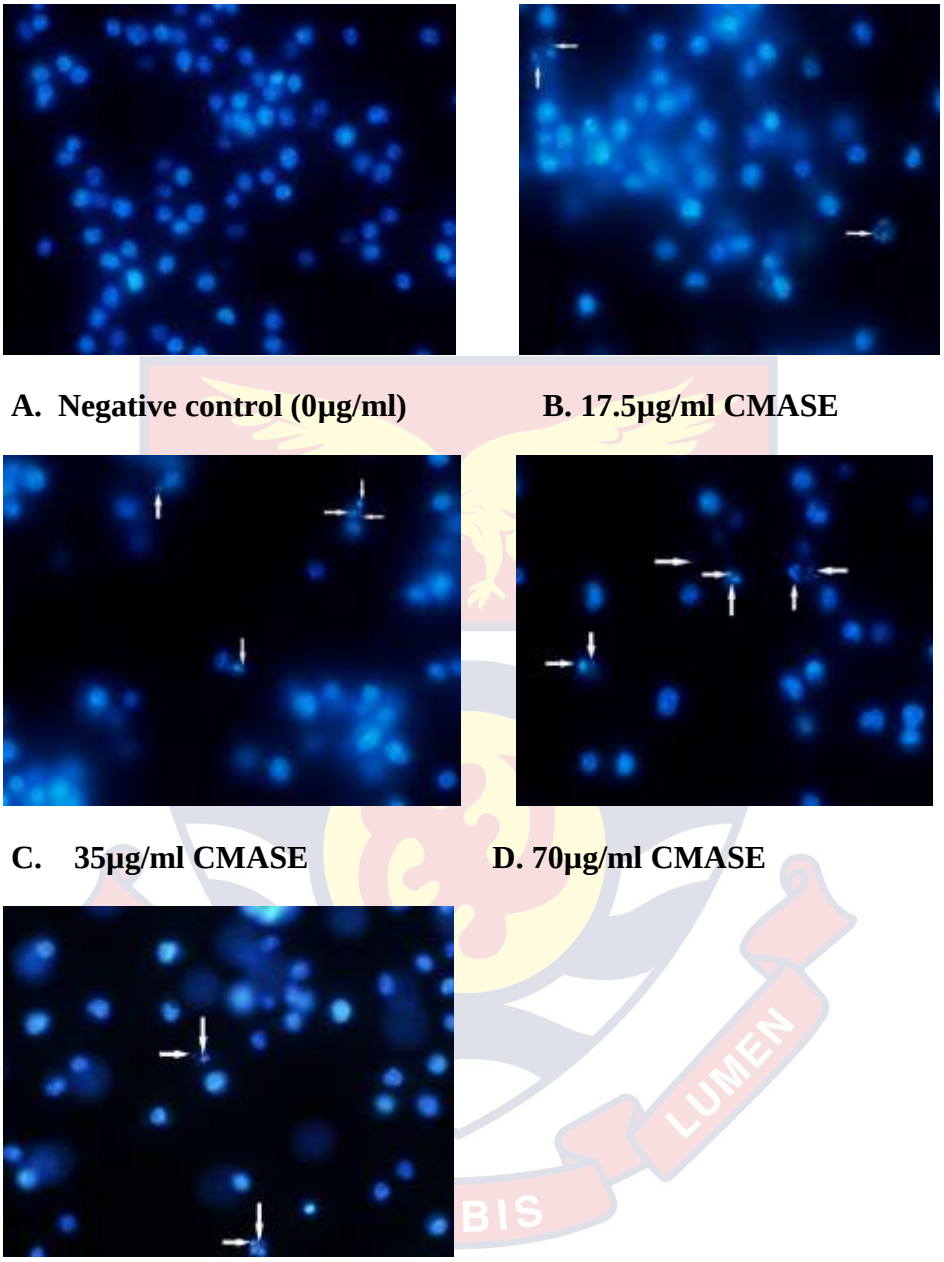
CC_{50} and IC_{50} values are mean of three independent experiments±S.E.M

Apoptosis –Hoechst Staining

Effect of CMASE on PC3 cells

Results obtained from the Hoechst 33258 examination showed induction of nuclear morphological changes of PC3 was characterized by marked nuclear condensation. There was apparent concentration-dependent increase in more apoptotic bodies after treatment of PC3 with concentrations of CMASE, and the positive control (curcumin) compared to the negative control (RPMI) cells. The negative control cells showed normal intact nuclear morphology. Figures 27: A, B, C, D and E shows induction of nuclear morphological changes of PC-3 cells by various concentrations of CMASE and the positive control.

Hoechst Stain



A. Negative control (0µg/ml) **B. 17.5µg/ml CMASE**

C. 35µg/ml CMASE **D. 70µg/ml CMASE**

E. 13.5 µM Curcumin (Positive control)

Figure 27: Apoptotic effect of CMASE on PC3 cells stained with Hoescht 33258 (magnification-40x). CMASE induces nuclei condensation and increase presence of apoptotic bodies (indicated by the arrows in PC3 cells). (a) RPMI (negative control) (b) 17.5µg/ml CMASE (c) 35µg/ml CMASE (d) 75µg/ml CMASE (e) 13.5 µM Curcumin (positive control).

DNA fragmentation and agarose gel electrophoresis

The results of DNA band pattern visualization of PC3 cells under UV illumination after 24 h treatment of 17, 35 and 70 µg/ml of CMASE is presented in Figure 28. It revealed presence of significant DNA fragmentation of PC3 cells compared to the negative control (RPMI only), and similar to the positive control (5 µg/ml) effect on PC3 cells.

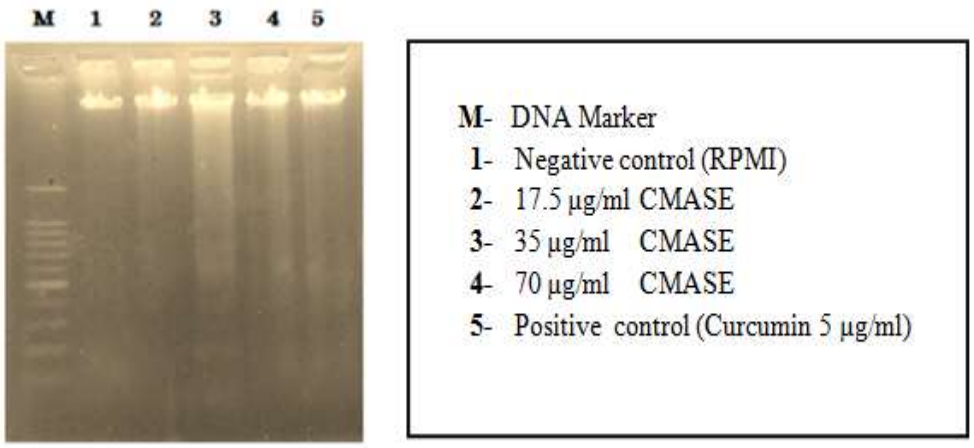


Figure 28: DNA fragmentation in PC3 cells after 24 h administration of various concentrations CMASE (17, 35 and 70 µg/ml). Lane M is the 100 bp DNA marker. CMASE induces DNA fragmentation in PC3 cells.

Apoptotic effects of CMASE on PC3 cell line from flow cytometry

Figure 29 shows Annexin V-FITC and propidium iodide (PI) staining in PC3 cells treated with 0, 17.5, 35 and 70 µg/ml of CMASE, and 5 µg/ml of curcumin for 24 h to induce apoptosis. PC3 apoptotic cells positive after Annexin V staining were seen in the bottom right quadrant, whilst late apoptosis in PC3 and dead PC3 cells positive for both AnnexinV and PI staining were observed in the top right quadrant. However, very late apoptosis and necrosis in PC 3 cells were observed in left top quadrant. Also, healthy or live PC3 cells which were negative for both stains were observed in the lower left quadrant.

Percentage viability of PC3 cells was lower in all the treated cells (CMASE and curcumin) than in the negative control (RPMI culture media only). Percentage viability of these PC3 cells in the control cells was 98.05%, compared to 78.79%, 75.86% and 72.06% for 17.5, 35, and 70 µg/ml, respectively in CMASE treated cells after 24 h. Whilst PC3 cell viability of 74.75% was obtained in the 5 µg/ml curcumin positive cells group after 24 h.

With respect to early apoptosis (lower right quadrant) which are cells bound only to Annexin V-FITC (excluding PI), percentage early apoptotic cells of 0.35%, 0.28% and 0.36% were obtained for 17.5, 35, and 70 µg/ml, respectively in the CMASE treated cells after 24 h compared to 0.25% obtained for the negative control cells. Percentage of early apoptotic PC3 cells after 24 h obtained for the positive control was 0.11%.

In the upper right quadrants where PC3 cells (were both Annexin-FITC and PI positive), higher percentages of late apoptotic/secondary necrotic cells in all the treated PC3 cells were observed compared to the control after 24 h. Percentage of late apoptotic/secondary necrotic cells in the control was 1.71% whilst that obtained for PC3 cells treated with 17.5, 35, and 70 µg/ml of CMASE were 3.22%, 2.72% and 2.74%, respectively. Percentage of late apoptotic/secondary necrotic cells obtained in the positive control was 3.19%.

At the very late stage of apoptosis (primary necrotic and some very late apoptotic cells) seen in the upper left quadrant, PC3 cells bind annexin V-FITC and stain brightly with PI. Results revealed that, percentage primary necrotic and very late apoptotic cells in the PC3 cells treated with 17.5, 35, and 70 µg/ml of CMASE were 17.64%, 21.11% and 24.81%, respectively whilst 21.95% was obtained in the positive control cells compared to the control value of 0.00%.

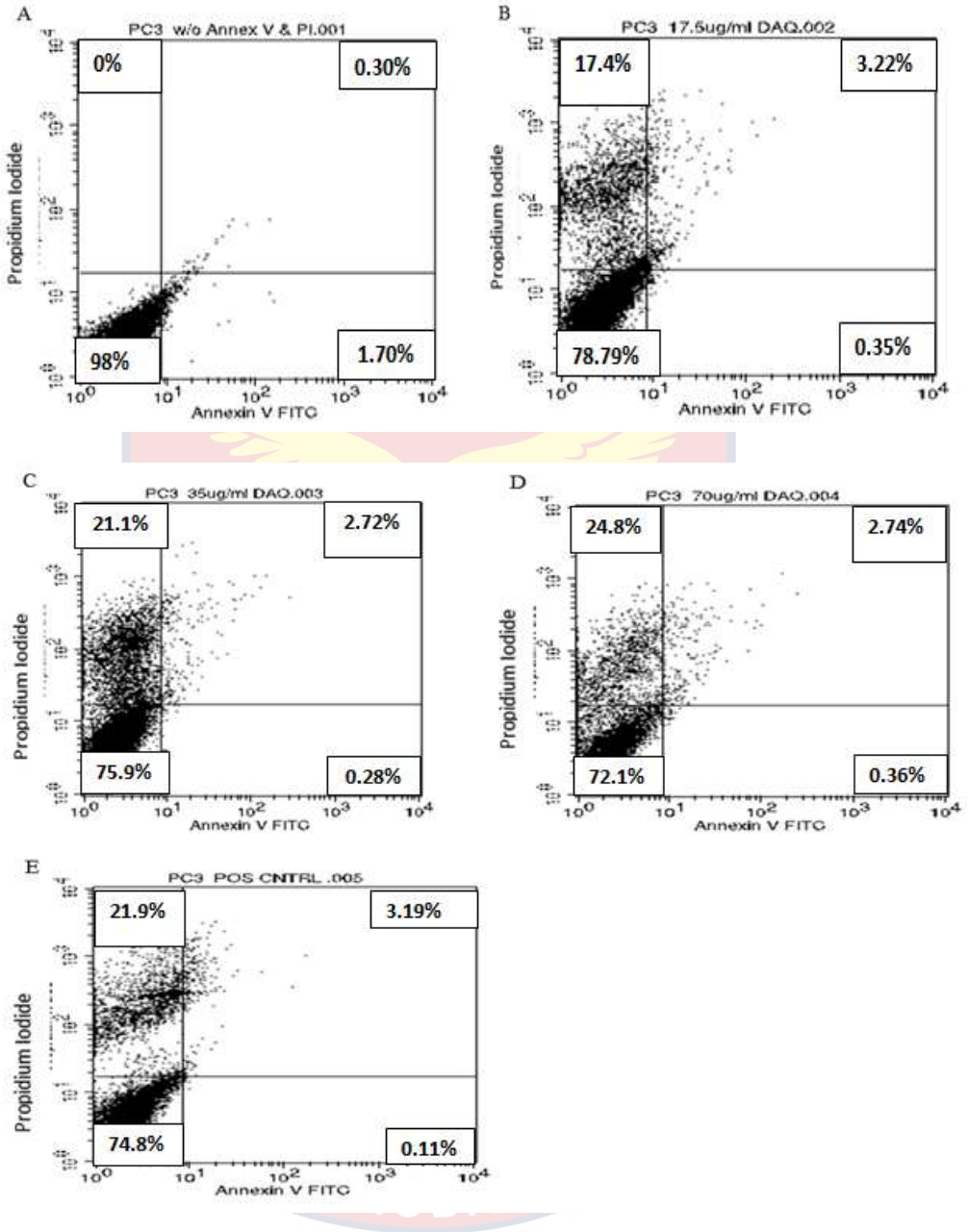


Figure 29: Flow cytometry plots showing CMASE inducing apoptosis in PC3 cells: (A) negative control (RPMI), CMASE: 17.5 µg/ml (B), 35 µg/ml (C) and 70 µg/ml (D) and positive control (curcumin 5 µg/ml). **Lower left quandrant**-Viable cells, **Lower right quadrant**-Early apoptotic cells. **Upper right quadrant**-late apoptotic cells. **Upper left quandrant**- very late apoptotic bodies.

In vivo Acute Toxicity Studies

Animal Survival and Clinical Observations

In this study, no clinical signs of toxicity such as grooming, restlessness, tremors, convulsion, dilation of pupils, pinna reflex, salivation, and lacrimation were observed in any of the treated groups during the period. All animals survived within the study period after administration of the various doses of the aqueous stem extract of *C. membranaceus*. Generally, there was an increase in body weight in all groups. The highest weight gains were observed in the low and median dose group, whilst the lowest weight gain was observed in the high dose group. However, at the end of 14-day experimental period, there were no significant differences in the body weight gained in rats treated with the aqueous stem extract of *C. membranaceus* (30, 150 and 300 mg/kg) compared to the control. All animals consumed virtually all their daily allocation of food during the study. Likewise, there was no significant difference in the average water consumption in the treated groups compared with control. The present study showed oral administration of doses of CMASE up to 5000 mg/kg did not illicit signs of toxicity or mortality in S-D rats during the period. Hence, LD_{50} value of extract was above 5000 mg/kg. The body weight changes of the various rat groups treated with acute doses of CMASE are presented in Table 9.

Table 9: Effect of extract of *CMASE* on male S-D rats body weights after 14 days of oral administration.

		Mean body weights		
Treatment	**Dose (mg/kg)**	**Day 0 (g)**	**Day 7 (g)**	**Day 14 (g)**
Control	-	116.70±5.58	138.9±3.99	163.3±3.16
	LD (1000)	123.30±6.67	139.3±5.02	169.5±6.09
C. membranaceus	MD (2500)	120.00±6.83	132.9±3.33	166.4±3.26
	HD (5000)	120.00±5.77	132.6±6.01	158.6±5.06

Data are presented as mean ± SEM. n = 5. LD=low dose, MD=median dose, HD= (High dose).

Heamatological indices

Detailed results on the haematological indices of S-D rats after 14 days administration of acute doses of CMASE are presented in Table 10. Results generally showed no significant ($p > 0.05$) changes in haematological indices among the groups. There was generally no dose-dependent pattern of effect of various doses of CMASE on the hematological parameters among the treated rat groups compared to control. Generally, values of HGB, RBC, WBC, HCT, MCV and MCH in the low and median groups were slightly lower than the control group, where as their respective values obtained in the high dose groups were slightly higher than their controls. Mean values of MCHC, LYM % and LYM count (#) in the treated groups were slightly higher than values obtained in the control group.

Mean WBC values of 12.53±2.21, 11.95±1.46 and 14.39±1.57 x 10^3 cells/µl were obtained for the low, median and high dose groups respectively, whilst 13.42±1.60×10^3 cells/µl was obtained for the control group. Furthermore, mean

RBC counts obtained at the end of this study were 7.99±0.29, 7.86±0.13 and 8.57±0.40 x 10^6/µl for low, median and high dose groups respectively compared to 8.35±0.15×10^6/µl observed in the control group. The values obtained for mean HGB values were 14.14±0.53, 13.84±0.31 and 15.28±0.25 g/dL for low, median and high dose group respectively compared to control value of 14.73±0.25 g/dL.

Table 10: Haematological parameters of S-D rats after 14 days of administration of CMASE

	Rat groups				
Heamatological Parameters	**Control**	**Low dose 1000 mg/kg**	**Median dose 2500 mg/kg**	**High dose 3000 mg/kg**	**p-value**
WBC×10^3/µl	13.42±1.60	12.53±2.21	11.95±1.46	14.39±1.57	0.770
RBC ×10^6/µl	8.35±0.15	7.99±0.29	7.86±0.13	8.57±0.40	0.285
HGB (g/dL)	14.73±0.25	14.14±0.53	14.00±0.31	15.28±0.25	0.077
HCT (%)	47.73±0.73	45.44±2.07	44.50±0.72	48.58±1.68	0.190
MCV (fL)	57.18±1.33	56.92±1.69	56.64±0.8	56.78±1.05	0.992
MCH (pg)	17.63±0.35	17.72±0.49	17.58±0.32	17.88±0.42	0.954
MCHC (g/dL)	30.85±0.22	31.18±0.26	32.48±1.2	31.45±0.15	0.323
PLT × 10^3/µl	767±96.7	576.8±26.85	533.8±50.97	631±29.37	0.059
LYM (%)	68.65±2.23	74.52±3.25	68.06±2.26	71.33±2.39	0.302
LYM(# ×10^3/µl)	9.25±1.24	9.31±1.75	8.40±0.93	10.38±1.46	0.792

Data are presented as mean ± SEM. n = 5.

MCV values in the high, median and low dose groups were 56.78±1.05, 56.64±0.8 and 56.92±1.69 fL, respectively and were all slightly lower than the control group value of 57.18±1.33 fL. Values obtained for PLTS in all the treated groups were also non-significantly ($p>0.059$) lower than the control. LYM % values for the low and high dose groups were 74.52±3.25 and 71.33±2.39%

respectively, and were slightly higher than the control (68.65±2.23%). However, the LYM % value of 68.06±2.26% obtained for median dose group was lower than other treated groups values and the control group.

Clinical Biochemical indices

Data on the effects of CMASE on biochemical parameters of rats at the end of the study period is presented in Table 11. No statistical significant differences were observed between serum levels among the treated groups compared to controls with respect to; gamma-GT, total bilirubin, direct bilirubin, indirect bilirubin (IBIL), total cholesterol (TC), total triglycerides, HDL and LDL indices. Also, no dose-dependent pattern of effect of CMASE was observed generally in these treated groups. Significant ($p<0.001$) differences were observed between the treated and control groups for all the liver enzymes indices; ALT, AST and ALP that were examined. However, no dose-dependent patterns of the effect of CMASE on these liver indices were observed. With respect to serum mean values obtained for total proteins, globulins and albumin, significant ($p<0.001$) differences were observed between in mean values obtained for the treated and control groups. Furthermore, values obtained for these indices in the treated groups did not show any dose-dependent pattern. Generally, the mean lipid indices for TG, TC, HDL and LDL obtained in the low and median dose group were lower than their respective control mean values, whilst the high dose values for these indices were slightly higher than their respective control group mean values.

Table 11: Biochemical indices of S-D rats treated with acute doses of *CMASE* after 14 days.

