ANTI-CANCER STUDIES OF *VITEX DONIANA* AND *FERETIA APODANTHERA* EXTRACTS ON MCF-7 CELL LINE AND RATS INDUCED WITH BREAST CARCINOGENESIS

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (PhD) DEGEREE IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,

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ZARIA

DECEMBER, 2019

DECLARATION

I declare that the work in this thesis entitled "Anti-cancer studies of *Vitex doniana* and *Feretia apodanthera* extracts on MCF-7 cell line and rats induced with breast carcinogenesis" has been performed by me in the Department of Biochemistry, Faculty of Life Sciences, Ahmadu Bello University. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

_____	_____	_____
Name of student	Signature	Date

CERTIFICATION

This thesis entitled ANTI-CANCER STUDIES OF *VITEX DONIANA* AND *FERETIA APODANTHERA* EXTRACTS ON MCF-7 CELL LINE AND RATS INDUCED WITH BREAST CARCINOGENESIS by Gilead Ebiegberi FORCADOS meets the regulations governing the award of a doctorate degree of philosophy (PhD) in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. D. B. James Chairman, Supervisory Committee	Signature	Date
Prof. A. B. Sallau Member, Supervisory Committee	Signature	Date
Dr. P. L. Mabeta Member, Supervisory Committee	Signature	Date
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ABSTRACT

Vitex doniana and *Feretia apodanthera* are two plants used traditionally for the treatment of inflammatory related disorders and cancer. This work investigated the*in vitro* antioxidant and *in vitro* cytotoxic effects of the extracts and fractions of *V. doniana* leaves and *F. apodanthera* root bark using diphenyl-β-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hematoxylin and eosin staining, xCELLigence and fluorescence activated cell sorting assays. Subsequently,*V. doniana* whole extract was used for an *in vivo* study in female Wistar rats aged 52 ± 2 days administered a single dose of 80 mg/kg DMBA (dimethyl benz[a]anthracene) by oral gavage and monitored for 150 days. Rats with relatively high levels of serum estrogen receptor-α were randomly assigned to treatment groups administered 0, 50, 100, 200 mg/kg *V. doniana* and 20 mg/kg Tamoxifen for 14 days. The *in vivo* experiment was then terminated and serum estrogen receptor-α (ER-α), interleukin-1β (IL-1β) and tumour necrosis factor -α (TNF –α) levels were determined using ELISA kits, some oxidative stress markers in mammary tissue homogenates were determined and expression of cyclooxygenase-2 (COX-2), caspase 3 and p53 in mammary tissue was done using immunohistochemistry. LCMS analysiswas done on the *V. doniana* extract to determine possible compounds present. The highest free radical scavenging potency from DPPH assay was observed in *V. doniana* Fraction 2 which had an IC_{50} of 98.12 ± 4.94 µg/ml. MTT results showed an IC_{50} of 26.11 ± 3.4 µg/mL and 66.5 ± 5.2 µg/mL values for *V. doniana* and *F. apodanthera* respectively, after treatment for 48 hours. MCF-7 cells treated with *V. doniana* extract showed cell shrinkage, nuclear condensation, increased nuclear-cytoplasmic ratio and arrest at the G_0/G_1 phase of the cell cycle. *In vivo*, significantly

($p<0.05$) lower serum ER-α, IL-1β, TNF –α and malondialdehydelevels were observed in Wistar rats administered *V. doniana* extract when compared to DMBA-induced non-treated control. Histological examination of the mammary tissue of *V. doniana* treated rats showed attenuation to malignant epithelial hyperplasia observed in mammary glands of the DMBA induced non-treated control. Immunohistochemical staining showed mild expression of COX-2 and moderate expression of caspase 3 and p53 in *V. doniana* treated rats in contrast to moderate expression of COX-2 and mild expression of caspase 3 and p53 observed in DMBA induced non-treated control. LCMS analysis of *V. doniana* extract showed 4',5-dihydroxy-7-methoxy-6-methylflavone, tartaric acid and vanillylamine which are compounds with reported anti-cancer, antioxidant and anti-inflammatory properties. The results suggest that*V. doniana* has great potential for the development of a therapeutic agent against estrogen receptor-positive breast cancer.

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Abbreviations

ABTS	-	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
AP-1	-	Activator protein -1
ATM	-	Ataxia telangiectasia mutated gene
Apaf-1	-	Apoptotic protease activating factor 1
Bax	-	Bcl-2 associated X protein
Bcl-2	-	B-cell lymphoma 2
BMI	-	Body mass index
BRCA 1	-	Breast cancer associated gene 1
BRCA 2	-	Breast cancer associated gene 2
BRIP1	-	BRCA1 Interacting Protein C-terminal Helicase 1
CDH1	-	Cell-cell adhesion gene 1
CHEK2	-	Checkpoint kinase 2
COX-2	-	Cyclooxygenase-2
DMBA	-	Dimethylbenz[a]anthracene
DPPH	-	Diphenyl-β-picrylhydrazyl
EGFR	-	Epidermal growth factor receptor
ELISA	-	Enzyme-linked immunosorbent assay
ER-α	-	Estrogen receptor –α
FACS	-	Fluorescence activated cell sorting
GCMS	-	Gas chromatography mass spectrometry
GSH	-	Reduced glutathione
GSSG	-	Glutathione disulphide (oxidized glutathione)
HIV	-	Human immunodeficiency virus
HNE	-	Hydroxynonenal

IGF-1	-	Insulin like growth factor-1
IL-1α	-	Interleukin-1α
IL-1β	-	Interleukin 1β
IL-6	-	Interleukin 6
IL-10	-	Interleukin 10
LCMS	-	Liquid chromatography mass spectrometry
MAPK	-	Mitogen activated protein kinase
MCF-7	-	Michigan Cancer Foundation - 7
MDA	-	Malondialdehyde
MLH1	-	MutL homolog 1
MPT	-	Mitochondrial permeability transition
MSH2	-	MutS homolog 2
MSH6	-	MutS homolog 6
MPT	-	Mitochondrial permeability transition pore
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	-	Sodium chloride
NF-kB	-	Nuclear factor kappa beta
PI3K	-	Phosphatidyl inositol 3 kinase
PMS2	-	Pair mismatch sequence 2
PTEN	-	Phosphatase and tensin homolog
PALB2	-	Partner and localizer of BRCA2
R_f	-	Retardation factor
ROS	-	Reactive oxygen species
SKT11	-	Serine/threonine kinase 11
SOD	-	Superoxide dismutase

TNF-α	-	Tumor necrosis factor-α
TP 53	-	Tumor protein 53
VEGF	-	Vascular endothelial growth factor

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Cancer is a disease characterized by an uncontrolled cell division, due to several alterations in genetic material and protein function, eventually resulting in a population of cells with a capacity to metastasize from the primary site to other organs of the body (Sreedhar and Zhao, 2018). Cancer metastasis is the main cause of mortality in patients (Ghoncheh *et al*., 2016). Although cancer was initially considered as a "Western" disease, it is now a major cause of morbidity and mortality in many countries(Forcados *et al*., 2017). In 2014, it was reported that on a global scale, cancer kills more people than malaria, tuberculosis and HIV combined (Moten *et al*., 2014).

Breast cancer is the leading cause of cancer related deaths among women globally, with an estimated incidence of 2.4 million cases each year and 523,000 deaths (Hu *et al*., 2019). There is a highprevalence of breast cancer in developed countries, a situation attributed to lifestyle and diet, as people in the developed countries tend to eat diets rich in calories and are more prone to a sedentary lifestyle (Ghoncheh *et al*., 2016). In developing countries, there is a relatively lower prevalence of cancer, but higher mortality rates are recorded (Pace and Shulman, 2016). In Africa, breast cancer incidence rates differ across regions with Eastern Africa, Western Africa and Southern Africa having an incidence rate of 30.4, 38.6 and 38.9 % respectively per 100,000 women (Brinton *et al*., 2014). In Nigeria, the incidence rate of breast cancer is 50.5 per 100,000, which implies that 1 out of every 2000 women in Nigeria stands the risk of been diagnosed with breast cancer each year (Bray *et*

al., 2018). The mortality rate among breast cancer patients in West Africa is the highest in Africa, and Nigeria contributes greatly to recorded mortality cases in the region (Azubuike *et al*., 2018). This lower survival chance among breast cancer patients in developing countries has been attributed to a number of factors among which are inadequate cancer treatment facilities, late disease prognosis and high costs of treatment (DeSantis*et al*., 2015).

Cancer pathogenesis is known to progress from initiation to promotion and progression stages, in which healthy cells undergo transformation into abnormal cells (Patel *et al*., 2011). In contrast to normal cells which regulate proliferation through controlled mechanisms, cancer cells acquire mutations to genes (tumor suppressor genes and proto-oncogenes) that normally regulate cell proliferation (Kane and Shcherbakova, 2014). Mutations in a gene can interfere with transcriptional and translational processes, resulting in altered protein function. Altered expression and function of tumor suppressor genes and proto-oncogenes are pointedly implicated in carcinogenesis (Lee and Muller, 2010).

Among cancers, breast cancer is reportedly the most traumatic among patients because of its psychological impact, as the disease can affect self-perception among female patients more than other cancers (Babita *et al*., 2014). The initiation of breast cancer is attributed to genetic and epigenetic alterations in a single cell, which successfully divides to give a number of altered cells, followed by clonal expansion due to an accumulation of acquired changes that favor tumorigenesis (Määttä *et al*., 2017). Breast tissue is vulnerable to carcinogenesis for two major reasons. First, in healthy females, estrogen activates downstream proteins required for normal mammary cell proliferation and tissue development (Anderson *et al*., 2014). This continuous cell division in the mammary tissue

increases the risk for cells with damaged DNA to escape cell cycle checkpoints (Macon and Fenton, 2013). Second, environmental carcinogens bind easily to immature breast cells in girls and young women who have not had their first full-term pregnancy. Such immature breast cells are also less efficient at repairing DNA damage compared to mature breast cells (Osborne *et al*., 2015).

Breast cancer is frequently diagnosed in women around menopause (Ghoncheh *et al*., 2016) accounting for about 500,000 deaths each year (Siegel *et al*., 2018). A study in Nigeria, used data from two cancer registries in Abuja and Ibadan, covering a 2-year period and found breast cancer to be the most diagnosed among Nigerian women (Jedy-Agba *et al*., 2012). Although the economic burden of breast cancer in Nigeria has not yet been fully ascertained, in the United States of America alone, the economic burden of breast cancer is estimated at $ 5.49 billion per annum (Ekwueme *et al*., 2014).

A number of breast cancer predisposing factors have been identified, prominent among which are family history, age, early menarche, genetics, breast feeding, exogenous hormone exposure, cigarette smoking and obesity(Vogel, 2017). Breast cancer is more prevalent among post-menopausal women, as women aged 60 – 70 years have the highest risk of developing breast cancer (McGuire *et al*., 2015). An increased risk for developing breast cancer is also associated with the number of first degree relatives of an individual who have been diagnosed with breast cancer (Colditz *et al*., 2012). Early menarche and late menopause have also been identified as a risk factor for breast cancer, due to longer duration of circulating estrogen in this category of women (Ritte *et al*., 2012). Women with no history of breast feeding are also at risk of developing breast cancer because they usually have continuous circles of estrogen synthesis (Babita *et al*., 2014). The genetic

makeup of an individual may also predispose to breast cancer as individuals who carry mutations in *BRCA1* and *p53* genes have a 65 – 81% risk of developing breast cancer (Shah *et al*., 2014).

The most commonly used treatment for breast cancer patients at present is chemotherapy (Collignon *et al*., 2016). The chemotherapeutic drugs in use include cyclophosphamide, doxorubicin, docetaxel, taxotere, tamoxifen, methotrexate, fluorouracil, vinblastine, cisplatin, and methotrexate, which are often used in combinations (Fan *et al*., 2017). These agents target and inhibit metabolic pathways implicated in carcinogenesis, but are associated with a number of side effects. For example, the anthracycline, doxorubicin exerts anti-cancer effects by intercalating with DNA and inhibiting the topoisomerase enzyme which controls DNA coiling and subsequent processes required for transcription (Lori *et al*., 2010). The major challenges associated with doxorubicin include myocardial damage and bone marrow suppression (Lori *et al*., 2010). Cisplatin is another drug used in cancer therapy which acts by crosslinking with purine bases on DNA, causing sustained DNA damage, thereby activating checkpoints that induce apoptosis in cancer cells (Dasari and Tchounwou, 2014). The side effects reported among patients on cisplatin therapy include severe kidney problems, allergy related reactions and altered immune response which makes the patients more prone to opportunistic infections, gastrointestinal disorders, and loss of organ function in some cases (Dasari and Tchounwou, 2014).

Other therapeutic agents used in cancer treatment are monoclonal antibodies, aromatase inhibitors and radiation therapy (Blumen *et al*., 2016). Currently, monoclonal antibody-based cancer therapy is regarded as a successful therapeutic strategy for both hematologic

malignancies and solid tumors. A major challenge associated with the use of monoclonal antibodies is high treatment costs which makes availability and use of the therapy very restrictive. Also, genetic polymorphism among cancer patients limits the expected optimal treatment response outcomes (Hsu and Lu, 2016). Aromatase inhibitors exert their anti-breast cancer effects by inhibiting aromatase, an enzyme which catalyzes the synthesis of circulating estrogen from androgen, thereby ameliorating the disease pathogenesis in patients with the estrogen receptor positive subtype of breast cancer (Chan, 2016). The major limitation for the use of aromatase inhibitors is that they are not given to premenopausal women, as the drugs cannot effectively inhibit rapidly synthesized aromatase in the ovaries of young women (Reinert and Barrios, 2015). Intrinsic and acquired resistance are also challenges associated with the use of aromatase inhibitors (Kümler *et al*., 2016). For radiation therapy, a major side effect is radiation dermatitis, as nearly all women who receive radiotherapy have reported experiencing some degree of radiation-induced dermatitis (Kole *et al*., 2017). Other side effects associated with radiation therapy include skin dryness and hair loss (Kole *et al*., 2017).

The challenges associated with current treatment options in use have necessitated research into the therapeutic potential of phytocompounds for the treatment of cancer (Levitsky and Dembitsky, 2014). Antioxidants and polyphenolic compounds in the plant,*Vernonia amygdalina* ("bitter leaf"), have been shown to exert anti-cancer effects on MCF-7 breast cancer cells (Yedjou *et al*., 2013). The activities of interleukin-1β and the tumor necrosis factor-α (pro-inflammatory cytokines), nuclear factor-kB and activator protein-1 (transcription factors), as well as other redox sensitive proteins associated with cell

survival and proliferation, can be altered by polyphenolic compounds (Kunnumakkara *et al*., 2008; Li *et al*., 2016). Phytocompounds have also been reported to activate caspase -3 and p53 (Ko *et al*., 2016). Caspase-3 plays an important role in inducing death of cancer cells by apoptosis (Olsson and Zhivotovsky, 2011). The p53 protein ensures that cells with damaged DNA do not progress to the division stage of the cell cycle, thereby protecting against proliferation of cancer cells (Park *et al*., 2016). Curcumin, a phytocompound obtained from *Curcuma longa*,has been shown to upregulate the expression of antioxidant enzymes by enhancing the activity of nuclear factor erythroid related factor-2, which is a transcription factor (Farombi *et al*., 2008). This property could partly be responsible for anti-cancer effects of curcumin (Kunnumakkara *et al*., 2008). Phytocompounds like saponin complexes with transmembrane cholesterol, which disrupts the mitochondrial membrane integrity (Gibellini *et al.*, 2015). Loss of transmembrane potential resulting in the release of cytochrome c eventually leads to death of such cancer cells by apoptosis via the intrinsic apoptotic pathway (Gibellini *et al*., 2015).

The potential targets of phytocompounds in ameliorating oxidative stress-mediated alterations implicated in carcinogenesis is illustrated in Figure 1.1. Phytocompounds, especially those rich in antioxidants, play a therapeutic role in cancer via different mechanisms (Forcados *et al*., 2017). Recent scientific studies have reported on the antioxidant and anti-inflammatory potency of two locally available plants; *Vitex doniana* and *Feretia apodanthera* (Lasekan, 2017; Owolabi *et al*., 2018). Both plants are used in traditional health care systems for the management of inflammatory related disorders (Lasekan, 2017; Owolabi *et al*., 2018).

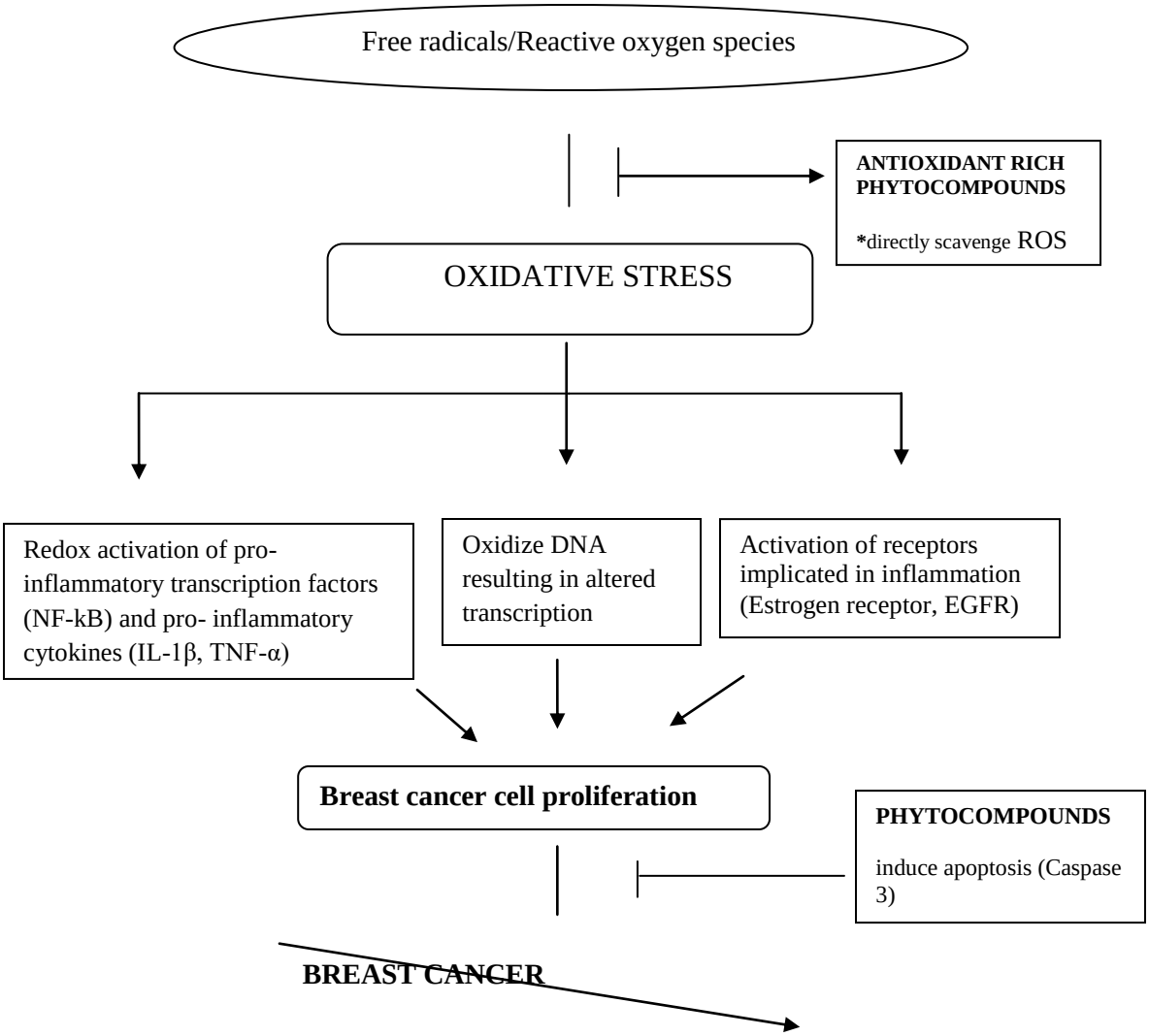


Figure 1.1: Potential targets of antioxidants in breast carcinogenesis (Forcados *et al*., 2017)

Vitex doniana is known as “Black plum” “Dinya”, “Ucha-koro” and “Oori-nla” in English, Hausa, Igbo and Yoruba languages respectively (Lasekan, 2017). Traditional medicine practitioners use various parts of the plant ranging from the leaves, stem and root bark for

the treatment of several disorders including hypertension, inflammatory related diseases and cancer (Agbafor and Nwachukwu, 2011). Studies on the plant show that the root bark, stem bark and leaves have antioxidant capacity and attenuated oxidative stress associated with carbon tetrachloridse induced hepatotoxicity in rats (Adetoro *et al*., 2013; James *et al*., 2014). A study on the phytochemical constituents of *V. doniana* leaves revealed the presence of saponins, tannins, anthraquinones, terpenoids, flavonoids and alkaloids, some of which could be responsible for the antioxidant effect of the plant leaves (Agbafor and Nwachukwu, 2011).

*Feretia apodanthera*is known as "Kuru-kuru", "Ishin" and "Okpu ulla" in Hausa, Yoruba and Igbo languages respectively (Owolabi *et al*., 2018). *F. apodanthera* is a bushy shrub of the family Rubiaceae (Coulibaly *et al*., 2014). The roots, root bark and leaves of this plant have been used locally to treat renal and urinary infections, stomach ache, nausea, syphilis, infective wounds and other disease conditions (Coulibaly *et al*., 2014; Owolabi *et al*., 2018). *F. apodanthera* extracts have been shown to have high antioxidant activity and relatively high flavonoid content (Coulibaly *et al*., 2014; Owolabi *et al*., 2018). Studies on the anti-inflammatory potential of *F. apodanthera* root bark showed it contains unsaturated steroids, triterpenes, cardiac glycosides, tannins, saponin and alkaloids, and had anti-inflammatory effects in Wistar rats (Owolabi *et al*., 2018).

1.2 Statement of the Problem

On a global scale, cancer associated deaths are responsible for more mortality cases than deaths from AIDS, malaria and tuberculosis combined (Angahar, 2017). Breast cancer accounts for approximately 25% of all female malignancies (Siegel *et al*., 2018). The

incidence, morbidity and mortality involving breast cancer is a worldwide challenge, as the condition affects people in both developed and developing countries (Siegel *et al*., 2018). Breast cancer is reportedly the second most diagnosed cancer worldwide, accounting for about 500,000 deaths yearly, with more deaths recorded in developing countries (Angahar, 2017; Siegel *et al*., 2018). In Nigeria, breast cancer is reportedly the most diagnosed cancer, contributing significantly to cancer related mortality (Jedy-Agba *et al*., 2012).The economical loss including medical bills and productivity loss associated with breast cancer in the United States of America alone, was put at $ 5.49 billion per annum (Ekwueme *et al*., 2014). Breast cancer is reported to affect perception of self-image among women, more than any other cancer (Babita *et al*., 2014).

1.3 Justification

Although the recent use of monoclonal antibodies has been successful in many patients, it is very expensive and not readily available (Moten *et al*., 2014; Hsu and Lu, 2016). Therefore, there is a need to discover other therapeutic agents from natural sources which could be cheaper, easily accessible and with lesser side effects. (Pan *et al*., 2012; Levitsky and Dembitsky, 2014). *Vitex doniana* leaves and *Feretia apodanthera* root bark are used traditionally for the treatment of wounds and inflammatory related ailments (Amegbor *et al*., 2012; Owolabi *et al*., 2018). Scientific investigations carried out on various parts of *V. doniana* showed that the leaves have high antioxidant capacity which could be due to the phytochemicals it possesses (James*et al*., 2014). *V. doniana* has also been shown to have *in vivo* anti-inflammatory effects (Iwueke*et al*., 2006). Studies carried out on extracts and fractions of*F. apodantheras*root bark have shown high antioxidant capacity and anti-

inflammatory effects (Owolabi *et al.*, 2018) possibly due to the phenolic compounds it contains. Since oxidative stress and inflammation are implicated in carcinogenesis, these plants may provide leads in the search for anti-cancer agents (Pan *et al*., 2012; Levitsky and Dembitsky, 2014).

1.4 Aim and Objectives

1.4.1 Aim of the Study

The aim of this research was to determine the anti-cancer potential of ethanol extract and fractions of *Vitex doniana* leaves and *Feretia apodanthera* root bark on MCF-7 breast cancer cell line and female Wistar rats induced with breast carcinogenesis using 7, 12 dimethylbenz[a]anthracene.

1.4.2 Objectives

The objectives of this work were to:

i. Determine the *in vitro* antioxidant capacity of *Vitex doniana* and *Feretia apodanthera* extracts and fractions
ii. Determine *in vitro* cytotoxic effects of *Vitex doniana* and *Feretia apodanthera* extracts and fractions on MCF-7 breast cancer cells
iii. Examine possible *in vivo* anti-cancer effects of the most potent in (ii) on 7, 12 dimethyl benz[a]anthracene-induced breast carcinogenesis in female Wistar rats
iv. Determine the *in vivo* toxicity/safety of the most potent in (ii) above
v. Identify components possibly present in the most potent in (ii) above

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Global and Regional Burden of Breast Cancer

There are a number of disparities in the global burden of breast cancer, attributable to genetics, social status, environment, lifestyle and differences in data collection efficiency across countries of the world (Hu *et al*., 2019). Records of cancer incidence and mortality are better documented in developed countries in contrast to developing countries with less efficient documentation (Azubuike *et al*., 2018). However, available records show that breast cancer remains a leading cause of cancer related deaths worldwide (Azamjah *et al*., 2019). Globally, 2.4 million new cases of breast cancer are diagnosed each year with about 523,000 deaths recorded each year (Hu *et al*., 2019).

In Africa, the highest incidence rate of breast cancer is recorded in Southern Africa, with an incidence rate of 38.9 per 100,000 followed by Western Africa, with an incidence rate of 38.6 per 100,000 (Brinton *et al*., 2014). In Nigeria, the incidence rate is 50.5 per 100,000 women (Ferlay *et al*., 2015). The mortality rate among breast cancer patients in West Africa is 20.1 per 100,000 for which Nigeria accounts for the highest mortality burden in the region due to the population size of the country (Azubuike *et al*., 2018).

Considering the limitations associated with data collection and storage in Nigeria and other West African countries, it is possible that the incidence, prevalence and mortality rates of breast cancer in documented literature may not even adequately portray the burden of breast cancer in the region. However, the published statistics shows that breast cancer is a threat to women in the region, and requires more attention from relevant stakeholders.

2.2 Habitat, Classification and General Description of *Vitex doniana*

2.2.1 Habitat

Vitex doniana is widespread in tropical Africa (Ruffo *et al*., 2002), occurring from Nigeria in West Africa through Somalia in Eastern Africa, to South Africa (Egbekun *et al*., 1996).

2.2.2 Botanical classification

According to the taxonomic System of the Angiosperm Phylogeny Group (APG III) (Chase *et al*, 2016) the taxonomic classification of *V. doniana* is:

Domain: Eukaryota

Kingdom: Plantae

Sub Kingdom: Viridaeplantae

Phylum: Tracheophyta

Sub phylum: Magnoliophyta

Subclass: Magnoliopsida

Super order: Lamiales

Order: Lamiales

Family: Lamiaceae

Genus: *Vitex*

Subspecies: *doniana*

Botanical name: *Vitex doniana*

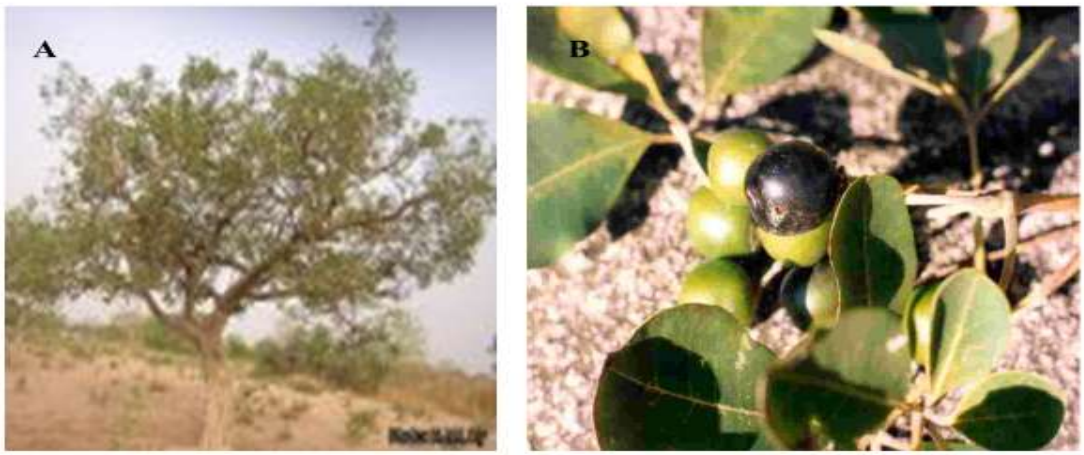
2.2.3 General description of *Vitex doniana*

Vitex doniana is a tree that sheds its leaves annually, with ability to grow to heights of about four to eight metres (Egbekun *et al*., 1996). The bark is light grey with numerous vertical fissures. The fruits of the tree are black, edible and sweet. The leaves are long stalked, more or less hairless and usually widest towards the tip (Egbekun *et al*., 1996). The flowers on the tree are numerous and usually white or tinged purple in colour. The tree bears fruits which areellipsoid to oblong in shape, approximately 3 cm long, containing one to four seeds. The fruits mature around April to June, during which they are collected as food (Ruffo *et al*., 2002).