Biochemical Parameters	Control	Low dose 1000mg/kg	Median dose 2500mg/kg	Group 3 5000mg/kg	p-value
T-bil (umol/l)	0.60±0.35	0.38±0.16	0.44±0.16	0.35±0.06	0.839
D-bil (umol/l)	1.28±0.24	1.23±0.16	1.00±0.02	1.51±0.16	0.228
IBIL (umol/l)	0.85±0.25	1.10±0.25	1.14±0.09	0.98±0.19	0.752
ALT (U/L)	78.15±0.92	60.75±0.57$^{!}$	87.83±0.13$^{!L}$	73.75±0.86^{aLM}	<0.001
AST (U/L)	267.70±0.52	245.30±0.50$^{!}$	395.38±0.04^{L}	301.90±0.59^{LM}	<0.001
ALP (U/L)	390.78±0.30	435.55±0.32$^{!}$	449.08±0.95^{L}	304.75±0.39^{LM}	<0.001
gamma-GT	1.38±0.27	1.60±0.52	1.90±0.58	1.75±0.19	0.843
TP (g/l)	66.73±0.71	58.55±0.25$^{!}$	63.23±0.64$^{!L}$	66.68±0.20^{LM}	<0.001
ALB	35.05±0.78	29.55±0.98$^{!}$	35.40±0.57^{L}	34.38±0.59^{b}	<0.001
Glo	31.68±0.89	28.13±0.40*	28.85±0.93	33.78±0.97^{LM}	<0.001
AST/ALT	3.45±0.17	4.53±0.46	5.15±0.67	3.40±0.17^{c}	0.025
TC	2.45±0.19	2.09±0.12	1.98±0.17	2.51±0.16	0.087
TG	1.46±0.15	1.27±0.12	1.44±0.14	1.80±0.29	0.279
LDL-C	0.59±0.09	0.46±0.02	0.45±0.03	0.58±0.06	0.198
HDL-C	0.99±0.04	0.89±0.04	0.91±0.08	1.09±0.04	0.064
CREA	64.30±0.80	52.39±0.95$^{!}$	56.55±0.53$^{!b}$	57.87±0.80$^{!L}$	<0.001
UREA	6.15±0.55	6.73±0.32	6.65±0.16	5.34±0.43	0.084
UA	211.60±0.19	148.33±0.65	175.35±0.02$^{!L}$	156.23±0.74$^{!LM}$	<0.001

Data are presented as mean ± SEM. n =5. *p<0.05 compared to control, $^{!}$p<0.001 compared to the control group. ^{a}p<0.001 median group compared to control. $^{L, M}$ p<0.001 compared to low and median dose group, ^{m}p<0.01 compared to median dose, ^{b} p< 0.01 compared to low dose.^{c}p<0.05 compared to the median dose group.

With respect to the kidney function indices, significant differences were observed between the treated and control groups for urea. Mean serum values for creatinine and uric acid were significantly lower ($p<0.001$) in the treated groups compared to control group.

Effect of CMASE on creatine kinase and lactate dehydrogenase

Administration of acute doses of CMASE did not significantly alter serum creatinine kinase and lactate dehydrogenase level after 14 days of administration. However, a dose-dependent elevation of serum levels for these indices was observed with increasing doses of CMASE. Mean serum values for creatine and lactate dehydrogenase levels are presented in Table 12.

Table 12: Effect of CMASE on serum creatinine kinase and lactase dehydrogenase values in S-D rats after 14th day of acute dose administration.

Groups	Creatine Kinase	p-value	Lactase Dehydrogenase	p-value
Control	613.8 ± 73.65		8197 ± 1052	
Low dose	671.0 ± 68.31		9101 ± 1537	
Median dose	719.6 ± 78.65	**0.5940**	9624 ± 1609	**0.3415**
High dose	748.8 ± 72.54		10077 ± 755	

Data are presented as mean ± SEM. n = 5.

Effect of CMASE on some serum markers of oxidative stress

Data obtained on effect of acute doses of CMASE on serum oxidative stress markers in this study revealed no significant difference in values between the treated and control groups for GSH and MDA. Also, there were marginal dose-dependent decline in serum GSH and MDA levels in treated groups was observed

with increasing dose of CMASE, 14 days post administration of CMASE. With respect to SOD, there was significant elevation (p=0.0002) in the serum levels of the treated groups compared to the control group values. *Post hoc* Tukey analysis revealed no significant difference between the low dose group value and control. However, both the median and high dose group values were significantly higher (p<0.001) respectively than the control group value. Further analysis revealed that, the median and high dose groups were significantly higher (p<0.01, p<0.001 respectively) than the low dose group values. Also mean SOD levels in high dose group was significantly (p<0.05) higher than median dose group levels. The detailed mean serum SOD, GSH and MDA values obtained from the various groups of rats 14 days post administration of acute doses of CMASE is presented in Table 13.

Table 13: Effect of acute doses of *CMASE* on SOD, GSH and MDA levels in serum of male S- rats after 14 days of oral administration.

Treatment Groups	**Dose (mg/kg)**	**SOD U/ml (x10 $^{-7}$)**	**GSH (mg/ml)**	**MDA[TBars] moles/mg (x10^{-7})**
Control	-	0.49±0.05	138.9±3.99	163.3±3.16
Low dose	1000	0.56±0.12	139.3±5.02	169.5±6.09
Median Dose	2500	0.92±0.09***	132.9±3.33	166.4±3.26
High dose	5000	1.06±0.01***^	132.6±6.01	158.6±5.06

Data are presented as mean ± SEM. n = 5. '***' p<0.001 compared to control and low dose group, '^' indicates that high dose group was significant (p<0.05) compared to median group.

Histological examination

Organ weights of male S-D rats

Examination of weights of targeted organs 14 days post administration of the acute doses did not result in any significant differences in their mean organ weights of the liver, heart and kidney of treated groups compared to the control group. Neither was there any dose-dependent effect of CMASE on any of the organs in the treated groups. The highest mean organ weights obtained for the kidney and the heart were observed in the low dose group, whereas the highest mean liver weight was observed in high dose group. The median dose group generally had the lowest organ weights among the treated and control groups. It is worth noting that, the prostate weights of the rats in all the groups were less than 0.1g at the end of the study. Table 14 presents the detailed mean organ weights obtained for the treated and control groups 14 days post administration of acute doses of CMASE.

Table 14: Mean organ weights of S-D rats on the 14th day after administration with oral acute doses of CMASE

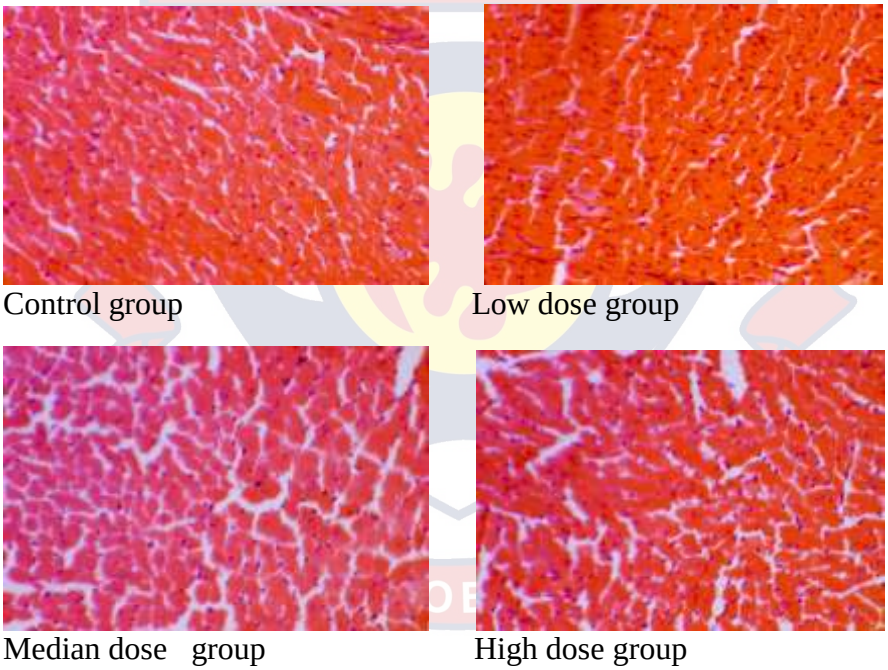
Organ	Control	Low dose	Median dose	High dose	p-values
Kidney (g)	1.20±0.08	1.24±0.06	1.04±0.08	1.16±0.07	0.97
Liver (g)	6.73±0.19	6.87±0.40	6.67±0.21	7.00±0.52	0.91
Heart (g)	0.62±0.02	0.64±0.04	0.56±0.03	6.00±0.05	0.52

Data are presented as mean ± SEM. n = 5.

Gross pathological and macroscopical examination

Gross pathological examination of the heart, liver and kidney of the treated groups compared to the control group generally did not reveal any sign of abnormality or damage. Further histological assessment of sections of these organs

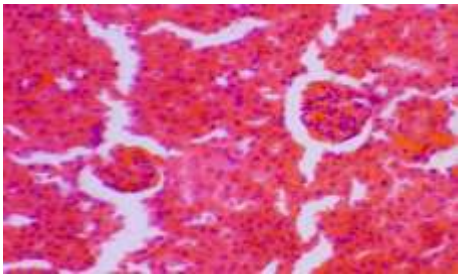
did not reveal any significant differences or abnormality. Histological examination of the hearts of rats in the treated groups showed normal cardiac tissue with no infarctions and leucocyte infiltrations. The kidneys of the treated groups showed normal renal tissue, with few congested renal tissues, dilated tubules, and the absence of tubular or glomerular necrosis. Examination of liver of treated groups revealed few areas containing dilated central veins but absence of any hepatocellular necrosis in the tissues. Presented in Figures 30, 31 and 32 are the histological photomicrographs of the heart, kidney and liver, respectively examined from the treated groups and the control groups.



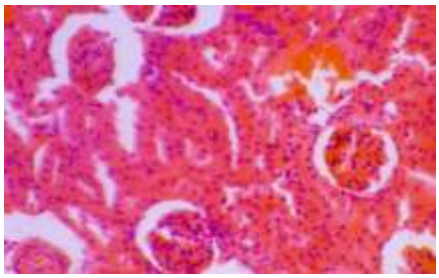
Control group Low dose group

Median dose group High dose group

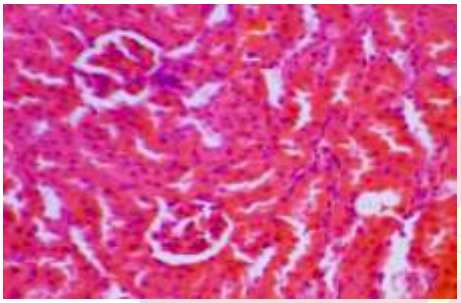
Figure 30: Photomicrograph of cross section of the cardiac muscle in S-D male rats of the control, low, median and high dose groups; showing normal features of myocardium. (H & E stain, x40).



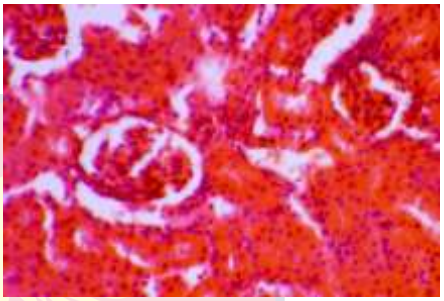
Control



Low dose group

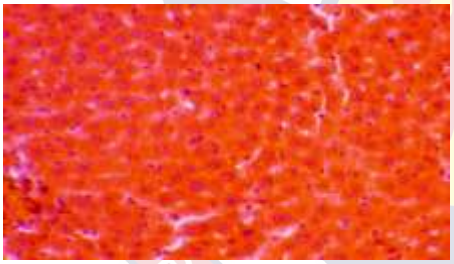


Median dose group

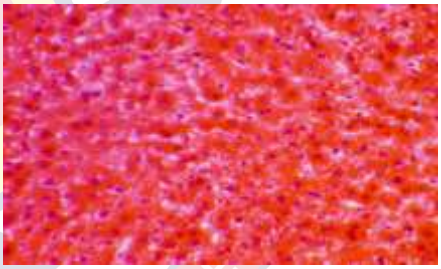


High dose group

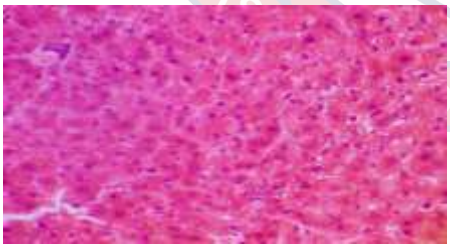
Figure 31: Photomicrograph of S-D rat kidney showing the normal architecture of the glomeruli (G) and tubules (T) of the control low, median and high dose groups animals (H & E stain, x40).



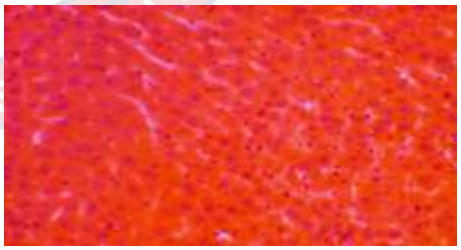
Control group



Low dose group



Median dose group



High dose group

Figure 32:Photomicrograph of cross section of the hepatic tissue in S-D rats showing normal features of hepatocytes in the control, low, median and high dose groups animals. (H & E stain, x40).

Sub-chronic oral toxicity

Rats' survival and clinical toxicity observations

Daily administration of CMASE daily by oral gavage for 90 days did not induce any clinical or behavioural signs of toxicity such as; changes in fur, salivation, sleep, mucous membrane, dilation of pupils, tremors, restlessness, grooming, and diarrhoea in the treated groups. No deaths were observed during the experimental period.

Body weights of rats in all groups generally increased with no significant difference ($p>0.05$) between treated groups compared to control during the 90 days experimental period (figure 33). The highest gain in body weight was observed in the control group, whilst the lowest weight gain was observed in the low dose group. No significant variation or reduction in food and water consumption was observed in all the treated rat groups compared to the controls throughout the study.

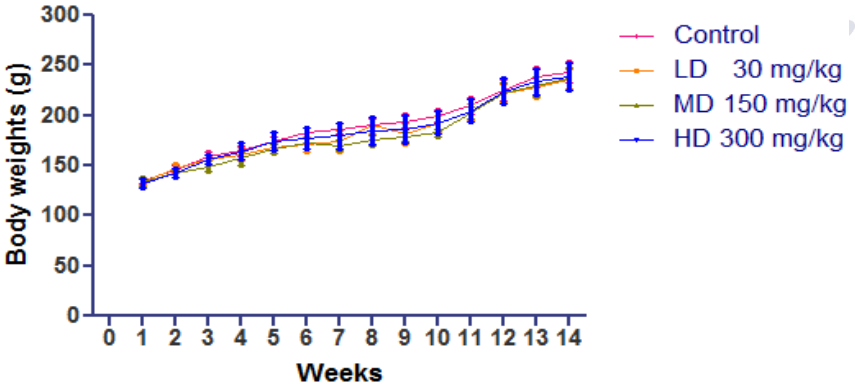


Figure 33:Effect of *C. membranaceus* on weights of Sprague-Dawley rats treated with aqueous stem extract for 90 days. Values are represented as mean ± SEM. n=6.

Effect of aqueous stem extract of *C. membranaceus* on haematological parameters of male S-D rats after 90 days

Oral administration of various doses of CMASE to male S-D rats for 90 days by gavage did not have significant effect on most of the haematological parameters assessed; RBC, WBC, HCT, MCV, MCH, PLT, LYM # and LYM %.

Table 15: Haematological parameters of S-D rats on 91st day after commencing oral administration of CMASE to S-D male rats.

Heamatological Parameters	Control	30 mg/kg Low dose	150 mg/kg Median dose	300 mg/kg High dose	p-values
WBC×10^3/µl	15.38±2.67	16.44±2.94	14.64±1.80	16.53±1.34	0.924
RBC ×10^6/µl	8.13±0.15	8.42±0.14	8.77±0.28	8.64±0.21	0.179
HGB (g/dL)	13.70±0.36	14.08±0.11*	14.54±0.39*	14.43±0.39*	0.019
HCT (%)	44.98±1.28	46.00±0.48	47.30±1.58	45.64±0.79	0.531
MCV (fL)	55.33±0.68	55.20±0.61	54.02±1.14	52.68±1.21	0.302
MCH (pg)	16.83±0.17	16.88±0.15	16.60±0.55	16.60±0.45	0.922
MCHC (g/dL)	30.47±0.26	30.65±0.17	30.76±0.31	31.36±0.37*	0.045
PLT × 10^3/µl	638.7±117.3	544.2±144.4	676.8±89.14	798.8±117.4	0.517
LYM (%)	76.02±3.43	77.42±2.04	83.76±1.03	80.62±1.39	0.095
LYM# ×10^3/µl	13.07±0.58	12.97±2.57	12.27±1.53	13.75±1.04	0.963

Data are presented as mean ± SEM. n = 5. '*' p <0.05 compared to control

However, significant elevation (p=0.019) of mean HGB values in treated groups was observed compared to their mean control group value. Tukey *post-hoc* analysis showed significant elevation (p<0.05) of all the treated group HGB values compared to control, but the effect was not dose-dependent. Also, significant elevation of MCHC values in the treated group was also observed compared to the control mean value. Tukey *post-hoc* analysis showed significant difference (p<0.05) between the high dose group and the control. The detailed results on the mean haematological parameters obtained from the treated and control groups after 90 days of oral administration of CMASE by oral gavage are presented in Table 15.

Clinical chemistry parameters after sub-chronic administration of CMASE

Effect of CMASE on kidney function parameters

During the experimental period, no significant differences (p>0.05) were observed between the treated and control groups for all the serum kidney function indices examined; creatinine, urea, potassium, sodium ions, and chloride ions. Generally, there were no dose-dependent effects of CMASE on almost all the kidney indices, except on the mean serum creatinine and sodium ion levels: which showed a marginal decline from the low dose groups to the high dose groups. Mean serum creatinine values obtained for low, median and high dose groups were; 46.14±1.11, 44.93±4.25 and 42.13±1.85 umol/l, respectively compared to the control group value of 44.10±2.30 umol/l. Blood mean urea values of 7.83±0.40, 7.32±0.42 and 7.63±0.71 mmol/l were obtained for the low, median and high dose groups compared to the control group value 7.42±0.58 mmol/l.

Analysis with respect of blood electrolytes after subchronic administration of CMASE revealed that, mean serum potassium ion values of 5.52±0.20, 5.25±0.25 and 5.31±0.42 mmol/l were obtained for the low, median and high dose group respectively, compared to the control value of 5.48±0.42 mmol/l. Mean serum sodium ions obtained for the treated groups were 141.30±0.48, 140.5±0.46 and 140.40±0.85 mmol/l, respectively for the low, median and high dose groups whilst that of the control value was 141.30±0.36 mmol/l. With respect to mean serum chloride ions, values of 107.10±0.42, 107.50±0.35 and 107.2±1.10 mmol/l were obtained in the low, median and high dose groups respectively, compared the control group result of 106.2±0.38 mmol/l. Summary of findings of the effects CMASE on serum kidney function indices are illustrated in Figure 34.

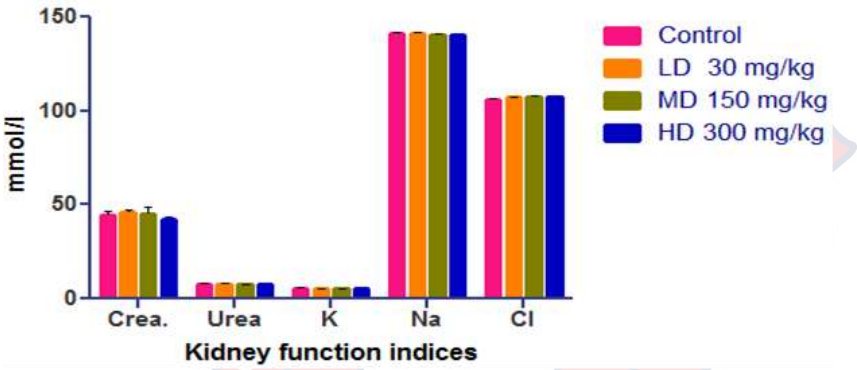


Figure 34: Effect of administration of subchronic treatment with CMASE on kidney function indices in male S-D rats compared to control groups. Values are represented as mean ± SEM, n=5.

Effect of aqueous stem extract of *C. membranaceus* on liver function indices

At the end of the study period, there was no significant ($p>0.05$) difference observed between the treated and control groups values for all the liver enzymes indices; ALT, AST and ALP that were examined. Mean values obtained for serum

ALT and AST, showed a general elevation in all the treated groups compared to the control. A dose-dependent elevation pattern was observed for the AST values, whilst mean serum ALP values in the treated groups except in the low dose were lower compared to the control group. Mean serum ALT values of 91.28±13.15, 86.75±10.33 and 91.37±20.69 U/L were obtained in the low, median and high dose groups, respectively, compared to the control value of 77.04±8.47 U/L. The mean serum AST values for the treated groups were 218.70±45.69, 248.80±11.29 and 282.70±19.25 U/L respectively, for the low, median and high dose groups compared to the control value of 201.10±23.34 U/L. Mean serum ALP values of 277.00±46.30, 230.00±32.12 and 222.50±35.77 U/L were obtained for low, median and high dose groups respectively, whilst control group value was 248.30±42.15 U/L. Effect of administering various doses of the aqueous stem extract of *C. membranaceus* on liver function parameters after 90 days is illustrated in Figure 40.