2.2.4 Common names of *V. doniana*

V. doniana is commonly known as Black plum in English. The fruit is commonly called ‘Ucha koro’ in Igbo, ‘Dinya’ in Hausa, ‘Oori-nla’ in Yoruba and ‘Mfudu’ or ‘Mfulu’ in

Swahili (Lasekan, 2017).



(**A)** **(B)**

Plate I: A cross section of the branches (A), leaves and fruits (B) of *Vitex doniana*

(Source: Photo taken during collection of the plant leaves)

2.2.5 Traditional uses of *V. doniana*

2.1.5.1 *Edible traditional uses*

The cooked leaves of *V. doniana* are eaten as a vegetable or used in the preparation of sauces. The black pulp of the fruits is sweet and can be eaten raw. The fruit is often used to make jam (Okigbo, 2018). A beverage is also made from the fruit juice, and boiled fruits are used to make fruit wine (Devika, 2017).

2.2.5.2 *Reported Efficacy of V. doniana in the Management of Diseases*

V. doniana leaves sap is used as an eye drop to treat conjunctivitis and other eye complaints (Rani and Sharma, 2013). The leaves juice can also be squeezed into eyes for the treatment of eye pain (James*et al*., 2014). Also, the leaves decoction is applied externally against headache, stiffness, measles, rash, fever, chickenpox and hemiplegia, and internally as a tonic for the treatment of respiratory diseases (Agbede and Ibitoye,

2007; Rani and Sharma, 2013). The leaves are also reportedly used in the improvement of fertility (Muanda *et al*., 2011). The leaves and bark are applied to wounds and burns, and the decoction is used to treat gastro-intestinal disorders and jaundice (James *et al*., 2015). A decoction of the chopped stem bark of *V. doniana* is taken orally for the treatment of gastroenteritis and other ailments including diarrhea and dysentery (Agbafor and Nwachukwu, 2011). Traditional healers also use *V. doniana* in the treatment of hypertension, cancer and inflammatory related diseases (Agbafor and Nwachukwu, 2011; Muanda *et al*., 2011).

2.2.6 Chemical constituents of *V. doniana*

A study on the phytochemical constituents of *Vitex doniana* leaves revealed the presence of saponins, tannins, anthraquinones, terpenoids, alkaloids and flavonoids (Agbafor and Nwachukwu, 2011). The fruit which has a unique sweet prune-like aroma when ripened, has been reported to contain ethylbutanoate, β-damascenone, ethyl-2-methyl propionate, linalool, hexyl acetate (Lasekan, 2017). Nutritional analysis of the fruit revealed the presence of protein, fibre, sugars, calcium, phosphorus, magnesium and low levels of tannin and phytate (Agbede and Ibitoye, 2007). Vitamin analysis of *V. doniana* leaves revealed the presence of vitamin A, B1, B2, B3, C, D, E and K, while elemental analysis found calcium, magnesium, potassium, sodium, iron, manganese, zinc and copper to be present (Adelodun *et al*., 2016)

2.3 Habitat, Classification and General Description of *Feretia Apodanthera*

2.3.1 Habitat

Feretia Apodanthera Del. is a member of the Rubiaceae family, found mostly in the savanna regions of the West African coast (Burkill and Dalziel, 1985; Taiwe *et al*., 2016).

The plant can grow both in riverine areas and dry hillsides, even at elevations of 1,400 metres above sea level (Burkill and Dalziel, 1985). *F. apodanthera* is widely distributed along Tropical Africa (Mauritania to Nigeria) and across the Congo basin to Sudan and East Africa.

2.3.2 Botanical classification

According to the taxonomic System of the Angiosperm Phylogeny Group (APG III) (Chase *et al*., 2016) the taxonomic classification of *F. apodanthera* is:

Domain: Eukaryota

Kingdom: Plantae

Sub Kingdom: Viridaeplantae

Phylum: Tracheophyta

Sub phylum: Eupyllophytina

Subclass: Asterideae

Super order: Gentiananae

Order: Gentiananae

Family: Rubiaceae

Genus: *Feretia*

Subspecies: *apodanthera*

Botanical name: *Feretia apodanthera*

2.3.3 General description of *F. apodanthera*

F. apodanthera is a bushy deciduous shrub with winding or twisting branches, growing from 2 to 6 m tall (Burkill and Dalziel, 1985). The fruits of *F. apodanthera* are about 3 to 7 mm in diameter (Owolabi *et al*., 2018). The leaves are elliptic to oval in shape with some

apiculate apex and rounded or cuneate base (Owolabi *et al*., 2018). The tree is moderately branched with branches carrying ripe fruits during the fruit bearing season. The fruits are red, green or wine in color depending on maturation levels (Burkill and Dalziel, 1985).

2.3.4 Common names of *F. apodanthera*

F. apodanthera is called "Kuru-kuru" "Ishin", "Okpu ulla" in Hausa, Yoruba and Igbo languages respectively.

(A) **(B)**

Plate II : A cross section of the branches, leaves, fruits (A) and the roots (B) of *Feretia apodanthera*(Source: Photo taken during collection of the plant roots and processing of the roots)

2.3.5 Traditional uses of *F. apodanthera*

2.3.5.1 *Edible traditional uses*

F. apodanthera is a fruit bearing tree of which the red fleshy pulp when ripe, is eaten raw especially by herdsmen and children, for quenching hunger and thirst (Maydell, 1990). The seeds can be roasted and used in making drinks, while the dried leaves are eaten as vegetable (Maydell, 1990).

2.3.5.2 *Reported Efficacy of F. apodanthera in the Management of Diseases*

Preparations from *F. apodanthera* roots are used in the management of epilepsy, convulsions, pain and inflammation (Taiwe *et al*., 2016). Decoction prepared from the roots are used in the treatment of gonorrhea, syphilis and leprosy (Maydell, 1990). The

root is used to treat stomach-ache, nausea, and is also used as a calming agent for agitated mental conditions, as well as for enhancing cognitive performance (Taiwe *et al*., 2016). The powdered root is reportedly used in the treatment of wounds and swellings (Inngjerdingen *et al*., 2004; Coulibaly *et al*., 2014).

In North Eastern Nigeria, the bark and roots of *F*. *apodanthera* is ground and taken orally with tea by the Kanuris to enhance male reproductive function (Ene and Atawodi, 2012). In Senegal, the leaves of *F*. *apodanthera* are used to treat different urinary and renal infections (Ene and Atawodi, 2012). The methanol extract of leaves of *F*. *apodanthera* have been found to have significant antimalarial activity (Ancolio *et al*., 2002).

2.3.6 Chemical constituents of *F. apodanthera*

A study on the phytochemical content of *F. apodanthera* showed that the plant contains phenols, flavonoids and β-Carotene (Coulibaly *et al*., 2014). Another study reported that the aqueous extract of *F. apodanthera* contained flavonoids, alkaloids, saponins, tannins, glycosides, anthraquinones, and phenols but not cardiac glycosides and lipids (Taiwe *et al*., 2016). Phytochemical analysis of the root bark of *F. apodanthera* showed it contains unsaturated steroids, triterpenes, cardiac glycosides, tannins, saponin and alkaloids (Owolabi *et al*., 2018).

2.4 Literature Review on the Antioxidant and Anti-inflammatory Effects of *V. doniana* and *F. apodanthera*

V. doniana and *F. apodanthera* have been extensively investigated for their antioxidant and anti-inflammatory effects(Iwueke*et al*., 2006;Adetoro *et al*., 2013; James *et al*., 2014; Owolabi *et al*., 2018). The anti-inflammatory effects of *V. doniana* leaves extract was

investigated in rats induced with paw edema (Iwueke*et al*., 2006). Treatment of the rats with *V. doniana* leaves extract inhibited the formation of paw edema in the rats with a corresponding decrease in the activities of phospholipase A2 and prostaglandin synthase, pointing to the anti-inflammatory potency of *V. doniana* leaves extract (Iwueke*et al*., 2006). In another study, the leaves of *V. doniana* were investigated for both *in vitro* and *in vivo* antioxidant potential (Agbafor and Nwachukwu, 2011). The extract was able to scavenge DPPH with inhibition values comparable to vitamin c, while rats induced with carbon tetrachloride associated tissue damage and treated with the *V. doniana* extract showed enhanced antioxidant capacity (Agbafor and Nwachukwu, 2011). Another study used different solvents to obtain extracts from the leaves, stem bark and root bark of *V. doniana* and evaluated the *in vitro* antioxidant potency of the obtained extracts, for which the ethanol leaves extract was found to have the highest antioxidant potency (James *et al*., 2014). Phytochemical analysis of the *V. doniana* leaves extract showed the presence of saponins, tannins, anthraquinones, terpenoids and flavonoids (Agbafor and Nwachukwu, 2011; James *et al*., 2014), while GCMS analysis of the extract showed the presence of phellandrene, phytol and caryophyllene (Sonibare *et al*., 2009)

The phytochemical, antioxidant and anti-inflammatory properties of *F. apodanthera* root bark has been investigated and reported (Owolabi *et al*., 2018). Phytochemical analysis revealed the presence of saponin, alkaloids, tannins and triterpenes, while DPPH results showed free radical scavenging capacity (Owolabi *et al*., 2018). Inhibition of right paw edema was observed in rats induced with inflammation and treated with *F. apodanthera*. Chromatographic analysis of the extract showed the presence of flavonoids and phenolics with reported antioxidant and anti-inflammatory effects (Owolabi *et al*., 2018).

Thus, scientific investigations on the antioxidant and anti-inflammatory potential of *V. doniana* and *F. apodanthera* point to the efficacy of the ethanol extract of the leaves of *V. doniana* and root bark of *F. apodanthera* (James *et al*., 2014; Owolabi*et al*., 2018).

2.5 Breast Cancer

2.5.1 Breast cancer predisposing factors

A number of factors which are briefly discussed below have been associated with an increased risk for developing breast cancer.

2.5.1.1 Age

The risk of developing breast cancer has been found to increase with age (McGuire *et al*., 2015). At present, about 40% of breast cancer patients are above 65 years of age and account for almost 60% of the total deaths from breast cancer (Siegel *et al*., 2018). A report shows that the estimated risk of developing breast cancer before 49 years of age is 1/53, between the ages of 50 – 59 years is 1/43, between the ages of 60 – 69 years is 1/23, while women at 70 years and above have the highest risk of 1/15 chance of developing breast cancer (McGuire *et al*., 2015). Considering the increased life span of people globally (Siegel*et al*., 2018), these statistics suggest that elderly patients with breast cancer are expected to increase in future, as more than 20% of the world's population are projected to attain 65 years of age and above by the year 2030 (McGuire *et al*., 2015).Theories have been put forward to explain the biochemical basis of aging and predisposition to cancer (Aunan *et al*., 2017). Mitochondria function has been found to deteriorate with age, resulting in increased generation of superoxide anion. High levels of

superoxide anion can lead to oxidative stress and subsequent oxidation of proteins, lipids and DNA which could predispose to cancer (Sun *et al*., 2016)

2.5.1.2 Family history

A woman's risk of developing breast cancer has been found to increase if her family has a history of the disease (Colditz *et al*., 2012). In a study, women with a mother diagnosed of breast cancer before the age of fifty had a relative risk of 1.69. Women with mothers diagnosed of breast cancer at an age above 50 years had a relative risk of 1.37 compared to women without a family history of breast cancer. Women who had a sister diagnosed with breast cancer showed a relative risk of 1.66 if the diagnosis was made before age 50, but if diagnosed after the age of 50, the relative risk was 1.52 when compared to breast cancer patients without a family history. Collectively, results from the study suggested that a higher risk of developing breast cancer is associated with an increased number of first degree relatives diagnosed with breast cancer at an age below 50 years (Colditz *et al*., 2012). In another study, women who had two or three first-degree relatives with breast cancer had relative risks of 2.93 and 3.90 respectively, when compared to women who had no affected relatives (Easton, 2002). A major contribution to cancer predisposition among first degree relatives has been attributed to inheritance of mutated BRCA1 and p53, which are tumor suppressor proteins responsible for ensuring fidelity of genetic material and cell duplication (Heisey *et al*., 1999; Wei *et al*., 2012). Studies have shown that about 65% of women with inherited deleterious mutations in BRCA1 will develop breast cancer at age 70 (Skol *et al*., 2016)

2.5.1.3 Early menarche

Age at menarche (first menstrual cycle or bleeding) is a reported risk factor for developing breast cancer among pre- and postmenopausal women (Terry *et al*., 2017). In a case-control study, women who had a two-year delay in menarche were associated with a corresponding breast cancer risk reduction of 10% (Hsieh *et al*., 1990). In a similar study carried out in the United States of America, early age of pubertal development was associated with an increased risk of breast cancer (Terry *et al*., 2017). A cohort study carried out in Europe, reported nearly two-fold increase in the risk of hormone receptor positive tumors among women who had early menarche (Ritte *et al*., 2012). The major factor implicated in early menarche predisposition to breast cancer is duration of estrogen exposure, as women with an early age at menarche and late age of menopause have longer periods of estrogen exposure (Dall and Britt, 2017). Higher circulating estrogen levels correlates with increased risk of breast cancer, as the hormone regulates breast cell proliferation (Chang, 2011).

2.5.1.4 Breast feeding

Breast feeding has been found from different studies to protect against breast cancer development in women (Babita *et al*., 2014). A number of factors could be responsible for this, as breast feeding may delay the return of regular ovulatory cycles and decrease endogenous sex hormone levels (Babita *et al*., 2014). Further protective effects of breastfeeding against breast carcinogenesis is inferred from a study which showed that exclusive breastfeeding among parous women reduced the risk of developing breast cancer when compared to parous women who did not exclusively breastfeed (Unar-Munguía *et al*., 2017). However, there are contradictory reports on the protective effects of breast

feeding duration against breast cancer development(Butt *et al*., 2014; Zhou *et al*., 2015). The molecular basis for breast feeding conferring protection against breast cancer is linked to enhanced differentiation of mammary tissue and reduction in the number of ovulatory cycles in women who have a history of breastfeeding (França-Botelho *et al*., 2012). Breast cells that are well differentiated are less susceptible to oncogenic proliferation, while fewer ovulatory cycles correlate with reduced exposure to estrogen and prolactin which are implicated in carcinogenesis (França-Botelho *et al*., 2012)

2.5.1.5 Genetic predisposition

Improved scientific methodology has enabled the identification of genetic predisposition alleles which are of clinical significance (Skol *et al*., 2016). These genes are broadly classified as either high risk or low risk susceptibility genes (Skol *et al*., 2016). So far, there are about seven breast cancer susceptibility genes or gene sets that have been identified, for which any germline mutations are often associated with an increased risk of developing breast cancer (Matthews and Thompson, 2016). These genes are: DNA damage repair genes *BRCA1/2*, tumor suppressor *TP53*, tumor suppressor phosphatase, cell–cell adhesion gene *CDH1*, tensin homolog *PTEN*, serine threonine kinase *SKT11* and DNA mismatch repair genes *MLH1*, *MSH2/6*, and *PMS2*(Matthews and Thompson, 2016). The lifetime breast cancer risk for BRCA1 mutation carriers ranges from 65% to 81% while the range for BRCA2 carriers is between 45% to 85% (Shah *et al*., 2014). The low risk breast cancer susceptibility genes include *CHEK2*, *ATM*, *BRIP1* and *PALB2* genes (Riaz*et al*., 2009).

The first moderate risk gene to be identified was *CHEK2* (Riaz *et al*., 2009). Mutations in *CHEK2* are identified in about 5% of families with breast cancer risk, though the prevalence has been found to vary widely among populations. Women who carry *CHEK2* mutations have a moderate two to three fold increased risk of developing breast cancer (Riaz *et al*., 2009).

2.5.1.6 Exogenous hormone exposure

Results from different studies suggest that a relationship exists between the use of hormone replacement therapy and risk of developing breast cancer (Jones *et al*., 2016). Hormone replacement therapy is common among menopausal women, due to its effectiveness in the treatment of symptoms like hot flashes, night sweats, and symptoms of urogenital atrophy associated with menopause (Velentzis *et al*., 2016; Yu *et al*., 2017). A study carried out among Japanese women showed that women who used hormone replacement therapy had a higher risk of developing breast cancer compared to women who never used it (Liu *et al*., 2016). Another means of exogenous hormone exposure among women is the use of hormonal contraceptives, as a study showed that women who used hormonal contraceptives were at a higher risk of developing breast cancer when compared to women who did not (Heikkinen *et al*., 2016). Estrogen is the major hormone used by women on hormone replacement therapy (Velentzis *et al*., 2016). Estrogen binds to receptors resulting in increased levels of AP-1 which could cause increased breast cell proliferation and predispose to breast cancer

2.5.1.7 Obesity

Obesity in postmenopausal women has been found to increase the risk of developing breast cancer (Fortner *et al*., 2016; Matthews and Thompson, 2016). The association between deregulated insulin signaling, obesity and breast cancer risk has long been recognized (Gunter *et al*., 2009). In breast cancer patients, increased circulating insulin is associated with increased expression of insulin like growth factor-1 (IGF-1) (Arcidiacono *et al*., 2012). Higher cellular levels of insulin and IGF-1 have the potential to create a pro-carcinogenic environment, because the binding of insulin or IGF-1 to their cognate receptors activates a series of events including the phosphatidyl inositol 3 kinase (PI3K)/Akt/ signal transduction cascade (Rose and Vona-Davis, 2012). This pathway climaxes in the activation of proteins like cyclin D1 and c-Myc which play roles in cell cycle progression (Arcidiacono *et al*., 2012).

Altered insulin signaling sometimes observed in obesity also promotes cancer progression through upregulation of vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1α, resulting in increased cell proliferation and promotion of vascular tube formation, which are key processes in angiogenesis (Rose and Vona-Davis, 2012). A cohort study reported that postmenopausal women with no history of exogenous hormone exposure, were associated with a higher breast cancer risk if they had relatively high body mass index (BMI) and hip circumference(Lahmann *et al*., 2004). A similar study carried out in Asia, observed that higher waist-to-hip ratio, waist circumference and body mass index were associated with a three-fold increased risk of breast carcinogenesis among premenopausal and postmenopausal women (Nagrani *et al*., 2016).

2.5.1.8 Cigarette smoking and alcohol consumption

The role of cigarette smoking on predisposition to breast cancer has generated a lot of controversies and research interest (Catsburg *et al*., 2015). This is due to a number of factors which includes quantity of cigarette content exposure and individual variations in levels of enzymes responsible for the metabolism of polycyclic aromatic hydrocarbons. A cohort study in Canada which followed about 89,000 women over a mean period of 22 years found an association between cigarette smoking and risk of developing breast cancer (Catsburg *et al*., 2015). The study also found a higher risk of developing breast cancer among women who smoked for a longer period before their first full-term pregnancy (Catsburg *et al*., 2015). Similar observations were also recorded in studies carried out in Canada (Cui *et al*., 2006), America (Gaudet *et al*., 2013), Serbia (Ilic *et al*., 2014) and Denmark (Andersen *et al*., 2017).

A study which examined if there was any correlation between cigarette smoking and mortality rates among breast cancer patients reported higher mortality among patients with a history of cigarette smoking before or after cancer diagnosis when compared to breast cancer patients with no history of cigarette smoking (Passarelli *et al*., 2016). A study on the effect of racial difference on etiology of breast cancer among smokers found higher breast cancer associated mortality among women of African-American descent who smoke when compared to non-African-American women who smoke (Parada *et al*., 2017). Cigarette smoke contains polycyclic aromatic hydrocarbons which undergo metabolism to yield epoxides that bind to DNA resulting in the formation of DNA adducts that could affect transcription and translation, leading to dysregulated function of proteins that regulate cell proliferation(Catsburg *et al*., 2015). Epoxides obtained from polycyclic

aromatic hydrocarbons can also bind to lipids and proteins resulting in oxidative stress that predisposes to cancer (Kispert and McHowat, 2017).

Alcohol consumption has also been reported to contribute to the risk of developing breast cancer (Scoccianti *et al*., 2014). A cohort study carried out in eight European countries found that alcohol consumption correlated with development of colorectal cancer among men and women (Schütze *et al*., 2011). A cohort study of 105, 986 women followed for 28 years showed a correlation between alcohol consumption and risk of developing breast cancer (Chen *et al*., 2011). The role of alcohol in carcinogenesis could be linked to acetaldehyde, a toxic product of ethanol metabolism, capable of causing oxidative damage to cellular macromolecules like DNA, proteins and lipids, eventually contributing to carcinogenesis (Seitz and Stickel, 2007). Women who smoke cigarette and also consume alcohol have been reported to be at an increased risk of developing estrogen receptor positive breast cancer (Kabat *et al*., 2011).

A proper understanding of the relationship between these breast cancer predisposing factors and the risk of developing breast cancer provides several advantages for patients, in terms of treatment planning and choices like whether or not to undergo mastectomy and/or prophylactic contralateral mastectomy.

2.5.2 Breast cancer pathogenesis

At present, two leading models are used to explain the etiology of breast carcinogenesis; the sporadic clonal evolution model and the cancer stem cell model (Bombonati and Sgroi, 2011). The sporadic clonal evolution hypothesis suggests that breast epithelial cells are prone to random mutation, and that over time, genetic and epigenetic alterations could

aggregate and contribute to tumor initiation and progression (Rodriguez-Brenes and Wodarz, 2015). The alternative cancer stem cell model postulates that the unique capacity to initiate and maintain tumor progression is limited to stem and progenitor cells (Bozorgi *et al*., 2015). Since stem cells have the capacity to divide and differentiate into other cells of the body, mutations in the DNA of such cells could be passed on to other daughter cells (Bozorgi *et al*., 2015). A large number of such cells with damaged DNA can then yield a number of potential cancer cells (Bozorgi *et al*., 2015). However, some scientists have maintained that the above hypothesis are not mutually exclusive, as stem cells could also undergo clonal evolution, thereby providing a link between the two models (Bombonati and Sgroi, 2011).

The basis for the above models, lies in the physiology of normal mammary tissue. The normal mammary tissue contains normal breast stem cells, which are long-term tissue resident cells (Xing *et al*., 2017). Breast stem cells are capable of self-renewal and multilineage differentiation, and are able to recapitulate the breast tubulo-lobular architecture that is composed of luminal and myoepithelial cells (Villadsen *et al*., 2007; Xing *et al*., 2017). Based on their phenotypic and functional features, normal breast stem cells can be identified and isolated, as they lack expression of epithelial cell adhesion molecule, hematopoietic and endothelial markers (Bombonati and Sgroi, 2011). The breast stem cells also lack estrogen and progesterone receptors, residing mostly in ducts in the suprabasal position, surrounded by proliferating progenitor cells (Bombonati and Sgroi, 2011). Since normal breast cancer stem cells are long-time tissue residents, they are susceptible to accumulating genetic and epigenetic modifications (Ercan*et al*., 2011). Such

molecular alterations could result in the development of a cancer stem cell that can undergo asymmetric division, thereby maintaining a stem cell population with molecular alterations (Bombonati and Sgroi, 2011). These altered stem cells also have the potential and capacity to differentiate into committed progenitor(s) cells that give rise to the different breast cancer cells (Ercan *et al*., 2011). Cancer initiating cells could also be derived from committed progenitor cells, as suggested from a study which showed that progenitor cells within the luminal compartment of the human breast represents a cancer initiating population in BRCA 1 mutation carriers (Lim *et al*., 2009).

In addition to the above models of carcinogenesis, which explain the role of cells in the initiation of cancer, studies have shown that the tumor microenvironment also plays a pivotal role in carcinogenesis (Wang *et al*., 2017). The tumor microenvironment consists of extracellular fluid and non-malignant cells such as fibroblasts, endothelial cells, immune and inflammatory cells (Artacho-Cordón *et al*., 2012). The interactions between cancer cells and the microenvironment contributes towards proliferation, alterations in tissue vasculature and metastasis in breast cancer (Artacho-Cordón *et al*., 2012; Wang *et al*., 2017). Scientific reports show that the tissue microenvironment becomes altered during tumorigenesis, characterized by interactions between stromal and tumor epithelial cells, which favors an environment that promotes cancer cell proliferation, invasiveness, tumorigenicity, and metastasis (Wang *et al*., 2017).

Although the above models are currently accepted as mechanisms of breast carcinogenesis, studies are still ongoing to further understand the dynamics of breast carcinogenesis. This

is important because if it is possible to reliably identify pro-cancer cells, strategies that successfully eliminate them quite early, would decrease the risk of mammary tumorigenesis and breast cancer recurrence.

2.5.3 Breast cancer molecular subtypes

Breast cancer has been found to consist of multiple subtypes with distinct morphologies (Prat *et al*., 2015). Accumulating evidence from molecular examination suggests that breast cancers with different histopathological and biological features exhibit varying levels of hormone receptor expression, proliferation patterns, metastasis and response to treatment(Dai *et al*., 2015). Below are the currently classified subtypes of breast cancer:

i. Luminal A tumours
ii. Luminal B tumours
iii. HER2 over expressing tumours
iv. Triple negative tumours
v. Quadruple negative tumours

The luminal-like tumors express hormone receptors with expression profiles similar to that of the luminal epithelial component of the breast (Lim *et al*., 2009). Luminal A tumour represents the estrogen receptor positive (ER+), progesterone receptor positive (PR+) and human epidermal growth factor receptor 2 negative (HER2-) subtype (Prat *et al*., 2015). The luminal B tumors represents the ER+, PR+ and HER+ subtype (Prat *et al*., 2015). These luminal tumors (especially luminal A) are the most common subtypes among breast cancer patients globally (Reis-Filho and Pusztai, 2011). Although treatment response has been found to differ between luminal subtypes, luminal tumors generally respond well to hormone therapy but respond poorly to conventional chemotherapy (Prat *et al*., 2015). The

luminal A tumors have higher expression of estrogen receptor related genes and lower expression of proliferative genes when compared to luminal B cancers (Reis-Filho and Pusztai, 2011).

Tumors classified as HER2 overexpressing are ER-, PR- and HER2 + with about 40% of these tumors showing TP53 mutation (Schramm *et al*., 2015). These tumors have been found to be sensitive to taxane-based neoadjuvant chemotherapy (Arteaga *et al*., 2012). The pathogenesis of breast cancer in these patients is usually characterized by poor prognosis and early relapse (Arteaga *et al*., 2012). Monoclonal antibodies like Trastuzumab are available for HER2 over expressing patients, although not all HER2 over expressing patients respond to Trastuzumab (Bartel and Jackson, 2017). Triple negative tumors are also referred to as basal tumors, with expression patterns similar to basal epithelial cells (Grubb*et al*., 2017). The tumors are ER-, PR- and HER2- showing low expression of hormone receptors and high expression of keratins and proliferation related genes (Shao*et al*., 2017). These tumors follow an aggressive clinical course and currently lack any form of standard targeted systemic therapy (Shao *et al*., 2017). This subtype is characterized by rapid growth and is commonly seen among younger women and women of African descent (Grubb *et al*., 2017). The tumors are reported to be larger than other subtypes and metastasis among patients is seen to have a tendency towards extending to visceral organs (Shao *et al*., 2017).

Recent studies examining molecular patterns among breast cancer patients has revealed quadruple negative tumours as a possible subtype of breast cancer, due to the potential prognostic role of androgen receptor (Bhattarai *et al*., 2019). These tumours are androgen

receptor negative in addition to being ER-, PR- and HER2-(Bhattarai *et al*., 2019). Androgens are hormones that control cell proliferation either by binding to the androgen receptor, resulting in activation of proteins implicated in breast cell proliferation or by serving as a pro-hormone for synthesis of estrogen (Rampurwala *et al*., 2016)Androgen receptors are a class of steroid receptor proteins and are members of the nuclear steroid hormone receptor family which act as transcription factors that regulate cell proliferation (Bleach and McIlroy, 2018). Since androgen receptors binds to ligands, identifying ligands that modulate its downstream proliferative targets, could have ameliorative effects for triple negative breast cancer patients expressing androgen receptor (Astvatsaturyan *et al*., 2018). Thus, current studies across geographical regions are examining androgen receptor expression patterns among triple negative breast cancer patients and possible factors associated with androgen receptor expression patterns among such patients (Bhattarai *et al*., 2019).

Extensive studies in sub-regions, aimed atfurther understanding the gene expression patterns among patients and epigenetic factors that couldbe responsible for the altered gene expression patterns among patients with different breast cancer subtypesare required. Due to current challenges with poor response to therapy observed among breast cancer patients, such studies couldlead to therapeutic options that are specific and efficacious for breast cancer patients.

2.5.4 Histological Classification of Breast Cancer

Breast cancers exhibit a wide range of morphological features characterized by specific clinical patterns and outcomes, which form the basis for their classification(Makki, 2015).

Histological classification broadly divides breast cancer into two groups; in situ carcinoma and invasive carcinoma (Malhotra *et al*., 2010). In situ carcinoma could be ductal or lobular. Ductal in situ carcinoma is characterized by non-invasive neoplastic epithelial cells within the mammary ductal system which do not extend to the basement membrane (Vaidya *et al*., 2015). Lobular in situ carcinoma is also characterized by non-invasive neoplastic epithelial cells centred in the terminal ductal lobular units that fill and expand most of the acini, sometimes growing underneath the ductal epithelium (Wen and Brogi, 2018). Both ductal and lobular in situ carcinoma show nuclear atypia with tendencies of subsequently developing into invasive breast cancer.

Invasive carcinoma is also referred to as infiltrating carcinoma and is characterized by a malignant proliferation of neoplastic breast cells which have penetrated through the duct and into the stroma (Makki, 2015). Invasive carcinoma could be ductal, lobular, tubular, infiltrating ductal or medullary (Malhotra *et al*., 2010). Invasive lobular carcinoma is characterized by cells that infiltrate the stroma and have low or no E-cadherin expression resulting in a non-cohesive phenotype (Rakha and Ellis, 2010). Invasive ductal carcinomas show a lot of heterogeneity with varying ductal differentiation. The cells are generally multi nucleated and vary in shape and size (Makki, 2015). In contrast tubular invasive carcinoma are well differentiated and less likely to have lymph node metastasis (Yilmaz *et al*., 2018). Medullary breast carcinoma is rare but mostly common in carriers of BRCA1 mutation (Makki, 2015; Zangouri *et al*., 2018)

Despite the advent of breast cancer classification based on recent molecular subtypes, the use of histological examination for classifying breast cancers and determination of

response outcomes to used treatment regimens continues to play an important role in breast cancer management.

2.6 Oxidative Stress, Inflammation and Carcinogenesis

2.6.1 Oxidative stress and carcinogenesis

Oxidative stress is defined as a physiological state in which systemic generation of reactive oxygen species (ROS) and free radicals overwhelms the cellular antioxidant capacity, resulting in an altered redox state of the cell, eventually causing damage to cellular macromolecules (Forcados *et al*., 2017). Processes that generate reactive oxygen species and free radicals occur during normal cellular metabolism and reactive species play important roles in cell signaling pathways (Alpay *et al*., 2015). Most reactive oxygen species are generated in the mitochondria, especially when electrons react with oxygen (O_2) during ATP generation, resulting in the formation of superoxide anion (Alkadi, 2018). Superoxide anion can react with transition metals like Fe,$^{2+}$ resulting in the generation of other reactive species like hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and organic peroxides (Pisoschi and Pop, 2015).

The major harmful effects of ROS in mammalian cells are caused by the hydroxyl radical (Catarino *et al*., 2015). The hydroxyl radical has a very unstable electron structure, easily reacting with cellular components, and is mainly produced via the Fenton reaction when hydrogen peroxide reacts with Fe^{2+} during hypoxic conditions, like prolonged exercise and disease conditions like diabetes (Pisoschi and Pop, 2015). The mitochondrial respiratory chain also produces nitric oxide (NO), which can generate other reactive nitrogen species

(Valko *et al*., 2007). Reactive species can react with cellular macromolecules like lipids, inducing lipid peroxidation that causes the formation of reactive intermediates like malondialdehyde (MDA) and 4-hydroxynonenal, which are mutagenic in bacterial and mammalian cells and carcinogenic in rats (Calaf *et al*., 2018).

An observable effect of lipid peroxidation by ROS in cells can be seen in the integrity of cell membranes (Valko *et al*., 2007). The cell membrane is made up of a phospholipid bilayer that is rich in polyunsaturated fatty acid residues that are extremely prone to oxidation (Pisoschi and Pop, 2015). Oxidation of membrane phospholipids by ROS causes alterations to the compactness and integrity of the membrane, which negatively affects normal functioning of the cell (Roque *et al*., 2015). A cell with altered mitochondrial membrane integrity is prone to apoptosis, as changes to the inner mitochondrial membrane integrity results in opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of cytochrome *c* which is normally a sequestered intermembrane pro-apoptotic protein (Yadav *et al*., 2015). The released cytochrome *c* binds and activates Apaf-1 and downstream procaspase-9, forming an apoptosome, thereby activating the intrinsic caspase dependent apoptotic pathway (Hill *et al*., 2004; White *et al*., 2017).

Studies examining the oxidation of proteins by ROS have shown that amino acids, simple peptides and proteins are prone to damage and altered function, when exposed to conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are present (Stadtman, 2004). Proteins rich in cysteine and methionine are particularly susceptible to oxidation by the action of ROS. The oxidation of cysteine residues by ROS

could cause the formation of disulphide bonds between protein thiol groups, possibly resulting in altered folding and function of the protein (Höhn *et al*., 2014). Proteins can also be oxidized by ROS to form carbonyl compounds, which are an irreversible form of modified protein (Weber*et al*., 2015). The concentration of such carbonyl groups generated can be used as a measure of ROS-mediated protein oxidation. (Weber *et al*., 2015).

During severe oxidative stress seen in certain disease conditions, increased protein oxidation, impaired proteolytic systems, accumulation of oxidized proteins and formation of non-degradable protein aggregates, could alter cellular homeostasis (Höhn *et al*., 2014). Advanced glycation end products, which are formed by a reaction between the free amino group of proteins and carbohydrateshave been reported to be mutagenic (Stopper *et al*., 2003). Glycation causes a modification of the structural properties of proteins such as albumin and haemoglobin leading to inflammation that further exacerbates oxidative stress (Younessi and Yoonessi, 2011). Glycated proteins form aggregations, which are insoluble and resistant to degradation, and also interact with MAPK and NF-kB signaling pathways implicated in carcinogenesis (Younessi and Yoonessi, 2011).

DNA is also susceptible to attack by free radicals, as the hydroxyl radical is reported to react with all components of the DNA molecule, damaging purine and pyrimidine bases as well as the deoxyribose backbone (Matsui *et al*., 2000). When such oxidative damage to DNA occurs, molecules like 8 -hydroxy deoxyguanosine (8-OHdG) are generated which increase the risk of mutagenesis (Matsui *et al*., 2000). The compound 8-OHdG is extensively used as a marker of free radical attack on DNA (Chu *et al*., 2012). 8-OHdG has been implicated in the initiation and promotion stages of carcinogenesis as it is reportedly increased eight to seventeen folds in breast tumors when compared to non-malignant breast

tissue (Matsui *et al*., 2000). Also, 8-OHdG can induce GC → TA transversion mutations and cause formation of DNA adducts which can predispose to cancer initiation if such oxidative lesions are not repaired(Sova *et al*., 2010). This is possible because formation of DNA adducts in the coding region of tumor suppressor proteins like TP53, causes altered function of the protein, leading toaltered cell proliferation(Rivlin *et al*., 2011).

In summary, an interplay exists between oxidative stress and carcinogenesis, as oxidative stress could result in oxidation of lipids to form mutagenic MDA and 4-hydroxynonenal. Oxidative stress can also cause oxidation of DNA, forming DNA adducts, resulting in altered transcription, translation and consequently dysregulated protein function (Forcados *et al*., 2017). Oxidative stress can also alter the redox status of cells causing oxidation of cysteine residues of antioxidants like glutathione and thioredoxin. Thus, mitigating oxidative stress could ameliorate molecular alterations and symptoms associated with breast carcinogenesis (Hecht *et al*., 2016).

2.6.2 Inflammation and carcinogenesis

The link between inflammation and cancer had been suggested as early as 1863 when it was observed that inflammatory cells (leukocytes which comprises of granulocytes, lymphocytes and monocytes) were present in cancerous lesions and that tumors could grow at sites of chronic inflammation (Povzun *et al*., 2011). Epidemiological and experimental data have also added to the hypothesis that inflammation is linked to cancer (Reuter, 2011). Due to the observation that inflammatory mediators contribute to carcinogenesis, cancer related inflammation is now regarded as the seventh hallmark of cancer (Colotta *et al*., 2009). During inflammation, mast cells and leukocytes are recruited to the site of damage (Multhoff *et al*., 2012). The activity of the recruited cells in an

attempt to correct the tissue damage leads to a 'respiratory burst' due to an increased uptake of oxygen and an increased accumulation of ROS at the site of damage (Multhoff *et al*., 2012).

Two stages of inflammation exist, acute and chronic inflammation (Reuter, 2011). Acute inflammation is regarded as the initial stage of inflammation, and is mediated through the innate immune response (Rehman and Akash, 2016). This type of inflammation usually persists for only a short time (between hours and few days) and is expectedly beneficial to the host (DeNardo and Coussens, 2007). However, if the cause of inflammation persists for a longer period of time, the second stage of inflammation (chronic inflammation) sets in and may predispose the host to various chronic conditions including cancer (Multhoff *et al*., 2012). Investigations on the role of inflammation in breast carcinogenesis observed that elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients, thereby suggesting that inflammation plays a major role in breast carcinogenesis (Cole, 2009; Pierce *et al*., 2009). Studies have also shown that inflammatory mediators promote mammary tumor development in animal cancer models (Kamel *et al*., 2012). Inhibition of inflammatory cytokines and transcription factors are reported to be protective against chemical induced mammary carcinogenesis (Kamel *et al*., 2012).

The pleiotropic cytokine tumor necrosis factor-α (TNF-α) can play dual roles of cell death induction or promotion of cell survival (Doss *et al*., 2014). Under certain conditions, TNF-α is destructive to tumor vasculature and induces necrosis (Doss *et al*., 2014). Yet, at doses of 10-120 ng/ml, an *in vitro* study showed that TNF-α promoted cell proliferation (Wang*et al*., 2015). Interleukin-1β (IL-1β) is another cytokine that plays a very important role in

inflammation (Dinarello, 2011). Aberrant IL-1β expression has been linked to breast carcinogenesis and poor response to therapy (Jeon *et al*., 2016). Clinical studies have also shown elevated circulating levels of IL-1β correlated with increased breast cancer progression and metastasis (Tulotta and Ottewell, 2018).

Cyclooxygenase 2 (COX-2), has emerged as an important mediator of inflammation in breast carcinogenesis (Krishnamachary *et al*., 2017). Increased COX-2 expression is associated with the severity of mammary cancer progression (Guler *et al*., 2016). COX-2 is the rate-limiting enzyme responsible for the conversion of arachidonic acid to prostanoids, which act as a key mediator of inflammation (Harris *et al*., 2014). Aberrant activity of COX-2 is implicated in the pathogenesis of breast cancer (Hoellen *et al*., 2011; Guler *et al*., 2016) through secretion of large amounts of vascular endothelial growth factor (VEGF) which is required in signalling pathways that promote carcinogenesis (Krishnamachary *et al*., 2017).

Considering the role of oxidative stress and inflammation in breast carcinogenesis, it would be necessary to examine locally available interventions that could mitigate these processes and ameliorate the condition in patients.

2.6.3 Biomarkers of oxidative stress

2.6.3.1 Malondialdehyde (MDA)

Malondialdehyde is a product of lipid peroxidation by free radicals(Niki, 2014). Lipids are very susceptible to peroxidation by oxidants, especially the polyunsaturated fatty acids that contain a number of double bonds (Ray *et al*., 2000). Since lipids constitute the membrane bilayer, attack on them by reactive intermediates leads to a cascade of reactions called lipid

peroxidation (Lykkesfeldt, 2007). This alters the permeability and function of the membrane, which could compromise cell integrity. MDA assays constitutes a marker of oxidative stress (Niki, 2014). Studies have shown elevated malondialdehyde levels among breast cancer patients (Zarrini *et al*., 2016)

2.6.3.2 Glutathione (GSH)

Glutathione is a tripeptide containing cysteine. In humans, this tripeptide is the most abundant endogenous cellular free radical scavenger (Allen and Bradley, 2011). The presence of cysteine in glutathione enables it to donate hydrogen that quenches reactive oxygen species, reducing them to less damaging forms (Pizzorno, 2014). The oxidized form of glutathione is glutathione disulphide (GSSG). Oxidative stress is usually characterized by relatively lower levels of reduced glutathione (GSH) and higher levels of the oxidized form (Schmitt *et al*., 2015). The ratio of GSH/GSSG can be used as an indication of oxidative stress in an animal (Schmitt *et al*., 2015). A low intracellular GSH level predisposes the cell to oxidative stress and disease (Schmitt *et al*., 2015). Moderate systemic levels of GSH is reportedly associated with favorable clinical characteristics and less aggressive breast carcinogenesis (Jardim *et al*., 2013)

2.6.3.3 Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) acts to scavenge superoxide anion, a reactive oxygen specie produced by one-electron reduction of an oxygen molecule (Sheng *et al*., 2014). SOD protects against spontaneous free radical toxicity and lipid peroxidation (Kumari *et al*., 2018). SOD also neutralizes excess superoxide anion generated by neutrophils (Sheng *et al*., 2014). The level of superoxide dismutase activity in serum or tissue homogenates,

provides some information about the enzymatic antioxidant capacity of the cell (Sheng *et al*., 2014). Some studies have reported that SOD expression and activity is associated with breast cancer prognosis and survival among patients (Cronin-Fenton *et al*., 2014). However, some other studies found no significant association between superoxide dismutase expression and breast cancer progression among patients (Weydert *et al*., 2006; Liu *et al*., 2012).

2.6.3.4 Catalase Activity

Catalase is located in the peroxisome of cells, detoxifying both intracellular and extracellular hydrogen peroxide to water and oxygen (Glorieux and Calderon, 2017). Since oxidative stress generates reactive intermediates, catalase activity is enhanced on exposure to reactive oxygen species by cells (Glorieux and Calderon, 2017). When the reactive intermediates are overwhelming, catalase activity becomes depleted (Goh *et al*., 2011). Catalase activity is reportedly down regulated in breast tumors (Glorieux *et al*., 2014). Treatment strategies that enhance catalase expression and activity are currently been examined as possible targets for breast cancer therapy (Glorieux *et al*., 2014).

2.7 Ex-vivo Breast Cancer Studies Using Cell Lines

MCF-7 is the acronym for Michigan Cancer Foundation-7, used to name a cell line first isolated in 1970 from the pleural effusion of a then Nun in Michigan, who was a patient with metastatic breast cancer (Lee *et al*., 2015). At present, the MCF-7 cell line is the most studied human breast cancer cell line in the world (Comşa *et al*., 2015). MCF-7 cells are relevant for *in vitro* breast cancer research because the cells retain certain ideal characteristics particular to the human mammary epithelium (Kamel *et al*., 2012). The most prominent characteristic of MCF-7 cells that makes it suitable for research in

humans, is the ability to process estrogen in the form of estradiol using estrogen receptors in the cytoplasm (Lee *et al*., 2015). A number of studies have been conducted to examine the inhibitory effects of test anti-cancer compounds on MCF-7 cell proliferation, providing results that could have a fundamental impact on breast cancer research (Forcados *et al*., 2017).

The use of cell lines in cancer research also provides the advantage of an abundant supply of a relatively homogeneous cell population, capable of speedy self-replication (Holliday and Speirs, 2011). This property of the cell lines facilitates replication studies using both synthetic and natural test compounds (Lacroix and Leclercq, 2004). A major contribution of the MCF-7 cell line to breast cancer research has been in the study of the role of estrogen and estrogen receptor-α (ER-α) in breast cancer pathogenesis, because this unique cell line expresses significant levels of ER-α, which mimics invasive ER-positive breast cancer (Lee *et al*., 2015). Hormone receptor positive breast cancer is the most common breast cancer among breast cancer patients in the world (Comşa *et al*., 2015).

In the search for anti-cancer agents, a number of phytochemicals have been evaluated for their cytotoxic effect on different cancer cell lines. The cytotoxicity of methanol and dichloromethane extracts of *Piper cubeba* seeds was evaluated against MCF-7 cell lines (Graidist *et al*., 2015). Using the MTT assay, it was observed that a chromatographic fraction of the plant extract had cytotoxic effects on breast cancer cells and normal epithelial breast cells, suggesting non-specific cytotoxicity (Graidist *et al*., 2015). Another study examined the anti-cancer effects and possible mechanism of action of *Mikania cordata* plant extract on MCF-7 cell line. The study also examined the cytotoxic effect of

the extract on normal J774A.1 murine macrophage cells (Uy *et al*., 2015). The results showed the limited potential of the extract as an anti-cancer agent, because the extract was cytotoxic to both the normal and cancer cells, indicating non-specific cytotoxicity.

In another study, the antioxidant potency and anti-cancer effects of extract and fractions of *Nardostachys jatamansi* DC were examined using MCF-7 and MDA-MB-231 cancer cell lines(Chaudhary *et al*., 2015). The most potent fraction caused cell cycle arrest at the G0/G1 phase in MDA-MB-231 cells (Chaudhary *et al*., 2015). *Centratherum anthelminticum* (L.), a medicinal herb in Indian sub-continent, was examined for its anticancer activity on the MCF-7 cell line (Looi *et al*., 2013). The results showed that the chloroform fraction of the plant seeds induced apoptosis, cell shrinkage, deformation of cytoskeletal structure and DNA fragmentation in MCF-7 human breast cancer cells (Looi *et al*., 2013). Dichloromethane fractions of *Scrophularia oxysepala* induced apoptosis in MCF-7 cells through increased expression of Caspase-3 and Caspase-9 mRNA (Hosseini *et al*., 2015). Treatment of MCF-7 cells with curcumin caused growth inhibition and induced apoptosis in MCF-7 cells (Koohpar *et al*., 2015). A decrease in Bcl-2 (anti-apoptotic protein) expression was observed, with a concomitant increase in Bax (pro-apoptotic protein) expression observed in the treated MCF-7 cells (Koohpar *et al*., 2015). In another study, metabolomics was used to investigate the anti-proliferative effects of flavopiridol on MCF-7 breast cancer cells (Shao *et al*., 2016). The results showed cell cycle arrest at the G_1 phase, reduced glutathione, and increased level of oxidized products in MCF-7 cells treated with flavopiridol, causing oxidative damage to mitochondrial membrane (Shao *et al*., 2016). The major limitation of these studies is that the experiments

were carried out only on MCF-7 cells, without examining any cytotoxic effects of the studied plant extracts/fractions in normal epithelial cells.

Most literature reports on anticancer studies in plants are examined using *in vitro* techniques, and are not followed by determination of *in vivo* anticancer effects in various models of breast cancer. Since Nigeria is blessed with a variety of plants, funding could be provided to examine the anticancer effects of some locally available plants. Isolation of the active ingredients present in the studied plants, followed by clinical trials, may lead to development of efficacious and relatively cheaper anti-cancer agents. Such a strategy will provide succor for the increasing number of cancer patients in Nigeria, who bear the burden associated with the currently available treatment regimen.

2.8 Estrogen Receptor as a Tumor Marker of Breast Cancer

A longer duration of exposure to estrogen has been found to correlate with an increased risk of breast cancer among women (Dall and Britt, 2017). Breast tissue development and function is particularly dependent on circulating estrogens, as experiments have revealed an absence of breast development in aromatase-deficient models (Chang, 2011). Interestingly, subsequent estrogen therapy in the same study cases, lead to normal post pubertal breast development (Chang, 2011). Estrogen effects are exerted through interaction with ER -α. ER -α acts mainly by regulating the expression of certain genes whose promoter region contain specific sequences called estrogen-responsive element (Yaşar *et al*., 2017).

After the binding of estrogen receptor to the estrogen-responsive element, altered transcription occurs through interaction with coactivators or corepressors, resulting in the recruitment of transcriptional machinery, subsequent modulation of chromatin structure and eventual regulation of gene expression (Yaşar *et al*., 2017). Chronic oxidative stress has been shown to alter the activity of ER -αin ER-positive breast cancer via estrogen (ligand) independent mechanisms, resulting in the activation of pro-carcinogenic and pro-inflammatory proteins (Mahalingaiah *et al*., 2015). This dysfunctional signaling is a feature of the aggressive subtype of breast cancer (Mahalingaiah *et al*., 2015).

Altered ER -αexpression and activity is observed in about 75% of human breast cancers (Yip and Rhodes, 2014). Under normal physiological conditions, ligand-bound ER activates gene expression through direct binding to specific DNA response elements or with co-activators at DNA response elements of genes which regulate cell proliferation (Yaşar *et al*., 2017). Also, through protein-protein interactions with other transcription factors like activation protein 1 (AP-1) and nuclear factor-kappa beta (NF-κB), estrogen receptor can alter the transcription and activation of downstream proteins required for breast cell proliferation (Zhao and Ramaswamy, 2014). However, during oxidative stress, ER-αcan be activated without ligand binding through signaling events mediated by the serine/threonine kinasesof the MAPK pathway, which are deregulated in breast cancer (Osborne and Schiff, 2011). Phytochemicals rich in antioxidants have been reported to protect against oxidative stress mediated activation of the redox sensitive estrogen receptor and downstream signaling pathways (Zhao and Ramaswamy, 2014).

2.9 Dimethylbenz[a]anthracene - induced Mammary Carcinogenesis

The use of chemicals to induce mammary carcinogenesis in rodents has been extensively studied for use as models in mimicking human breast carcinogenesis (Kerdelhu *et al*., 2016; Ananda *et al*., 2018). As early as the 1960's, studies showed that the polycyclic aromatic hydrocarbon 7, 12 dimethylbenz[a]anthracene (DMBA) induces mammary carcinogenesis in female Spraque-Dawley rats (Heise and Görlich, 1966). Mammary carcinogenesis induced by DMBA has been found to progress from precancerous lesions (hyperplasia) to malignant tumor (Feng *et al*., 2015). Basically, DMBA is a pro-carcinogen, undergoing metabolic activation by CYP 1A1 (Cytochrome P 450 1A1) to yield the potent dihydrodiol epoxide, which is the potent carcinogen. Dihydrodiol epoxide is reported to bind adenine and guanine residues of DNA, resulting in the formation of DNA adducts (Benakanakere *et al*., 2006). Successful induction of mammary carcinogenesis in female Wistar rats is achieved when DMBA is administered to young animals (aged 60 ± 5 days), as the mammary glands in these animals are undifferentiated and undergoing high rates of cell division (Kerdelhu *et al*., 2016).

Administration of DMBA in rodents has also been reported to cause dysregulated aryl hydrocarbon receptor function associated with specific transcription regulatory sequences in DNA, resulting in altered function of growth factors and proto-oncogenes implicated in carcinogenesis (Kerdelhu *et al*., 2016). Some of the reported proteins include c-myc, c-fos, c-Jun and H-ras (Kerdelhu *et al*., 2016). Higher expression of Cyclin D1 and Rb protein has also been observed in rodents induced with mammary carcinogenesis using DMBA (Kerdelhu *et al*., 2016). Taken together, these scientific findings suggest that DMBA induced mammary tumor development is associated with dysregulated function of pathways controlling inflammation, cell growth and apoptosis.

Epigenetic changes attributed to transcription products of oxidized bases have also been found in DMBA administered rats resulting in altered function of tumor suppressor genes like p53 (Miliaras *et al*., 2011). Inactivation of p53 in such cases causes spontaneous mutations due to sustained errors in cell replication or errors in DNA damage repair (Todorova *et al*., 2006; Miliaras *et al*., 2011). Oxidative stress has also been observed in rodents administered DMBA (Frenkel *et al*., 1995; Periyasamy *et al*., 2015). Administration of DMBA caused oxidative damage to DNA bases, subsequent oxidation of other macromolecules and increased levels of hydrogen peroxide and other reactive species resulting in oxidative stress (Frenkel *et al*., 1995).

Scientific investigations into alterations associated with DMBA administration have linked the carcinogen to increased pro-inflammatory cytokines expression and activity (Li *et al*., 2002; Gasparoto *et al*., 2012; Ahn *et al*., 2014). A study on IL-1αexpression in DMBA administered rodents showed that DMBA administration is associated with increased expression and activity of IL-1α(Li *et al*, 2002). A similar study observed increased levels of IL -6 and decreased levels of IL -10 in rodents administered DMBA (Gasparoto *et al*., 2012). Thus, dimethylbenz[a]anthracene induced mammary carcinogenesis in rodents, mimics conditions associated with mammary carcinogenesis in humans, and is suitable for studies on mammary carcinogenesis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

All chemicals and reagents were of analytical and molecular grade. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), fetal bovine serum (FBS), 2-thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Ellman's Reagent [5′, 5′-Dithiobis- (2-nitrobenzoate) DTNB], 4% Sulphursalicyclic acid and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were purchased from sigma chemical company St. Lious, U.S.A. 7, 12 Dimethylbenz[a]anthracene was purchased from Tokyo Chemical Industry Co., Ltd. ELISA kits for estrogen receptor – α, Interleukin-1β and tumor necrosis factor – α antibody were purchased from Wuhan Fine Biotech Co., Ltd. Goat polyclonal anti COX-2, Caspase 3 and p53 antibody were purchased from Elab Science Wuhan China. Kits for Alanine aminotransferase, Gamma glutamyltransferase, Blood urea nitrogen and Creatinine were purchased from RANDOX, UK. Lactate dehydrogenase was purchased from AGAPPE Diagnostics, Switzerland.

3.1.2 Equipment

FD 8518 ilShin freeze dryer, Nikon E 100 Microscope, Sony A7 III camera, Sherwood colorimeter 257, Grant JB series water bath, RS-232C weighing balance, Model 680 XR

Micro plate reader (Bio-Rad),xCELLigence RTCA DP (Roche, Applied Science Basel, Switzerland), GCMS-QP2010SE Shimadzu Gas chromatography mass spectrometer, LCMS-8050 Shimadzu Liquid chromatography mass spectrometer.

3.1.3 Plant material collection and identification

The leaves of *V. doniana* were collected from a farm in Toro, Bauchi State (10.0596^0N, 9.0709^0E) while *F. apodanthera* root bark was collected from a farm in Katsina, Katsina State (12.5139^0N, 7.6114^0E), Nigeria. The plants were identified at the Federal College of Forestry, Jos with voucher numbers FHJ 189 and 194 obtained for *V. doniana* and *F. apodanthera* respectively from the Herbarium for future references.

3.1.4 MCF-7 Cells

Estrogen receptor (ER)-positive MCF-7 breast cancer cells were purchased from the American Type Culture Collection, USA.

3.1.5 Experimental animals

3.1.5.1 Experimental animals for anti-cancer study

Fifty-five (55) female Wistar rats aged 45 ± 2 days, weighing 57 ± 5 grams were purchased from the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The animals were acclimatized for a period of 1 week under ambient environmental conditions in well aerated cages at the Department of Anatomy Animal House, Ahmadu Bello University, Zaria. They were allowed free access to grower's mash (Vital feeds, Grand Cereals Plc) and water ad libitum. The use of animals for this study was approved by Ahmadu Bello

University ethics committee for animal studies (Reference number: ABUCAUC/2018/004).

3.1.5.2 Experimental animals for sub-acute toxicity study

Twenty (20) female Wistar rats aged 180 ± 10 days, weighing 165 ± 10 grams were purchased from the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The animals were acclimatized for a period of 1 week under ambient environmental conditions in well aerated cages at the Department of Anatomy Animal House, Ahmadu Bello University, Zaria. They were allowed free access to grower's mash (Vital feeds, Grand Cereals Plc) and water ad libitum. The use of animals for this study was approved by Ahmadu Bello University ethics committee for animal studies (Reference number: ABUCAUC/2018/004).

3.2 Methods

3.2.1 Preparation and extraction of plant material

The collected leaves and root bark were washed with distilled water and air dried at room temperature. The dried leaves and root bark were pulverized to obtain a dry powder which was subjected to ethanol extraction. Two hundred grams (200g) of the obtained powder was transferred to volumetric flasks and soaked with 70% ethanol to the 1000 ml mark. The powder in ethanol was left for 48 h (James *et al*., 2014). The dissolved extracts were filtered using an ash less Whatman filter paper (size 1) and the filtrate was allowed to freeze dry at -40^0C for 72 h. The freeze dried extract was weighed to determine percentage yield and then transferred into labelled containers for further analysis.

The percentage yield was calculated using the following formula

Yield (%) = (Weight of extract/Weight of dry powder used for extraction) × 100

3.2.2 Separation using chromatography

Principle:

Chromatography is a technique that utilizes adsorption, ion exchange or affinity, to separate compounds in a liquid phase as they pass through a packed column (adsorbent). The substance intended for separation is dissolved in the liquid phase, which forms different partitions in the column, depending on the chemical or physical nature of the compounds to be separated(Coskun, 2016). The most strongly adsorbed component will remain near the top of the column, while the least strongly adsorbed component will elute the column fastest, as the different components to be separated will elute based on their affinity to the adsorbent (Coskun, 2016).