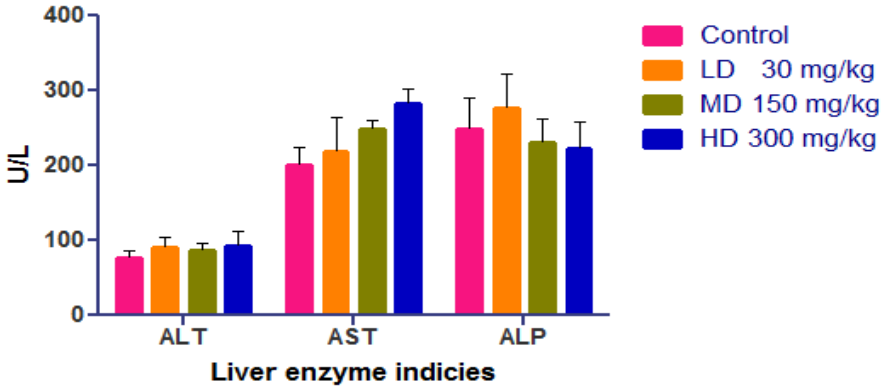


Figure 35: Effect of sub-chronic administration of CMASE on liver function indices in male S-D rats compared to control group. Values are represented as mean ± SEM, n=5.

Effect of *C. membranaceus* on serum protein, globulin and albumin indices

Oral administration of sub-chronic doses of CMASE did not have any significant effect on the mean serum values of total proteins, globulins and albumin, obtained for treated groups and compared to the control after 90 days. Furthermore, there were no dose-dependent trends observed in the treated groups. The effect of the sub-chronic oral administration of various doses of CMASE on serum protein, albumin and globulin parameters after 90 days is presented graphically in Figure 35.

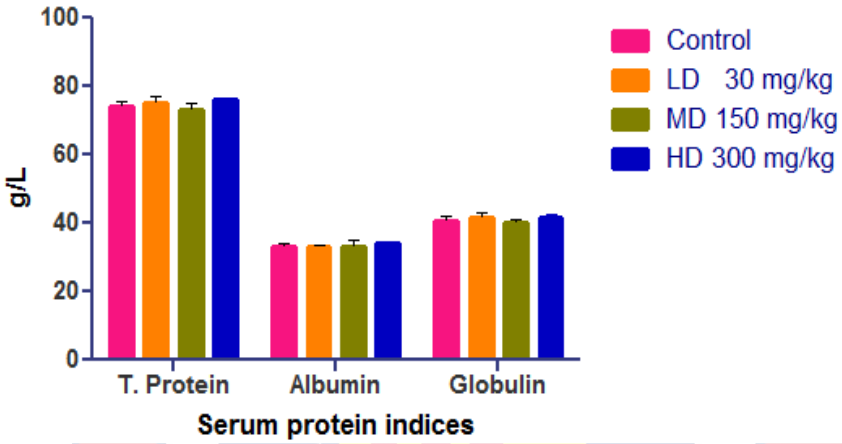


Figure 36: Effect of sub-chronic oral administration of aqueous stem extract of *C. membranaceus* on serum total protein, albumin and globulins in male S-D rats compared to control group. Values are represented as mean ± SEM, n=5.

The mean serum total protein values obtained in low, median and high dose groups were 75.28±1.60, 73.18±2.14 and 76.05±0.56 g/l, respectively compared to the 74.05±1.71 g/l for the control group. The mean serum albumin values obtained for the low, median and high dose group were 33.25±0.24, 33.18±1.74, and 34.20±0.61 g/l, respectively compared to the control mean value of 33.33±0.82 g/l. Mean serum albumin values of 32.25±0.70, 31.97±0.38 and 32.35±0.42 g/l were

obtained for the low, median and high dose groups, respectively whilst that of the control group was 30.73±0.54 g/l.

Effect of *C. membranaceus* on serum lipid profile parameters

No statistically significant differences ($p>0.05$) were observed between the treated and control groups for all the serum levels of total cholesterols, triglycerides, HDL and LDL after 90 days oral administration of sub-chronic doses of CMASE. Generally, they were marginally lower serum mean levels of all the lipid indices in the treated groups compared to control, except with respect to total serum mean triglycerides which showed marginal elevations in the low and high dose group values. Serum mean total cholesterol obtained for the low, median and high dose groups were 1.56±0.10, 1.50±0.17 and 1.52±0.15 mmol/l, respectively compared control value of 1.67±0.09 mmol/l. Mean serum triglyceride values of 0.52±0.08, 0.44±0.07 and 0.54±0.12 mmol/l were obtained for low, median and high dose groups, respectively compared to the control value of 0.40±0.03 mmol/l. Mean serum HDL values for low, median and high dose groups were 1.10±0.06, 0.10±0.10 and 1.12±0.07 mmol/l, respectively compared to 1.18±0.10 mmol/l obtained in the control group. The mean serum LDL values obtained in the low, median and high dose groups were 0.40±0.05, 0.39±0.05 and 0.33±0.04 mmol/l, respectively whilst their control group mean value was 0.40±0.03 mmol/l. Sub-chronic effects of oral administration of CMASE to S-D rats for 90 days on serum lipid indices examined is illustrated graphically in Figure 37.

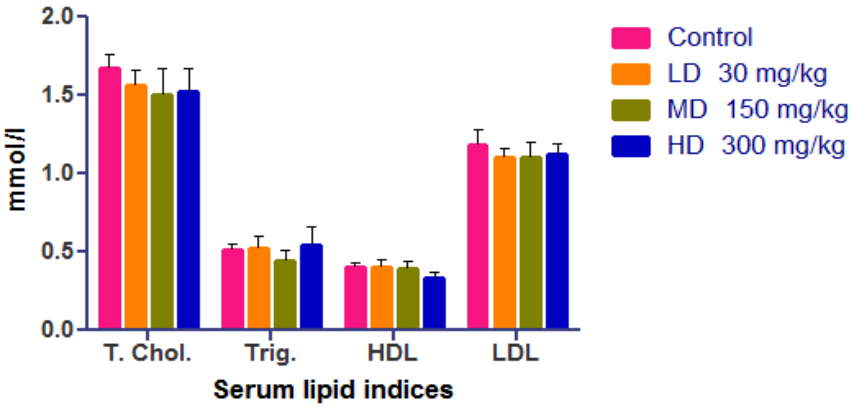


Figure 37: Effect of sub-chronic oral administration of CMASE on serum total cholesterols, triglycerides, HDL and LDL in male S-D rats compared to control group. Values are represented as mean ± SEM, n=5.

Effect of *C. membranaceus* on the skeletal and heart muscles biochemical markers

Summary on findings of the effect of oral sub-chronic doses of CMASE on rat serum LDH, skeletal and heart muscle markers in treated and the control groups after 90 days of administration is presented in Table 16.

No statistical significant differences ($p>0.05$) were observed between the treated and control groups in the serum levels of lactate dehydrogenase and creatine-kinase total indices examined, except in creatine-kinase-MB index. Furthermore, it was only with creatine-kinase-MB that the treated group showed some sort of dose-dependent pattern. That is, decreasing serum levels with increasing dose of CMASE, even though the low dose group value was higher than the control.

Mean serum creatine kinase total values obtained for low, median and high dose groups were 771.3±33.89, 777.7±40.19 and 733.5±99.35 u/l, respectively and were

higher than the control group mean value of 671.5±49.13 u/l. The serum mean creatine-kinase-MB values obtained in the low, median and high dose groups were 1536±58.26, 1424±80.29 and 676.7±11.67 u/l, respectively whilst that of control group was 1347±78.07 u/l. The results of the latter revealed that, there was significant ($p < 0.001$) difference between the serum mean value obtained in treated groups compared to the control group. Tukey's *post hoc* analysis revealed signficant ($p<0.001$) higher values in the low and median groups compared to the high dose group values. The serum mean lactate dehydrogenase values obtained for the low, median and high dose groups were 1276±24.17, 1175±80.69 and 1177±69.92 u/l, respectively and that of the control group was 1228±55.40 u/l.

Table 16: Effect of CMASE on muscle enzymes after 90 days oral administration in S-D rats

	Cardiac Enzymes and Lactate Dehydrogenase (LDH) Markers					
Rat Groups	**Creatine Kinase (U/L)**	**p-value**	**Creatine Kinase-MB (U/L)**	**p-value**	**Lactate dehydrogenase (U/L)**	**p-value**
Control	671.5±49		1347±78.07		1228±55.40	
Low dose (30 mg/kg)	771.3±33.89	0.681	1536±58.26	<0.001	1276±24.17	0.668
Median dose (150 mg/kg)	777.7±40.19		1424±80.29		1175±80.69	
High dose (300 mg/kg)	733.5±99.35		676.7±11.67		1177±69.92	

Data are presented as mean ± SEM. n = 5. P <0.001 for control, low and median values compared to high group.

Effects of CMASE on fasting blood glucose levels

Baseline fasting blood glucose values obtained in control group was comparable to those obtained in rat groups assigned to be treated with CMASE on day 0, and there was no significant difference (p=0.3512) between them. Administration of oral sub-chronic doses of CMASE to the rats after 90 days, resulted in a slight dose-dependent decline in the fasting blood glucose values from the low dose group (7.48±0.64 mmol/L) to the high dose group (6.53±0.27 mmol/L). However, there was no significant difference (p=0.4213) were observed in the values obtained in the treated group compared to the control. The detailed results on the potential antihyperglycaemic effect of CMASE in normal S-D rats after 90 days of oral administration is presented in Table 17.

Table 17: Effect of CMASE on fasting blood sugar of S-D rats after 90 days oral (*p.o*) gavage.

	Fasting Blood Sugar values (mmol/L)			
Rat Groups	**Day 0**	**p-value**	**Day 90**	**p-value**
Control	5.45±0.21		7.64±0.57	
Low dose	5.83±0.20	0.3512	7.48±0.64	0.4213
Median dose	5.30±0.20		6.68±0.42	
High dose	5.47±0.22		6.53±0.27	

Data are presented as mean ± SEM. n = 5.

Effect of *C. membranaceus* on liver antioxidative stress markers

Oral administration of sub-chronic doses of CMASE to S-D male rats during the 90 days did not have any significant effect on liver antioxidative stress markers SOD, GSH, CAT and MDA examined. Furthermore, dose-dependent elevations of

mean liver values were observed in SOD, GSH and CAT, whilst there was a dose-dependent decline in mean MDA values. Mean SOD values obtained showed marked elevations in the low, median and high dose group values of 3.40±0.90 3.70±0.47 and 4.84±0.96 U/ml, respectively compared to the control value of 2.00±0.00 U/ml. Mean serum GSH values obtained also showed consistent increase in values in the low, median and high dose group being 2.75±0.18, 2.82±0.33 and 3.24±0.25 x 10^{-7}mg/ml, respectively whilst control value was 2.21±0.14x 10^{-7} mg/ml. Also, mean serum catalase values obtained in the low, median and high dose group were 2.32±0.16, 3.22±0.87 and 3.23±0.48 kU/L, respectively compared to control value of 2.08±0.75 kU/L.

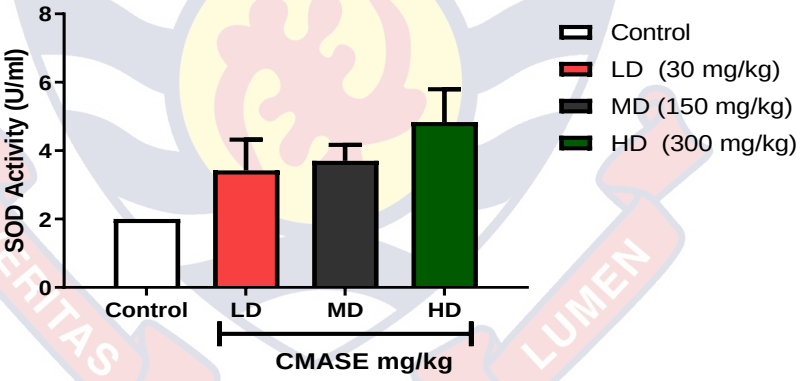


Figure 38: Effect of sub-chronic oral administration of CMASE on liver SOD levels in male S-D rats. Values are represented as mean ± SEM, n=5.

The mean serum MDA (thiobarbituric [TBars]) control value at the end of this study was 3.16 ±0.07*10^{-7} moles/mg, whilst values obtained in low, median and high dose group values of 3.26±0.17, 3.15±0.12 and 3.09±0.04*10^{-7} moles/mg respectively. However, there was no significant (p=0.7556) difference between the

control and treated groups values of MDA. Data on oral subchronic effects of CMASE to rats for 90 days on liver antioxidative stress markers examined in S-D male rats is illustrated graphically as Figures 38-41.

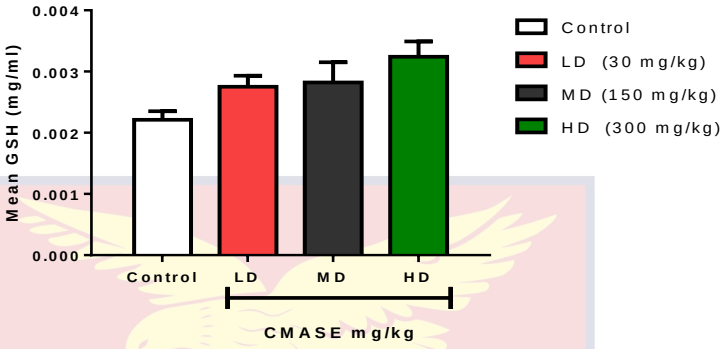


Figure 39: Effect of sub-chronic oral administration of CMASE on liver GSH levels in male S-D rats. Values are represented as mean ± SEM, n=5.

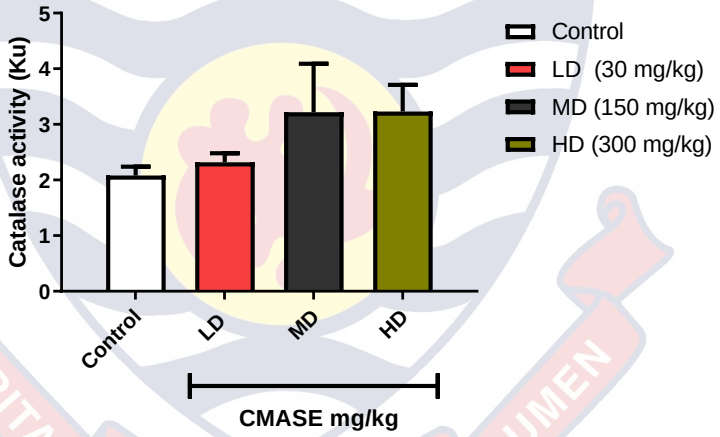


Figure 40: Effect of sub-chronic oral administration of CMASE on liver CAT levels in male S-D rats. Values are represented as mean ± SEM, n=5.

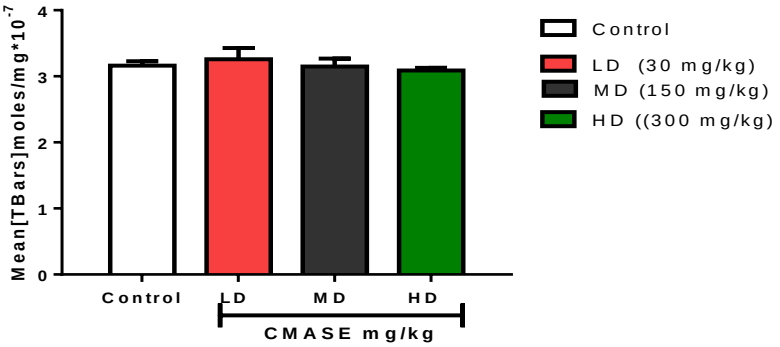


Figure 41: Effect of sub-chronic oral administration of CMASE on liver MDA [TBars] levels in male S-D rats. Values are represented as mean ± SEM. n=5.

Histopathological examination of organs after sub-chronic toxicity

Organ weights and relative organ to body weights of male S-D rats

Oral administration of the aqueous stem extract of C. *membranaceus* by gavage for 90 days in all treatment groups did not result in any significant difference in mean organ weights of the liver, heart, kidney nor the prostate of treated groups compared to the control group. The highest mean organ weights obtained for the liver, prostate and the heart were observed in the control dose group whereas with the kidney, highest mean weight was observed in the high dose group. All the mean weights of the prostate in the treated groups were slightly lower than the control group. However, the lowest mean organ weight for the prostate was also observed in the low dose group. Dose-dependent effect of CMASE was observed in the mean liver weights. The rest of the mean organ weights did not show any dose-dependent pattern. The detailed mean organ weights obtained for the treated and control groups after 90 days of administration of the aqueous stem extract of *C. membranaceus* is shown in Table 18.

Table 18:Mean organ weight of S-D rats treated with oral doses of CMASE for 90 days

	Mean organ weights				
Organs	**Control (g)**	**Low dose (g)**	**Median dose(g)**	**High dose (g)**	**p -values**
Liver	8.46 ± 0.48	8.42 ± 0.45	8.20 ± 0.90	7.98 ± 0.47	0.94
Heart	0.84 ± 0.50	0.72 ± 0.06	0.76 ± 0.09	0.82 ± 0.08	0.75
Kidney	1.30 ± 0.08	1.26 ± 0.05	1.26 ± 1.00	1.40 ± 0.09	0.61
Prostate	0.56 ± 0.09	0.40 ± 0.05	0.42 ± 0.04	0.52 ± 0.09	0.31

Data are presented as mean ± SEM. n = 5.

Also, analysis of organ to body weight ratio revealed that, there was no significant gain or loss in the relative organ weights in any of the treated groups compared to the control group. The highest relative organ weights with respect to the prostate showed that, the relative organ mean value of the low dose group was the lowest compared to the other groups, whilst that of the median and high dose groups had similar relative mean weight values. The average organ to body weight ratios of S-D rats given sub-chronic doses of CMASE orally for 90 days is presented in Table 19.

Table 19: Relative organ to body weight ratios of S-D rats orally gavaged with extract of *C. membranaceus* for 90 days

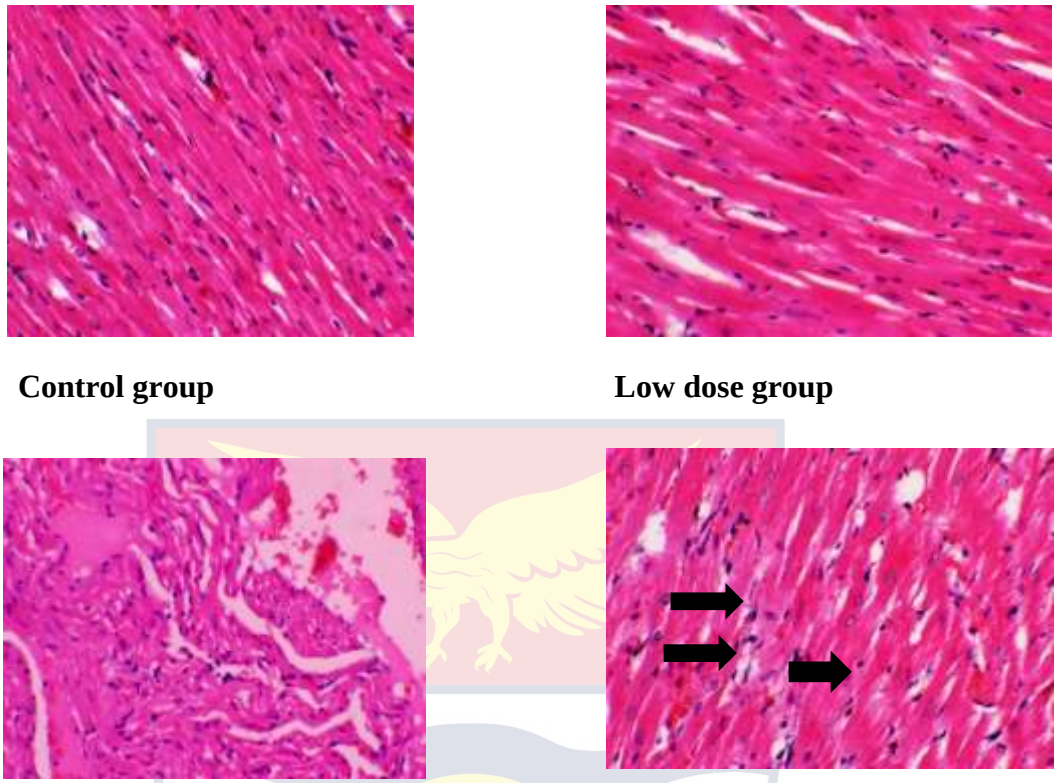
	Mean organ to body ratios				
Organs	**Control**	**Low dose**	**Median dose**	**High dose**	**p-values**
Kidney(g)	0.55±0.04	0.56±0.04	0.58±0.01	0.59±0.04	0.790
Liver(g)	3.56±0.16	3.58±0.14	3.60±0.16	3.54±0.14	0.993
Heart(g)	0.36±0.03	0.34±0.04	0.36±0.03	0.36±0.02	0.948
Prostate(g)	0.20±0.03	0.18±0.01	0.19±0.02	0.19±0.01	0.894

Data are presented as mean ± SEM. n = 5.

$$\text{Relative organ weight to body weight} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100$$

Gross pathological and macroscopical examination

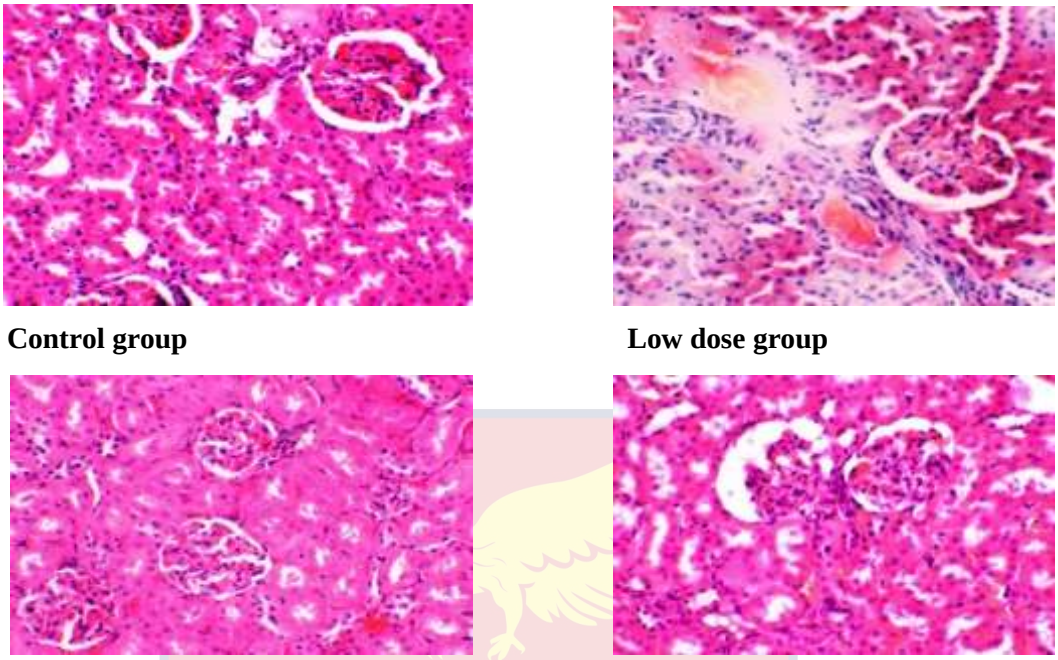
Generally, gross pathological examinations of the heart, liver, kidney and prostate of the treated groups compared to the control group did not reveal any sign of abnormality or damage after 90 days of oral administration of CMASE. Also, histological examination of sections of these organs except for the prostate, did not show any significant differences or abnormality which could be attributed to the extract. Histological assessment of liver sections showed that, micro structures were generally normal in all groups indicating regular hepatocyte. Inflammatory cells were found on both control and test group slides which was indicative of ongoing condition in the experimental subjects prior to the study. Heart sections had generally normal architecture of the heart muscle, and inflammatory cells were generally absent in all groups. However, mild nuclear changes were seen in the treated groups. Kidney sections revealed there were no marked extensive changes in the kidneys. Tubular integrity and glomerular structure (glomerulus and renal tubules) were mostly normal. Tubular cast with epithelial sloughing was evident in very few rats including some of the controls. Prostate sections revealed variable grades of changes in secreting epithelium ranging from normal columnar glandular epithelial cells in the controls transitioning (reduction of this columnar epithelium gradually) and finally squamous epithelial cells (very flattened) in treated groups. Photomicrographs of the histological sections of the heart, liver, kidney and prostate of the control and treated groups respectively are presented in Figures 42-45.



Control group **Low dose group**

Median dose group **High dose group**

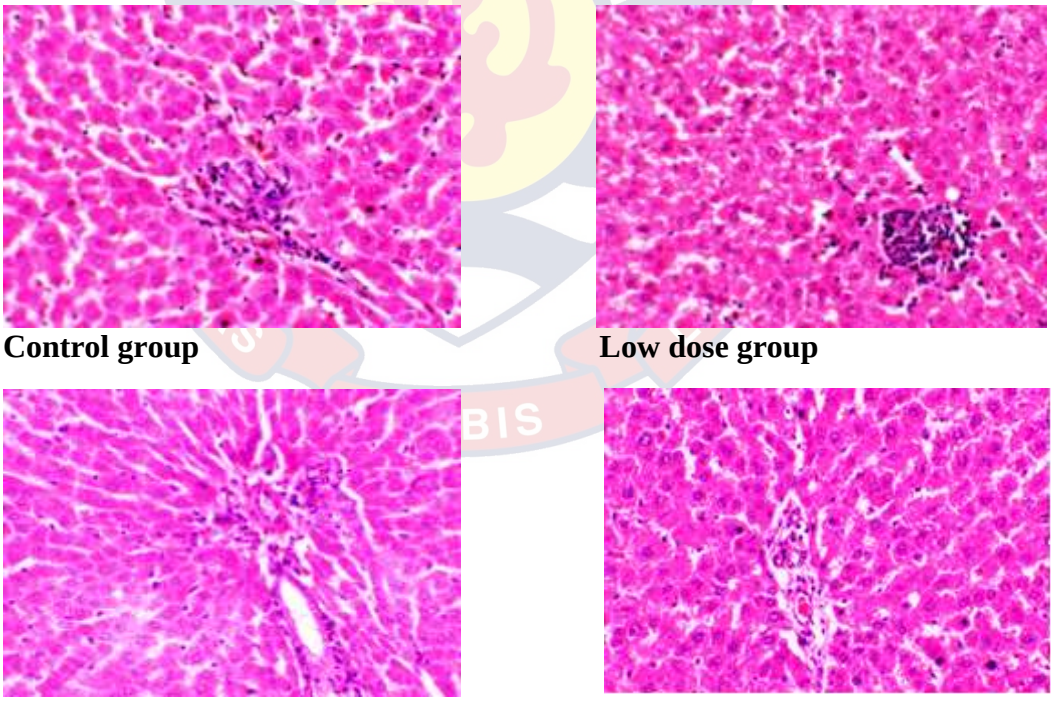
Figure 42: Photomicrograph of the cross section of the cardiac muscle in S-D male rats for control group and treated groups after 90 days of sub-chronic administration of CMASE orally; showing normal myocardial fiber with nuclear integrity maintained in the various groups, with arrows in high dose group showing mild changes in nuclear integrity. (H & E stain, x40).



Control group **Low dose group**

Median dose group **High dose group**

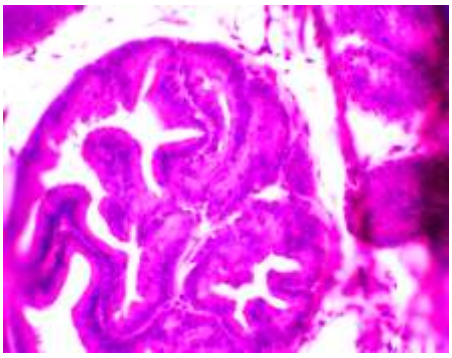
Figure 43: Photomicrograph of S-D male rats kidney above showing the normal architecture of the glomeruli and tubules of the control and treated groups after 90 days of CMASE administration orally. (H & E stain, x40).



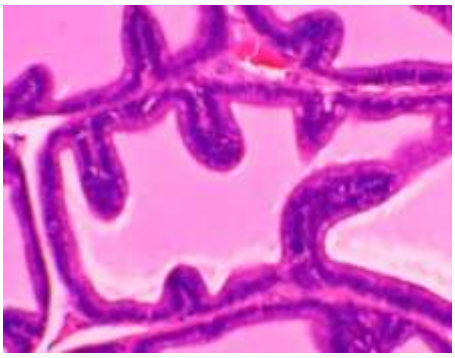
Control group **Low dose group**

Medium dose group **High dose group**

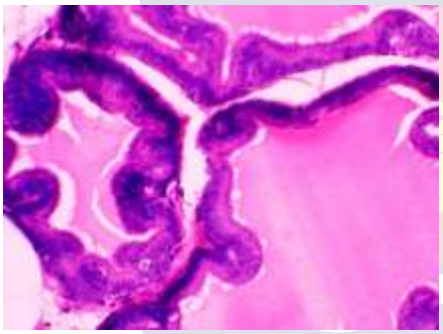
Figure 44: Photomicrograph of cross section of the hepatic tissue in S-D rats showing normal features of hepatocytes in the control and treated groups after 90 days of CMASE administration orally (H & E stain, x40).



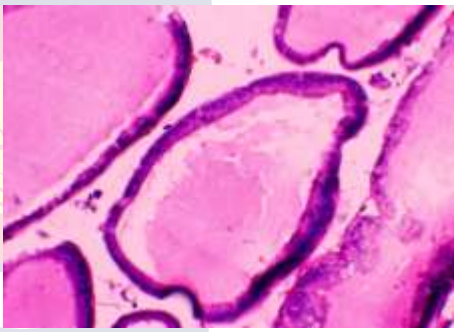
Control group



Low dose group



Median dose group



High dose group

Figure 45: Photomicrograph of S-D rat prostate after 90 days sub-chronic administration of CMASE; showing normal architecture of the columnar glandular epithelium, stroma and minimal acini in the control group, and a diminishing of the columnar epithelium and increasing clear acini in treatment groups (H & E stain, x40).

Efficacy of CMASE on testosterone-induced BPH rats

Effect of CMASE on S-D rats body weights, prostate weights and prostatic index

Daily food consumption among castrated S-D rats after induction of BPH reduced during the early phase (two weeks) of the study, and this resulted in loss of body weights in these rats. Improvement in appetite with gradual increase in body weights were later observed in the BPH rats treated with various doses of CMASE and positive control (finasteride) till the end of the study. The mean body weight of negative control was 257.7±6.49 g, whilst those of the model BPH group, low,

median, high and finasteride groups were 248.0±3.78, 224.7±12.82, 227.9±9.18, 241.6±2.58, and 228.4±4.98 g respectively at the end of the study. No dose-dependent or significant changes in mean body weight effects were observed among the treated groups compared to model group. The effect of various doses (30, 150 and 300 mg/kg) of CMASE, and finasteride on body weights of testosterone-induced BPH rats is presented Figure 46.

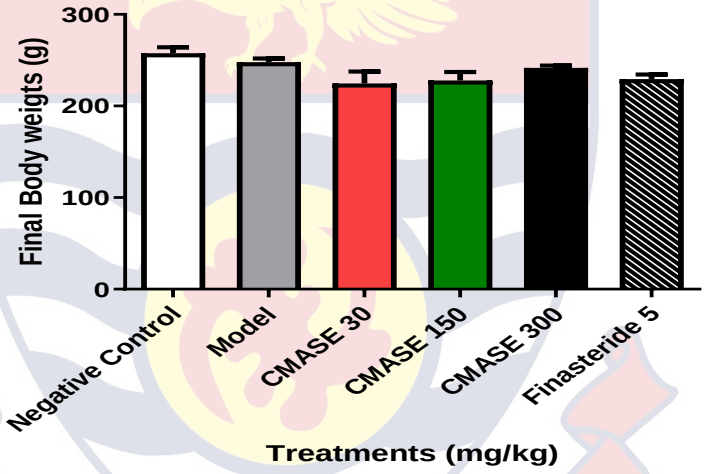


Figure 46: Effect of oral administration of CMASE (30, 150 and 300 mg/kg) and finasteride (5 mg/kg) on body weights in BPH induced male S-D rats after 28 days. Values are represented as mean ± SEM, n=5.

With respect to effects of CMASE on prostatic weights of rats, the model showed significant increase ($p<0.01$) in prostate weight of 1.64±0.13 g compared to negative control (1.10±0.06 g). Furthermore, all the CMASE and finasteride treated groups showed decrease in prostate weights at the end of the study period. However, no significant differences in reduction in the prostate weights of the CMASE treated groups were observed compared to model group, except in the

finasteride group ($p<0.01$). Among the CMASE treated groups, lowest mean prostate weight of 1.26±0.09 g was observed in the median dose group compared to the 1.36±0.11 and 1.38±0.08 g obtained for the low and high dose groups respectively. Further analysis revealed, treatment with 30, 150 and 300 mg/kg of CMASE reduced the prostate weight by 51.9, 70.4 and 48.1 %, respectively. The finasteride group's mean prostate weight of 1.16±0.11 observed in this study reduced by 88.8% in weight compared to the model group prostate weight. The effect of various doses of CMASE (30, 150 and 300 mg/kg) and finasteride on testosterone induced BPH rat prostate weights is presented in Figure 47.

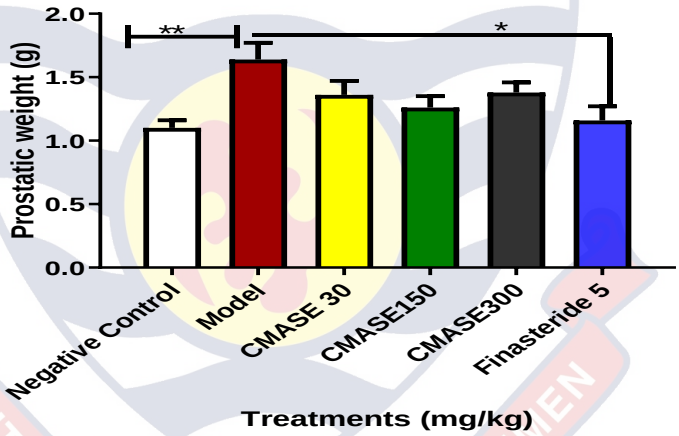


Figure 47: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on prostatic weights in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. Significant **$p<0.01$ model group compared to negative control, *$p<0.05$ model compared finasteride group.

Relative prostate weight (prostatic index) is a common index used to assess BPH development. Analysis of the prostatic indices of the harvested prostates from the various rat groups revealed, significant increase ($p<0.05$) in the prostate index in the

model BPH group value of 0.66 ±0.05 compared to that of the negative control (0.42±0.02). Mean prostatic indices of 0.61±0.07, 0.56±0.05 and 0.56±0.38 were obtained for the low, median and high dose CMASE treated groups respectively, whilst that of finasteride group showed marked reduced prostatic index of 0.49±0.05. No significant difference in prostatic indices were observed among the treated groups compared to the model group. Lowest prostatic index was observed in the finasteride treated group. Details on the prostatic indices of the various treated and control groups of rats at the end of the study period is presented in Figure 48.

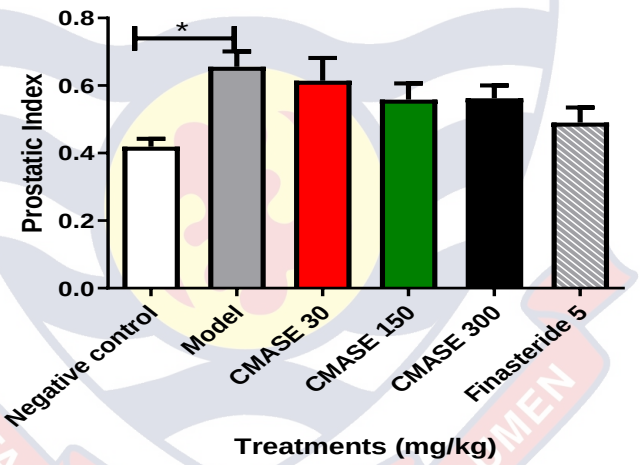


Figure 48: Effect of oral administration of of CMASE (30-300 mg/kg) and finasteride (5 mg/kg) on prostatic indices in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. *p<0.05 model group control compared to negative control.

Mechanistic *in vivo* screening for (anti)androgenic properties of CMASE

Effect of CMASE on prostatic dihydrotestosterone (DHT) level

Effect of various doses of CMASE on prostatic DHT levels is presented in Figure 49. There was marked elevation of mean prostatic DHT value from 2.65 ±0.15 pg/ml in the negative control to 3.44±0.23 ng/ml in model BPH group. The prostatic DHT levels in the CMASE and finasteride treated groups showed marked reduction compared to the model BPH group. Mean values of 2.92±0.23, 2.72±0.19 and 2.86±0.09 pg/ml were obtained for low, median and high CMASE dose groups, respectively whilst that of the finasteride group was 2.41±0.19 pg/ml. No dose-dependent effect of the various doses of CMASE were observed on the prostatic DHT levels. Significant reduction ($p<0.05$) of prostatic DHT levels was observed in only the finasteride among treated groups compared to model group.

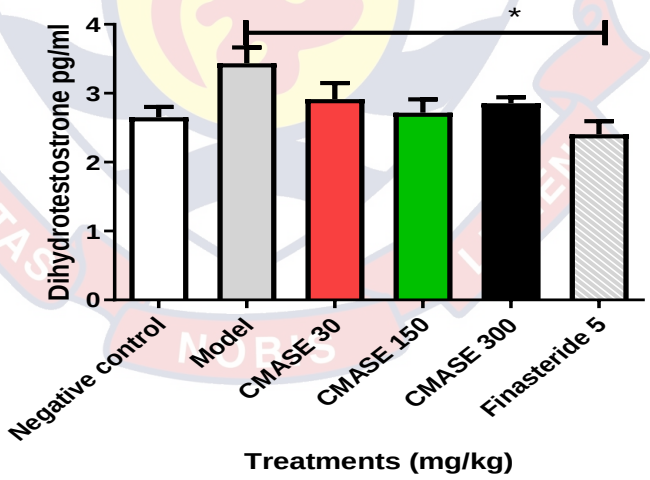


Figure 49: Effect of oral administration of various doses of CMASE (30, 150& 300 mg/kg) and finasteride (5 mg/kg) on prostatic DHT levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. *$p<0.05$ model group compared to positive control.

Effect of CMASE on prostatic 5-alpha reductase levels

Results of the effects of various doses of CMASE on prostatic 5-alpha reductase levels is presented in Figure 50. Generally, significant (p=0.0081) non-dose dependent reductions in prostatic 5-alpha reductase activity were observed in CMASE and finasteride treated groups compared to model group. Significant reduction ($p<0.01$) in 5-alpha reductase levels was observed in the median dose group compared to the model group. Also, significant reductions ($p<0.05$) in prostatic 5-alpha reductase levels were observed in the high dose group and positive controls compared to model. Marked elevation of prostatic 5-alpha reductase mean value of 65.63±1.86 ng/ml was observed in model compared to negative control value of 48.56±4.19 ng/ml. Prostatic mean values of 49.70±5.77, 43.68±2.99 and 45.32±3.49 ng/ml were obtained in the low, median and high CMASE dose groups, respectively whilst that for the finasteride group was 44.41±4.92 ng/ml.

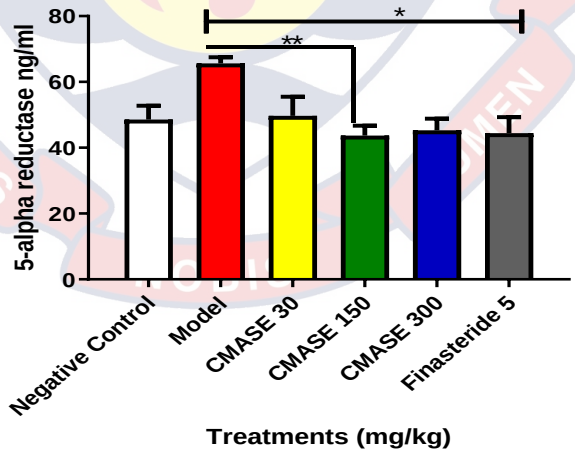


Figure 50: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on prostatic 5-alpha reductase levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. **p<0.01 model compared to median group, and *p<0.05 model compared to high and positive group.

Effect of CMASE on prostatic testosterone level

The results of the effects of administration of various doses of CMASE on serum testosterone levels are presented in Figure 51. Marginal elevation in prostatic mean testosterone levels of 0.017±0.00 pg/ml was obtained in model group compared to the negative control value of 0.015±0.00 at the end of the study. Mean values of 0.016±0.00, 0.010±0.00 and 0.012±0.00 pg/ml were obtained for low, median and high CMASE dose groups, respectively whilst that of the finasteride group was 0.011±0.00 pg/ml. No dose-dependent pattern was observed in the extract treated groups, except for the significant reduction ($p<0.01$) observed in only the median dose group compared to model group.