Thin Layer ChromatographyProcedure for the Determination of Best Solvent System

A preparative TLC was carried out on the crude ethanol extracts of *V. doniana* and *F. apodanthera*to determine the best solvent system that will give the highest number of distinct band of components.A pre-coated aluminium chromatographic plate with silica gel was used. The crude ethanol extract was dissolved in the solvent used for extraction and applied several times on individualplates using a micro haematocrit capillary tube until the quantity loaded was adjusted sufficiently for the experiment. Each plate was then placed in separate chromatographic tanks and eluted with different solvent systems (n-hexane: ethyl acetate; n-hexane: acetone; n-hexane: methanol; and n-hexane: ethanol) as the mobile phase in order to get the suitable solvent system. Thereafter the plates were removed, air

dried and developed using 20% sulphuric acid in methanol to identify most suitable solvent system. The best solvent systems determined were acetone: ethylacetate and n-hexane: acetone for *V. doniana* and *F. apodanthera* respectively.

Column Chromatography Procedure for Obtaining Fractions from the Extracts

Preparatory to the column chromatography separation, the column was conditioned using n-hexane to pack the silica gel of 50-200µm mesh size. The crude extracts(2.0g) of *V. doniana* and *F. apodanthera* wereemptied into a porcelain mortar, 5 g of silica gel was added and a pestle was used to macerate the mixture to homogeneity, to ensure that the extract does not pack on one side of the column. Each mixture was separately packed on top of the column to partially separate the compound(s) present in the extracts. The column was then eluted with the identified solvent systems of acetone: ethylacetate and n-hexane: acetone for *V. doniana* and *F. apodanthera*respectively using 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (v/v) and the column was finally washed with ethanol. Approximately a 50 ml per 18min flow rate was maintained. The fractions collected were evaporated to near dryness. Each of the fractions was weighed and spotted on TLC plate and developed, and viewed under UV radiation (λ=254 nm and 365 nm). The plate was further sprayed with 20% sulfuric in methanol solution and dried for 15 min at 110°C. This post-column chromatography separation TLC procedure enabledsimilar fractions obtained after column chromatography to be pooled together based on the pattern and R_f values of the spots on the TLC plate into main fractions respectively. The R_f values was calculated using the formula:

R_f =Dc (distance travelled by component) /Ds (distance travelled by solvent front).

The combined yield of fractions with the same R_f value and combined yield as percentage (%) of the total crude fraction fractionated were calculated. The pooled fractions were kept in a refrigerator for further analysis.

3.2.3 *In vitro* antioxidant Assays

The free radical scavenging activity of the extract and fractions of *V. doniana* and *F. apodanthera* were evaluated using DPPH and ABTS free radicals.

3.2.3.1 Diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity

Principle:

The molecule 1,1-diphenyl- β -picrylhydrazyl (DPPH) is a stable free radical due to the delocalisation of the spare electron over the compound which is responsible for the colored complex which can be measured spectrophotometrically (Brand-Williams *et al*., 1995; Kedare and Singh, 2011; El-Sayed *et al*., 2013). In the presence of an antioxidant that donates a hydrogen atom, DPPH is reduced in a process characterized by loss of the initial color (Akar *et al*., 2017). The change in color is directly proportional to a change in absorbance which is measured spectrophotometrically (Alam *et al*., 2013)

Procedure:

One ml of 0.1 mM DPPH solution in methanol was added to 1.0 mL of standard/extract solution at different concentrations. The mixture was incubated for 20 min and the absorbance recorded at 517 nm. Ascorbic acid was used as positive control. DPPH radical

scavenging activity was calculated using the formula: Percent scavenging = ((A_o – A_t)/A_o) × 100; where A_o = Absorbance of control (without extract) and A_t = Absorbance of sample. All determinations were carried out in triplicate.

3.2.3.2 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

Principle:

This method measures the loss of color when an antioxidant is added to $ABTS^{*+}$, a blue-green chromophore. The antioxidant reduces $ABTS^{*+}$ to ABTS, and decolorizes it. When used to determine the antioxidant capacity of a plant extract or fraction, the antioxidant capacity of the plant extract is determined by its ability to reduce $ABTS^{*+}$ to ABTS, measured by the color change observed at 750 nm wavelength (Dong *et al*., 2015).

Procedure:

ABTS free radical scavenging activity of the extract and fractions of *V. doniana* and *F. apodanthera* were evaluated by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate. The mixture was allowed to stand for 15 h in dark at room temperature. ABTS solution was diluted with methanol to obtain the absorbance of 0.7 ± 0.2 units at 750 nm. The standard/extract solutions were prepared at different concentrations in methanol and 20 µl of test solutions were added to 180 µl of ABTS free radical solution. The absorbance was measured after 20 min incubation at 750 nm. Ascorbic acid was used as control. The ABTS free radical scavenging activity was calculated using the formula:

% scavenging = $((A_o - A_t)/A_o) \times 100$

where A_o = Absorbance of control (without extract) and A_t = Absorbance of sample.

All the tests were performed in triplicate.

3.3 *In vitro* Cytotoxicity Studies

3.3.1 Preparation of MCF-7 cells

The MCF-7 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and1% penicillin-streptamycin. The cells were incubated at 37°C in CO_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by sub-culturing in 25 cm^2 tissue culture flasks. Cells growing in the exponential phase were used for cell viability. The cells were counted after splitting by adding 1 ml of media to the splitted cells. After shaking properly, cells (20µl) were then added to 20µl of trypan blue, of which 15µl was taken out and placed on a hemocytometer, covered using a cover slip and then mounted on a microscope. The number of cells counted in each of the four segments of the hemocytometer was added (a + b + c + d) and the formula below was used to determine the number of cells per ml (Mphahlele *et al*., 2014).

Number of cells/ml = (a + b + c + d) × 2500 (dimensions) × 2 (dilution factor)

3.3.2 Cell viability assay

MTT cytotoxicity assay was used to determine the inhibition of cancer cell proliferation by extract and fractions of *V. doniana* and *F. apodanthera.*

3.3.2.1 Cell viability by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) Assay

Principle:

MTT is reduced in the presence of mitochondrial dehydrogenase enzymes (Chaudhary *et al*., 2015; Sliwka *et al*.,2016) which generate reducing equivalents such as NADH + H^+ and NADPH + H^+ from viable cells that cleave the tetrazolium rings of the pale yellow MTT, forming dark blue formazan crystals (McCauley *et al*., 2013). Thus, MTT assay quantifies cell viability. The MTT dye is reduced when metabolic events are obstructed, possibly leading to apoptosis or necrosis.

Procedure:

Exponentially growing MCF-7 cells were seeded into 96-well plates (5×10^3 cells/well in 100 µl of media) and allowed to attach for 24 h. Test extract/fractions were prepared in 0.1% DMSO, serially diluted with serum free media and filtered using a 0.22 µm sterile filterto obtain appropriate concentrations. Cells were treated with 12.5, 25, 50 and 100 µg/ml of the extract and fractions of *V. doniana* and *F. apodanthera* and incubated for 24 and 48 h. Cells in the control group received only media containing 0.1% DMSO. The test

compound containing media was removed and washed with 200 µl of PBS followed by addition of 20 µl of MTT reagent (5 mg/ml MTT in PBS) and incubated for 4 h at 37°C. The medium was removed and 100 µl DMSO was added and the absorbance measured using a micro plate reader at 540 nm followed by the calculation of percentage viability.

Relative percentage cell viability = [(Absorbance of sample)/(Absorbance of control) × 100]

Where control cells were treated with 0.1% DMSO medium. Each treatment was performed in triplicate. Vinblastine was used as control. The results were recorded as half maximal inhibitory concentration(IC_{50}), which provides information on the effectiveness of the extracts/fractions in inhibiting mitochondrial enzyme functions of MCF-7. The experiment was done thrice.

3.3.3 Cellular growth patterns by xCELLigence

Principle:

The xCELLigence System is a new equipment invented by ACEA Biosciences, which records cellular parameters such as cell attachment, cell viability and cell death in culture using electrical impedance measurements as cell index values (Ke *et al*., 2011; Kustermann*et al*., 2013; Martinez-Serra *et al*., 2014; Mphahlele*et al*., 2014; Moniri *et al*., 2015). The effect of *V. doniana* and *F. apodanthera*extracts on MCF-7 attachment and growth patterns was studied.

Procedure:

Media was pre-warmed to 37^0C and 100 µl serum free medium with growth factor was added into the lower chamber of a 16-well E-Plate which was incubated for 30 min to calibrate. A plate was used for each extract. A 100µl cell suspension which included 6000 cells/well and treatments of 0, 1, 10 and 50 µg/ml of the extract was added to each well and loaded into the xCELLigence machine. The experiment was done three times. Cell kinetics was monitored and data analyzed by the machine.

3.3.4 Cell morphology by hematoxylin and eosin (H and E) staining

Principle:

The use of hematoxylin and eosin (H and E) is a widely accepted staining technique for cell morphology analysis (Ding *et al*., 2011; Feng*et al*., 2015). Hematoxylin is a dye with a deep blue-purple color and stains nucleic acids, while eosin is pink and stains proteins. After the staining process, nuclei are stained blue, while the cytoplasm is stained pink. Well-fixed and properly stained cells could provide information on morphological changes due to the cytotoxicity of a test compound (Fischer *et al*., 2008; Alturkistani *et al*., 2015). H and E staining was used to determine the effect of administration of *V. doniana* and *F. apodanthera* extracts and fractions on MCF-7 cell morphology.

Procedure:

Exponentially growing MCF-7 cells were seeded into 6-well plates with cover slips at the base (5×10^3 cells/well in 100 µl of media) and allowed to attach for 24 h. Test extract/fractions were prepared in 0.1% DMSO and serially diluted with media to obtain appropriate concentrations. After the cells attached, medium was removed from each well and the cells were treated with 0, 50 and 100 µg/ml of *V. doniana* and *F. apodanthera*

extracts, and incubated for 48 h. Medium was then removed and Bouin's fixative was added for 30 min. This was rinsed out and 70% ethanol was added for 20 min after which the cover slips were rinsed in tap water. Mayer's Haemalum reagent was added for 20 min and rinsed in tap water for 2 min, after which the cells were rinsed with 70% ethanol. 1% eosin was then added to the cells for 5 min after which the cells were rinsed twice for 5 min with 90%, 96%, 100% ethanol and then with xylol. The slips were treated with resin and allowed to dry after which they were examined using a microscope. The experiment was done thrice.

3.3.5 Cell cycle analysis

Principle:

The analysis is based on the ability of propidium iodide to stain cellular DNA in a stoichiometric manner. The amount of stain is directly proportional to the amount of DNA within the cell (Schorl and Sedivy, 2007; Kim and Sederstrom, 2015)

Procedure:

MCF-7 cells were seeded at density of 1×10^6 cells/well in 6-well plates. After 24 h, cells were treated with *V. doniana* at doses of 12.5 and 50 µg/ml. Then, after 48 h treatment and incubation at 37^0C and 5% CO_2, cells were washed with PBS harvested with trypsin and centrifuged, fixed with ice-cold 100% ethanol overnight. The fixed cells were centrifuged for 1 h at 300 × g and washed with PBS twice. The cells were then treated with 50 μl of 100µg/ml RNase A solution in PBS and incubated at 37^0C for 15 min. Thereafter, 400µl of 50µg/ml solution in PBS was added and allowed to stand at room temperature for 10 min. Cell cycle distribution was analyzed by fluorescence activated cell sorting. Excitation wavelength was 488 nm, and fluorescence analyzed at 620 nm. A total of 10,000 events in

each sample were acquired. Using Cellquest Pro software, the percentages of cells at different phases of the cell cycle were determined.

3.4 In vivo Anti-cancer Studies

3.4.1 Induction of mammary carcinogenesis

Mammary carcinogenesis was induced in 45 female Wistar rats using 7, 12 Dimethylbenz[a]anthracene. Rats were administered 80 mg/kg of DMBA in olive oil once via oral gavage. The rats were then monitored for 150 days. Ten (10) normal rats served as normal control

3.4.2 Animal grouping and treatment

After 150 days monitoring of carcinogenesis induction by DMBA, estrogen receptor-alpha was determined, after which, the rats were randomly divided into six groups of five rats each. Since*V. doniana* whole extract showed the best anti-cancer effect from the *in vitro* study, the *V. doniana* whole extract only was used for the*in vivo* experiment at doses of 50, 100 and 200 mg/kg. The groups and respective treatments are as follows:

Group 1: Normal rats administered the vehicle (olive oil) only

Group 2: DMBA-induced rats administered the vehicle (olive oil) only (negative control)

Group 3: DMBA-induced rats treated with 50 mg/kg of *V. doniana*

Group 4: DMBA-induced rats treated with 100 mg/kg of *V. doniana*

Group 5: DMBA-induced rats treated with 200 mg/kg of *V. doniana*

Group 6: DMBA-induced rats treated with 20 mg/kg of Tamoxifen (standard drug)

The treatment lasted for 14 days

3.4.3 Collection and treatment of samples

At the end of the experiment, the rats were sacrificed humanely and blood was collected in non-heparinized tubes and allowed to stand then centrifuged at 10,000 × g for 10 min to obtain serum. The serum was then stored in labelled containers for estrogen receptor-α, IL-1β and TNF-α analysis. Mammary tissues were also harvested at the same time.

3.4.4 Oxidative stress assays

Mammary tissues were collected and washed in ice cold 1.15% KCl solution, blotted with filter paper and weighed. The tissues were then chopped into bits and homogenized in four volumes of the homogenizing buffer (0.05M, pH 7.4) using a Teflon homogenizer. The resulting homogenate was centrifuged at 10,000 ×g for 10 min in a cold centrifuge at -4^{0}C. The supernatant was then collected for analysis.

3.4.4.1 Assessment of lipid peroxidation

Lipid peroxidation was determined by measuring the malondialdehyde level (thiobarbituric acid reactive substances) present in the sample which reacts with thiobarbituric acid to form a pink colored adduct (Niehaus and Samuelsson, 1968).

Principle:

This method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxidation. On heating in acidic pH, the product (a pink complex) is produced, which absorbs maximally at 532nm and which is extractable into organic solvents such as butanol.

Procedure:

An aliquot of 0.4 ml of the sample was mixed with 1.6 ml of Tris-KCl buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% TBA was added and placed in a water bath for 45 min at 80^0C. This was then cooled on ice and centrifuged at 3000 ×g. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm. The MDA level was calculated according to a standard method of (Adam-Vizi and Seregi, 1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 105 $m^{-1}cm^{-1}$.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{\text{E532nm x volume of sample x mg protein}}$$

3.4.4.2 Reduced glutathione assay

The method of (Ellman, 1959) was used in estimating the level of reduced glutathione (GSH).

Principle:

The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable (yellow) colour when 5′, 5′–dithiobis-(2-nitrobenzoic acid) (Ellman‘s reagent) reacts with sulfhydryl compounds. The chromophoric product resulting from the

reaction of Ellman reagent with the reduced glutathione, 2 – nitro-5-thiobenzoic acid possesses a molar absorption at 412nm which was read at 412nm in a colorimeter.

Procedure:

An aliquot of the sample was deproteinated by the addition of an equal volume of 4% sulfosalicyclic acid. This was centrifuged at 4000 × g for 5 min. Thereafter, 0.5ml of the supernatant was added to 4.5ml of Ellman‘s reagent. A blank was prepared with 0.5ml of the diluted precipitating agent and 4.5ml of Ellman‘s reagent. Reduced glutathione (GSH) concentration was proportional to the absorbance of the colored product at 412nm.

3.4.4.3 Determination of superoxide dismutase activity

The level of SOD activity was determined using a standard protocol (Misra and Fridovich, 1972).

Principle:

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide (O_2• −) radical generated by the xanthine oxidase reaction causes the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 • − introduced increases with increasing pH and concentration of epinephrine (Valerino and McCormack, 1971).

Procedure:

Sample (0.2ml) was diluted in 0.8ml of distilled water to make a 1 in 5 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction was started by the addition of 0.3 ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline)

and 0.2ml of water. The increase in absorbance at 480 nm was monitored every 30 s for 150 s.

Calculation

Increase in absorbance per minute = $\frac{A3 - A0}{2.5}$

Where A0 = absorbance after 30 seconds, A3 = absorbance after 150 seconds

% inhibition = [(100)–(100 x increase in absorbance for substrate)]

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

3.4.4.4 Determination of catalase activity

Catalase activity was determined according to the method of (Sinha, 1972).

Principle:

This method is based on reduction of dichromate in acetic acid to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate produced is measured at 570-610nm. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate at 570 nm after heating the reaction mixture.

Procedure:

Supernatant fraction (0.2ml) of the organ homogenate was mixed with 0.8ml distilled water to give a 1: 5 dilution. The assay mixture contained 2ml of H_2O_2 solution (800 μmole) and 2.5ml of 0.05 M phosphate buffer, pH 7.0 in a 10ml flat bottom flask. One ml (1ml) of properly diluted sample was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. One ml (1 ml) of the reaction mixture was withdrawn and blown into 2ml dichromate/acetic acid reagent at 60 s intervals.

Calculation

Catalase activity was calculated by plotting the standard curve and the concentration of the remaining H_2O_2 was extrapolated from the curve.

H_2O_2 consumed = 800μmoles – H_2O_2 remaining

$$\text{Catalase activity} = \frac{H_2O_2 \text{ consumed}}{\text{mg protein}}$$

3.4.5 Determination of estrogen receptor-α, interleukin-1β and tumor necrosis factor-α using sandwichELISA kits

3.4.5.1 Determination of estrogen receptor-α (ER-α)

Principle:

The ER– α present in a serum sample conjugates with an immobilized ER– α antibody when added into wells. Addition of a secondary antibody conjugated with horse radish peroxidase and TMB substrate to the well results in an antibody-antibody colored complex (Gaastra, 1984) which is measured at 450 nm.

Procedure:

First, the serum sample was diluted 1:2. Before adding reagents into wells, equilibration was done using TMB substrate for 30 min at 37^0C. Aliquot of 0.1 ml standard solution (2.5ng/ml) was added into the wells for standard, while 0.1 ml of diluted sample was added into the wells for control and test sample wells. The plate was then sealed with a cover and incubated at 37^oC for 90 min. The plate was then carefully washed twice with wash buffer(0.01 M phosphate buffer, pH 7.4). Biotin-labeled antibody (1 mg/ml, 0.1 ml), working solution(20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, and 0.312 ng/ml) was carefully added to the standard, test sample and zero wells. The plate was then sealed with a cover and incubated at 37^oC for 60 min. Next, the cover was removed and the plate washed thrice with wash buffer(0.01 M phosphate buffer, pH 7.4), after which 0.1ml of HRP-Streptavidin conjugate (1 mg/ml) working solution was added into each well. The plate was covered and incubated at 37°C for 30 minutes. The cover was removed; the plate washed again and about 90µl of TMB was added into each well. The plate was sealed with a cover and incubated at 37°C in dark within 15-30 minutes (depending on the intensity of the blue colour developed). The reaction was stopped by adding 50 µl of stop solution (0.18 M sulpuric acid solution) into each well and the colour changed to yellow immediately.Absorbance was read at 450 nm and the corresponding ER– α values (ng/ml) interpolated from a standard curve.

3.4.5.2 Determination of Interleukin 1beta (*IL-1β)*

The serum was used for assaying IL-1βusing specific ELISA kits.

Principle:

The IL-Iβ present in a serum sample conjugates with an immobilized IL-Iβ antibody when added into wells. Addition of a secondary antibody conjugated with horse radish peroxidase and tetramethylbenzidine (TMB) substrate to the well results in an antibody – antibody coloured complex reaction (Gaastra, 1984). The intensity of colour developed at 450nm is directly proportional to the concentration of IL-Iβ (measured in ng/ml) in the sample.

Procedure:

First, the serum sample was diluted 1:2. Before adding reagents into wells, equilibration was done using TMB substrate for 30 min at 37^0C. Aliquot of 0.1 ml standard solution (2.5 ng/ml) was added into the wells for standard, while 0.1 ml of diluted sample was added into the wells for control and test sample wells. The plate was then sealed with a cover and incubated at 37^0C for 90 min. The plate was then carefully washed twice with wash buffer (0.01 M phosphate buffer, pH 7.4). Biotin-labeled antibody (1 mg/ml, 0.1 ml), working solution (20 ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, and 0.312ng/ml) was carefully added to the standard, test sample and zero wells. The plate was then sealed with a cover and incubated at 37^0C for 60 min. Next, the cover was removed and the plate washed thrice with wash buffer (0.01 M phosphate buffer, pH 7.4), after which 0.1ml of HRP-Streptavidin conjugate (1 mg/ml) working solution was added into each well. The plate was covered and incubated at 37°C for 30 min. The cover was removed; the plate washed again and about 90µl of TMB was added into each well. The plate was sealed with a cover and incubated at 37°C in dark within 15-30 min (depending on the intensity of the blue colour developed). The reaction was stopped by adding 50 µl of stop solution (0.18 M sulpuric acid solution) into each well and the colour changed to yellow immediately.

Absorbance was read at 450 nm and the corresponding IL-Iβ values (ng/ml) interpolated from a standard curve.

3.4.5.2 Determination of tumor necrosis factor(TNF-α)

The serum was used for assaying TNF-α using specific ELISA kits.

Principle:

TNF-α present in a serum sample conjugates with an immobilized TNF-α antibody when added into wells. Addition of a secondary antibody conjugated with horse radish peroxidase (HRP) and tetramethylbenzidine (TMB) substrate to the well results in an antibody-antigen antibody coloured complex (Gaastra, 1984). The intensity of the colour developed at 450nm is directly proportional to the concentration of TNF-α (measured in ng/ml) in the samples.

Procedure:

First, the serum sample was diluted 1:2. Before adding reagents into wells, equilibration was done using TMB substrate for 30 min at 37^0C. Aliquot of 0.1 ml standard solution (2.5 ng/ml) was added into the wells for standard, while 0.1 ml of diluted sample was added into the wells for control and test sample wells. The plate was then sealed with a cover and incubated at 37^0C for 90 min. The plate was then carefully washed twice with wash buffer (0.01 M phosphate buffer, pH 7.4). Biotin-labeled antibody (1 mg/ml, 0.1 ml), working solution (20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, and 0.312 ng/ml) was carefully added to the standard, test sample and zero wells. The plate was then sealed with a cover and incubated at 37^0C for 60 min. Next, the cover was removed and the plate washed thrice with wash buffer (0.01 M phosphate buffer, pH 7.4), after

which 0.1ml of HRP-Streptavidin conjugate (1 mg/ml) working solution was added into each well. The plate was covered and incubated at 37°C for 30 min. The cover was removed; the plate washed again and about 90µl of TMB was added into each well. The plate was sealed with a cover and incubated at 37°C in dark within 15-30 min (depending on the intensity of the blue colour developed). The reaction was stopped by adding 50 µl of stop solution (0.18 M sulpuric acid solution) into each well and the colour changed to yellow immediately. Absorbance was read at 450 nm and the corresponding TNF-α values (ng/ml) interpolated from a standard curve.

3.4.6 Histological Examination of Tissues

Histological analysis was carried out on excised mammary tissue (DMBA-induced experiment and sub-acute toxicity study) as well as liver and kidney tissues(sub-acute toxicity study) according to standard protocol (Fischer *et al*., 2008). The excised mammary tissues were fixed in 10% buffered formal saline (137 mM Sodium chloride) dehydrated in 95% ethanol, cleared in xylene and embedded in paraffin wax. Micro sections of 3μm thickness were then prepared on slides. The slides were de-paraffinized using xylene after which hydration was done by dipping in 100%, 95% and 70% ethanol. The slides were then rinsed carefully by dipping in tap water. Next, staining was done using hematoxylin, which was allowed for 5 min, followed by rinsing by dipping in tap water. Next slides were stained with eosin and allowed for 10 min. Dehydration was then carried out by dipping in 95% ethanol followed by 100% ethanol and xylene. The slips were then treated with resin and allowed to dry, after which they were examined under a light microscope

3.4.7 Determination of COX-2, Caspase-3 and p53 expression using immunohistochemical technique

Principle:

Immunohistochemistry is the most common application of immunostaining. The method is based on the selective binding of antigens in cells of a tissue section to antibodies. The antibody-antigen binding produces a coloration, through conjugation of the antibody to an enzyme such as a peroxidase, that catalyzes a color producing reaction that can be visualized using a microscope (Ramos-Vara, 2011).

3.4.7.1 Procedure for Determination of COX-2 Expression

Micro sections (3 μm) were obtained from excised mammary tissues and fixed in 10% formalin (4% w/v formaldehyde) at 25^0C for 48 h. Next the tissues were rinsed in tap water for 30 min. The slides were then dehydrated in 100, 95, 70 and 50% alcohol for 3 min each. Antigen retrieval was done by immersing the slides in 10mM citrate buffer (pH 7.4) for 25minutes with subsequent peroxidase quenching in 3% H_2O_2/methanol. The respective sections were blocked by adding 100 µl of 10 % fetal bovine serum in PBS (137 mM NaCl) and incubated for 12 h. Next 100 µl of anti-COX 2 diluted 1: 500 with 0.5 % bovine serum albumin in PBS (137 mM NaCl) was added to the sections and incubated in a humidified chamber at 25^0C for 1 h. The slides were then washed in 300 ml PBS (137 mM NaCl), followed by addition of 100 µl of anti-COX 2 diluted 1: 800 with 0.5 % bovine serum albumin in PBS (137 mM NaCl) to the sections and incubated in a humidified chamber at 25^0C for 30 min. The slides were then dipped in 300 ml PBS (137 mM NaCl)

for 5 mins in dark. Detection of band antibody was done by adding 100 µl of HRP-Streptavidin conjugate (1 mg/ml). Coloration of the reaction product was enhanced by adding 100 µl of diaminobenzidine (0.05 % in hydrogen peroxide) for 10minutes, with subsequent dehydration by dipping in 300 ml of100 % ethanol. Slides were then mounted on a microscope and images acquired using adigital camera at × 400 magnification.

3.4.7.2 Procedure for Determination of Caspase 3 Expression

Micro sections (3 μm) were obtained from excised mammary tissues and fixed in 10% formalin (4% w/v formaldehyde) at 25^0C for 48 h. Next the tissues were rinsed in tap water for 30 min. The slides were then dehydrated in 100, 95, 70 and 50% alcohol for 3 min each. Antigen retrieval was done by immersing the slides in 10 mM citrate buffer (pH 7.4) for 25 min with subsequent peroxidase quenching in 3% H_2O_2/methanol. The respective sections were blocked by adding 100 µl of 10 % fetal bovine serum in PBS (137 mM NaCl) and incubated for 12 hours. Next 100 µl of anti-caspase 3 diluted 1: 500 with 0.5 % bovine serum albumin in PBS (137 mM NaCl) was added to the sections and incubated in a humidified chamber at 25^0C for 1 h. The slides were then washed in 300 ml PBS (137 mM NaCl), followed by addition of 100 µl of anti-caspase 3 diluted 1: 800 with 0.5 % bovine serum albumin in PBS (137 mM NaCl) to the sections and incubated in a humidified chamber at 25^0C for 30 min. The slides were then dipped in 300 ml PBS (137 mM NaCl) for 5 mins in dark. Detection of band antibody was done by adding 100 µl of HRP-Streptavidin conjugate (1 mg/ml). Coloration of the reaction product was enhanced by adding 100 µl of diaminobenzidine (0.05 % in hydrogen peroxide) for 10minutes, with subsequent dehydration by dipping in 300 ml of 100 % ethanol. Slides were then mounted on a microscope and images acquired using adigital camera at × 400 magnification.

3.4.7.3Procedure for Determination of COX-2 Expression

Micro sections (3 μm) were obtained from excised mammary tissues and fixed in 10% formalin (4 % w/v formaldehyde) at 25^0C for 48 h. Next the tissues were rinsed in tap water for 30 min. The slides were then dehydrated in 100, 95, 70 and 50 % alcohol for 3 min each. Antigen retrieval was done by immersing the slides in 10 mM citrate buffer (pH 7.4) for 25minutes with subsequent peroxidase quenching in 3% H_2O_2/methanol. The respective sections were blocked by adding 100 µl of 10 % fetal bovine serum in PBS (137 mM NaCl) and incubated for 12 hours. Next 100 µl of anti-p53 diluted 1: 500 with 0.5 % bovine serum albumin in PBS (137 mM NaCl) was added to the sections and incubated in a humidified chamber at 25^0C for 1 h. The slides were then washed in 300 ml PBS (137 mM NaCl), followed by addition of 100 µl of anti-p53 diluted 1: 800 with 0.5 % bovine serum albumin in PBS (137 mM NaCl) to the sections and incubated in a humidified chamber at 25^0C for 30 min. The slides were then dipped in 300 ml PBS (137 mM NaCl) for 5 mins in dark. Detection of band antibody was done by adding 100 µl of HRP-Streptavidin conjugate (1 mg/ml). Coloration of the reaction product was enhanced by adding 100 µl of diaminobenzidine (0.05 % in hydrogen peroxide) for 10minutes, with subsequent dehydration by dipping in 300 ml of 100 % ethanol. Slides were then mounted on a microscope and images acquired using adigital camera at × 400 magnification.

3.5 Sub-acute Toxicity on *V. doniana*

3.5.1 Animal Grouping and Treatment

The rats were randomly divided into four groups of five rats each, as follows:

Group 1: Normal rats administered distilled water only

Group 2: Normal rats administered 50 mg/kg *V. doniana*

Group 3: Normal rats administered 100 mg/kg *V. doniana*

Group 4: Normal rats administered 200 mg/kg *V. doniana*.

The treatment lasted for 28 days.

3.5.2 Collection of and Treatment of Samples and Tissues

At the end of the experiment, the rats were sacrificed humanely and blood was collected in non-heparinized tubes and allowed to stand after which centrifugation was done at 10,000 × g for 10 min to obtain serum, which was stored in labelled containers. Liver, kidney and mammary tissues were excised and stored in labelled containers containing buffered formalin.

3.5.3 Determination of Tissue Damage Using Serum Markers

The serum obtained was used to determine levels of the following: alanine aminotransferase, γ-glutamyltransferase, blood urea nitrogen, creatinine and lactate dehydrogenase. Procedures were carried out using kits according to Manufacturer's instructions.

3.5.3.1 Determination of Alanine aminotransferase activity

ALT activity was determined following a standard protocol (Reitman and Frankel, 1957).

Principle:

Alanine aminotransferase catalyzes the reaction between alanine and α-ketoglutarate to produce pyruvate and glutamate. When phenylhydrazine is added, the generated pyruvate

reacts with phenylhydrazine to form phenylhydrazone. Phenylhydrazone is reddish brown under alkaline conditions and absorbs at 540 nm (Reitman and Frankel, 1957).

Procedure:

Briefly, 0.1ml of diluted sample was mixed with phosphate buffer (100mmol/l, pH 7.4), L-alanine (100mmol/l), and α-oxoglutarate (2mmol/l). The mixture was then incubated for exactly 30 min at 37^{o}C. Then, 0.5ml of 2, 4-dinitrophenylhydrazine (2mmol/L) was added to the reaction mixture and allowed to stand for exactly 20min at 25^{0}C. Next, 5.0ml of NaOH (0.4mol/L) was added and the absorbance read against the reagent blank after 5 min at 546 nm. The corresponding concentrations were obtained from a standard curve for ALT.

3.5.3.2 Determination of γ-glutamyl transferase activity

Gamma glutamyl transferase (GGT) activity was determined following a standard protocol (Szasz, 1969).

Principle:

The substrate (L-γ-glutamyl-3-carboxy-4-nitroanilide), in the presence of glycylglycine is converted by gamma glutamyl transferase (γ-GT) in the sample to 5-amino-2-nitrobenzoate where absorbance can be measured at 405 nm (Szasz, 1969).

Procedure:

Briefly, 0.1ml of diluted sample was mixed with 0.5 ml of tris buffer (100mmol/l, pH 8.25) and incubated at 25^{o}C. The mixture was carefully shaken and 0.5 ml of L-γ-glutamyl-3-carboxy-4-nitroanilide (2.9 mmol/L) was added. The reaction mixture was then mixed thoroughly, initial absorbance read, and then absorbance values were recorded after 1, 2 and 3 min at 405 nm wavelength.

GGT (U/L) = 1158 × change in absorbance at 405 nm/min

3.5.3.3 Determination of Urea Concentration

Principle:

Urea in serum is hydrolyzed to ammonia and carbon dioxide in the presence of urease. The ammonia formed can react with a phenolic chromogen and hypochlorite to form a green colored complex which is measured spectrophotometrically at 546 nm by Berthelot's reaction (Wilcox*et al*., 1966)

Procedure:

Briefly, three cuvettes for Blank, Standard and Sample were used. For the blank, 10 µl of distilled water was added to 100 µl of Reagent 1 (Urease reagent). The cuvettes for standard contained 10 µl of standard and 100 µl of Reagent 1, while the cuvette for sample contained 10 µl of sample and 100 µl of Reagent 1. The contents of the three cuvettes were mixed and incubated at 37^{0}C for 10 min. After that, 2.5 ml of Reagent 2 (phenolic chromogen) and Reagent 3 (standard urea, 40 mg/dl) were added to the three cuvettes, mixed and incubated at 37^{0}C for 15 min. Absorbance of sample and standard were then read against the blank at 546 nm.

Urea concentration (mmol/L) = (Absorbance of sample)/(Absorbance of standard) × standard concentration

3.5.3.4 Determination of creatinine concentration

Creatinine concentration was determined in serum using a standard protocol (Fossati *et al*, 1983).

Principle:

Creatinine in alkaline solution reacts with picric acid to form a colored complex which absorbs between 492 and 510 nm. The amount of the complex formed is directly proportional to the creatinine concentration.

Procedure:

Briefly, for sample cuvette, 1 ml of Reagent 1 (picric acid) and 2 (chromogen) were pipetted into cuvette and 0.1 ml of sample was added, mixed and absorbance read (A1) after 2 min, the absorbance was read again (A2). The same was done for standard but without addition of sample. All absorbance were read at 492nm.

Creatinine (mg/dl) = dAsample/dAstandard × standard concentration (mg/dl)

3.5.3.5 Determination of Lactate dehydrogenase activity

Lactate dehydrogenase activity was determined in serum according to a standard protocol (Vanderlinde, 1985).

Principle:

Lactate dehydrogenase catalyzes the conversion of pyruvate to lactate with NADH + H^+ oxidized during the process to NAD^+ which absorbs at 340 nm. The rate of NAD^+ generation and pyruvate reduction is directly proportional to the activity of LDH present.

Procedure:

Briefly, 1000 µl of R1 (pyruvate, 0.2 µmol/ml) and 250 µl of R2 (chromogenic agent) was pipetted into a cuvette, shaken, then 10 µl of sample was added, mixed and incubated at 37^0C for 1 min. Change in absorbance per min during a period of 3 min was measured. All absorbance were determined at 340 nm.

LDH (U/L) = change in absorbance/min X 16030 (conversion factor obtained from manual)

3.6 Chromatographic Determination of Possible Components Present in *V. doniana* Extract

3.6.1 Liquid chromatograpy - mass spectrometry (LCMS)

Principle:

Liquid chromatography mass spectrometry (LCMS) combines two approaches to separate and identify compounds present in a sample. High performance liquid chromatography (HPLC), which separates the components of a mixture, and mass spectrometry (MS) which identifies them. In the first phase (HPLC), a liquid mixture is passed through a column which separates components based on hydrophobic interactions or ion exchange. The time

taken for a solute to pass through the column (retention time) is characteristic of each component of the mixture but could be similar between different components. Thus, MS is employed. The separated components are converted to an ionized state. MS uses mass to charge ratio which is a more specific characterizing factor to identify components (Pitt, 2009)

Procedure:

One microliter (1µl) of *V. doniana* extract (2mg) dissolved in ethanol (1 ml) was injected via a loop into a RTX-7 column of LC-MS. The operating parameters were as follows: Pump A: LC-2030 Pump, Flow: 0.2 mL/min, B Conc.: 95 %; C Conc.: 0%; D Conc.: 0%; Mobile Phase A: Water; Mobile Phase B: Methanol; Start Wavelength: 190 nm; End Wavelength: 800 nm; Cell Temp.: 40^{o}C; Start Time: 0 min; End Time: 4 min; Acquisition Mode: Scan Polarity: Positive; Event Time: 1 sec; Detector Voltage: +1 kV; Threshold: 0; Start m/z: 50; End m/z: 1700; Scan Speed: 1667 u/sec. Compounds were identified by direct comparison of mass spectral data with those in the NIST library and Food Database.

3.7 Statistical analysis

All statistical analysis were done using SPSS 20.0 software for windows. Where appropriate, the results are expressed as Mean ± Standard deviation (SD). Data was analyzed by one way analysis of variance (ANOVA). Differences between treatment groups were compared using the Duncan Multiple Range Test. P-values less than 0.05 were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Characterization of *V. doniana* and *F. apodanthera* Extracts and Fractions

4.1.1 Percentage (%) yield of *V. doniana* and *F. apodanthera*

The % yield of the two extracts is presented in Table 4.1. Ethanol extract of *F. apodanthera* had the highest yield (9.21%), while ethanol extract of *V. doniana* had the lowest yield (7.80%), which shows that more of the *F. apodanthera* was extracted given the same initial quantity.

4.1.2 Acute toxicity (LD_{50}) of *V. doniana* and *F. apodanthera* extracts

The results for acute toxicity determination is presented in Table 4.2 and shows that the LD_{50} of both *V. doniana* and *F. apodanthera* extracts were greater than 5 mg/g.

4.1.3 Thin layer chromatography

The thin layer chromatogram used to determine the most suitable solvent for the partial purification of both ethanol extracts of *V. doniana* and *F. apodanthera* is shown in appendix II. The best solvent system determined was acetone: ethyl acetate for *V. doniana* which gave 4 different components. For *F. apodanthera*,n-hexane: acetone was the best solvent system with 4 different components.

4.1.4 Column chromatography and Rf values

The column chromatography setup and Rf values of pooled fractions of the extracts is presented in Appendix IIIand Table 4.3 respectively. Thirteen fractions were collected for *V. doniana* while 16 fractions were collected for *F. apodanthera*. The fractions with similar Rf values were pooled together to give four pooled fractions and five pooled fractions for *V. doniana* and *F. apodanthera* respectively. The Rf values obtained show that for *V. doniana*,pooled fractions 2 (*V. doniana* Fraction 2*)* had the highest value while the lowest value was that of fraction 1 (*V. doniana* Fraction 1). For *F. apodanthera*, pooled fractions 1 (*F. apodanthera* Fraction 1) had the highest Rf value, while that of pooled fractions 5 (*F. apodanthera* Fraction 5) had the lowest value. In comparing the Rf values of fractions obtained from both extracts, *V. doniana* pooled fractions 2 (*V. doniana* Fraction 2*)* had the highest value while the lowest value was seen in *F. apodanthera* Fraction 5. Based on abundance, *F. apodanthera* fraction 1 was more abundant with the

highest percentage yield of 40.50%, while the least abundant was *F. apodanthera* Fraction 5 with a yield of 4.50%.

Table 4.1 Percentage (%) Yield of *V. doniana* and *F. apodanthera* ethanol extracts

Extract	Amount recovered (g)	% Yield
V. doniana	15.60	7.80
F. apodanthera	18.41	9.21

Table 4.2 Acute Toxicity (LD_{50}) of Ethanol Extracts of *V. doniana* and *Feretia apodanthera*

PHASE I			
Dosage of *V. doniana* and *F. apodanthera* extracts (mg/g)	**Rat I**	**Rat II**	**Rat III**
0.01	No death	No death	No death

0.1	No death	No death	No death
1	No death	No death	No death
	PHASE II		
1.2	No death		
1.6	No death		
2.9	No death		
5	No death		

Since there was death recorded in rats administered 5 mg/g, the LD_{50} was considered to be> 5mg/g body weight

Table 4.3 Rf-values of Fractions Obtained from *V. doniana* and *F. apodanthera* Extracts

Fraction Number	**Combined Fractions**	**Solvent System (%)**	**Fraction Weight (g)**	**Yield (%)**	**R_f-value**
V. doniana Pool1	V1	A20: 80EA	0.61	30.5	0.45
*V. doniana*Pool2	V2, V3, V4, V5	A70: 30EA	0.17	8.5	0.80

V. doniana Pool3	V6, V7, V8 V9	A50: 50EA A60: 40EA	0.34	17	0.62
V. doniana Pool4	V10, V11, V12, V13	A40: 60EA A30: 70EA	0.45	22.5	0.78
F. apodanthera Pool 1	F1, F2, F3	nH80: 20A	0.81	40.5	0.77
F. apodanthera Pool 2	F4, F5, F6	nH80: 20A	0.22	11	0.69
F. apodanthera Pool 3	F7, F8, F9, F10	nH70: 30A nH60: 40A	0.13	6.5	0.66
*F. apodanthera*Pool4	F11, F12, F13, F14	nH50: 50A	0.36	18	0.12
F. apodanthera Pool5	F15, F16	nH60: 40A	0.09	4.5	0.07

Keys: A – Acetone, EA – Ethyl acetate, nH – n-hexane

V – *V. doniana* fractions, F – *F. apodanthera* fractions

4.2 *In vitro* Antioxidant Activity of *V. doniana* and *F. apodanthera* Extracts and Fractions

The results of the *in vitro* antioxidant activities of *V. doniana*and *F. apodanthera* extract and fractions are presented in Table 4.4. A graph of percentage inhibition of free radicals versus extract concentration depicts antioxidant capacity of the extracts and fractions based on their scavenging of DPPH and ABTS radicals. The results show that fraction 3 of *V.*

doniana gave the highest antioxidant activity (lowest IC_{50}) of 72.27 ± 6.23 µg/mland42.81 ± 4.22 µg/ml for DPPH and ABTS respectively. The lowest antioxidant activity (highest IC_{50}) was obtained from pooled fraction 1 of *F. apodanthera*

4.3 *In vitro* Anti-cancer Effects of *V. doniana* and *F. apodanthera* on MCF-7 Cells

4.3.1 Cytotoxic effects of *V. doniana* and *F. apodanthera* on MCF-7 cells using MTT assay

The cytotoxic effects of *V. doniana* and *F. apodanthera* extract and fractions on MCF-7 cells from MTT results is presented as IC_{50} values shown in Table 4.5. The IC_{50} values for *V. doniana* and *F. apodanthera* extracts were found to be 26. 11 ± 3.4 µg/mL and 66.51 ± 5.2 µg/mL respectively, after treatment for 48 hours. These values were higher than that of vinblastine (standard drug). Of the two extracts, the IC_{50} of *V. doniana* was lower than that of *F. apodanthera*.

Table 4.4 Antioxidant Activity of *V. doniana* and *F. apodanthera* Extracts and Fractions

Extract/Fractions	**DPPH** Scavenging IC_{50} (µg/ml)	**ABTS** Scavenging IC_{50} (µg/ml)
V. doniana Extract	107.12 ± 8.51^{c}	65.58 ± 4.17^{c}
V. doniana Fraction 1	101.55 ± 7.42^{c}	71.15 ± 5.41^{c}

V. doniana Fraction 2	98.12 ± 4.94^{b}	59.18 ± 4.34^{b}
V. doniana Fraction 3	72.27 ± 6.23^{b}	42.81 ± 4.22^{b}
V. doniana Fraction 4	125.81 ± 9.62^{c}	69.06 ± 5.33^{c}
F. apodanthera Extract	118.36 ± 9.86^{c}	69.14 ± 5.25^{c}
F. apodanthera Fraction 1	186.33 ± 10.12^{d}	91.07 ± 4.19^{d}
F. apodanthera Fraction 2	106.15 ± 8.77^{c}	85.12 ± 4.56^{d}
F. apodanthera Fraction 3	83.12 ± 5.17^{b}	45.47 ± 4.27^{b}
F. apodanthera Fraction 4	88.93 ± 5.92^{b}	43.96 ± 4.05^{b}
F. apodanthera Fraction 5	96.40 ± 6.22^{b}	51.15 ± 5.21
Ascorbic acid	24.81 ± 3.62^{a}	7.67 ± 0.84^{a}

Values are mean ± SD of three determinations: Values with different superscripts down the column differ significantly ($p<0.05$). Values with the same superscripts down the column do not differ significantly ($p>0.05$)

Table 4.5 Cytotoxic Effects of *V. doniana* and *F. apodanthera* Extracts and Fractions

	IC_{50} after 24 hours treatment (µg/ml)	IC_{50} after 48 hours treatment (µg/ml)
V. doniana extract	95.43 ± 8.23^{c}	26.11 ± 3.4^{b}

V. doniana Fraction 1	109. 76 ± 5.85[d]	69.44 ± 8.95[c]
V. doniana Fraction 2	84.28 ± 6.63[c]	34.83 ± 5.21[b]
V. doniana Fraction 3	75.55 ± 6.12[c]	37.79 ± 8.93[b]
V. doniana Fraction 4	89.34 ± 5.67[c]	41.73 ± 4.88[c]
F. apodanthera Extract	133.75 ± 8.45[b]	66.51 ± 5.24[c]
F. apodanthera Fraction 1	101.18 ± 8.76[d]	54.26 ± 4.49[c]
F. apodanthera Fraction 2	144.05 ± 9.29[b]	69.96 ± 5.08[c]
F. apodanthera Fraction 3	71.83 ± 5.65[d]	35.05 ± 4.94[b]
F. apodanthera Fraction 4	95.08 ± 8.35[c]	59.95 ± 6.19[c]
F. apodanthera Fraction 5	149.74 ± 10.84[b]	88.74 ± 7.97[c]
Vinblastine	8.21 ± 0.87[a]	4.62 ± 0.75[a]

Values are mean ± SD of three determinations: Values with different superscripts down the column differ significantly ($p<0.05$). Values with the same superscripts down the column do not differ significantly ($p>0.05$)

* IC_{50} values of other cell lines are presented in Appendix X

4.3.2 Effect of *V. doniana* and *F. apodanthera* on MCF-7 morphology

The effects of *V. doniana* and *F. apodanthera* treatment on MCF-7 cell morphology are presented in Plates III and IV respectively. Control cells showed normal morphology with

multi nucleated cells. MCF-7 cells treated with 50 µg/ml of *V. doniana* showed cells with increased nuclear-cytoplasmic ratio and presence of interstitial spaces were observed.MCF-7 cells treated with 50 µg/ml *F. apodanthera* showed normal morphology with multi nucleated cells while cells treated with 100 µg/ml of *F. apodanthera* showed dense cytoplasm.

4.3.3 Effect of *V. doniana* and *F. apodanthera* on MCF-7 Cell Kinetics

The effect of *V. doniana* and *F. apodanthera* treatment on MCF-7 cell kinetics are shown in Figure 4.1 and 4.2 respectively. The results from MCF-7 cells treated with *V. doniana* showed a dose dependent reduction in cell growth, with 50 µg/ml of *V. doniana* showing the most significant potency with the lowest cell index after treatment for 120 hours. For cells treated with *F. apodanthera* at 1µg/ml,comparable kinetics to control was observed. A reduction in cell kinetics was observed in cells treated with 10 and 50 µg/ml of *F.* apodanthera.

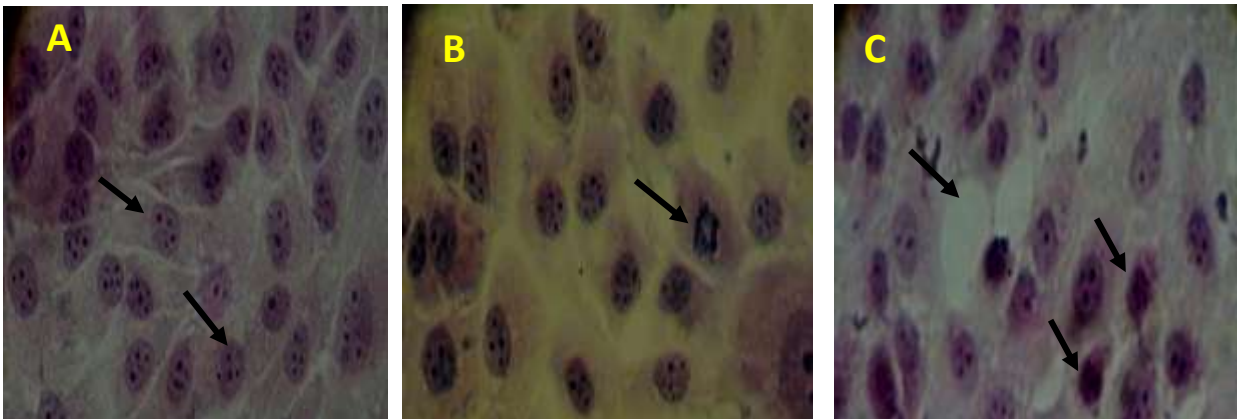


Plate III: Effect of *V. doniana* Extract on MCF-7 Morphology (×40)

A - Control : Untreated MCF-7 cells show normal morphology, multi nucleated

B - *V. doniana (*12.5 µg/ml) : Cells show increased nuclear-cytoplasmic ratio

C - *V. doniana (*25 µg/ml) : Cells show nuclear condensation with interstitial spaces observed

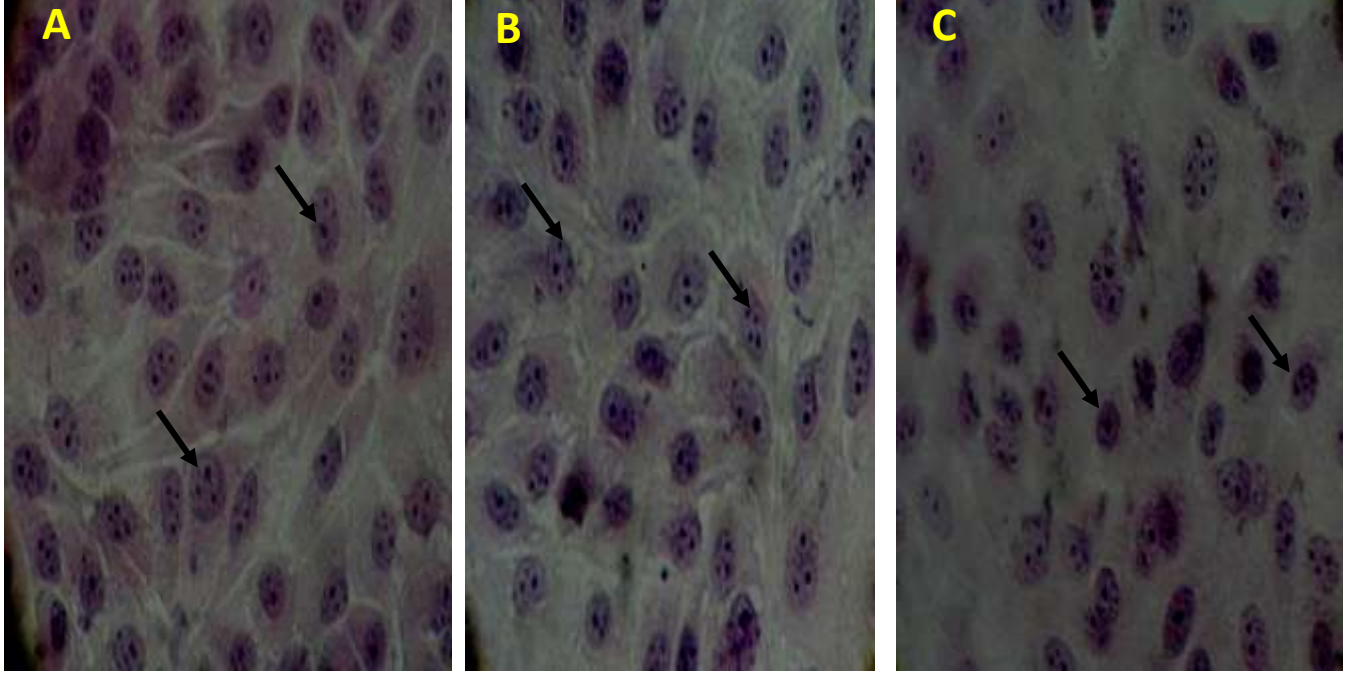


Plate IV: Effect of *F. apodanthera* Extract on MCF-7 Morphology (× 40)

A - Control : Untreated MCF-7 cells show normal morphology, multi nucleated

B - *F. apodanthera (*12.5 µg/ml) : Cells are multi nucleated, dense cytoplasm

C - *F. apodanthera (*25 µg/ml) : Cells are multi nucleated, dense cytoplasm

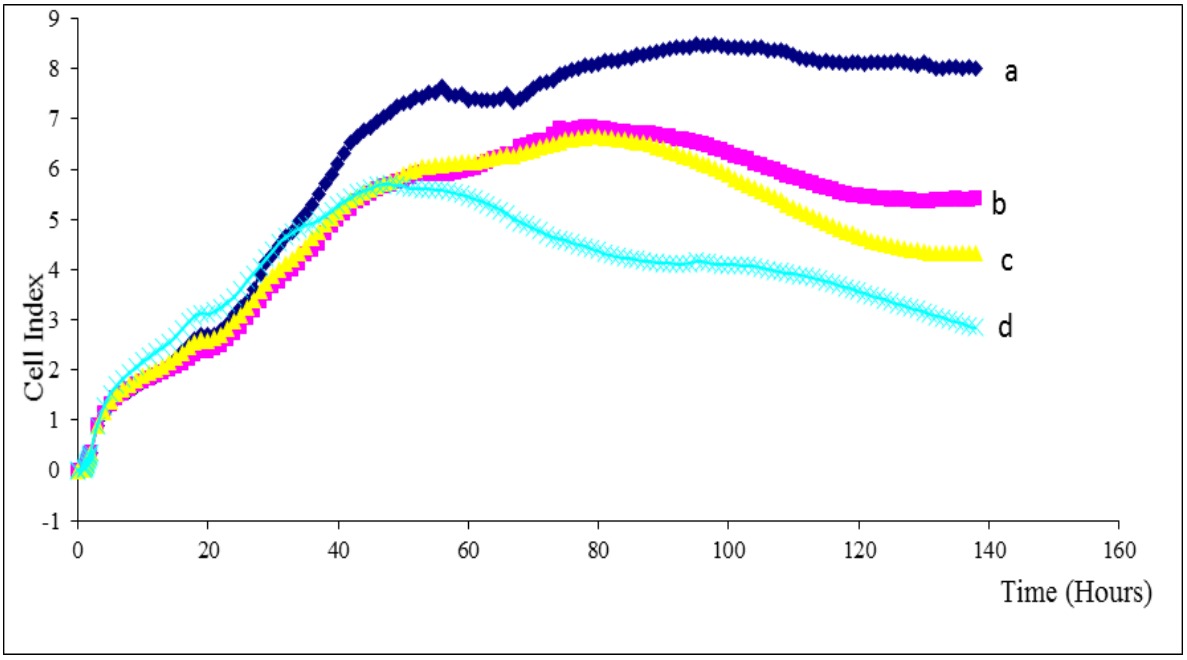


Figure 4.1: Effect of *V. doniana* on MCF-7 Cell Index

Keys: a - Control, b - 1 µg/ml, c - 10 µg/ml, d - 50 µg/ml

* Mean values are presented in Appendix XI

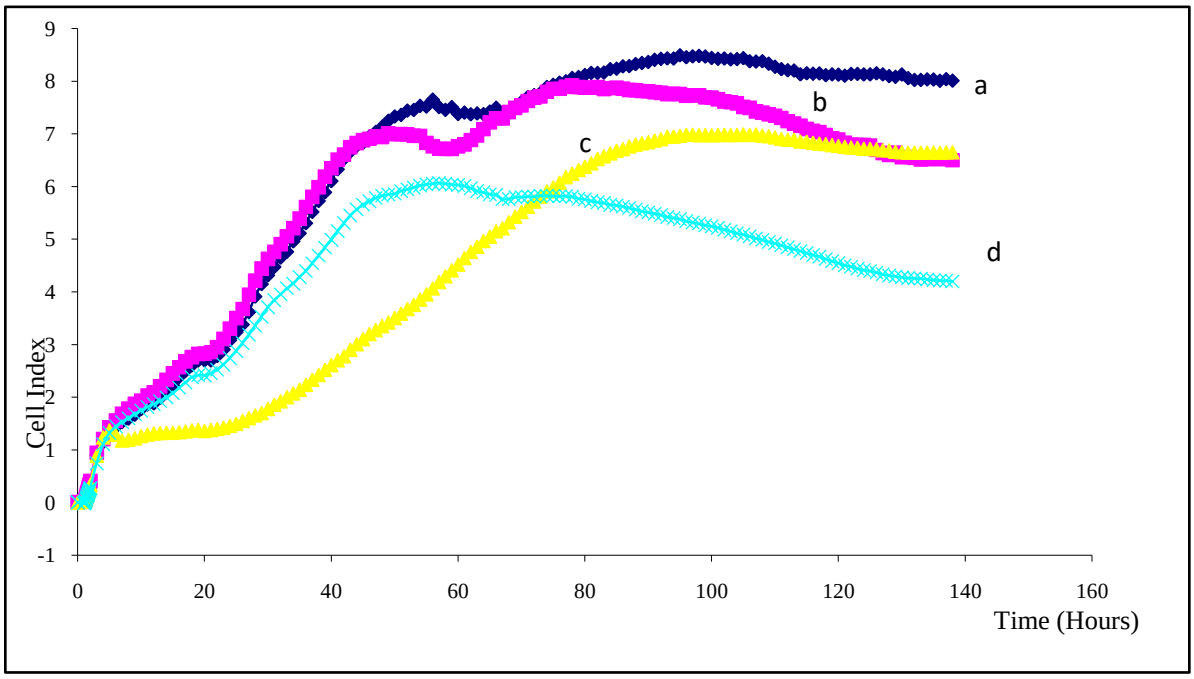


Figure 4.2: Effects of *F. apodanthera* on MCF-7 Cell Index

Keys: a - Control b - 1 µg/ml c - 10 µg/ml d - 50 µg/ml

* Mean values are presented in Appendix XI

4.3.4 Effect of *V. doniana* on MCF-7 cell cycle

The effect of *V. doniana* treatment on MCF-7 cell cycle progression is shown in Figure 4.3. A dose dependent accumulation of cells was observed in the MCF-7 cells treated with 12.5 µg/ml and 50 µg/ml of *V. doniana* after. The observation shows that *V. doniana* caused arrest of MCF-7 cells at G_0/G_1 phase of the cell cycle with a dose dependent reduction in the percentage of cells in S and G2/M phases after treatment for 48 h.