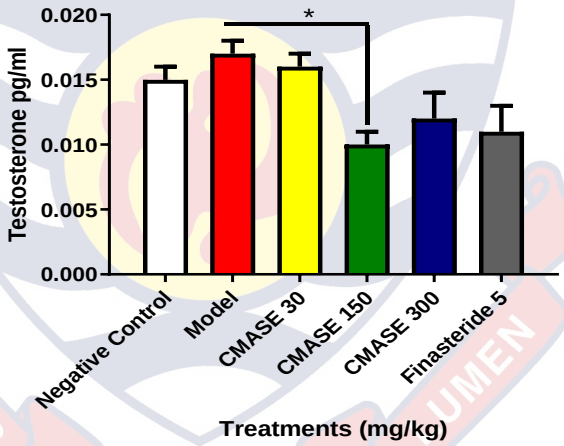


Figure 51: Bar chart showing the effect of oral administration of various doses of CMASE (30, 150 & 300 mg/kg) and Finasteride (5 mg/kg) on prostatic testosterone levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. **p<0.05 model group compared to median dose group.

Effect of CMASE on prostatic prostate specific antigen (PSA) levels

The effect of administration various doses of CMASE on prostatic PSA levels is presented in Figure 52. Significant elevation ($p<0.001$) of prostatic PSA levels was

observed in the model group compared to the negative control. Administration of various doses of CMASE to testosterone-induced BPH rat groups resulted in significant dose-dependent reduction ($p<0.001$) in prostatic PSA levels in all groups compared to the model. Mean prostatic PSA values of 0.08±0.01, 0.05±0.01 and 0.04±0.00 ng/ml were obtained for low, median and high CMASE dose groups, respectively compared to model group value of 0.12±0.01 ng/ml. Mean prostatic PSA value of the finasteride group was 0.03±0.00 ng/ml at the end of study.

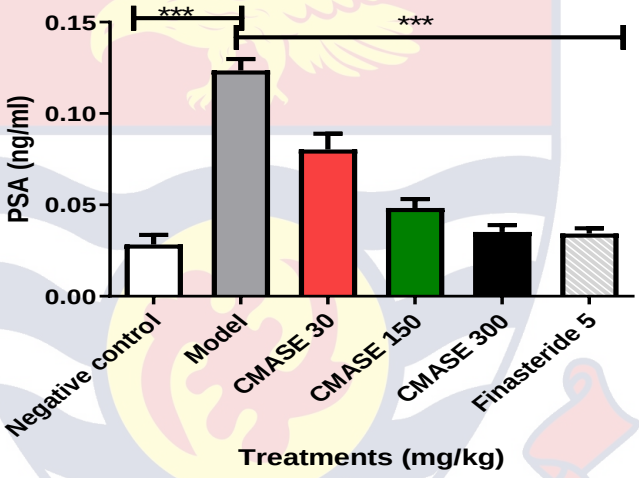


Figure 52: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on PSA levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. ***$p<0.001$ model group compared to negative control, and all the treated (CMASE and finasteride) groups.

CMASE effects on antioxidative stress markers in the prostate of BPH rats

Effect of CMASE on prostatic SOD levels

Effects of administration various doses of CMASE on testosterone induced BPH rats is presented in Figure 53. Administration of testosterone to male S-D rats

induced significant decrease ($p<0.001$) in prostatic SOD values from 2.00±0.00 U/ml (100%) in the negative control group to 0.48±0.07 U/ml (28.9%) in model group. Administration of various doses of CMASE caused a dose-dependent significant increase in prostatic SOD levels in the treated groups.

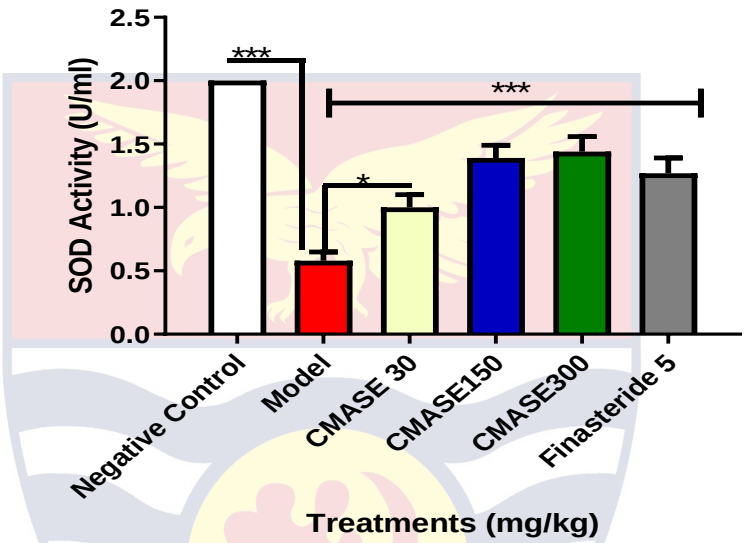


Figure 53: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on prostatic SOD levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. ***$p<0.001$ model group compared to negative control, median, high and finasteride groups. *$p<0.05$ low dose compared to model.

Mean SOD values (with percentage increase in SOD levels) of 1.00±0.10 (72.4 %), 0.64±0.12 (139.7 %) and 0.59±0.12 (148.3 %) U/ml were obtained in the low, median and high dose groups, respectively compared to model group. Mean SOD values of finasteride group was 1.27±0.12 (119 %) U/ml compared to model group. Significant increase ($p<0.001$) in SOD levels were observed between the median, high dose and finasteride groups compared to model, whilst significant ($p<0.05$) elevation was observed in the low dose group compared to model SOD levels.

Effect of CMASE on prostatic GSH levels

The effect of administration of various doses of CMASE (30, 150 and 300 mg/kg) to testosterone-induced BPH S-D rats is presented in Figure 54. Subcutaneous administration of testosterone to castrated S-D rats reduced significantly ($p<0.001$) prostatic GSH levels in the negative control group value from 6.64±0.61 mg/ml to 3.88±0.23 mg/ml in the model group. The decrease in prostatic GSH levels was reversed in a dose-dependent manner with various doses of CMASE resulting in marked increase to 4.62±0.44 and 5.17±0.51 mg/ml in the median and high dose groups respectively. No significant difference was observed in the prostatic GSH values in the treated groups compared to the model group. Mean prostatic GSH value of 3.66±0.31 mg/ml obtained in the low dose group was lower than the model group value, whilst mean prostatic GSH value was obtained 4.34±0.08 mg/ml was obtained for the positive (finasteride) control group.

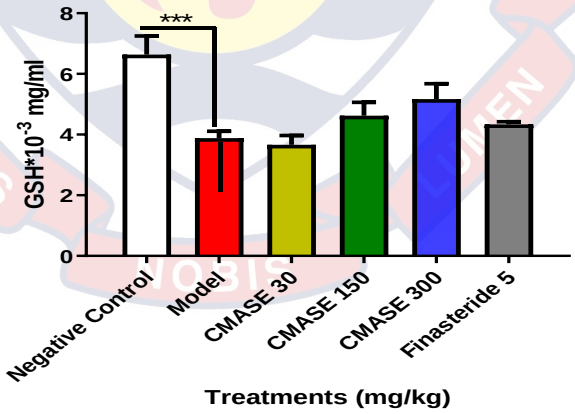


Figure 54: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on prostatic GSH levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. ***$p<0.001$ model group compared to negative control.

Effect of CMASE on prostatic MDA levels

Subcutanoeus administration of testosterone to castrated male S-D rats induced significant elevation ($p<0.001$) of malondialdehyde (MDA) prostatic levels in the model BPH group value of $5.23\pm0.22*10^{-7}$ moles/mg compared to the negative control value of $3.43\pm0.13*10^{-7}$ moles/mg. Furthermore, there was decrease in the prostatic MDA levels in the various CMASE treated and positive control groups. However, significant decline ($p<0.001$) in MDA levels was only observed in the finasteride group compared to the model group. Mean MDA values obtained for low, median, high and finasteride groups were 4.90±0.21, 5.15±0.05, 4.82±0.06, and $3.48\pm0.10*10^{-7}$ moles/mg, respectively. No dose-dependent pattern was observed in the CMASE treated groups. Effect of administration of various doses of CMASE on prostatic MDA levels is presented in Figure 55.

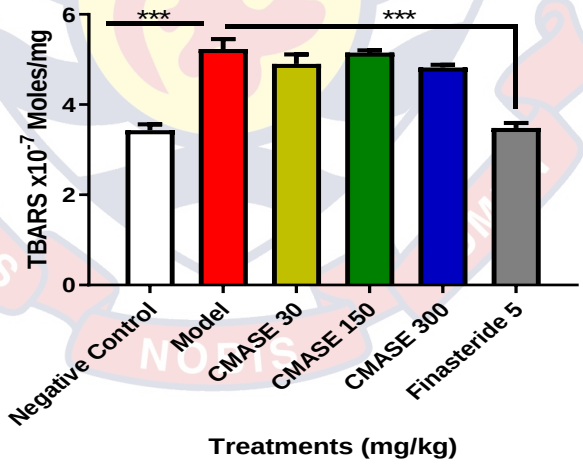


Figure 55: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on prostatic MDA levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. ***p<0.001 model group compared to negative control, and positive (finasteride) control compared CMASE (30,150, 300 mg/kg) and model groups.

CMASE effects on antioxidative stress markers in the liver of BPH rats

Effect of CMASE on liver SOD levels

The effect of administering various doses of CMASE on liver SOD levels in testosterone-induced BPH S-D rats is presented in Figure 56. Subcutanoeus administration of testosterone to male S-D rats induced marked but non-statistically significant decrease of liver SOD levels in negative control group from 0.86±0.09 moles/mg to 0.31±0.09 moles/mg in model group. Administration of various doses of CMASE caused elevations of liver SOD levels in a non-dose dependent manner. Mean liver SOD values of 1.16±0.29, 1.17±0.25 and 1.04±0.09 moles/mg were obtained in the low, median and high dose groups, respectively. Significant increased ($p<0.05$) in SOD levels were observed were observed only in the low and median dose groups compared to model group. The mean liver SOD value of 0.86±0.09 moles/mg was observed in the positive control group was the lowest among the treated groups.

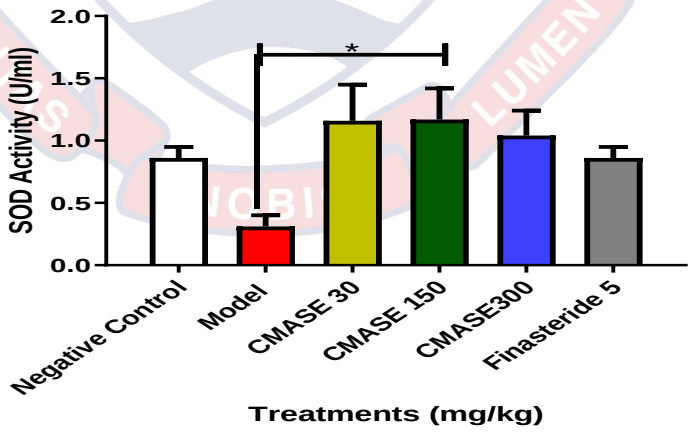


Figure 56: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on liver SOD levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. *p<0.05 model group compared to median and high dose CMASE groups.

Effect of CMASE on liver GSH levels

Subcutaneous administration of testosterone to castrated male testosterone-induced BPH S-D male rats caused a non-significant reduction in serum GSH levels to 2.10±0.50*10^{-3} mg/ml in the model group compared to negative control group value of 2.50±0.20 mg/ml. However, administration of various doses of CMASE caused signficant increased in liver GSH levels in treated groups. Mean liver GSH values increased to 2.90±0.70, 3.70±0.60 and 3.70±0.51 mg/ml in the low, median and high dose groups, respectively. *Post hoc* tukey analysis revealed highest and significant elevation ($p<0.001$) in liver GSH levels (6.00±0.31mg/ml) was observed in the positive control compared to model group. Other significant differences between CMASE and positive control groups were also observed. Effect of various doses of CMASE on rat liver GSH levels is presented in Figure 57.

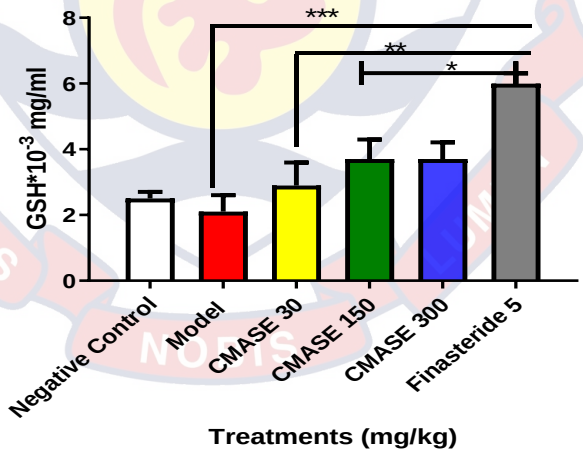


Figure 57: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on liver GSH levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. ***p<0.001 model group compared to positive control. **p<0.01 low dose compared to positive control, and *p<0.05 median and high dose groups compared to positive (finasteride) control.

Effect of CMASE on liver MDA levels

Subcutaneous administration of testosterone to castrated male S-D rats induced significantly elevatin ($p<0.001$) of liver mean MDA values from 5.82±0.21 moles/ng in the negative control group to $7.52\pm0.20^{*}10^{-7}$ moles/mg in the model group after induction of BPH. Mean liver MDA values of 8.68±0.23, 8.12±0.43 and $7.70\pm0.18^{*}10^{-7}$ moles/mg were obtained in the low, median and high dose groups, respectively. Means serum MDA values in treated groups were generally higher than the model group MDA levels, with the low dose value being significantly ($p<0.05$) higher than model group. Mean serum MDA value of 7.22±0.81 moles/ng obtained in the positive control group was significantly lower ($p<0.01$) than the low dose group, but comparable to model group. Effect of administration of various doses of CMASE on rat liver MDA levels is presented in Figure 58.

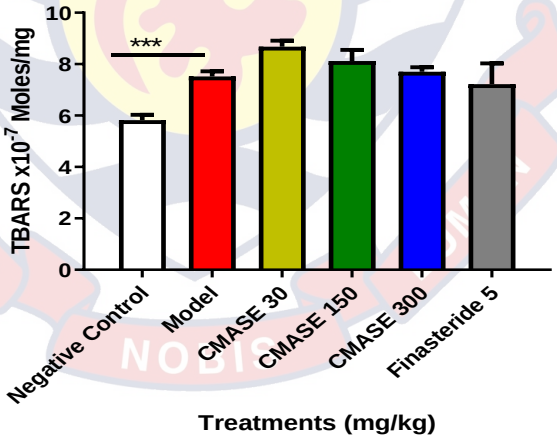
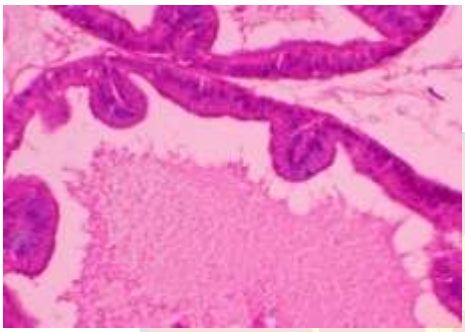


Figure 58: Effect of oral administration of CMASE (30, 150 & 300 mg/kg) and finasteride (5 mg/kg) on liver TBARS levels in male S-D rats after 28 days. Values are represented as mean ± SEM. n=5. ***p<0.001 model group compared to negative control group. **p<0.01 finasieride group compared to low dose group. *<0.05 LD compared to model group.

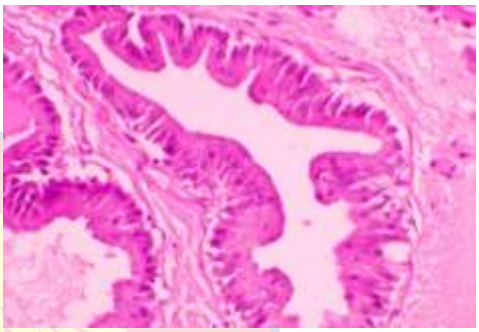
CMASE effects on prostatic histopathology of BPH male S-D rats

In this study, with the exception of the negative control male S-D rats which did not receive testosterone injection during the study period of 28 days, all the treated groups exhibited benign prostatic hyperplasia confirmed by the photomicrograph (x40) of the model BPH group (Figure 59B). Photomicrograph of cross section of the prostate gland of the negative control group revealed a thick layer of involuntary muscles (stromal cells) surrounding the prostate gland, thick intraglandular epithelial with few columnar infoldings of these epithelial cells and a clear gland lumen as presented in figure 59A. Figure 59B shows a photomicrograph (x40) of cross section of testosterone-induced model BPH gland of S-D rats indicating thin connective tissues (fibrous stromal cells) due to enlarged tubules, proliferation of epithelial with many columnar infoldings, reduced volume gland lumen compared to negative control, and increased presence of basal and secretory cells. The photomicrographs (Figure 59C and 59D) of the cross sections of the prostate glands treated with low dose (30 mg/kg) and median dose (150 mg/kg) of CMASE revealed reduced intraglandular epithelium, much less coloumnar infoldings of the epithelium compared to model BPH group, increased gland lumen, reduced stroma, and thicker connective tissue compared to model (naive control) group. Features indicated normal recovery in the texture of tubules but less than the negative control group. Photomicrographs of cross section of prostate glands treated with high dose (150 mg/kg) of CMASE and positive control appeared similar. High dose group (figure 59E) showed simple thick layered intraglandular epithelium without any infoldings into the lumen, clear acini, presence of basal cells and increased density

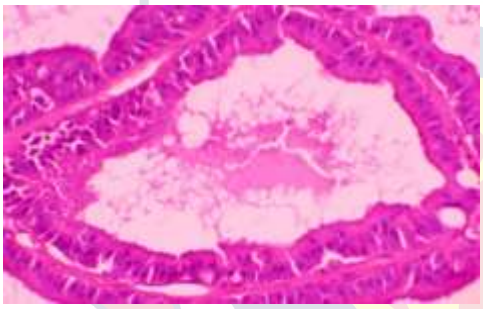
of interglandular smooth muscles fibre and stromal cells. A cross section of positive control prostate gland showed reduced intraglandular epithelium, absence of columnar epithelium infoldings, increased intraglandular lumen and density of fibromuscular matrix as seen in Figure 59 F.



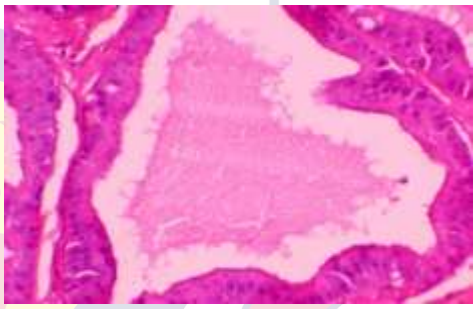
Negative control group (A)



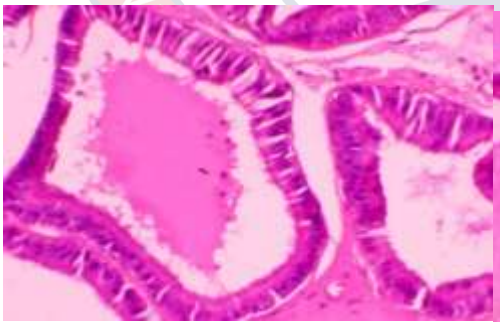
Model BPH group (B)



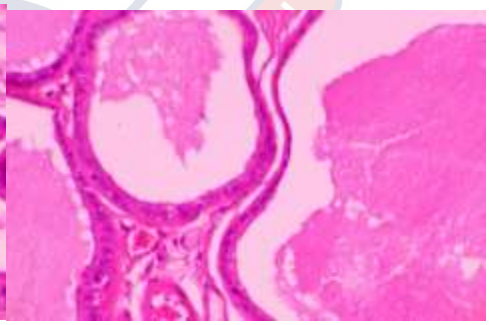
Low dose group (C)



Median dose group (D)



High dose group (E)



Positive control group (F)

Figure 59 (A-F): Photomicrograph of S-D rat prostate showing normal columnar architecture in control (A), and increased infolding columnar glandular epithelium, stroma and minimal acini and model group (B), with diminishing of the columnar epithelium and increasing clear acini in treatment and positive (finasteride) control (F) groups (H &E stain, x40).

DISCUSSION

Majority of men will develop benign prostatic hyperplasia at the age of 50 years and beyond. It is related to prostate cancer which is the second most prevalent cancer in men globally (Ferlay *et al*., 2013; Smith, 2012). BPH and PCa often affect the quality of life (QoL) of patients with its bothersome LUTS and sometimes deaths associated with PCa. This study on the stem extracts of *C. membranaceus* sought to establish; its safety, identify potential phytochemicals contributing to the activity of the plant against BPH and PCa management, antioxidant and anticancer properties, mechanisms of action and establish its efficacy in management of BPH using *in vitro* and *in vivo* systems.