4.4 *In-vivo* Anti-cancer Effects of *V. doniana*

4.4.1: Estrogen receptor – α (ER– α) levels in female Wistar rats after induction of mammary carcinogenesis using 7, 12 Dimethylbenz[a]anthracene (DMBA)

Estrogen receptor– α (ER– α) levels in female Wistar rats induced with mammary carcinogenesis using DMBA is presented in Figure 4.4. There were significantly ($P<0.05$) higher ER– α levels in the DMBA-induced group when compared to normal control.

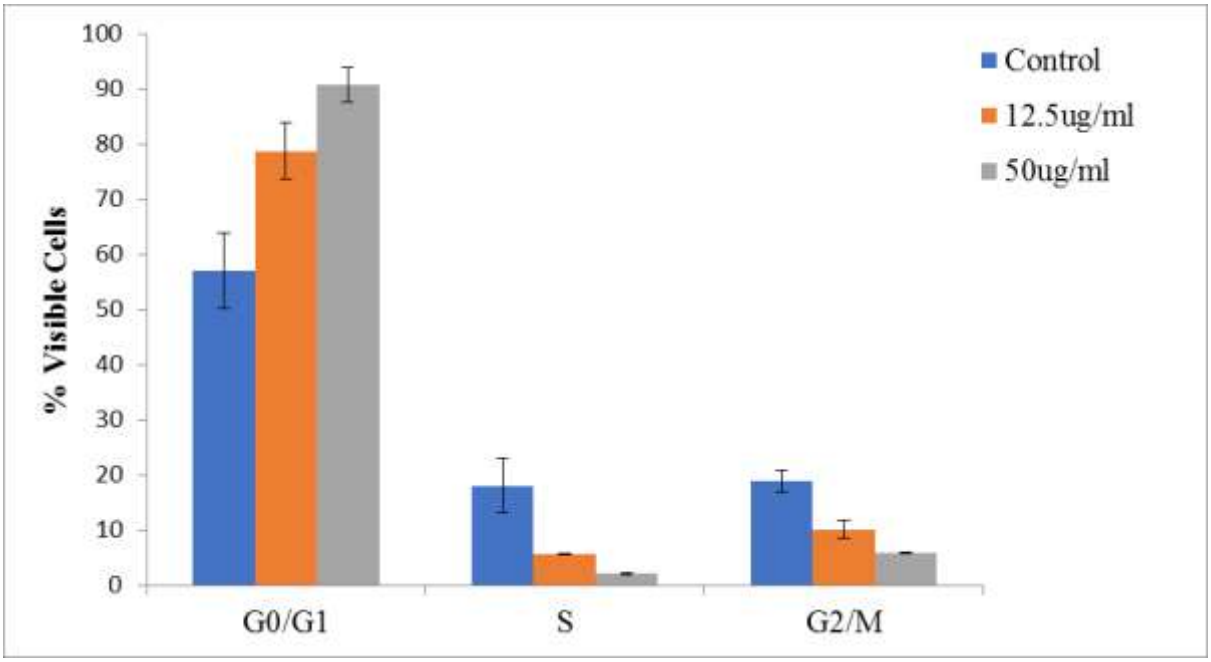


Figure 4.3: Effect of *V. doniana* Treatment on MCF-7 Cell Cycle Progression

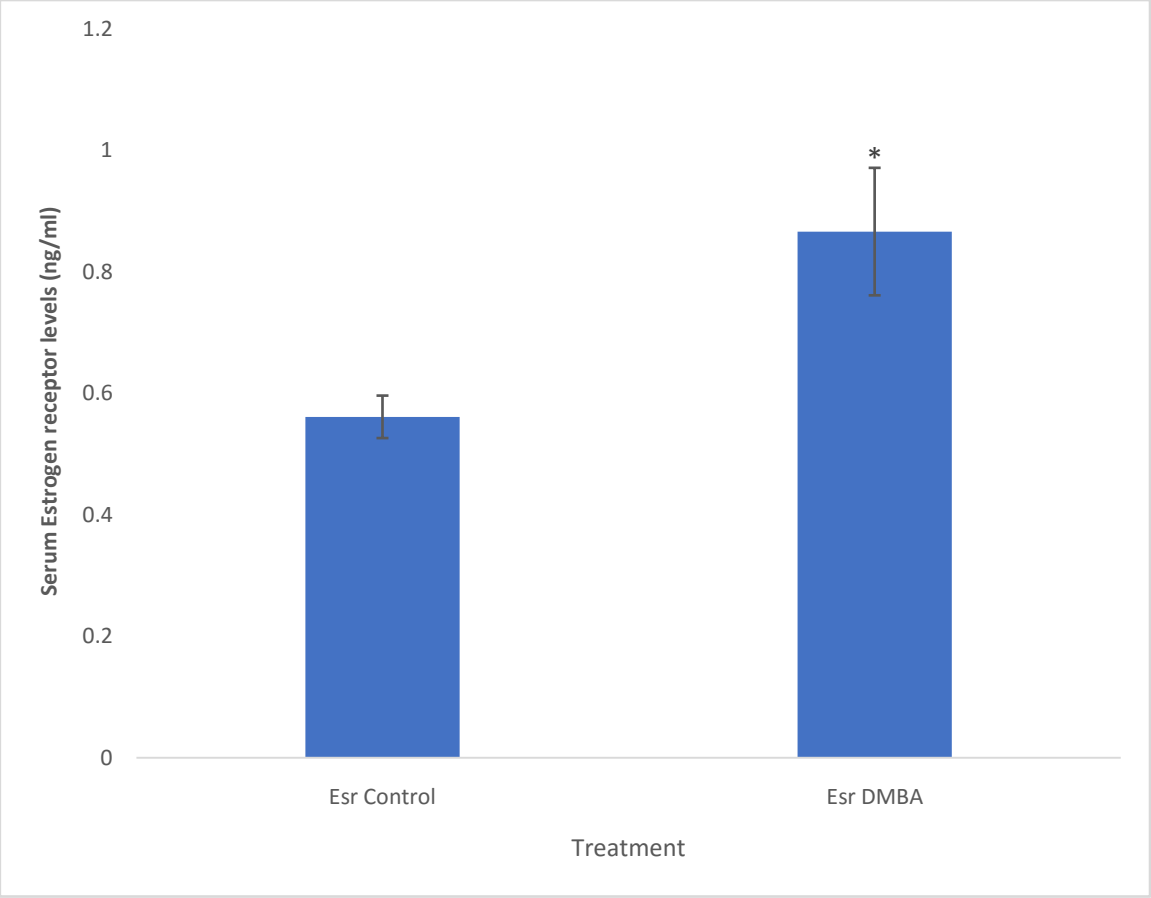


Figure 4.4: Estrogen Receptor– α levels in DMBA-induced and Normal (Control) Rats

n = 20 for DMBA-induced and n = 5 for normal (control) groups

* indicates significant ($p<0.05$) difference

4.4.2 Estrogen receptor – α (ER– α) levels in DMBA-induced rats before and after treatment with *V. doniana*

The estrogen receptor– α (ER– α) levels in the rats randomly assigned to treatment groups is shown in Table 4.6. The lowest ER– α levels were seen in the normal control group. Although the ER– α levels in the other groups were significantly ($P<0.05$) higher than the normal control, there was no significant ($P<0.05$) difference between ER– α levels in the groups before treatment.

After 14 days of treatment, there was no significant ($p<0.05$) reduction in ER– α levels in the *V. doniana* treated groups except for the 200 mg/kg treated group (also shown in Table 4.6). The lowest ER– α level was obtained in the tamoxifen (standard drug) treatment group which was significantly ($p<0.005$) lower than before the treatment.

Table 4.6: Estrogen Receptor – α (ER– α) Levels in DMBA-induced and Treated Rats

	ER– α levels across the groups (ng/ml)	**ER– α levels after Treatment (ng/ml)**
Normal	0.56 ± 0.04^{a}	0.57 ± 0.03^{a}
Untreated	0.91 ± 0.09^{a}	0.96 ± 0.06^{a}
DMBA-induced + 50 mg/kg *V. doniana*	0.77 ± 0.09^{a}	0.69 ± 0.09^{a}
DMBA-induced + 100 mg/kg *V. doniana*	0.87 ± 0.08^{a}	0.80 ± 0.09^{a}
DMBA-induced + 200 mg/kg *V. doniana*	0.90 ± 0.14^{b}	0.63 ± 0.13^{a}
DMBA-induced + Tamoxifen (20 mg/kg)	0.89 ± 0.09^{b}	0.39 ± 0.13^{a}

Values are mean ± SD of three determinations: Values with the same superscripts across the row do not differ significantly ($p>0.05$) before and after treatment. Values with different superscripts across the row differ significantly ($p<0.05$) before and after treatment.

4.4.3: Effect of *V. doniana* treatment on some oxidative stress markers in DMBA-induced rats

Effect of *V. doniana* treatment on some oxidative stress markers is presented in Table 4.7. Malondialdehyde (MDA) levels were significantly ($P<0.05$) higher in the DMBA induced-untreated rat group when compared with normal control and all the DMBA-induced and treated rat groups. MDA levels in the 100 mg/kg *V. doniana* treated group was significantly ($P<0.05$) lower compared with all the treated rat groups and was also statistically ($p>0.05$) similar to normal control.

Reduced glutathione (GSH) level was significantly ($P<0.05$) lower in the DMBA induced-untreated rat group when compared to normal control and DMBA administered-treated rat groups. Of all the groups, rats treated with 100 and 200 mg/kg of *V. doniana* extractshowedsignificantly ($P<0.05$) higher GSH levels.

Catalase activity was significantly ($P<0.05$) lower in the DMBA induced-untreated rat group when compared with normal control and treated groups, except the 100 mg/kg treated group. Treatment with 50 and 200 mg/kg of *V. doniana* enhanced the catalase activity, which was comparable to normal control.

Superoxide dismutase activity showed no significant ($P>0.05$) change across the treatment groups and the normal control.

Table 4.7: Effect of *V. doniana* Treatment on Some Oxidative Stress Markers in DMBA-induced Rats

	Malondialdehyde (mmol/g of tissue)	**Reduced glutathione (µg/g of tissue)**	**CAT (µmole H_2O_2/min/mg protein)**	**SOD (µmole SOD/min/mg protein)**
Normal	1.06 ± 0.04^{a}	4.40 ± 0.29^{c}	1.06 ± 0.15^{b}	14.52 ± 2.07^{a}
Untreated	1.77 ± 0.01^{d}	2.94 ± 0.24^{a}	0.81 ± 0.07^{a}	15.09 ± 0.91^{a}
DMBA-induced + 50 mg/kg *V. doniana*	1.27 ± 0.13^{b}	$4.0 \pm 0.35^{b,c}$	$1.17 \pm 0.02^{b,c}$	15.44 ± 2.69^{a}
DMBA-induced + 100 mg/kg *V. doniana*	1.11 ± 0.08^{a}	5.25 ± 0.41^{d}	0.88 ± 0.01^{a}	15.01 ± 2.54^{a}
DMBA-induced + 200 mg/kg *V. doniana*	$1.38 \pm 0.06^{b,c}$	5.31 ± 0.43^{d}	1.05 ± 0.08^{b}	15.20 ± 0.68^{a}
DMBA-induced + Tamoxifen (20 mg/kg)	1.48 ± 0.14^{c}	3.69 ± 0.24^{b}	1.22 ± 0.11^{c}	15.53 ± 1.54^{a}

Values are mean ± SD of three determinations: Values with different superscripts down the column differ significantly ($p<0.05$). Values with the same superscripts down the column do not differ significantly ($p>0.05$)

4.4.4: Effect of *V. doniana* treatment on serum cytokine (Interleukin-1β and Tumor necrosis factor-α) levels in DMBA-induced rats

Effect of *V. doniana* treatment on interleukin -1β (IL-1β) and tumor necrosis factor - α (TNF-α) levels are presented in Table 4.8. Levels of IL-1β and TNF-α were significantly ($P<0.05$) higher in the DMBA induced-untreated rat group when compared to normal control and all the DMBA induced- treated rat groups. Levels of IL-1β and TNF-α were significantly ($P<0.05$) lower in the 200 mg/kg *V. doniana* administered group when compared with the other treatment groups. Levels of IL-1β and TNF-α in all the treatment groups were higher than normal control.

4.4.5 Effect of *V. doniana* treatment on mammary histology of DMBA-induced rats

The effect of *V. doniana* treatment on mammary histology of DMBA-induced untreated rats is shown in Plate V. Mammary cells of the gland in normal control showed normal epithelial cells. For the DMBA induced-untreated group, mammary cells of the gland showed malignant epithelial hyperplasia. Generally, attenuation to atypia (abnormal cell morphology) was observed across the *V. doniana* treated groups. However, the best cell morphology among the treatment groups was observed in the tamoxifen (standard drug) treated group.

Table 4.8: Effect of *V. doniana* Treatment on Serum Cytokine (IL-1β and TNF-α) Levels in DMBA-induced rats

	IL-1β (ng/ml)	TNF-α (ng/ml)
Normal	45.04 ± 7.81^{a}	77.43 ± 8.17^{a}
Untreated	91.15 ± 6.90^{d}	139.27 ± 9.21^{e}
DMBA-induced + 50 mg/kg *V. doniana*	74.02 ± 4.52^{c}	121.77 ± 6.16^{d}
DMBA-induced + 100 mg/kg *V. doniana*	70.52 ± 5.57^{c}	110.94 ± 6.05^{cd}
DMBA-induced + 200 mg/kg *V. doniana*	60.73 ± 5.16^{b}	88.96 ± 7.41^{b}
DMBA-induced + Tamoxifen (20 mg/kg)	54.46 ± 3.59^{b}	100.56 ± 6.24^{c}

Values are mean ± SD of three determinations: Values with different superscripts down the column differ significantly ($p<0.05$). Values with the same superscripts down the column do not differ significantly ($p>0.05$)

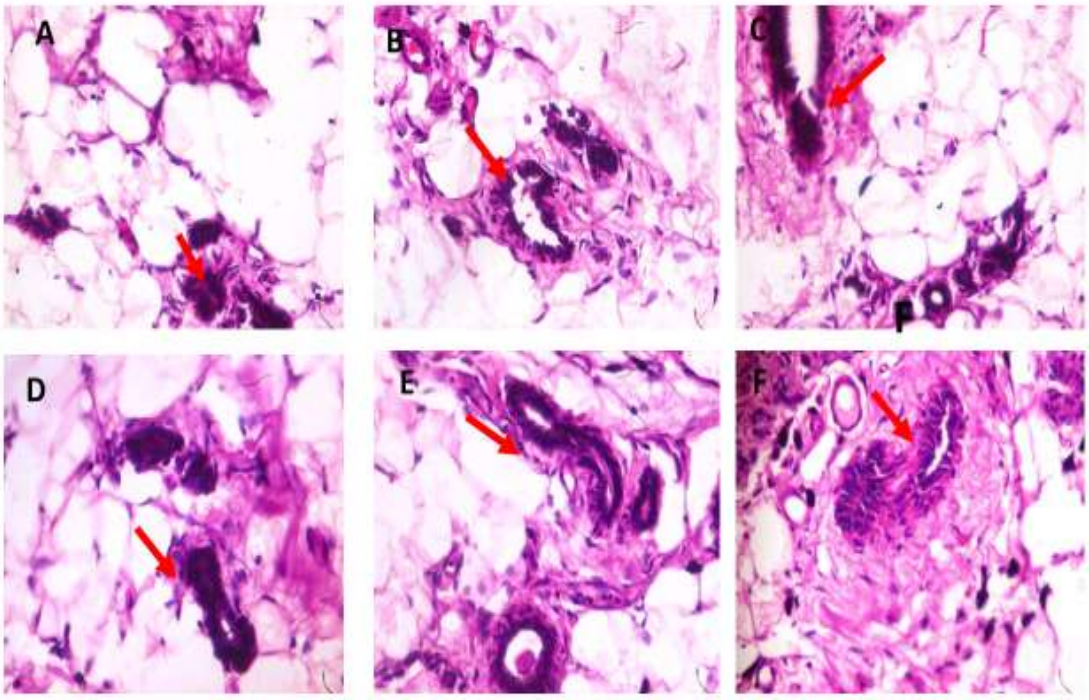


Plate V: Effect of *V. doniana* Treatment on Mammary Histology using H and E staining (× 100)

A- Mammary histology of the glands of normal (control) rats, B - Mammary histology of the glands of DMBA-induced untreated rats (negative control), C - Mammary histology of the glands of DMBA-induced rats treated with 50 mg/kg of *V. doniana*, D - Mammary histology of the glands of DMBA-induced rats treated with 100 mg/kg of *V. doniana*, E - Mammary histology of the glands of DMBA-induced rats treated with 200 mg/kg of *V. doniana*, F - Mammary histology of the glands of DMBA-induced rats treated with 20 mg/kg of Tamoxifen

4.4.6 Effect of *V. doniana* Treatment on the expression of COX-2 in DMBA-induced rats

The effect of *V. doniana* treatment on the expression of COX-2 in DMBA-induced rats is shown in Plate 4.8. Mammary adipose tissues in the normal control showed mild expression of COX-2. Mammary adipose tissues in the DMBA-induced untreated group showed moderate expression of COX-2, while the groups treated with *V. doniana* showed mild to moderate COX-2 expression.

4.4.7 Effect of *V. doniana* treatment on the expression of Caspase-3 in DMBA-induced rats

The effect of *V. doniana* treatment on Caspase-3 expression is shown in Plate VII. Mammary adipose tissues in the normal control showed moderate expression of Caspase-3. Mammary adipose tissues in the DMBA-induced untreated group showed mild expression of Caspase-3, while the groups treated with *V. doniana* showed mild to moderate expression of Caspase-3.

4.4.8 Effect of *V. doniana* treatment on the expression of p53 in DMBA-induced rats

The effect of *V. doniana* treatment on p53 expression is shown in Plate VIII. Mammary adipose tissues in the normal control showed moderate expression of p53. Mammary adipose tissues in the DMBA-induced untreated group showed mild expression of p53, while the groups treated with *V. doniana* showed mild to moderate expression of p53.

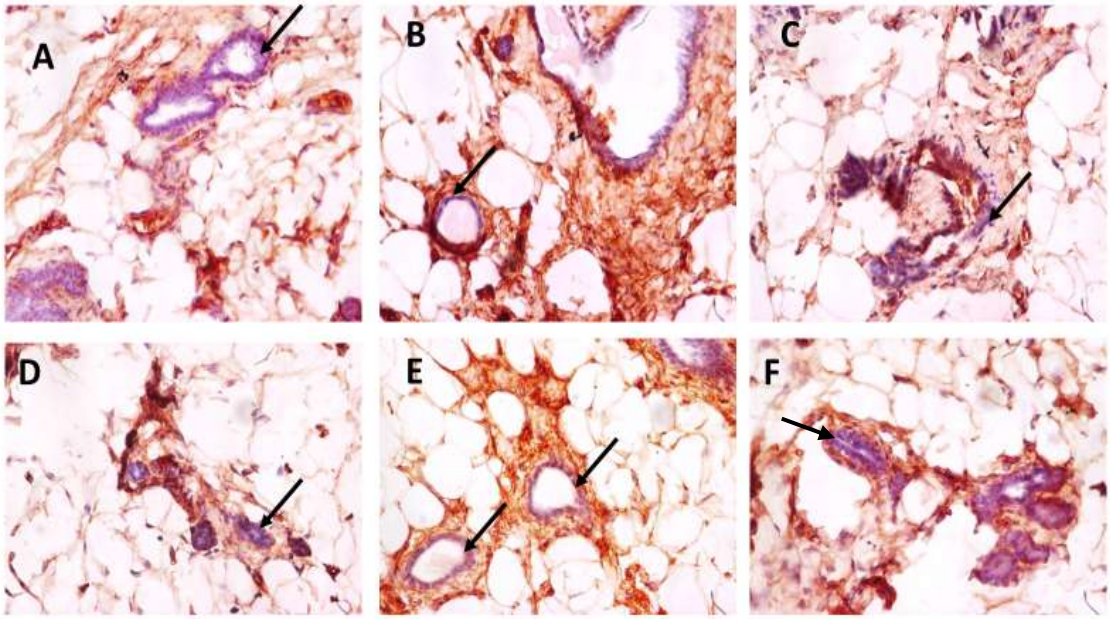


Plate VI: Effect of *V. doniana* Treatment on the Expression of COX-2 in DMBA-induced Rats (× 400)

A –Mild COX-2 expression in Mammary tissue of normal (control) rats, B -Moderate COX-2 expression in Mammary tissue of DMBA-induced Untreated (negative control) rats, C –Moderate COX-2 expression in 50 mg/kg *V. doniana* treated group, D - Mild COX-2 expression in 100 mg/kg *V. doniana* treated group, E - Mild COX-2 expression in 200 mg/kg *V. doniana* treated group, F - Mild COX-2 expression in 20 mg/kg Tamoxifentreated group

Keys:

mild	-	< 5 per high power field
moderate	-	5 to ≤ 25 per high power field
severe	-	> 25 per high power field

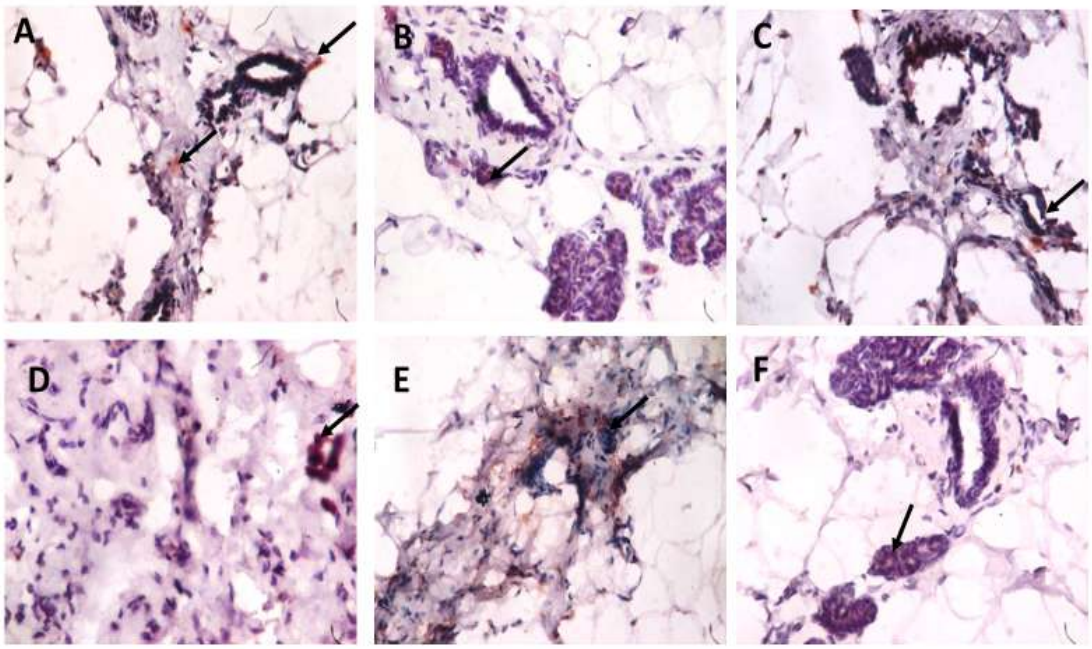


Plate VII: Effect of *V. doniana* Treatment on the Expression of Caspase-3 in DMBA-induced rats (× 400)

A –Moderate caspase-3 expression in Mammary tissue of normal (control) rats, B –Mild caspase-3 expression in Mammary tissue of DMBA-induced Untreated (negative control) rats, C –Mild caspase-3 expression in 50 mg/kg *V. doniana* treated group, D - Moderate caspase-3 expression in 100 mg/kg *V. doniana* treated group, E - Mild caspase-3 expression in 200 mg/kg *V. doniana* treated group, F –Moderate caspase-3 expression in 20 mg/kg Tamoxifentreated group

Keys:

mild	-	< 5 per high power field
moderate	-	5 to ≤ 25 per high power field
severe	-	> 25 per high power field

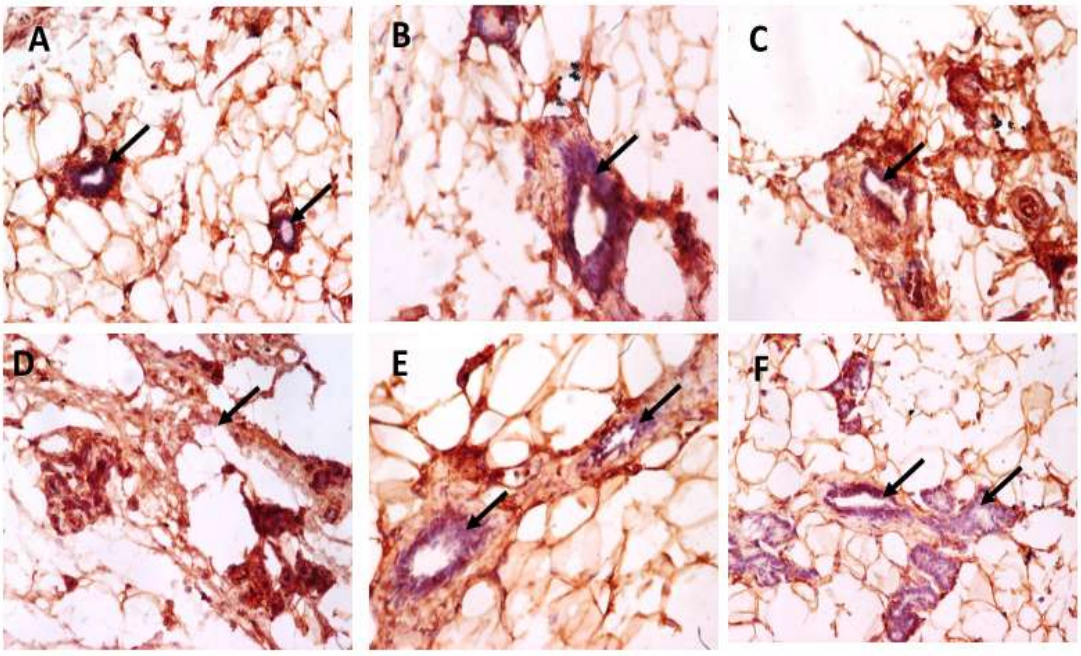


Plate VIII: Effect of *V. doniana* Treatment on the Expression of p53 in DMBA-induced Rats (× 400)

A –Mild p53 expression in Mammary tissue of normal (control) rats, B -Moderatep53 expression in Mammary tissue of DMBA-induced Untreated (negative control) rats, C –Moderate p53 expression in 50 mg/kg *V. doniana* treated group, D - Mild p53 expression in 100 mg/kg *V. doniana* treated group, E – Moderate p53 expression in 200 mg/kg *V. doniana* treated group, F –Moderate p53 expression in 20 mg/kg Tamoxifentreated group

Keys:

mild	-	< 5 per high power field
moderate	-	5 to ≤ 25 per high power field
severe	-	> 25 per high power field

4.5 Effect of Sub-acute Administration of *V. doniana* on Normal Rats

4.5.1 Effect of sub-acute administration of *V. doniana* on some serum markers of tissue damage in normal rats

The effect of sub-acute administration on some serum markers of tissue integrity are presented in Tables 4.9 to 4.10. After *V. doniana* administration for 28 days, there was no significant ($P>0.05$) difference in serum Alanine aminotransferase (ALT), Gamma glutamyltransferase (GGT) and Lactate dehydrogenase levels (LDH) activity between the control group and all the *V. doniana* administered groups. Also, there was no significant ($P>0.05$) difference in urea and creatinine levels between the control group and all the *V. doniana* administered groups.