Many factors such as season for harvesting, region, ecology and topography of plant location are likely to influence the quantity, types and pharmacological properties of phytochemicals in medicinal plants, including those belonging to the same species of a plant genus. Moreover, the therapeutic efficacy or pharmacological activities of medicinal plants has a direct correlation with the presence of several phytochemicals (Somit *et al*., 2014). Results of the sequential extraction of fractions of the pulverized stem of *C. membranaceus* with different solvents yielded an overall of 3.14% w/w whilst that of the direct aqueous extract (DAQ) was 6% w/w. Further analysis of the sequential extraction revealed that, the aqueous fraction had the highest yield, indicating the stem of *C. membranaceus* possessed more polar consitutents. Also, there was more yield of the sequential aqueous fraction (AQ) than the DAQ. This indicates that, sequential extraction increased selectivity and solubility of polar substances from the pulverized stem of

C. membranaceus. Also, the overall yield of *C. membranaceus* using the sequential method is likely to result in about five times the overall DAQ yield, hence increasing extraction efficiency similar to studies by Jin *et al*. (2011). Further phytochemical, HPLC, antioxidant, and cytotoxicity analysis among others were performed to determine the similarity and differences in the extracts of *C. membranaceus* to draw appropriate conclusion on the most cost-effective extract in managing BPH and PCa.

Preliminary phytochemical analysis on the aqueous stem extract of *C. membranaceus* revealed the presence of alkaloids, saponins, tannins, phenols and terpenoids. In a study by Sarkodie *et al*. (2014a) on the root of *C. membranaceus*, they revealed the presence of alkaliods, terpeniods, reducing sugars, polyamides and absence of tannins. However, tests for reducing sugars in this study were negative in CMASE, and could due to heating during its extraction. Also, there was absence of sterols in CMASE whereas the ethyl acetate fraction from root of *C. membranaceus* indicated the presence of beta-sitosterols (Bayor *et al*., 2007; 2009). The presence of terpeniods in the root and stem of *C. membranaceus* extracts appears to support the observation that, they are the predominant secondary metabolites in the *Croton* species, of which diterpenoids are the characteristic chief component (Xu *et al*., 2018). Furthermore, the presence of alkaliods and terpeniods in CMASE was similar to preliminary phytochemical findings on ethanolic root extract of *C. membranaceus* as reported by Sarkodie *et al.* (2014a).

Inspite of the growing medicinal or herbal plant industry across the world, the issue of quality control and or standardization of plant material remains a challenge

in assuring potential users of the authenticity of these plants (and their extracts). Thus, concerns of their safety often deter their acceptance and marketing vis-a vis conventional drugs. HPLC is employed in phytochemical and analytical chemistry analysis to identify, quantify and purify individual components of plant extracts or formulations; and has been primarily developed for the purposes of fingerprinting and quality control of herbal plants (Su *et al* 2007; WHO, 2000). Furthermore, HPLC analysis of medicinal plant products or extracts is very useful for academicians, researchers and industrialists those who focus on quality of these herbal extracts or products. A HPLC analysis of the aqueous root extract of *C. membranaceus* in a study by Afriyie *et al*. (2014b) revealed four main peaks (active phytochemicals). In this study, the analysis of HPLC chromatograms of the various stem fractions of *C. membranaceus* revealed 12 peaks in the aqueous fraction (AQ) whereas 9 peaks were identified in direct aqueous extract (DAQ). The main peaks for both aqueous extracts were recorded at retention time (RT) 5, 14 and 20 mins. For the fractionated aqueous extract (figure 1A), the peak with RT of 20 min had the highest peak area of 20396.7mAU and a peak height of 169.129mAU with overall percentage peak area of 62%, whereas the direct aqueous extract had the highest peak area of 9703.9mAU and a peak height of 58.17mAU with overall percentage peak area of 40.9%. Other peaks were also identified with their corresponding low peak area and peak height which indicates low concentration compounds in fraction/extract. These high peak areas are directly proportional to high levels of that compound and might have contributed to the anti-cancer properties of the fractions/extract in the cytotoxity studies. However, it is worth

noting that, compounds present in low concentration may exert more potent biological activity than those present in greater amounts (Lukasz, 2012; Yu *et al.*, 2010; Su *et al.*, 2007). It is, therefore, important to introduce screening of the biological activity into the chromatographic fingerprinting analysis. The ethanolic extract recorded 10 peaks with the peak at RT 20 min recording the highest peak area of 1738.92 mAU and a peak height of 169.129mAU with overall percentage peak area of 23.11%. However, under conditions which Lambert *et al*. (2005) conducted HPLC analysis on the ethanolic root bark extract of *C. membranaceus*, showed one constituent eluted at retention time *t*RD 14.3 min. The identity was determined as scopoletin, and was unambiguously identified as the major extract constituent using HPLC–SPE–NMR analysis. The ethyl acetate fraction recorded 13 peaks with the peak at RT 27.8 min recording the highest peak area of 6007.88mAU and a peak height of 71.97mAU with overall percentage peak area of 34.91%. The hexane extract recorded the least number of peaks (8) with small peak areas. The established chemical profile of these crude extracts in this study provides guidance for quality control of similar extracts of stem of *C. membranceus* obtained under similar extraction conditions. The peaks identified in the HPLC analysis should be isolated and tested for biological activity.

GC-MS analysis is a useful analytical technique used in the identification, quality control and standardization of phyto-therapeuticals (Marton, 2007). GC-MS analysis on methanolic-dichloromethane CMASE solution revealed the presence of 15 main phytochemical constituents. Of the 15 compounds, the nine major phytochemicals present among the identified compounds in the stem of *C.*

membranaceus and the order of identification were; 2,3-Butanediol (**1**), 2-butoxy-ethanol (**2**), 4-bromo-3,3,4,4-tetrafluoro-2-butanol (**3**), 2-(2-butoxyethoxy)-ethanol (**4**), Bacchotricuneatin c (**5**), N-(1-[1-(4-Amino-furazan-3-yl)-1H] (**6**), 1-[-]-4-hydroxy-1-methylproline (**7**), n-hexadeconoic acid (**11**), (Z) 6-octadecanoic acid (**12**). The percentage compositions of these major compounds ranged from 3 to 32.58 %, and 7 (1-[-]-4-hyroxy-1-methylproline was the most abundant (32.58%). Futhermore, n-hexadecanoic acid constituted 5.11% whilst 6-octadecaenoic acid constituted 3.99%. It worth noting that, both of the latter constituents are fatty acids. Studies have shown that, n-hexadeconoic acid has anti-androgenic, hypocholesterolemic, antioxidant, and 5-alpha reductase inhibitory activities (Sermakkanni and Thangapandian, 2012; Jananie *et al*., 2011). A similar study on octadecanoic acid found it to exhibit hypocholesterolemic and 5-alpha reductase inhibitory activity (Basu *et al*., 2013). Furthermore, a related compound to the latter compound, 9-octadecanoic acid methyl ester have been found to possess anticancer properties (Asghar *et al*., 2011; Ha *et al*., 1989). Findings from this aspect of the study, also tends to support observations made from related studies on the efficacy of some lipids from herbal therapies such as Saw palmetto (*Serenoa repens*), Cuban Royal palm and pumpkin seed oil found to contain active constituents useful for BPH treatment (Tsai *et al*., 2006; Carbajal *et al*., 2004; Strauch *et al*., 1994). This is the first phytochemical study reporting the presence of these constituents in the stem of *C. membranaceus* which could be responsible for the anti-benign prostatic hyperplasic activity, the 5-alpha reductase inhibitory, antioxidant, anti-androgenic and anticancer activities revealed in this study. According to Kalu *et al*. (2016a), the

presence of these bioactive compounds could confer synergistic anti-benign prostatic hyperplasia activity to the extract. Furthermore, the presence of naturally occurring constituent such as 2, 3 butanediol in CMASE also present in cocoa butter, and in the fraction of kolaviron extract (obtained from *Garcinia kola*) is worth noting. The latter extract is known for its anti-BPH activities (Kalu *et al*., 2016a). Hsin-Chin *et al*., (2012) have reported that, 2,3 butanediol enhances or activates NK cells (activates innate immunity cells) cytotoxic to human pNK cells and NK92 cells in a dose-dependent manner via NKG2D/NCR pathways. The latter study mentioned 2,3 butanediol as a known detoxifying product of liver metabolism in humans which ameliorates endotoxin-induced acute lung injury. Hydroxyl amino acids are essential in multiple critical functions in organisms. Also, they have physiological functions such as being components of glycopeptide antibiotics, cyclodepeptides and collagen; and possess antifungal, antibacterial, antiviral and anticancer properties (Sun *et al*., 2018). In this study, 7 (1-[-]-4-hyroxy-1-methylproline which is a hydroxyl-amino-acid was the most abundant (32.58%) constituent in CMASE. Thus, the presence of this related hydroxyl amino-acid in high proportions in CMASE could contribute to its anticancer activity observed in this study.

Phenolics or polyphenols in plants are known for their wide spectrum of physiological functions such as antioxidant, antimutagenic, antitumor activities and their ability to modify gene expression (Othman *et al*., 2007; Nakamura *et al*., 2003). Phenolic compounds are known to possess antioxidant activity based on their scavenging and chelating properties (Mitic *et al*., 2014). In this study, results

showed the presence of phenols in all the fractions, with ethanolic and aqueous fractions possessing the highest contents. The phenolic contents of the sequential aqueous fraction and direct aqueous extract were found to be similar, suggesting the different extraction method does not significantly affect the phenolic contents of extracts from the stem of *C. membranceus*. Furthermore, its presence in all the fractions underpins the antioxidant activities observed in these fractions and CMASE. However, Javanmardi *et al*. (2003) reported that, the presence of other secondary metabolites in extracts such as volatile oils, carotenoids, and vitamins could also complement or augment the antioxidant capacity of phenolic and flavonoid compounds.

Flavonoids are the most common group of polyphenols (such as flavonols, flavones and condensed tannins), and this class of phenolic compounds have hydroxyl groups which are responsible for their radical scavenging and chelating activities. In this study, flavonoids were present in all the fractions except the sequential aqueous fraction and the direct aqueous extract of *C. membranaceus.* This observation from the quantitative assay was similar to that qualititative phytochemical screening of CMASE which showed negative presence of flavonoids. The flavonoid content in the ethyl acetate fraction was the highest among the fractions assayed and may be the main secondary metabolite responsible for its antioxidant activity, as its phenolic content was relatively low. The absence of flavonoids in the sequential aqueous and direct extracts of the stem suggests their potential antioxidant activities may be attributed to other secondary metabolites other than flavonoids, as observed in this study. The study revealed a relatively high

phenolic content, and additional flavonoid contents in the ethanolic and ethyl acetate fractions compared to the hexane fractions. This observation corroborates the relatively strong antioxidant activities observed in the ethanolic and ethyl acetate fractions compared to the aqueous fractions similar to observations in a related antioxidant study by Javanmardi *et al*. (2003).

DPPH is used to evaluate the free radical scavenging capacity of several natural or synthetic compounds through the donation of proton to reduce DPPH (Prasad *et al*., 2010; Bhasker *et al*., 2007). Extracts (and isolated pure compounds) of the stem, leaf and essential oils obtained from several species of *Croton* such *Croton lechleri, Croton zenthmeri, Croton nepetaefolius, Croton argyrophylloides* have been found to possess good antioxidant activities (Simionatto *et al*. 2007; Morais *et al*., 2006; Lopes *et al*., 2004). In this study, all sequential fractions and direct aqueous extract from the stem of *C. membranaceus* possessed some antioxidant activities. The ethanolic and ethylacetate fractions exhibited the highest antioxidant activities with EC_{50} values of 0.35 and 1.41 mg/ml, respectively whilst the hexane fraction had significantly weak antioxidant activity than the rest of fractions. Furthermore, the EC_{50} value obtained for the ethanolic fraction revealed that, it possessed significantly higher antioxidant properties compared to the rest of the fractions and CMASE. Also, the sequential aqueous fraction possessed almost twice antioxidant activity compared to the direct aqueous extract from the stem of *C. membranaceus*. Mohd *et al*. (2012) reported that, DPPH assay of the ethanolic extracts leaf, stem and root of *C. argyratus* revealed that, the leaf extract possessed the highest total phenolic and total flavonoid contents. Hence, the leaf extract was a potential source

of natural antioxidant. In this study, both the qualitative and quantitative screening for flavonoids in the CMASE and AQ were negative. Therefore, the antioxidant potentials of CMASE and AQ could be attributed to its phenolic and other contents, as several studies have shown a linear correlation between antioxidant activity and phenolic content of plant extracts (Altemimi *et al*., 2017; Kolli *et al*., 2015).

According to Blois (1958), EC_{50} values of plants extracts lower than 50 µg/ml are considered very strong antioxidants, values of 50-100 µg/ml are considered strong antioxidant, values between 101-150 µg/ml possess medium antioxidant activity, whilst greater than 150 µg/ml are considered weak antioxidants. Per Blois (1958) categorization of the plant antioxidant activity levels, even the EC_{50} value obtained for the fraction with the highest antioxidant activity in this study was greater than 150 µg/ml, thus suggesting all the fractions analysed in this study could be considered weak antioxidants. A similar study by Sarkodie *et al*. (2014a) on the ethanolic root extract of *C. membranaceus* revealed it's antioxidant activity with EC_{50} value of 0.100 mg/ml. Comparison of the results of the latter study to this study in which EC_{50} of ethanolic stem fraction was 0.35 mg/ml, tends to revealed that, the root ethanolic extract has about 3.5 times more antioxidant activity than the ethanolic stem fraction. Some studies have reported the potential of antioxidants of plant origin in reducing risk of arteriosclerosis, cardiovascular diseases and some forms of cancer (Rekha *et al*., 2012; Choi *et al*., 2007). Thus, the presence of antioxidant activity in all fractions and direct aqueous extract of the stem of *C. membranaceus* suggests its potential in reducing the risk of these diseases among patrons of the aqueous and ethanolic extracts.

To assess the therapeutic potential of anticancer drug candidate(s) during preclinical studies, their ability to inhibit cancer cells proliferation at low concentrations, coupled with selective inhibition of benign (non malignant) or tumor growth in animal models, are the most common indices used by researchers (Calderón-Montaño *et al*., 2014; Mahapokai *et al*., 2000). Furthermore, if the selectivity index of a potential anticancer drug is higher (or at least similar) than that of the standard (reference) anticancer drug, then it has chemotherapeutic potential, which could be confirmed using further *in vivo* studies (Calderón-Montaño *et al*., 2014). Previous *in vitro* studies have revealed the cytotoxic and growth inhibitory activity of the methanolic root extract of *C. membranaceus* on human cancer lines DLD-1(colon), MCF-7 (breast) and M14 (melanoma) with IC_{50} values of 16, 17.4 and 33.5 µg/ml, respectively (Bayor *et al*., 2007). Bioassay guided fractionation of the methanolic extract from the root of *C. membranaceus* revealed that, its cytotoxic activity was due to bioactive compounds which mainly resided in the ethyl acetate fraction (Bayor *et al*., 2007). Furthermore, three bioactive compounds isolated from the ethyl acetate fractions were crotomembranafuran, p-sitosterol-3-D-glucoside and DL-threitol and showed anticancer activity against human PC3 cells, with IC_{50} values of 4.1, 9.7 and 6.6 µg/ml, respectively. Findings from this study showed that, all SECM and CMASE showed cytotoxic activities against PC3 cells, however, their IC_{50} values were far higher than those obtained for the isolated compounds in the latter study. Futhermore, the aqueous fraction and the direct aqueous extracts exhibited the

highest and comparable cytotoxic activities among the fractions against PC3 cells with IC_{50} values 13.26 ±1.30 and 11.75 ± 1.15 µg/ml, respectively.

In this study, curcumin the reference compound exhibited relatively strong inhibitory activity against PC3 and LNCaP cells with IC_{50} values of 3.58 ± 1.18 µg/ml (SI=1.61) and 8.01 ± 1.15 µg/ml (SI=0.72), respectively with low therapeutic indices. Hence, per the recommendations for by Calderón-Montaño *et al*. (2014), all the stem extracts (SECM and CMASE) except the ethyl acetate fraction could be considered as potential chemotherapeutic agents as their S.I were higher than that of curcumin (standard reference) with respect to PC3 cell lines. And the highest SIs was exhibited by the aqueous fractions (from SECM [SI=75.41], and CMASE [SI=85.11]), suggesting these fractions could be best chemotherapeutic candidates for PC3 cells among the fractions. With regards to LNCaP cells, all the stem extracts of *C. membranaceus* except the ethyl acetate fraction exhibited higher SIs above that of the reference compound. But in this aspect, it was the hexane fraction which exhibited highest SI (4.42), followed by the aqueous fraction and direct aqueous extracts (SI=2.2 and 2.1), respectively. Per the criteria suggested by Calderón-Montaño *et al*. (2014), the aqueous stem extracts of *C. membranaceus* tend to possess the best SI values for both cell lines, thus the best chemotherapeutic potential extracts for further *in vivo* studies in animals. Further analysis of the cytotoxic assay results was done to prove the latter assertion.

According to AI-Rashidi *et al*. (2011), plant extracts that exhibit high cytotoxic activity against cancer cells with low cytotoxicity to human non-malignant cells tend to have a good selective indices, and are potential anticancer agents. The

United States National Cancer Institute plant program also recommends extracts with IC_{50} values less than 30 µg/ml as potential anticancer agents or candidate for drug development (Boik, 2001). Itharat (2004) also stated that, extracts with IC_{50} values between 30 and 200 µg/ml are considered having moderate potential for anticancer drug development, whilst those with IC_{50} > 200 µg/ml are unlikely to be selected for further drug development studies. Another study recommends that, extracts with SIs > 2 as having good chemotherapeutic potentials for further *in vivo* studies and drug development (Basida *et al*., 2009). This study revealed that, the aqueous extracts showed the highest cytotoxicities with IC_{50} for AQ being 13.26 ±1.30 µg/ml and CMASE was 11.75 ± 1.15 µg/ml). The IC_{50} of the rest of the extracts (hexane, ethanolic, ethylacetate) ranged between 55.21 to 68.30 µg/ml against PC3 cells. However, only the aqueous extracts (AQ and CMASE), ethanolic and hexane extracts exhibited SI >2, with the SI values of the aqueous extracts being the highest, followed by the ETOH and hexane extracts. Thus, the aqueous extracts exhibiting the highest cytotoxic activities among the rest of the extracts examined, and also having the best SIs against PC3 cells, makes them good chemotherapeutic candidates for drug development for management non androgen dependent prostate cancer (Basida *et al*., 2009; AI-Rashidi *et al*. 2011; Itharat, 2004; Boik, 2001).

With reference to LNCaP cells, all the stem extracts of *C. membranaceus* with exception of ethyl acetate fraction which showed weak cytotoxicities with their IC_{50} >200 µg/ml. Thus, these extracts may not be suitable anticancer candidates for development (Itharat, 2004; Boik, 2001). Further analysis revealed that, the highest

cytotoxicity among extracts examined was exhibited by the ethyl acetate fraction (IC_{50}=144.3±1.44 µg/ml). Hence, this makes it the only fraction with moderate potential for development as a candidate for management prostate cancer (Itharat, 2004). Furthermore, only the hexane and the aqueous stem extracts of *C. membranaceus* had SIs >2 with hexane being the highest (SI=4.42). Thus, only the aqueous stem extracts of *C. membranaceus* and hexane fractions qualify as potential agents for development as anticancer agents as recommended in related studies (AI-Rashidi *et al*. 2011; Basida *et al*., 2009; Itharat, 2004; Boik, 2001). Overall, the study revealed the aqueous stem extracts of *C. membranaceus* had the best S.I values among the rest of the extracts against both PC3 and LNCaP cells. Furthermore, they possessed much higher selective preference for non-androgen dependent human prostate cancers (PC3) than androgen dependent human prostate cancers (LNCaP). These observations also tend to suggest that, the aqueous extracts may have both non androgen dependent and androgen dependent pathways or mechanism of action against prostate cancer. Also, the cytotoxic observations made from previous studies on the root extracts (Bayor *et al*., 2007), and the current findings on the stem extracts of *C. membranaceus*, lends credence to the use of the stem and root extracts for treatment or as an adjunct in the management of prostate cancers. Thus, CMASE (which is based on the folkloric formulation) was used for the subsequent *in vitro* and *in vivo* studies as there was not much difference between the SIs and IC_{50} values obtained between the aqueous extracts.

Several studies have demonstrated that reduction of apoptosis is associated with BPH and its related cancer development (Kyprianou *et al*., 1996; Mc Neal, 1981).