Table 4.9: Effect of Sub-acute Administration of *V. doniana* on Activity of Some Serum Enzyme Markers of Tissue Damage

Treatment	Alanine aminotransferase (IU/l)	Gamma glutamyltransferase (IU/l)	Lactate Dehydrogenase (IU/l)
Control	49.20 ± 3.03^{a}	1.67 ± 0.14^{c}	155.81 ± 5.42^{a}
50 mg/kg *V. doniana*	48.2 ± 5.81^{a}	1.59 ± 0.12^{a}	154.14 ± 4.45^{a}
100 mg/kg *V. doniana*	48.4 ± 2.88^{a}	1.57 ± 0.12^{a}	154.06 ± 4.18^{a}
200 mg/kg *V. doniana*	47.4 ± 2.61^{a}	1.56 ± 0.12^{a}	152.25 ± 3.53^{a}

Values are mean ± SD of three determinations: Values with different superscripts down the column differ significantly ($p<0.05$). Values with the same superscripts down the column do not differ significantly ($p>0.05$)

Table 4.10: Effect of Sub-acute Administration of *V. doniana* on Levels of Some Serum Markers (Urea and Creatinine) of Tissue Damage

Treatment	Urea (mmol/L)	Creatinine (µmol/L)
Control	6.81 ± 0.52^{a}	64.69 ± 6.83^{a}
50 mg/kg *V. doniana*	6.67 ± 0.83^{a}	$64. 85 \pm 2.53^{a}$
100 mg/kg *V. doniana*	6.57 ± 0.42^{a}	65.26 ± 7.32^{a}
200 mg/kg *V. doniana*	6.35 ± 0.37^{a}	69.11 ± 3.54^{a}

Values are mean ± SD of three determinations: Values with different superscripts down the column differ significantly ($p<0.05$). Values with the same superscripts down the column do not differ significantly ($p>0.05$)

4.5.2 Effect of sub-acute administration of *V. doniana* on liver histology

The effect of sub-acute administration of 50, 100 and 200 mg/kg of *V. doniana*on liver histology is shown in Plate IX. The plates show normal architecture of the liver in all the *V. doniana* administered groups. Normal architecture of the liver was also observed in the normal control group.

4.5.3 Effect of sub-acute administration of *V. doniana* on kidney histology

The effect of sub-acute administration of 50, 100 and 200 mg/kg of *V. doniana* on kidney histology is shown in Plate X. The plates show normal architecture of the kidney in all the *V. doniana* administered groups. Normal architecture of the kidney was also observed in the normal control group.

4.5.4 Effect of sub-acute administration of *V. doniana* on mammary tissue histology

The effect of sub-acute administration of 50, 100 and 200 mg/kg of *V. doniana* on mammary tissue histology is shown in Plate XI. The plates show normal architecture of mammary tissue in all the *V. doniana* administered groups. Normal architecture of mammary tissue was also observed in the normal control group.

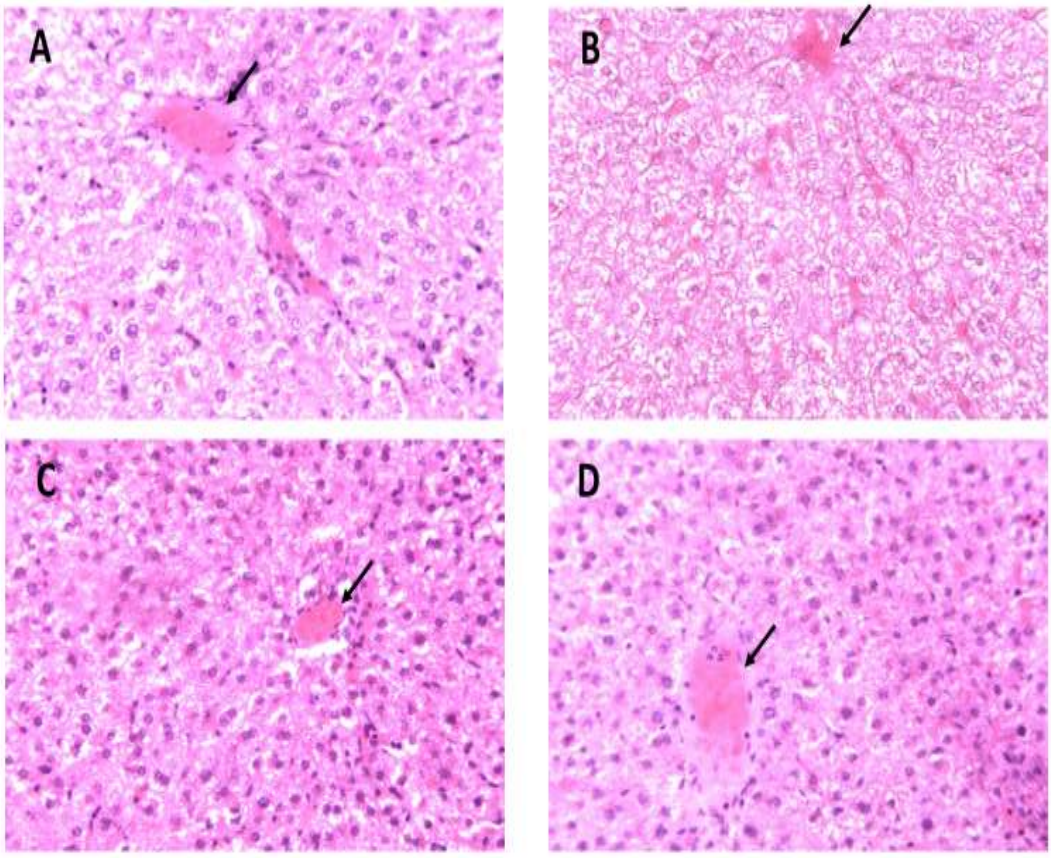


Plate IX: Effect of Sub-acute Administration of *V. doniana* on Liver Histology using H and E staining (× 400)

A - Liver histology of control, B - Liver histology of 50 mg/kg *V. doniana* administered (sub-acute) group, C -Liver histology of 100 mg/kg *V. doniana* administered (sub-acute) group, D - Liver histology of 200 mg/kg *V. doniana* administered (sub-acute) group

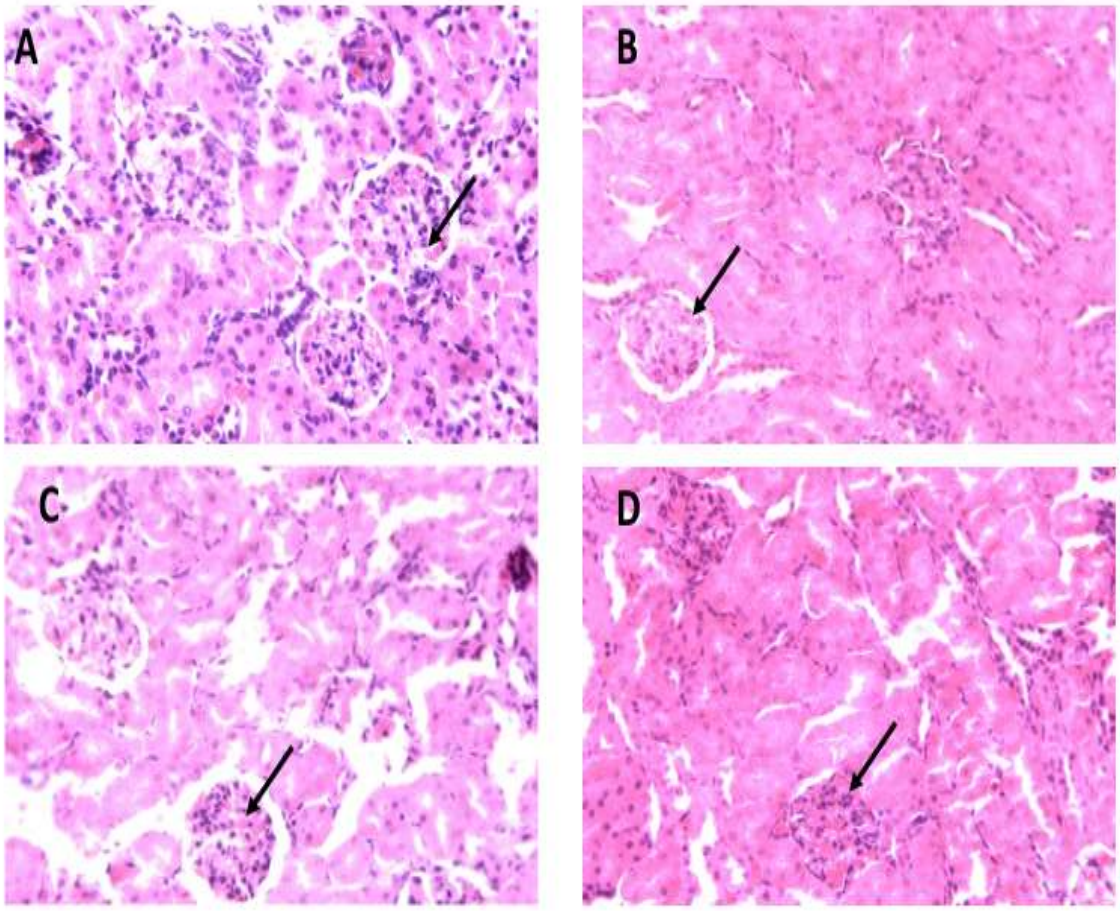


Plate X: Effect of Sub-acute Administration of *V. doniana* on Kidney Histology using H and E staining (× 400)

A - Kidney histology of control, B - Kidney histology of 50 mg/kg *V. doniana* administered (sub-acute) group, C - Kidney histology of 100 mg/kg *V. doniana* administered (sub-acute) group, D - Kidney histology of 200 mg/kg *V. doniana* administered (sub-acute) group

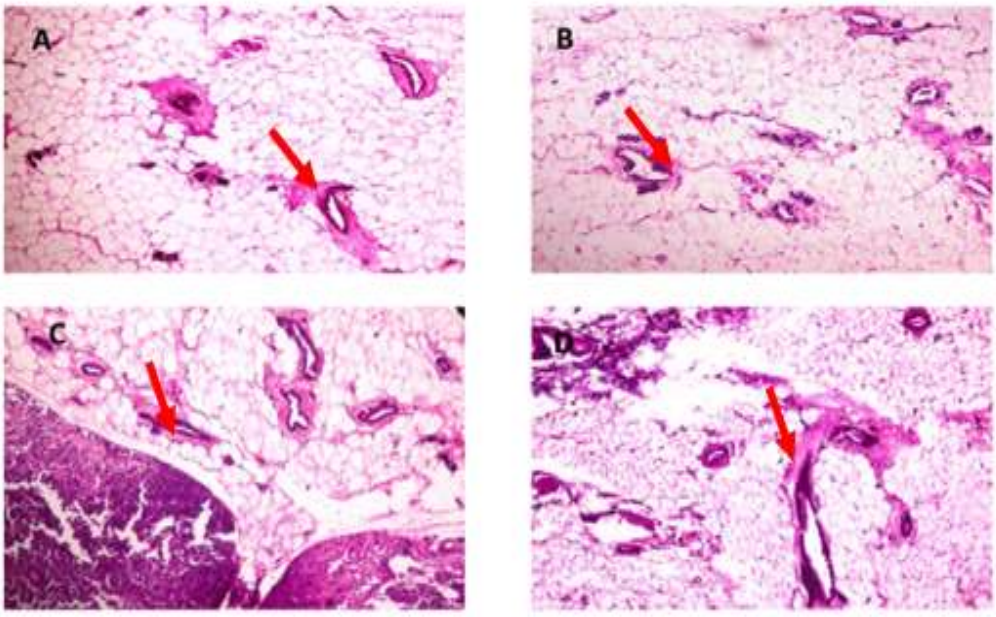


Plate XI: Effect of Sub-acute Administration of *V. doniana* on Mammary Tissue Histology using H and E staining (× 400)

A - Mammary tissue histology of control, B - Mammary tissue histology of 50 mg/kg *V. doniana* administered (sub-acute) group, C - Mammary tissue histology of 100 mg/kg *V. doniana* administered (sub-acute) group, D - Mammary tissue histology of 200 mg/kg *V. doniana* administered (sub-acute) group

4.6 Chromatographic Analysis of *V. doniana*

4.6.1Liquid chromatography mass spectrometry (LCMS)of *V. doniana*

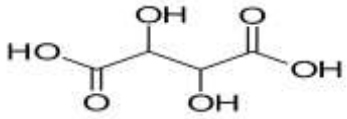
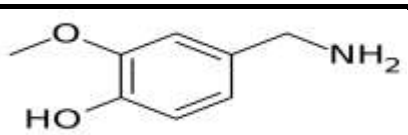
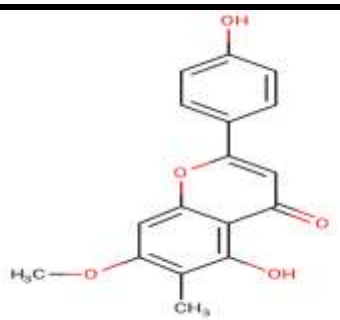
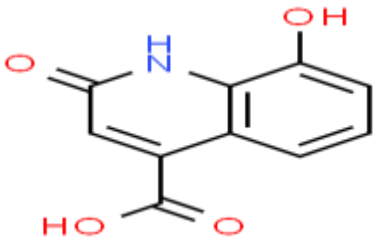
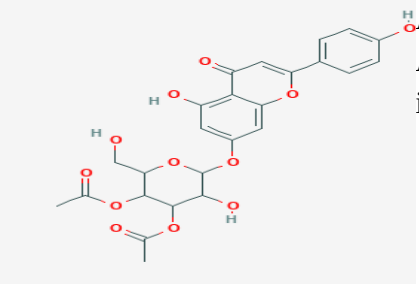
The results for Liquid chromatography mass spectrometry (LCMS) analysis of *V. doniana* extract is shown in Table 4.11. LCMS analysis revealed the presence of L-tartaric acid, vanillylamine, 4',5-dihydroxy-7-methoxy-6-methylflavone, zeanic acid, 2',3'-diacetylcosmosiin.

The structure of the LCMS identified compounds with reported biological activity is shown in Table 4.12.

Table 4.11: Proposed Compounds Present in *V. doniana* Using LCMS

Compound	Relative abundance (%)
L-Tartaric acid	29.87
Vanillylamine	6.93
4',5-Dihydroxy-7-methoxy-6-methylflavone	17.32
Zeanic acid	26.41
2',3'-Diacetylcosmosiin	19.48

Table 4.12: Reported Biological Activity of Some Compounds Identified Using LCMS

S/No.	Name of Compound	Structure	Biological Activity	Reference
1	L-Tartaric acid		Antioxidant	DeBolt *et al*., 2006; Cholet*et al*., 2016
2	Vanillylamine		Antioxidant	Weber *et al*., 2014
3	4',5-Dihydroxy-7-methoxy-6-methylflavone		Antioxidant, Anti-inflammatory	Catarino *et al*., 2015
4	Zeanic acid		Growth regulator	Fukumi *et al*., 1974
5	2',3'-Diacetylcosmosiin		Antioxidant, Anti-inflammatory	Min *et al*., 2018

CHAPTER FIVE

5.0 DISCUSSION

Globally, breast cancer is reported to account for 25% of all new cancer cases and 15% of cancer mortality cases (Azubuike *et al*., 2018). In Africa, Sub-Sahara Africa is reported to currently record the highest incidence and mortality rates of breast cancer (Pace and Shulman 2016; Azubuike *et al*., 2018). This rising incidence of breast cancer among women in Nigeria and Sub-Sahara Africa has become a source of concern to relevant authorities (Pace and Shulman 2016; Adeloye *et al*., 2018). The challenges associated with breast cancer in Sub-Sahara Africa have been attributed to changes in diet in favor of a more westernized diet, increased exposure to carcinogens, a more sedentary lifestyle, poor cancer awareness among women, inadequate cancer care facilities, high cancer care costs and delay in seeking available cancer care facilities (Adeloye *et al*., 2018; Azubuike *et al*., 2018).

Carcinogenesis of the breast or mammary tissue has been found to be influenced by genetic and epigenetic factors (Coyle *et al*., 2017), with recent emphasis also pointing to the role of exposure to chemicals and toxins in predisposition to breast cancer (Siddique *et al*., 2016; Rodgers *et al*., 2018). Heritable changes to DNA sequence and composition that causes altered protein function, which promotes mammary cell proliferation has been observed among breast cancer patients (Määttä *et al*., 2017). The breast cancer associated gene 1 and 2 (*BRCA1* and *BRCA2*) are anti-oncogenes that code for tumor suppressor proteins under normal conditions (Godet and Gilkes, 2017). However, when these genes are mutated in individuals, they exhibit altered function which increases the susceptibility

of such individuals to breast cancer (Mehrgou and Akouchekian, 2016). Also, mutations to the tumor suppressor gene *TP53* have been shown to increase susceptibility to different cancers (Schon and Tischkowitz, 2018). Breast cancer is the most frequently diagnosed neoplastic disease among women with *TP53* mutation (Schon and Tischkowitz, 2017).

Epigenetic factors reported to influence carcinogenesis includes DNA methylation, histone modification and altered expression of non-coding RNAs (Yen *et al*., 2016). Epigenetic alterations refer to changes in gene expression which are not a result of changes to the inherent DNA sequence of the individual (Romagnolo *et al*., 2016). A number of breast cancer tumor suppressor genes have been found to undergo DNA methylation resulting in a masking of the gene, hindering the binding of transcription factors to the promoter regions and reducing the expression of important proteins implicated in carcinogenesis (Long *et al*, 2017). These proteins include p21, ATM, BRCA1, APC, BIN1, ESRb, GSTP1, P16, BMP6 and CST6 (Askari *et al*., 2013; Begam *et al*., 2017).

Oxidative stress, a condition characterized by increased generation of reactive oxygen species and free radicals beyond cellular antioxidant capacity, resulting in damage to cellular macromolecules, has been implicated in the initiation, promotion and progression stages of mammary carcinogenesis (Hecht *et al*., 2016). Oxidative stress alters the redox state of cells, causing dysregulated activation of redox sensitive proteins, oxidation of polyunsaturated fatty acids resulting in lipid peroxidation, and oxidation of guanosine residues in DNA, ultimately promoting dysregulated cell function and proliferation (Lee*et al*., 2017). The tumor environment of breast tissue is characterized by elevated levels of cytokines and growth factors causing altered cellular function that promotes tumorigenesis (Bussard *et al*., 2016).

Currently used models for studying mechanisms of carcinogenesis and potency of potential anti-cancer compounds can broadly be divided into *in vitro* and *in vivo* models (Holliday and Speirs, 2011; Russo, 2015). A number of breast cancer cell lines are available for research, prominent among which are the MCF-7, MDA-MB-468 and MDA-MB-231 cell lines (Holliday and Speirs, 2011). Cell lines offer the advantage of cell uniformity but are easily prone to contamination if proper quality control measures are not adopted. *In vivo* studies generally use either genetically modified animals or chemically induced models of carcinogenesis. Dimethylbenz[a]anthracene, Dimethyl nitrourea and Benzo[a]pyrene are some commonly used carcinogens in animal studies of chemically-induced carcinogenesis (Alvarado *et al*., 2017).

Current research in breast cancer is examining the anti-cancer potential of natural compounds, due to the challenges associated with therapies presently used in the treatment of breast cancer (Ali *et al*., 2016). Phytocompounds rich in antioxidants can counteract oxidative stress mediated processes in carcinogenesis through reduction of reactive oxygen species to neutral compounds thereby regulating the cellular redox state (Pisoschi and Pop, 2015). Some phytocompounds can chelate transition metals that contribute to increased generation of reactive oxygen species (Winiarska-Mieczan, 2018). Certain phytocompounds have also been found to bind transmembrane lipids, resulting in altered membrane integrity of cancer cells, leading to death of the cells (Podolak *et al*., 2010). Thus, studies across the globe are examining the anti-cancer potential of locally available plants (Yedjou *et al*., 2008; Fadeyi *et al*., 2013; Koohpar *et al*., 2015). *Vitex doniana* and *Feretia apodanthera* are locally available plants that have reported usage in herbal medicine for the treatment of inflammation related disorders and have been subjected to

scientific investigations for their antioxidant and anti-inflammatory potency (James *et al*., 2015; Owolabi *et al*., 2018).

In this study, *V. doniana* and *F. apodanthera* extracts and fractions showed free radical scavenging ability by reducing DPPH and ABTS free radicals (Table 4.4). Both DPPH and ABTS are free radicals, and their level of reduction by a plant extract or fraction, is an indication of the antioxidant capacity. The results thus suggest that *V. doniana* has a higher *in vitro* antioxidant capacity than *F. apodanthera*. The results obtained are corroborated by earlier studies which showed that *V. doniana* leaves and *F. apodanthera* root bark are potent sources of natural antioxidants, due to their high free radical scavenging activity and presence of phytochemicals like flavonoids and polyphenols (James *et al*., 2014; Owolabi *et al*., 2018). The higher antioxidant potency of *V. doniana* extract could be due to the varying nature and abundance of flavonoids contained in the plants (Sonibare *et al*., 2009; Owolabi *et al*., 2018)

The IC_{50} results obtained from extracts and fractions of *V. doniana* and *F. apodanthera* showed dose and time-dependent cytotoxic effects on MCF-7 cells (Table 4.5). *V. doniana* extract was also cytotoxic to B16-F10 melanoma cell line but not as cytotoxic to sEnd.2 endothelial cell line (Appendix XXIX). MTT assay provides information on cell proliferation rates which are reduced when metabolic events in the mitochondria of a cell are obstructed. The results suggest that *V. doniana* with a comparatively lower IC_{50} was more cytotoxic to MCF-7 cells than *F. apodanthera*. The results also suggest that the whole extract of *V. doniana* was more cytotoxic than the fractions tested. These results can be explained by a similar study which showed that different components of a plant extract can inhibit MCF-7 proliferation through different mechanisms of action and cytotoxicity

(Uy *et al*., 2015). The results obtained could be due to the different compounds present in the whole extract of *V. doniana*which inhibited different targets of the MCF-7 proliferative pathway, causing the whole extract to show more potency than individual fractions tested. The IC_{50} value of 26. 11 ± 3.4 µg/ml obtained for *V. doniana* extract further shows the cytotoxic potency of the extract, because the American National Cancer Institute (NCI) guidelines states that for crude extracts, IC_{50} values less than 30 µg/ml after exposure for 72 h are cytotoxic. Saponin and tannins are phytocompounds with reported cytotoxic effects on cancer cells, which were found to be present in *V. doniana* and *F. apodanthera* extracts (James *et al*., 2014; Owolabi *et al*., 2018). The higher cytotoxic effects of *V. doniana* extract on MCF-7 cells could be due to the varying nature and abundance of saponin and tannin contained in the plants (James *et al*., 2014; Owolabi *et al*., 2018).

Treatment of MCF-7 cells with *V. doniana* extract caused some alterations to cell morphology which included decrease in cell number, cell shrinkage and nuclear condensation in cells treated with 50 µg/ml of *V. doniana*(Plate III). However, MCF-7 cells treated with *F. apodanthera* showed cells with relatively similar morphology to untreated MCF-7 cells (Plate IV). The decrease in cell number, cell shrinkage and nuclear condensation in *V. doniana* treated cells observed on Hematoxylin and Eosin staining suggests that the extract had cytotoxic effects on the cells, as cells subjected to altered membrane and nuclear integrity are susceptible to death by apoptosis or necrosis (Ghobrial *et al*., 2005). Saponins, which were found in both extracts, can bind to membrane cholesterol, resulting in altered membrane integrity that could be responsible for the altered membrane integrity of MCF-7 cells treated with the extracts (Podolak *et al*., 2010)

MCF-7 cells treated with *V. doniana* showed similar adhesion time when compared to untreated control. However, there was a dose dependent reduction in cell growth, characterized by a reduced cell index in *V. doniana* treated cells compared to untreated control cells.Although there was a reduction in MCF-7 cell index in F. *apodanthera* treated cells, the reduction was not comparable to *V. doniana* (Appendix XXIX).Since cell index is a measure of the growth and proliferation pattern of cells, the reduced MCF-7 cell index observed from xCELLigence results for cells treated with *V. doniana*, further suggests that the plant extract had cytotoxic effects (Kho *et al*., 2015). Tannins, which were present in both extracts have been found to alter protein function, resulting in death of cancer cells exposed to different levels of tannins (Levitsky and Dembitsky, 2014). The varying levels of tannins in the extracts could be responsible for the reduced metabolic and proliferative activity observed in the treated MCF-7 cells (James *et al*., 2014; Owolabi *et al*., 2018).

Since the above results showed that *V. doniana* whole extract showed more potency against MCF-7 cell, the study focused on *V. doniana* whole extract. Cell cycle analysis indicated that *V. doniana* extract inhibition of MCF-7 cell growth involves induction of G_0/G_1 arrest with a concomitant reduction in the proportion of cells in S and G2/M phases. Cell cycle arrest at G_0/G_1 phase is associated with apoptosis (Pucci *et al*., 2000; Aliyu *et al*., 2013). Treatment of MCF-7 cells with *V. doniana* also increased the duration of cells at G_0/G_1 phase, showing that this extract delayed the entry of MCF-7 cells into mitosis, thereby inhibiting MCF-7 growth and proliferation. After treatment with anti-cancer agents, cells can exit the cell cycle without undergoing cytokinesis. Accumulation of cells at G_0/G_1 activates G_0/G_1 cell cycle checkpoint proteins, resulting in death of a cell (Aliyu *et al*., 2013).

For the *in vivo* study, estrogen receptor-α (ER-α) levels in blood of rats administered DMBA and monitored for 150 days was significantly ($p<0.05$) higher than normal control rats which is similar to other studies which have shown that the model of DMBA-induced mammary carcinogenesis is ER-positive (Bishayee *et al*., 2013). Although there was no significant ($p>0.05$) reduction in ER-α levels in DMBA-induced rats after treatment with 50 and 100 mg/kg of *V. doniana*, there was a significant ($p<0.05$) decrease in ER-α levels after treatment with 200 mg/kg of *V. doniana* when compared to ER-α levels in the group before the treatment commenced (Table 4.6). The lowest ER-α level was observed in the tamoxifen treated group, which could be due to the anti-estrogenic effects of tamoxifen (Bishayee *et al*., 2013). Studies have shown that phytochemicals can protect against oxidative stress mediated activation of the redox sensitive estrogen receptor (Kumar *et al*., 2018).Since oxidative stress causes elevated ER-α levels, the reduction in ER-α levels in the 200 mg/kg *V. doniana* treated group could be due to a reduction in some markers of oxidative stress also observed in the group (Table 4.7).High ER-α levels have been found to correlate with increased breast cancer progression in hormone receptor positive breast cancer patients (Yip and Rhodes, 2014). Since estrogen receptor activates downstream proteins like AP-1 that regulate cell proliferation, treatments which lower ER-α levels could have therapeutic effects in breast cancer (Mahalingaiah *et al*., 2015).

The significantly ($p>0.05$) high malondialdehyde levels (Table 4.7) observed in DMBA-induced untreated rats agrees with previous studies which showed elevated MDA levels in DMBA-induced rats due to oxidative stress(Kalaiselvi *et al*., 2013). Treatments with antioxidants have been found to lower elevated MDA levels observed in breast cancer patients (Zarrini *et al*., 2016). The reduction in MDA observed in the treated rats could be

due to the antioxidant effects of *V. doniana* as seen earlier from the MTT results. The rats treated with 100 and 200 mg/kg *V. doniana* showed significantly ($p<0.05$) higher GSH levels in mammary tissue homogenates when compared to untreated control and even normal rats (Table 4.7), suggesting antioxidant enhancing effects (Adetoro *et al*., 2013). This is important as relatively higher GSH levels have been associated with less aggressive breast cancer pathogenesis (Jardim*et al*., 2013). The non-significant ($p<0.05$) difference in SOD activity between the groups is in line with some studies which found no significant ($p>0.05$) association between superoxide dismutase expression and breast cancer (Liu*et al*., 2012). Catalase activity was lowest in the DMBA-induced untreated group, which agrees with reports of down regulated catalase activity in breast cancer (Glorieux *et al*., 2014), and the enhanced catalase activity in the 50 and 200 mg/kg *V. doniana* treated groups could be indicative of positive treatment effects (Glorieux and Calderon, 2017). One of the side effects associated with the treatment of breast cancer patients with tamoxifen is oxidative stress (Silva *et al*., 2017), which possibly explained the relatively higher MDA and lower GSH levels in Tamoxifen treated group when compared to *V. doniana* treated groups and normal control. These results suggest that treatment with 100 and 200 mg/kg of *V. doniana* possibly enhanced the antioxidant capacity of the rats, which is beneficial, as oxidative stress is implicated in the initiation, promotion and progression stages of carcinogenesis (Reuter, 2011). The results also agree with similar studies which showed that phytochemicals can ameliorate oxidative stress in mammary carcinogenesis (Tabaczar *et al*., 2015).