Imbalance in the molecular mechanisms that regulate cell proliferation and apoptosis in stromal and epithelial cells has been implicated in both the development and progression of BPH, and prostate cancer (Kyprianou *et al*., 2000). Therefore, focusing on induction of apoptosis or pathways of apoptosis has emerged as a potential target for optimizing the medical treatment of BPH and prostate cancer. Consequently, several medicinal plants found to contain active biological compounds with protective and disease preventive potentials, strong antioxidant, anticancer and apoptosis activities are also seen as viable alternatives for the management of BPH and its cancer (Jacobs, 2018; Madhuri and Padley, 2009; Chottanapund *et al*., 2014; Grossarth-Maticek *et al*., 2001).

Cellular and morphological alterations associated with apoptosis are established hall marks, and is characterized by features such as; cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and are eventually engulfed by macrophages (Bayne *et al*., 2000; Majno and Joris, 1995; Collins *et al*., 1997). Related studies on extracts from several Croton species used in the management of tumours such as *C. zambesicus, C. argyratus,* and *C. pierrei* have confirmed their potential to induce apoptosis in cells (Sandoval *et al*., 2002; Block *et al*., 2005; Morales *et al*., 2005). In this study, the Hoescht results on the effect of CMASE on PC-3 cells showed marked apoptosis induction, with nuclear condensation of the PC3 cells chromatin, and fragmented nuclear in an apparent concentration-dependent manner. This finding was similar to observations made in a related study on BPH-1 cells with the aqueous root extract of *C. membranaceus* (Afriyie *et al*., 2015). DNA ladder assay is a very useful method for rapid screening of apoptotic

changes in cells, as DNA fragmentation remains a hall mark of apoptosis (Bayne *et al*., 2000; Majno and Joris, 1995; Collins *et al*., 1997). Analysis of the DNA ladder after administering various concentrations of CMASE on PC3 cells revealed intense pattern of laddering, indicative of apoptosis induction in the treated cells. Further annexin V-FITC/PI staining and flow cytometric analysis of effect of CMASE on PC3 cells, indicated a general concentration dependent increase in apoptotic bodies to very late apoptosis and or necrosis in treated cells. This also was indicative of presence of cell membrane disruption or blebbing (compromised cell membrane) enabling the binding of propidium iodide dye to DNA during its fragmentation (and not necrotic cells), characteristic of late stages of apoptosis. Hence, the observations on effects on PC3 cells with various concentrations of CMASE using the Hoescht, DNA laddering and Annexin V/PI assays tend to agree with the hall marks of apoptosis seen in related studies. Furthermore, findings from this study are similar to previous *in vitro* studies on the aqueous root extract of *C. membranaceus* on human benign prostatic cells (Afriyie *et al*., 2015). The latter study revealed suppressed proliferation of BPH-1 cells through apoptosis via mitochondria-dependent pathway; this was characterized by loss of membrane potential, significant nuclear condensation, DNA fragmentation, and significant upregulation of Bax proteins. Thus, the cytotoxic activity of CMASE on PC3 could be attributed to presence of phytochemicals with apoptotic potentials. Furthermore, the presence of fatty acids in CMASE such as octadecanoic acids (stearic acid) and hexadeconoic acid (palmitic acids) could account for its apoptotic activity in PC3 as observed in other cell lines, and possibly with the involvement of the Fas receptor

and ligand in this process (Ulloth *et al*., 2003; Mu *et al*., 2001). Studies have also shown that oxidative stress plays a primary role in the pathophysiology of age-induced apoptosis via accumulated free-radical damage to mitochondrial DNA (Harman, 1992; Ozawa, 1995). However, the presence of phytochemicals with antioxidant activity in CMASE as confirmed by the DPPH assay, rules out the possibility of induction of apoptosis by CMASE through oxidative stress. Furthermore, the *in vivo* study of CMASE effect on testosterone-induced BPH male S-D rats confirmed mild antioxidant potentials of CMASE. To the best of our knowledge, this is the first study on CMASE which has revealed its apoptotic potentials *in vitro*.

The use of ethnomedicinal plants in the management of diseases has been practiced over centuries across the world since the existence of man. However, pharmacological and toxicological evaluations of medicinal plant extracts or potential drug molecules are essential for drug development (Mushtaq *et al*., 2003; Ibarrola *et al*., 2000). Currently validation for their safety with toxicological data, quality and efficacy are required by regulatory agencies in many countries before their marketing and use (Morgan *et al*., 2004; Gericke, 1995).

In this study, the aqueous stem extract of *C. membranaceus* was virtually non-toxic following single oral administration of acute doses to male S-D rats. No signs of behavioral or clinical signs of toxicity, or mortality were observed during the period of the study after administering CMASE. Findings in this aspect of the study were similar to that reported in related acute toxicity studies using the aqueous, ethanolic and aqueous-ethanolic root and stem extracts of *C. membranaceus* in S-D

rats (Sarkodie *et al*., 2014a; Appiah, 2011; Asare *et al*., 2011). Body and internal organ weight changes are sensitive and indicative markers of toxicity when exposed to toxic substances (Tan *et al.*, 2008; Raza *et al.*, 2002; Auletta, 1995). In this study, there were no significant changes in the body and organ weights between treated and control groups of rats, and gradual body weight gain was observed in all groups. Furthermore, the normal consumption of food and water during the study period by the animals might have also contributed to the gain in body weights of the rats in both treated and control groups. The observations on the effect of acute doses of CMASE on body and organ weights, as well on food and water consumption S-D rats were indicative of its safety potentials.

Analysis of blood parameters is relevant in toxicological risk evaluation, as changes in the haematological system had higher predictive value for toxicity in humans in assays involving rodents and non-rodents (Adeneye, *et al*., 2006; Olson *et al.*, 2000). In this study, assessment of haematological parameters (RBC, WBC, PLT, HGB, HCT, MCV, MCH, and MCHC) between control and treated groups revealed that, CMASE was non-toxic to the haemopoietic system as there was no significant variation between the indices.

Significant increases in creatinine and urea levels in the blood are indicative of abnormal kidney function (Sireeratawong *et al.*, 2008b; Gad, 1994). However, plasma urea concentrationis a less reliable than creatinine as an index of glomerular filtration rate (Mckie and Parkar, 2006; Tilkian *et al.*, 1979). In this study, creatinine and uric acid levels were reduced significantly among the treated group, however, there was no significant change in urea levels. Findings from this aspect

suggests kidney protective or toxic activity of CMASE, hence monitoring renal function parameters is suggested for users with kidney problems and healthy subjects due to the observed significant reduction in creatinine. Studies have shown that, liver injury caused by hepatotoxic drugs or extracts could lead to elevated ALT, AST and total proteins levels (Ozer *et al*., 2008). Results from this study showed that acute oral administration of CMASE with doses between 1000-5000 mg/kg could result in significant non dose-dependent alterations (elevation or decrease) of serum ALT, AST and total proteins indices within the treated groups. Thus, indicative of the liver being a possible target organ of toxicity, which should be established with repeated toxicity study using sub-chronic doses of CMASE. Furthermore, a significant decline in ALT, AST and TP were observed in the low dose groups, and vice versa effects in the median and high dose groups. These findings were contrary to observations in oral acute and sub-chronic toxicity studies on male S-D rats using hydroethanolic and aqueous root extracts of *C. membranaceus* (Afriyie *et al*., 2013; Asare *et al*., 2011).

Triglycerides are the most common lipids and higher than normal range levels suggest higher risk of atherosclerosis, heart disease or stroke (Austin, 1989; Wilhelmsen *et al*., 1973; Goldstein *et al*., 1973). In this study, CMASE did not cause any significant effect on lipid parameters, though the low and median doses appeared to have slightly reduced these lipid parameters, an indication that acute oral doses of CMASE does not increase risks of atherosclerosis, heart disease or stroke. Serum creatine kinase (CK), CK-MB and lactate dehydrogenase assays in toxicity studies have been used clinically as biomarkers to assess the extent of

damage to heart and skeletal muscles, as well as the brain (JiPing, 2011; Moss and Henderson, 1999; Bessman and Carpenter, 1985). Administration of acute oral doses of CMASE in this study did not cause any significant alteration of serum creatinine kinase and lactate dehydrogenase level. Findings from this aspect of the study suggest, acute oral doses CMASE does not cause harm to heart and skeletal muscles, nor brain.

Studies have revealed that, there is overproduction of ROS the main mediators of oxidative stress, and reduction of antioxidant enzymes such CAT, GST, GPx, SOD and GR during oxidative stress (Cederbaum, 2001). In this study, there was significant elevation of serum SOD, and non-significant, non dose-dependent elevation and decline of serum GSH in treated groups compared to controls. Also, there was non-significant decline in mean serum MDA levels among the treated rat groups compared to controls were observed. These findings tend to confirm the mild antioxidant potential of CMASE observed in the DDPH assay. Furthermore, the antioxidant potential of CMASE in reducing oxidative stress, could be attributed to the presence of phenolic compounds (Altemimi *et al*., 2017; Kolli *et al*., 2015).

No gross lesions were observed in the gross pathological and histopathology examinations of the tissues of the heart, liver, kidney compared to the control group organs. Inspite of the significant elevation and decrease of some renal and liver function serum parameters, no pathological lesions were observed in the kidneys and liver of treated groups. Findings from this aspect of the study indicate that, CMASE could be considered relatively safe on acute exposure, and the LD_{50} value could be greater than 5000 mg/kg. However, further repeated toxicity studies are

recommended for CMASE in rats due to the observed significant non-dose dependent reduction and elevations in serum liver indices, as it could suggest potential target organ of toxicity. Previous acute studies by Appiah (2011) on the aqueous stem extract with doses up to 5000 mg/g in rats reported only on clinical observations. To our knowledge, this is the first toxicity study on the aqueous stem extract of *C. membranaceus* in rats which has provides indepth acute toxicological (clinical, haematological, biochemical and histopathological) data.

Plant-derived medicines are the first line of treatment for various diseases, and are used by 75-80% of the world populations especially from the developing countries (Pandey *et al*., 2011: Monteagudo *et al.*, 2006). Many naturally occurring substances present in the human diet have been identified as potential chemopreventive agents, and the consumption of relatively large amounts of vegetables and fruits is believed to prevent the development of cancer (Vecchia and Tavani, 1998). However, toxicity reports on medicinal plant or their extracts are well known, and even from the same plant could be variable depending on the plant part used, and the solvent employed (Coria-Tellez *et al*., 2016). Thus, repeated or sub-chronic exposure to a test substance or medicinal plant extract provides information on potential health hazards that are likely to arise and or possible target organs of toxicity. Currently, data on the repeated safety profile of the CMASE is lacking. Thus, limiting the safety and efficacy profile on the CMASE, and consequently its marketing and use. In this present study, the sub-chronic toxicological effects of CMASE in male S-D rats were evaluated.

Alteration (gain or reduction) in general behavior, body weight and internal organ weights of experimental animals are sensitive indices of toxicity after exposure to toxic substances (Tan *et al.*, 2008; Raza *et al.*, 2002; OECD, 1998). The present study demonstrated the safety profile of CMASE in experimental animals, as there were no clinical signs of toxicity, alteration in their food and water consumptions, and increase in body weights were observed in all the treatment groups. These findings were similar to observations made from related acute and sub-chronic toxicity studies on the stem and root extracts (aqueous and ethanolic) of *C. membranaceus* and *C. zambesicus* (Afriyie *et al*., 2013, Asare *et al*., 2011; Okokon *et al*., 2010).

Assay and interpretation of haematological parameters in test animals during the course of a toxicity study is a common practice (Frith *et al.*, 1980). In this study, there was significant increase in HGB and MCHC indices, coupled with non-significant changes in RBC, HCT, MCV, MCH, and WBC in treated groups. Findings revealed CMASE was safe on blood indices examined similar to observations in the sub-chronic toxicity study on the aqueous root extract of *C. membranaceus* (Afriyie *et al*., 2013), and related sub-acute studies with the ethanolic root extract of *C. zambesicus* (Okokon *et al.*, 2010).

The kidney plays an important role in regulating the excretion and reabsorption of body wastes (and toxins) including drug metabolites, osmolarity, ions, pH and production of hormones among other functions. Serum urea, uric acid and creatinine are considered as important markers for kidney function (Mckie and Parkar, 2006; Gad, 1994; Tilkian *et al.*, 1979). Also, serum electrolytes such as

sodium, potassium and chloride have been used with urea and creatinine indices in several toxicity studies as markers of kidney function, (Hutailok-Towatana *et al.*, 2010). In this study, the non-significant changes in the serum levels of urea, potassium, sodium and chloride was observed in treated groups compared to controls. Furthermore, there was significant decline in creatinine levels in only the high dose group. These findings sugguests that, CMASE was generally not nephrotoxic after oral sub-chronic administration. Observations in this study was also similar to findings from the acute toxicity effects of CMASE on kidney functions which revealed significant reduction in serum creatinine and urea levels. These results on CMASE corroborate with the findings from its acute toxicity study, and related toxicity studies on root extracts of *C. membranaceus* and *C. zambesicus* (Afriyie *et al*., 2013; Asare *et al*., 2011; Okokon *et al*., 2010). Further, histopathological examination of kidney tissues of treated rats revealed no abnormalities and findings corroborated with the serum hepatic function indices.

Liver is the major organ involved in drug metabolism, and increased significant serum levels of AST and ALT indicates possible liver toxicity (OECD, 1998; Tilikian *et al.*, 1979). Severe hepatic injury due to the metabolism of toxic phytochemicals, and failure of the metabolic products to be eliminated by the liver, could be associated with marked distortion of serum enzyme activities (Geidam *et al.*, 2004). In this study, marked non significant and non dose-dependent increases in serum ALT, AST and ALP levels in treated groups, compared to the controls. Findings from this aspect of the study suggest CMASE is not hepatotoxic. Findings from a related sub-chronic study by Afriyie *et al*. (2013) using aqueous root extract

of *C. membranaceus* was contrary to this study, as ALT and AST levels were found to be reduced by more than 50%. Hence, the latter study also revealed the aqueous root extract was hepatoprotective. It would be worth monitoring liver function parameters of potential patrons of CMASE due to the generally non-significant elevations of these serum; ALT, AST and ALP markers. This view is also supported by the observation of significant non dose-dependent elevations and decreases observed among the treated groups with respect with serum ALT, AST and ALP compared to controls during the acute toxicity study on CMASE.

High serum levels of cholesterol, VLDL and triglycerides are associated with cardiovascular diseases (Austin, 1989; Wilhelmsen *et al*., 1973). Studies by Edijala *et al*. (2005) revealed that, reduction of serum total cholesterol and low-density lipoprotein is a primary factor for prevention of cardiovascular disease. Sub-chronic toxicity study on the aqueous root extract of *C. membranaceus* revealed that, there were non-significant changes in serum total cholesterol, LDL and HDL (Afriyie *et al*., 2013). However, there were significant reductions in TG and VLDL levels suggesting it had cardioprotective properties. Studies on *Croton pendilflorus* oil showed significant reduction in total cholesterol, LDL, HDL/LDL ratio but a non-significant elevation in triglycerides (Ojokuku *et al.,* 2011). Thus, suggesting the oil had hypocholesterolemic and cardiac protective activity. In this study, the non significant reduction in serum cholesterol, LDL, coupled with non-significant changes in serum HDL and triglycerides in treated groups compared to controls suggests that CMASE use may not be associated with hypercholesterolemia, cardiovascular diseases or atherogenic risk. And histological examination of livers

of treated groups compared with controls corroborated with this clinical biochemical observation. Observations on the extracts from other species of Croton on serum lipid indices and *C. membranaceus* suggest these species may not be atherogenic or increase risk of cardiac diseases as observed in this study.

Most plasma proteins are synthesized and degraded in the liver, and significant reduction in the blood plasma could suggests either impaired hepatocellular production and or increased catabolism as observed in various physiological or pathological processes (Hutadilok-Towatana *et al.*, 2010; Gatsing *et al*, 2005). Adeyemi *et al.* (2010) reported that, a significant decline in serum total protein due to administration of an extract in toxicity studies should be of concern as it tends to reflect chronic liver disease. In this study, non significant changes of serum total proteins, albumin, and globulins was not quite different from the findings on the sub-chronic study on the aqueous root extract of *C. membranaceus* in which there was a general non significant increase in all the serum protein indices (Afriyie *et al*., 2013). Findings from this aspect of the study tend to rule out the possibility of CMASE to induce hypoalbuminemia often seen in clinical conditions such as congestive heart failure, chronic liver diseases (hepatitis), liver cirrhosis, and nephritis (Burtis and Ashwood, 1994). Furthermore, the non-induction of hyperlipemia by CMASE observed in this study, appears to support findings from a study that associated clinical situations of hypoalbuminemia with hyperlipemia (Busher, 1990).

High production levels of ROS results in significant decrease in cell antioxidant defense mechanisms with subsequent damage to protein, lipid, DNA, disruption of

cellular functions and cell death (Udensi and Paul, 2016). Enzymes such as SOD, CAT, GPx and GST are vital antioxidant enzymes that scavenge reactive oxygen species (ROS) such as superoxide, hydroxy radicals and other reactive products produced in mammalian tissues during biochemical processes (Valko *et al*., 2007; Singhal *et al*., 1987). Studies have revealed that, there is overproduction of ROS, and reduction of antioxidant enzymes such as CAT, GST, SOD and GR during oxidative stress (Udensi and Paul, 2016; Cederbaum, 2001). After sub-chronic administration of oral doses of CMASE to S-D rats for 90 days, there was marked dose-dependent elevations of liver enzmymatic levels of SOD, CAT and GSH. However, these elevations were not statistically significant in all the treated groups compared to controls. Also, there was no significant difference in MDA levels in the treated groups compared to controls. Findings from this aspect appears to partly support the observations from the acute toxicity study of CMASE in S-D rats which also showed that, CMASE's had some antioxidant potentials. Studies have reported the association between the development, progression of BPH and PCa, and response to drug therapy with oxidative stress (Aryal *et al*., 2007). Observations of the antioxidant potentials of CMASE from previous DPPH assay, acute toxicity study and in this sub-chronic toxicity study suggests it could be effective in the management of BPH, PCa and other oxidative stress-related chronic diseases.

Hypoglycemic activities of extracts various plant parts from species of Croton such *C. zambesi*, *Croton klozchianus*, *C. lobatus* and *Croton cajucara* has been well documented (Fasola *et al*., 2016; Govindarajan *et al*., 2008; Okokon *et al*., 2006; Farias *et al*., 1997). Also, the antihyperglycaemic activity of the ethanolic root

extracts of *C. membranaceus* has been reported using streptozotocin-induced diabetic rats (Sarkodie *et al*., 2014a; 2014b). A related study using the aqueous root extract in genetically modified diabetic mice by Asare *et al.* (2016a) also confirmed the antidiabetic properties of the aqueous root extract of *C. membranaceus*. On the contrary, in this study, none of the doses administered to the treated groups produced any significant decrease in fasting blood glucose level compared to the controls during the period. This suggests CMASE may not have the potential of induce hypoglycemia in healthy subjects. Studies have reported that, the terpenoid t-DCTN isolated from *C. cajucara* exhibited hypoglycemic activity in alloxan-induced diabetic rats, but not did replicate similar effect in normal rats (Farias *et al*., 1997). Hence, further studies to ascertain the hypoglycemic potential of CMASE in drug-induced diabetes or genetically modified diabetic animal models are recommended.

Lactate dehydrogenase (LDH) is a cytoplasmatic enzyme present primarily in all major organ systems, and its appearance in the serum (total serum LDH) is often used as a sensitive indicator to detect cell damage, cytotoxicity or cell death (Lott and Nemensanszky, 1987; Moss and Henderson, 1986). Creatine kinase (CK) is an intracellular enzyme expressed by various tissues and cell types, and present in greatest amounts in skeletal muscle, myocardium, and to a lesser extent in the brain (JiPing, 2011). Disruption of cell membranes due to hypoxia, heat stress, dehydration, drugs, alcohol abuse, chemical poisonings, crush trauma and or other injury releases CK and LDH from the cellular cytosol into the systemic circulation (Clarkson *et al*., 2006; Terpilowski and Criddle, 2004; Lott andNemensanszky,

1987; Moss and Henderson, 1986). Elevated serum levels of total CK have been associated with many conditions but primarily, cardiac and muscle diseases (Chattington *et al*., 1994). CK is a muscle enzyme which exists in three recognised isoenzymes: CK- MM (skeletal muscle); CK-MB (myocardium muscle and brain) (Moss and Henderson, 1999; Bessman and Carpenter, 1985). The MM fraction is present in both cardiac and skeletal muscle, significant concentration of CK–MB isoenzyme is found almost exclusively in the myocardium, whilst elevation in total CK can be associated with many conditions but primarily, cardiac and muscle diseases (Chattington *et al*., 1994). Reduced significant serum levels of these markers (CK, CK-MB and LDH) suggests some protections against cardiac and skeletal diseases related to the muscles (Zhang *et al.*, 2011; Adeboyo *et al.*, 2010). Furthermore, elevated serum CK–MB levels are highly specific and sensitive for myocardial cell wall injury (Al Gani *et al*, 2011). In this study, non statistical significant alterations were observed in the serum elevation of lactate dehydrogenase levels, and also marginal elevation and decline in serum creatine-kinase total levels in the treated groups compared to controls. However, significant reduction in creatine-kinase-MB index in high dose group was observed, whilst slight elevations in the median and low dose groups were observed. This, suggests that CMASE may not induce the disruption of skeletal and heart muscles, and may possess cardiac muscle protective properties when used for prolong periods in high doses. Findings were similar studies on the root extract of *C. membranaceus* which showed signficant reduction of CK-MB in high dose group (Afriyie *et al*., 2013).

Furthermore, LDH levels in all dose groups, and CK-total in the high dose group significantly reduced contrary to the observations from this study.

Gross pathological examinations of the heart, liver, kidney and prostate of the treated groups compared to the control group did not reveal any sign of abnormality (no change in color, shape, size, and texture). Neither were there signs of damage to the architecture of the tissues which could be attributed to the extract during the sub-chronic toxity study. Studies have showed that, alterations (gain or reduction) in body and internal organ weights are simple and sensitive indices of toxicity after exposure to toxic agents (Tan *et al.*, 2008; Raza *et al.*, 2002). In this study, there was no significant change in weights of liver, heart, prostate and kidneys of treated groups compared to controls at the end of the study period. The non-significant differences may be attributed to individual variation in size of internal organs as reported by Bailey *et al.* (2004). These findings were similar to histopathological results from related toxicity studies on the leaf and root extracts of *C. membranaceus* and *Croton zambesicus* (Ofusori *et al.*, 2008; Okokon *et al.*, 2010; Asare *et al.*, 2011; Afriyie *et al.*, 2013). However, the columnar glandular epithelium infoldings were observed in the prostates of rats in the controls, which generally decreased or thinned with increasing dose in the treated groups. It was also observed that, the stromal cells appeared to reduce (appeared darker) with increasing dose of CMASE in the treated groups. The latter observations were similar to findings in a related study using oral administration of sub-chronic doses of the aqueous root extract of *C. membranaceus* (Afriyie *et al.*, 2013). The findings from pathological and histological examinations on the heart, liver, prostate and

kidney generally collaborates with the clinical chemistry markers for their respective organs. Thus, the absence of degenerative alterations, necrosis, lesions, inflammation or any abnormality as seen in the treated groups suggests that, CMASE is not nephrotoxic nor hepatotoxic, and could be cardioprotective. In conclusion, no serious adverse pre-clinical symptoms of toxicity were observed on oral administration of the aqueous stem extract of *C. membranaceus* during the sub-chronic toxicity study. To the best of our knowledge, this is the first sub-chronic toxicity *in vivo* profiling of the CMASE and finding provides valuable data which could be useful in future chronic toxicity and clinical studies.

Conventional treatment regimens and procedures for BPH management with orthodox therapy such as α-adrenergic blockers, 5 α-reductase inhibitors, antimuscarinic agents, β-adrenergic agonists, phosphodiesterase type 5 inhibitors, prostatectomy, radiation, besides being expensive are associated with adverse effects (Loblaw *et al*., 2014; Singh *et al*., 2006; Steineck *et al*., 2002). Thus, there is an increase in search of natural products of plant origin for the treatment of BPH as they are considered natural, much safer, easily accessible and affordable alternatives. In this study, the efficacy and possible mechanism of action of the aqueous stem extract of *C. membranaceus* using *in vivo* BPH S-D rats was investigated.

Though increased prostatic weight also remains a key indicator of BPH (Lu *et al*., 2014; Pias, 2010), relative prostate weight (prostatic index) is a more sensitive index used to assess BPH development (Arruzazabala *et al*., 2006). This study revealed significant increase in both mean prostatic weight and prostaic index (PI,

0.66) of model group compared to the negative control (PI, 0.42), which indicated successful induction of BPH in the model group and the rest of the testosterone injected castrated S-D rats. Histological examination of prostates in the model group confirmed the proliferation of glandular epithelium with infoldings into the lumen, thinned stroma and reduced lumen, which are characteristic of BPH induction. However, the latter observations were contrary to histological observations made in the negative control group. Furthermore, the reduction of prostatic weights and PI by 51.9% (PI, 0.61), 70.4% (PI, 0.56) and 48.1% (PI, 0.56) in the low, median and high dose groups, respectively after administration of CMASE suggests reversal of BPH development in these groups (Lu *et al*., 2014; Pias, 2010; Arruzazabala *et al*., 2006). However, the extent of reversal was not as prominent as the effect in the positive control group. Histopathological examinations appeared to agree with the latter observations as, photomicrographs of cross sections of treated group prostates showed, reduced or mild hyperplasia of the glandular epithelium and increased lumen. Findings with regards to prostatic weights and indices in this study were similar to testosterone-induced BPH studies in S-D rats using the aqueous stem and root extracts of *C. membranaceus* (Appiah, 2011; Afriyie *et al*., 2014b).

Natural plant extracts have been useful for the prevention and treatment of BPH through their ability to regulate the expressions of DHT, 5α-reductase, androgen receptors, and prostate-specific antigen in the prostate and serum (Lee *et al*., 2014; Kang *et al*., 2007). Proliferation of smooth muscle and epithelial primarily within the prostatic zone results in BPH and its associated symptoms LUTS (Minutoli *et*

al., 2014). However, it is reported that, only about 25% to 50% of BPH cases with clinical gland enlargement manifest LUTS (Roehrborn, 2012; Oesterling, 1995a). Conversion of testosterone to dihydrotestosterone by 5 alpha-reductase type 2 which is found on the nuclear membrane of both the stroma and epithelium of the prostate has been documented as a major pathway for BPH development (Aleksandra *et al*., 2015; Roehrborn and McConnell, 2002). Thus, 5-alpha reductase inhibitors are employed therapeutically to suppress production of prostatic DHT levels, and subsequently this results in reduction of prostate volume and LUTS (Alberto *et al*., 2009). In this study, CMASE in a non-dose dependent pattern reduced prostatic testosterone levels in the treated groups, with significant prostatic testosterone observed only in the median dose group compared to the model group's prostatic testosterone levels. The general marked decline in prostatic testosterone in the CMASE and finasteride groups compared to the model observed in this study was similar to findings in anti-BPH efficacy studies of *Veratrum maackii* and *Mangifera indica* in rats (Ishola *et al*., 2019; Park *et al*., 2018). This, suggests that, CMASE suppresses prostatic testosterone availability in treated groups to reduce its conversion into DHT, the potent substrate hormone for proliferation of the prostate. Thus, it is indicative of the antiproliferative potential of CMASE.

Dihydrotestosterone an important metabolite of testosterone is considered the most potent androgen in men with a higher affinity for androgen receptors in the prostate than testosterone (Grino *et al*., 1990). Binding of DHT with the androgen receptors results in transcription of various genes, leading to production of proteins

such as prostate specific antigen and other essential proteins (and or growth factors), required for cellular proliferation and differentiation of epithelial, stromal cells, and an overall enlargement of prostate (Briganti *et al*., 2009; Griffiths and Denis, 2000). The present study found treatment of testosterone-induced BPH rats with CMASE/finasteride markedly reducing prostatic levels of DHT in a non-dose dependent manner compared to model group, with significant reduction observed only in the finasteride group. Similar non-significant reduction in serum DHT levels was found in related experimental studies in rats using the root extracts of *C. membranaceus* and *Boerhaavia diffusa* (Afriyie *et al*., 2014b; Vyas *et al*., 2013). On the contrary, significant reduction in prostatic DHT was observed in a similar recent study using *Veratum maackii* on rats (Park *et al*., 2018)

Five-alpha reductase inhibitors are principally used therapeutically to suppress production of prostatic levels of DHT. This leads to the reduction of prostate volume and associated LUTS (Alberto *et al*., 2009). Other studies have revealed that, there is significant reduction in serum and prostatic DHT levels and associated reduction in prostate size as a result of inhibition of 5-alpha reductase which converts testosterone to DHT (Gormley *et al*., 1992). In this study, administration of CMASE and finasteride markedly reduced prostatic 5-alpha reductase levels, with significant reductions observed in the median and high dose groups, comparable to the finasteride group. Furthermore, the pattern of 5-alpha reductase levels in the prostate was similar to that of DHT in this study. These observations tend to confirm that, CMASE contains constituents (n-hexadecanoic and 6-octadecanoic acid) which have inhibitory potentials against 5-alpha reductase as

detected in the GC-MS analysis. Studies on some medicinal plant extracts have also found the presence of constituents that inhibit 5-alpha reductase, and prevents dihydrotestosterone from binding to androgen receptors in the prostate gland (Sermakkanni and Thangapandian, 2012; Jananie*et al*., 2011; Plosker and Brogen, 1996).

PSA is among the key biochemical markers available for diagnosis of BPH and prostate cancer (McPartland and Pruitt, 2000). PSA test is useful in the assessment of LUTS by acting as a surrogate marker in BPH diagnosis. Furthermore, evidence suggests that men with a PSA >1.4 ng/ml may be considered as having an increased risk of developing BPH (Melia, 2005; Bartsch *et al*., 2004). Elevated levels of PSA in serum are associated with prostate cancer, and could be used to diagnose clinically significant prostate cancer at an early and potentially curable stage (Oesterling, 1995b; Stamey *et al*., 1987). Recent sub-chronic toxicity and experimental hyperplasic rat studies using the aqueous root extract of *C. membranaceus* revealed significant decline in serum PSA in treated groups compared to controls (Afriyie *et al*., 2014a; Afriyie *et al*., 2014b). In this study, significant elevation of prostatic PSA was observed in the testosterone induced model BPH group compared to the negative controls. On the contrary, significant reduction in prostatic PSA levels were seen in all the CMASE and finasteride treated groups. CMASE's ability to significantly reduce serum PSA as observed in this study was similar to findings in related studies using the aqueous root extract of *C. membranaceus.* This suggests that CMASE possesses antiproliferative activity against BPH using *in vivo* models, and possible PCa cells. This observation was

confirmed with the histological examinations on the prostates of the experimental animals.

Many medicinal plants have antioxidant phytochemicals with therapeutic potentials which have been employed to mitigate oxidative stress associated with BPH (Suffredini *et al*., 2004: Jakubowski and Bartosz, 1997). Oxidative stress has been associated with mechanisms involved in the development and progression of BPH and PCa, including the response to drug therapy (Duru *et al*., 2014; Battisti *et al*., 2011; Aryal *et al*., 2007). For instance, a study revealed that, phytochemicals from *Vernonia amygdalina* complements the therapeutic effects PCa drugs like Paclitaxel (Cameron *et al*., 2013). Recent human and animal studies provide credence to the role of prostatic MDA, antioxidants and defense enzyme levels as markers in the development of BPH and PCa (Udensi and Paul, 2016; Kalu *et al*., 2016a; Khandrika *et al*., 2009; Arsova-Sarafinovska *et al*., 2009). Glutathione-S-transferase (GST)-P1 and SOD have been found to be the main scavengers of free radicals in the prostate tissue during oxidative stress (Naber and Weidner, 2000). A study on finasteride and kolaviron treated BPH rat models revealed significant decline in prostatic GSH, GPx, SOD and catalase levels, with significant elevation in malondialdehyde (MDA) levels compared to model BPH group (Kalu *et al*., 2016a). This present study showed that, in the BPH model group there was significant decline in prostatic SOD and GSH levels, and marked reduction of liver levels of these enzymes compared to negative controls. Furthermore, both prostatic and liver MDA were significantly elevated in BPH model group compared to negative controls. The liver and prostatic levels of SOD, GSH and MDA in the

model BPH group tends to confirm the assertion of oxidative stress being implicated in BPH development (Udensi and Paul, 2016; Kalu *et al*., 2016a; Khandrika *et al*., 2009). Also, the generally marked or significant elevation of prostatic and liver SOD, GSH, and mild reduction of MDA in the CMASE treated groups, are indicative of some antioxidant potential of CMASE in ameloriating BPH development. The latter observation of changes in liver and prostatic SOD, GSH and MDA associated with BPH development and management, were similar to findings on related studies, except for MDA levels in the treated groups in this study (Ishola et al., 2019; Naber and Weidner, 2000). The antioxidant potentials of CMASE observed in this aspect of the study, was generally consistent with observations made during the acute and sub-chronic toxicity studies.

To the best of our knowledge, this is the first study on the aqueous stem extract of *C. membranaceus* which has established its efficacy in management of BPH development. It revealed that, CMASE has the ability to inhibit 5-alpha reductase, induce antioxidant enzymes (SOD, GSH), poor inhibitor of lipid peroxidation in oxidative stress associated BPH, and could possibly be used in PCa management. Thus, these findings support the recent use of the stem extract of *C. membranaceus* in the management of BPH and prostate cancer.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

This study was undertaken to establish the pharmacological and toxicological activities of the aqueous stem extract of *C. membranaceus* in the management of BPH and prostate cancer.

1. The phytochemical analysis of the CMASE revealed the presence of phenols, alkaloids, saponins, terpeniods and tannins, whilst reducing sugars, anthraquinones, phytosterols, and flavonoids were absent. The hexane, ethyl acetate, ethanol and aqueous fractions showed presence of 8, 13, 10, 12 peaks in their chromatographs, whilst CMASE showed presence of 9 peaks.

2. Further GC-MS analysis of chromatograph obtained on microwave assisted dichloromethane-methanol extract of CMASE revealed the presence of 15 main phytochemical constituents. The major nine phytochemicals present were; 2,3-Butanediol (**1**), 2-butoxy-ethanol (**2**), 4-bromo-3,3,4,4-tetrafluoro-2-butanol (**3**), 2-(2-butoxyethoxy)-ethanol (**4**), Bacchotricuneatin c (**5**), N-(1-[1-(4-Amino-furazan-3-yl)-1H] (**6**), 1-[-]-4-hydroxy-1-methylproline (**7**), n-hexadeconoic acid (**11**), 6-octadecanoic acid (**12**). The highest constituent was 1-[-]-4-hyroxy-1-methylproline (32.58%).

3. Quantitative analysis on sequential fractions of pulverized stem of *C. membranaceus* revealed presence of phenols in all the sequential fractions.

Also, analysis of total flavonoids content of AQ and CMASE revealed absence of flavonoids in both aqueous extracts, but present in the other sequential fractions. Furthermore, CMASE and all the extracts possessed mild antioxidant activities.

4. Cytotoxicity results obtained from the MMT assay using various concentrations) of SECM and CMASE after 72 h of treatment of PC3 and LNCaP cell lines revealed, all the extracts/fractions exhibited antiproliferative activities. With regards to PC3, the AQ and CMASE extracts exhibited strongest antiproliferative activity with median inhibitory concentrations (IC_{50}) values of 13.26±1.30 and 11.75±1.15 µg/ml, respectively and the weakest cytotoxic effect was exhibited by the ethyl acetate fraction with mean IC_{50} value of 68.3±1.15 µg/ml. The IC_{50} values of the fractionated ethanolic and hexane extracts were virtually the same (about 55.0 µg/ml).

5. Cytotoxicity assay on LNCaP cells revealed that, there was a general concentration dependent decrease in cell viability with increasing concentration of the various extracts (CMASE and SECM). Hexane and ethyl acetate solvent extracts exhibited the strongest cytotoxic activities with IC_{50} values of 144.3±1.44 and 226.2±1.31 µg/ml, respectively. The ethanolic, fractioned aqueous (AQ) and CMASE had IC_{50} values of 366.3±1.33, 482.9±1.38 and 453.3±1.32 µg/ml, respectively.

6. Based on the IC_{50} values of extracts obtained for the PC3 and LNCaP, and CC_{50} on normal cells (PNT2) obtained, selectivity indices were calculated indicated

CMASE and AQ were had the best selective indices (SIs) for both cell lines. The SI values of AQ and CMASE for PC3 were 85.11 and 75.41, respectively whilst the values obtained for LNCaP cells were 2.2 and 2.07, respectively.

7. The SI values obtained for hexane extract against PC3 and LNCaP were 18.11 and 4.42, respectively. The ethanolic fraction showed good selective index for PC3 (10.07) but not in the case of LNCaP cells (1.52), whereas the ethyl acetate fractions did not exhibit acceptable SIs for both PC3 (1.28) and LNCaP (0.61) cells.

8. Examination of the apoptotic potentials of various concentrations of CMASE on PC3 cells revealed nuclear condensation and increased apoptotic bodies in a concentration dependent manner. Analysis with annexin V-FITC/PI staining with flow cytometry revealed that, there was concentration dependent increase in the presence of apoptotic bodies to the very late stage of apoptosis (primary, late, mostly very late apoptotic cells) in all CMASE treated cells. Thus, CMASE has apoptotic potentials as one of its mechanisms of action in BPH or PCa management.

9. Toxicity (acute and sub-chronic) studies done on the extract suggest that CMASE was relatively safe, may have kidney protective properties, and some antioxidant potentials *in vivo*.

10. Administration of sub-chronic doses of CMASE to testosterone-induced BPH treated rats revealed; significant reduction of prostate specific antigens levels, marked reduction in; prostatic indices, decline in prostatic 5-alpha reductase

activities, and reduced prostatic DHT and testosterone levels. Furthermore, there was marked significant elevation of prostatic and liver SOD and GSH, with negligible changes in reducing elevated prostatic and liver MDA levels. Dose-dependent reduction in epithelial and stroma cells by CMASE was confirmed by histological examination in the treated. This also confirmed the potentials of CMASE in the management of BPH, and possibly its cancer *in vivo*.

Conclusion

The study concluded that, CMASE and SECM possessed antioxidant potentials *in vitro* and *in vivo*, and could be attributed to the presence of phytochemicals such as phenols and flavonoids, though flavonoids were absent in the aqueous extracts (AQ and CMASE).

GC-MS analysis on CMASE revealed presence of 15 constituents. Three of the identified consituents being 1-[-]-4-hydroxy-1-methylproline, n-hexadeconoic acid, 6-octadecanoic acid are known for their anti-androgenic, hypocholesterolemic, antioxidant, 5-alpha reductase inhibitory, antifungal, antibacterial, antiviral or anticancer properties.

The study also established that, CMASE and SECM possessed cytotoxic activities against PC3 and LNCaP cells, and the aqueous extracts had the best selective indices for both PC3 and LNCaP cells. Apoptosis was also established as one of the mechanisms of action of CMASE *in vitro* in PC3 cells.

Acute and sub-chronic oral toxicity studies on CMASE in S-D rats showed that, the extract did not illicit clinical signs of toxicity. Overall biochemical, haematological and histological observations in the liver, heart and kidney suggests CMASE was relatively safe, with LD_{50} greater than 5000 mg/kg.

Finally, this study establish that, CMASE has anti-BPH potentials such as significantly reducing prostatic PSA, reduced prostatic testosterone and dihydrotestosterone levels. CMASE was found to markedly inhibit 5-alpha reductase activity, and upregulated liver and prostatic antioxidant levels without marked effect in reducing elevated MDA levels in treated groups (as similarly observed in toxicity studies). Histological examination on prostates of CMASE treated BPH rats also confirmed its ability to reduce proliferation of epithelial and stromal cells.

Recommendations

Based on the conclusions of this study, the following recommendations are made:

1. Further phytochemical analysis on CMASE (extend to possibly to AQ) such as column chromatography and LC-MS to identify and or isolate potential (novel) anti-BPH and anti-prostate cancer drug candidates.

2. Further studies to establish the apoptotic pathways (intrinsic or extrinsic) *in vitro* or *in vivo*.

3. A similar study using combination of the stem and root extract of *C. membranaceus* for toxicity studies in rats to establish its safety and efficacy.

4. Further observation studies using encapsulated CMASE in BPH and prostate cancer patients to establish its safety/efficacy in humans.

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APPENDIX A

UNIVERSITY OF GHANA

University of Ghana Institutional Animal Care and Use Committee
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07/05/2019

ETHICAL CLEARANCE
(UG-IACUC 003/18-19)

On February 13th, 2019 the University of Ghana – Institutional Animal Care and Use Committee (UG-IACUC) at a full committee meeting reviewed and approved your protocol as follows:

TITLE OF PROTOCOL: Pharmacological and Toxicological evaluation of stem extracts of Croton Membranaceus used in Benign Prostatic Hyperplasia and related cancer in Ghana

PRINCIPAL INVESTIGATOR: Mr Daniel Kwame Afriyie

Please note that the final review report must be submitted to the Committee at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to UG-IACUC for review and approval prior to implementation.

Please report all serious adverse events related to this study to UG-IACUC within seven days verbally and in writing within fourteen days.

This certificate is valid till 6th May, 2020. You are to submit annual reports for continuing review.

Signature of Chairperson:.....

Prof. Major (Rtd.) George A. Asare