The observation that levels of the pro-inflammatory cytokines Interleukin-1β and Tumor necrosis factor-α were significantly ($p<0.05$) high in DMBA-induced untreated rats when

compared to normal control agrees with experimental studies which have shown elevated levels of cytokines in mammary cancer (Kamel *et al*., 2012; Tulotta and Ottewell, 2018). Elevated circulating levels of IL-1β have been reported to correlate with increased breast cancer progression and poor prognosis (Tulotta and Ottewell, 2018). Tumor necrosis factor (TNF-α) is a pleiotropic cytokine with contrasting effects in different cancers, functioning sometimes as a necrotic factor and other times as a tumor promoting factor. In breast carcinomas, TNF-α is highly expressed, stimulating mammary cell proliferation through an NF-κB-dependent increase in cyclin D1 (Kamel *et al*., 2012). Inhibition of TNF-α has been shown to be protective against breast tumorigenesis (Baumgarten and Frasor, 2012). The results obtained suggests that *V. doniana* administration at all the treatment doses reduced IL-1β and TNF-α levels in DMBA induced rats and could protect against mammary cancer associated inflammation. The reduction in the levels of TNF-α and IL-1β, which are pro-inflammatory cytokines could be due to the anti-inflammatory potency of *V. doniana* which has been previously reported (Iwueke *et al*., 2006). Oxidative stress can also result in increased levels of TNF-α (Doss *et al.,* 2014) and the reduction in oxidative stress markers as shown in Table 4.7, could protect against oxidative stress mediated activation of TNF-α. Some phytocompounds which exert anti-inflammatory effects through reduction of cytokine activity have been reported to have anti-cancer potential (Cho *et al*., 2018).

The histology of the mammary tissue of DMBA-induced untreated rats showed cells with malignant epithelial hyperplasia which was less visible in the rats administered 50 and 100 mg/kg of *V. doniana*. However, the situation appeared to be attenuated in rats administered 200 mg/kg, quite similar to rats administered tamoxifen. The mammary tissue is composed of a glandular ductal network (Wen and Aguirre-ghiso, 2014). These ducts are lined by a

protective layer of epithelial cells, and also contain myoepithelial cells that aid in contraction. The terminal duct-lobular unit, which makes up the terminal collecting duct within the breast is thought to be where breast cancer initiates. The mammary adipose tissue is a bioactive endocrine organ that secretes hormones, growth factors and cytokines, that contribute significantly to the composition of the extracellular matrix and the tumor microenvironment, promoting carcinogenesis (Ercan *et al*., 2011). The results thus suggest that treatment with *V. doniana* at 200 mg/kg ameliorated malignant epithelial hyperplasia and fibrous connective tissue in the mammary glands and stroma respectively.

Since cyclooxygenase-2 (COX-2) over expression has been associated with hormone receptor positive breast cancer (Subbaramaiah *et al*., 2002; Jana *et al*., 2014), it was not surprising that COX-2 expression was higher in the DMBA-induced untreated group compared to the other groups. Cyclooxygenase-2 (COX-2) is an inducible protein implicated in inflammation and mammary carcinogenesis (Jana *et al*., 2014). COX-2 expression has also been shown to contribute to inflammation (Harris *et al*., 2014) and alteration of cellular matrix structure and function (Krishnamachary *et al*., 2017) in breast cancer patients. Inhibition of COX-2 could serve as a viable therapeutic target in breast cancer (Davies, 2002; Jana *et al*., 2014). Treatment with *V. doniana* showed a reduction in COX-2 expression which agrees with the lowered levels of pro-inflammatory cytokines (Table 4.8), that could protect against inflammation implicated in mammary carcinogenesis.

Caspase-3 is an important protein in the regulation of cell death via the apoptotic pathway (Julien and Wells, 2017) and this could account for the mild to moderate expression of Caspase-3 observed in the *V. doniana* treated groups when compared to DMBA-induced

untreated group which showed mild expression of Caspase-3. Dysregulated expression of Caspase-3 has been implicated in carcinogenesis, through enhancing the survival of aberrant cells that are supposed to be tagged for death by apoptosis (Yang *et al*., 2018). In breast cancer, Caspase-3 over expression is associated with poor overall survival among patients (Yang *et al*., 2018). Compounds that enhance Caspase-3 activity have been reported to possess chemotherapeutic potential (Souli *et al*., 2008; Bishayee *et al*., 2013; Looi *et al*., 2013) suggesting that Caspase-3 could serve as a therapeutic target. The observation that Caspase-3 expression was enhanced in the 200 mg/kg *V. doniana* treated group further suggests that *V. doniana* could exert anti-cancer effects.

The guardian of the genome, p53, protects against proliferation of damaged cells (Gasco *et al*., 2002). Examination of p53 expression in mammary tissue showed mild to moderate expression in the *V. doniana* treated groups while the DMBA-induced untreated group showed mild expression of p53 which could be due to the fact that p53 is observed to have altered functions due to down regulation or inactivation in mammary carcinogenesis resulting in uncontrolled cell proliferation (Dworkin *et al*., 2009). Altered p53 expression and function is considered a biomarker of breast cancer risk (Terry *et al*., 2016) and has potential for use as a therapeutic target (Ali *et al*., 2016). The observation of a mild to moderate p53 expression in the *V. doniana* treated groups could contribute to the anti-carcinogenic effects of *V. doniana*.

Results from the sub-acute toxicity study showed no significant difference ($p>0.05$) in serum Alanine aminotransferase (ALT) and Gamma glutamyltransferase (GGT) activity in *V. doniana* administered groups compared to control. This suggests that administration of *V. doniana* extract did not have deleterious effects on the liver. Serum ALT and GGT are

used as markers of liver damage, because damage to hepatocytes during exposure to certain concentrations of xenobiotic can cause increased serum levels of ALT and GGT(McGill, 2016). There was also no significant ($p>0.05$) difference in serum urea and creatinine levels between the treatment groups, suggesting that the treatment did not cause damage to the kidney of the rats. Serum urea and creatinine are used to estimate glomerular filtration efficiency(Ferguson and Waikar, 2012). Any condition that causes damage to the nephrons affects kidney reabsorption, resulting in decreased clearance of urea and creatinine(Wasung *et al*., 2015). That the histological examination of liver, kidney and mammary tissue of the rats after administration of *V. doniana* for 28 days showed normal architecture in all the administered doses, similar to control, indicates that the extract caused no damage to liver, kidney and mammary tissue, suggesting safety of *V. doniana* extract at the doses of this study.

Chromatographic analysis of the *V. doniana* extract using Liquid Chromatography – Mass Spectrometry (LCMS) showed some phenolic compounds. The compound, 4',5-dihydroxy-7-methoxy-6-methylflavone is a flavone belonging to the class of flavonoids known for potent antioxidant capacity due to their ability to chelate metals, and quench reactive oxygen species (Catarino *et al*., 2015). Dihydroxy flavones have been reported to exert antioxidant, anti-inflammatory and anti-cancer effects (Mani and Natesan, 2018) via mechanisms involving the mitogen activated protein kinase (MAPK) and Nrf-2 signaling pathways (Wang *et al*., 2018). Studies on natural plant flavonoids and their synthesized derivatives have shown *in vitro* antioxidant activity, tumor necrosis factor – α and interleukin inhibitory activity (Nile*et al*., 2018). Tartaric acid is synthesized from ascorbic acid (vitamin C) and is reported to possess antioxidant activity (DeBolt *et al*., 2006;

Melino *et al*., 2009). Vanillylamine is an alkaloid found in plants and is an intermediate in the biosynthesis of caspsaicin (Weber*et al*., 2014) which has shown anti-cancer effects in different studies (Chapa-Oliver and Mejía-Teniente, 2016; Clark and Lee, 2016). These phenolic compounds identified by LCMS could be responsible for the antioxidant and anti-inflammatory effects observed in the DMBA-induced rats treated with *V. doniana*.

From the results discussed, we propose a possible mechanism through which *V. doniana* ethanol leaves extract exerts *in vitro* and *in vivo* anti-cancer effects. Treatment of MCF-7 cells with *V. doniana*extract caused reduced proliferation and death to the cancer cells via reduced cell proliferation kinetics, alterations to cell membrane integrity, and cell cycle arrest at the G_0/G_1 phase. *In vivo,* in the rats induced with mammary carcinogenesis using DMBA, it is converted to the potent dihydrodiol epoxide by CYP 1A1 leading to oxidative damage to macromolecules which results in oxidative stress and inflammation characterized by increase in ER-α, IL-1β, TNF-α and COX-2 expression which may lead to dysregulated cellular proliferation implicated in mammary carcinogenesis. However, administration of *V. doniana* leaves extract attenuated these processes thereby protecting against DMBA-induced mammary carcinogenesis (Figure 5.1).

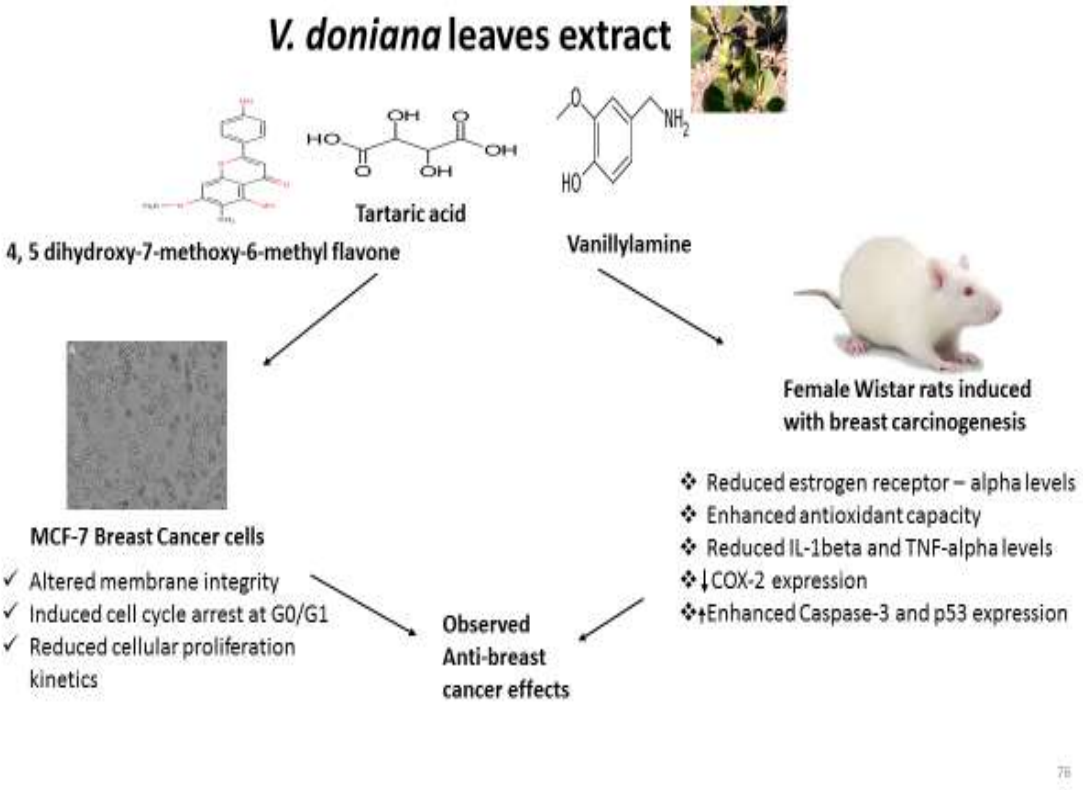


Figure 5.1: Proposed Mechanism by which *V. doniana* exerts Anti-cancer Effects

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The results from this study showed that:

i. *V. doniana* and *F. apodanthera* extracts and fractions have *in vitro* antioxidant capacity

ii. *V. doniana* and *F. apodanthera* extracts and fractions showed cytotoxicity to MCF-7 cells

iii. MCF-7 cells treated with *V. doniana* showed altered membrane integrity and nuclear condensation, typical of apoptotic changes

iv. MCF-7 cells treated with *V. doniana* showed reduced cell proliferation implying reduced cellular metabolic activities

v. Treatment of MCF-7 cells with *V. doniana* caused cell cycle arrest at G_0/G_1 phase with a concomitant reduction in the proportion of cells in S and G_2/M phases of the cell cycle

vi. Female Wistar rats induced with mammary carcinogenesis and treated with *V. doniana* showed reduced estrogen receptor –α levels, enhanced attenuation to oxidative stress, reduced pro-inflammatory cytokine levels, mild expression of cyclooxygenase-2, moderate expression of p53 and caspase -3

vii. Sub-acute administration of *V. doniana* did not cause any notable alterations to liver, kidney and mammary tissues of normal rats, suggesting safety at the doses used

viii. LCMS analysis of *V. doniana* extract showed the presence of compounds with reported anti-cancer and antioxidant properties

6.2 Conclusion

V. doniana ethanol leaves extract was cytotoxic, induced alteration in cellular morphology and reduced the growth kinetics of MCF-7 breast cancer cells. Therefore, the whole extract was further tested in an *in vivo* model of breast cancer. In female Wistar rats induced with mammary carcinogenesis and treated with *V. doniana*,reduced estrogen receptor-α levels, attenuation to malignant epithelial hyperplasia and oxidative stress as well as reduced levels of pro-inflammatory cytokines wereobserved. Furthermore, sub-acute toxicity results showed that *V. doniana* is relatively safe for use at the doses of the study. Chromatographic analysis showed phenolic compounds which could be responsible for the antioxidant and anti-cancer effects observed. Thus, *V. doniana* leaves extract has potential for the development of a therapeutic agent against breast cancer.

6.3 Recommendation

Vitex doniana leaves may ameliorate oxidative stress and inflammation which contribute to mammary carcinogenesis in breast cancer patients.

Further studies can examine if co-administration of *V. doniana* with chemotherapeutic drugs can ameliorate the side effects of oxidative stress mediated toxicity currently observed in breast cancer patients on chemotherapy.

Contribution to knowledge

i. Treatment with *V. doniana* caused cytotoxicity to MCF-7 cells with an IC_{50} of 26. 11 ± 3.4 µg/ml after treatment for 48 hours, which is within internationally accepted range for cytotoxic extracts.

ii. Treatment of DMBA-induced rats with *V. doniana* leaves extract at 200 mg/kg caused a 30.3%, 33.37% and 36.12% reduction in ER-α, IL-1β and TNF-α levels respectively when compared to DMBA-induced untreated rats.

iii. Treatment with *V. doniana* leaves extract showed moderate expression of Caspase-3 and p53 in contrast to mild expression in DMBA –induced untreated rats.

iv. LCMS analysis of *V. doniana* extract revealed compounds (4',5-Dihydroxy-7-methoxy-6-methylflavone, Tartaric acid, Vanillylamine) with reported antioxidant, anti-inflammatory and anti-cancer effects.

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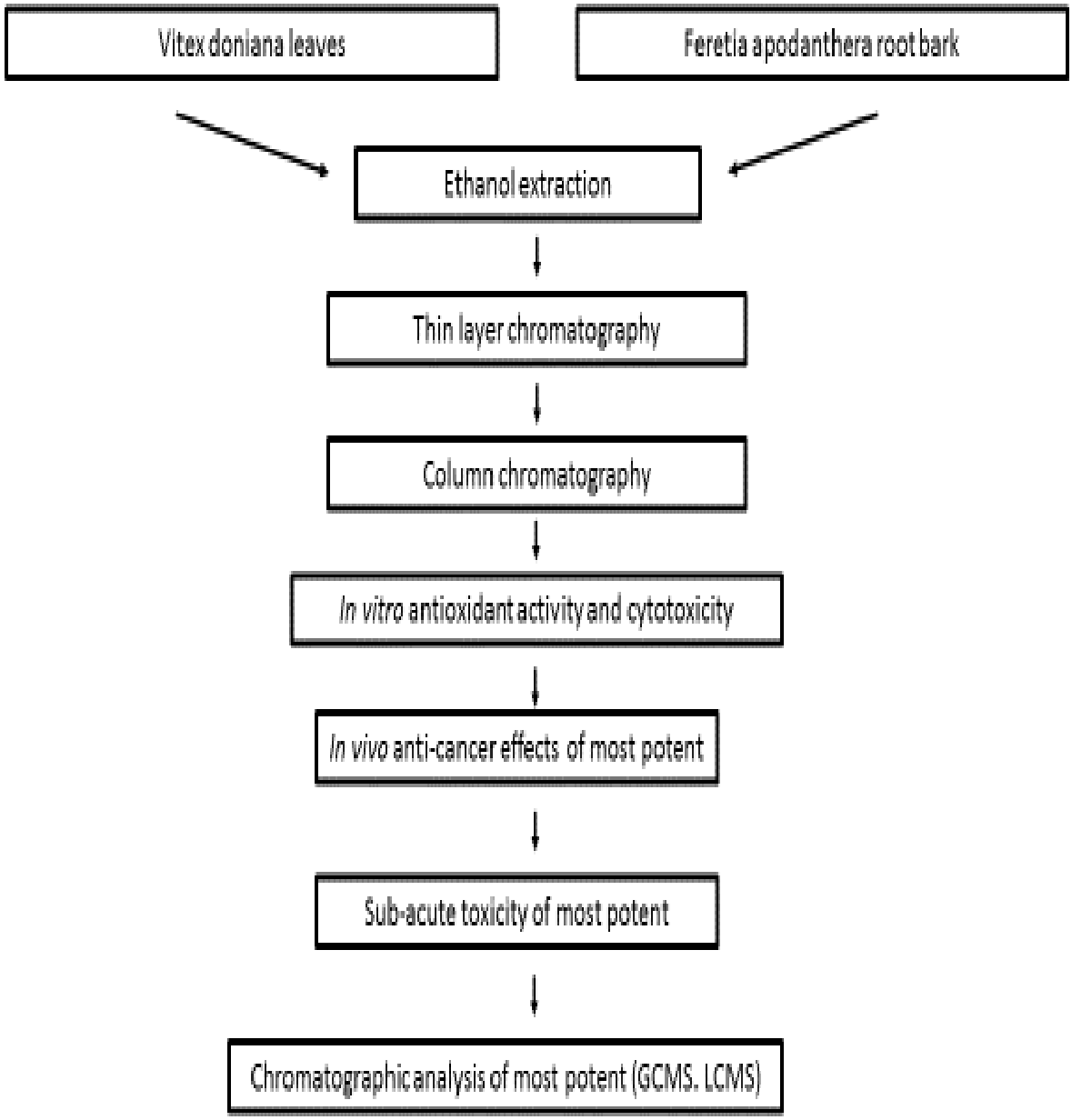
APPENDIX I



Experimental Design for the study

APPENDIX II

TLC chromatogram of *V. doniana* and *F. apodanthera* Extracts

APPENDIX III

Set up for separation of *V. doniana* and *F. apodanthera* extracts using Column chromatography

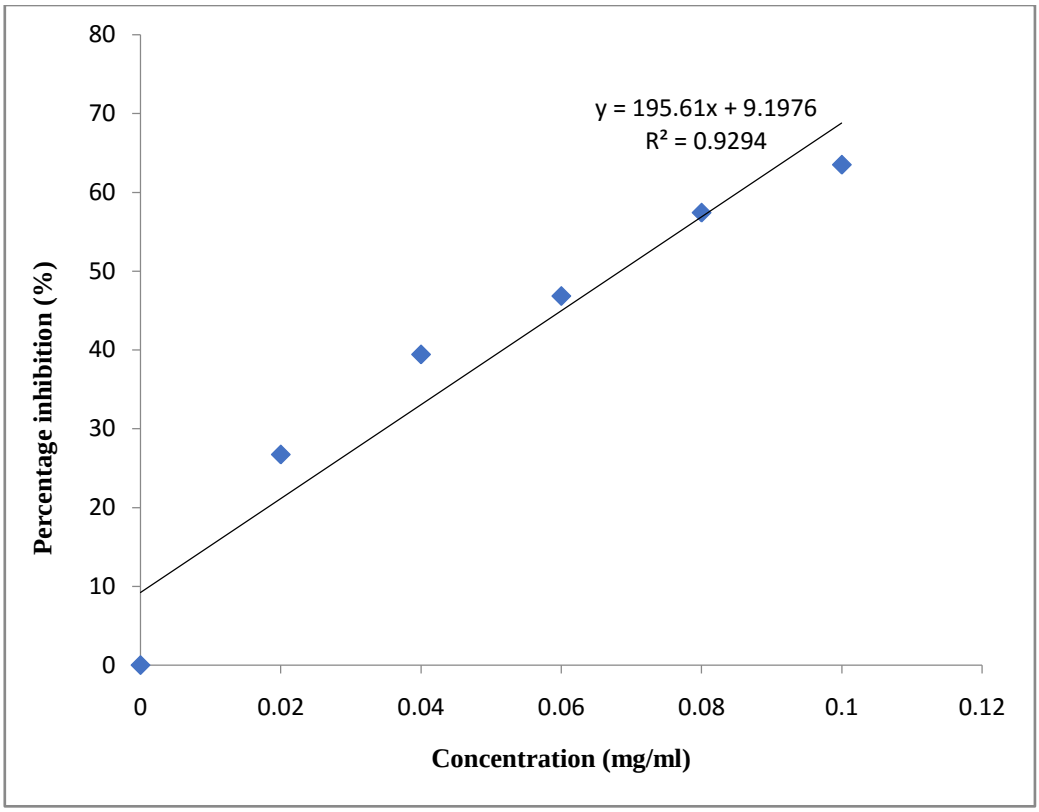
APPENDIX IV

Fractions of *F. apodanthera* extract

APPENDIX V

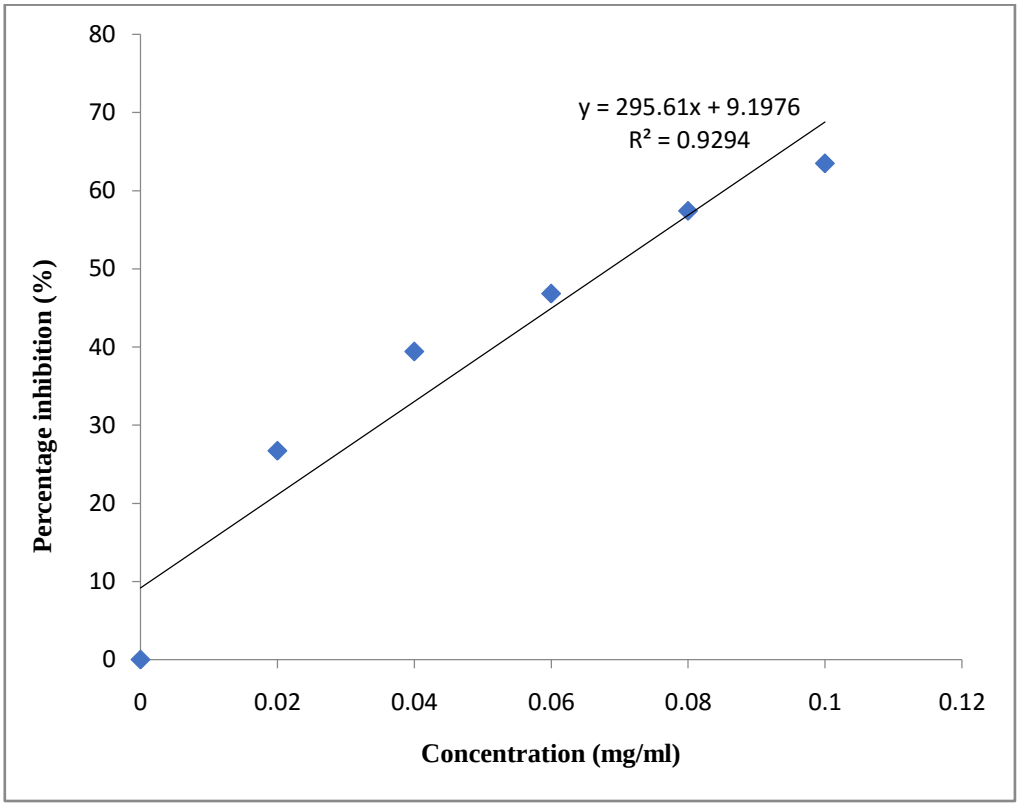
Setup for column chromatography separation

APPENDIX VI



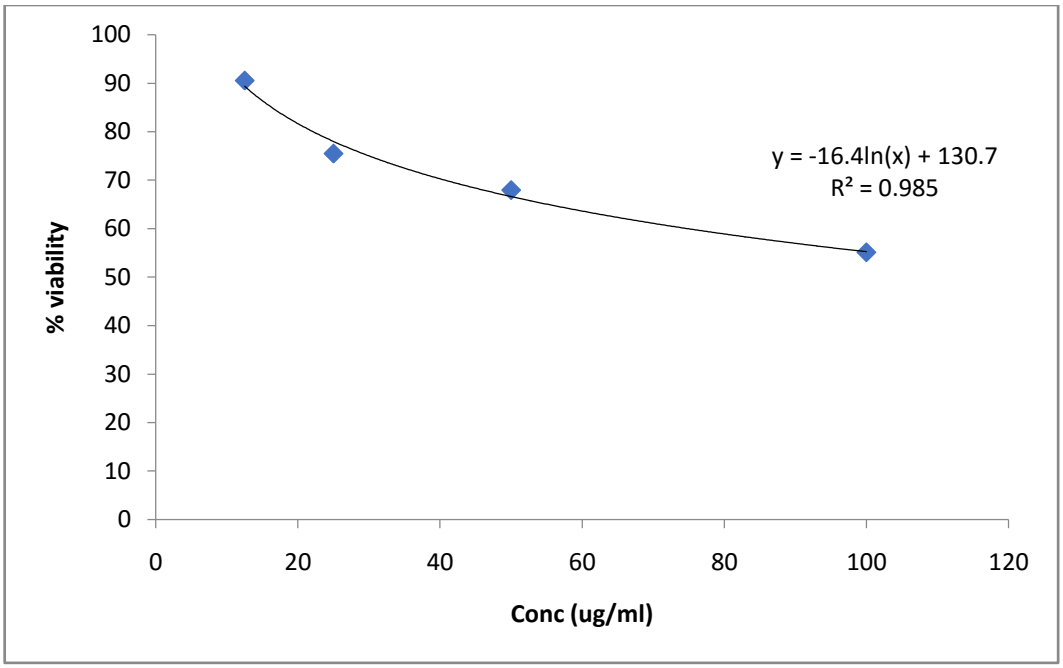
Plot of Percentage Inhibition of DPPH by *V. doniana*.

APPENDIX VII



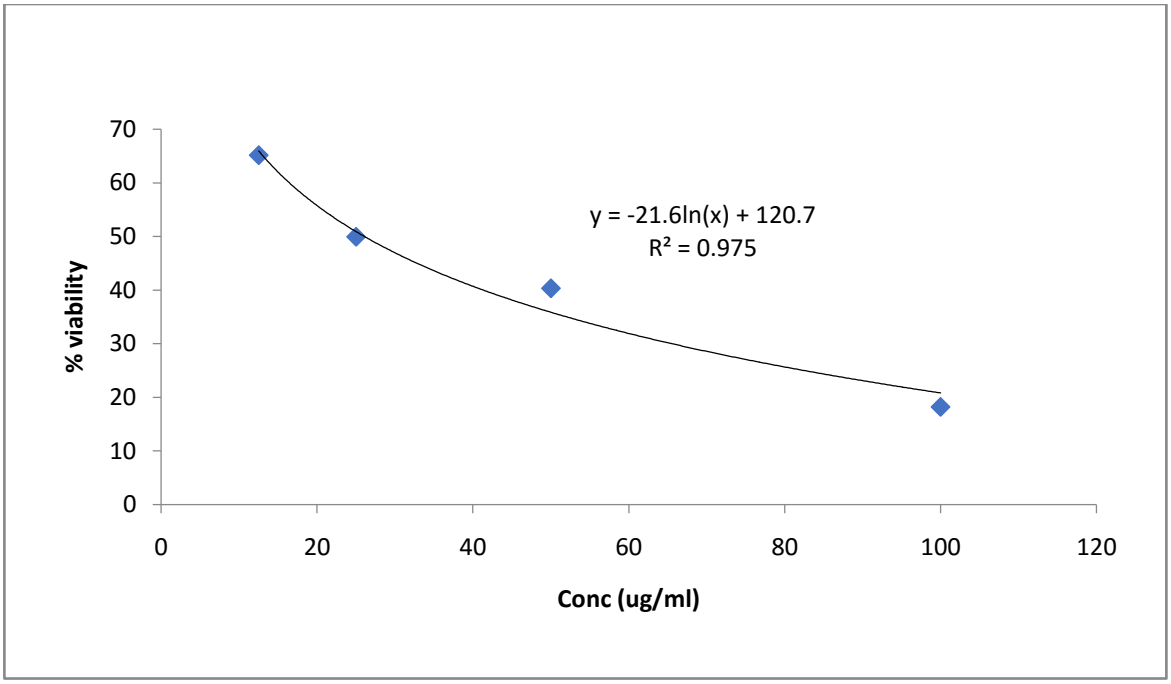
Plot of Percentage Inhibition of DPPH by *F. apodanthera*

APPENDIX VIII



Percentage Viability of *V. doniana* from MTT results

APPENDIX IX



Percentage viability of *F. apodanthera* from MTT results

APPENDIX X

IC_{50} values of *V. doniana* treated B16-F10 and aEnd.2 cells

Cell line	IC_{50} for 48 h treatment (µg/ml)
B16-F10	19.65 ± 2.1
sEnd.2	48. 93± 1.6

Values are expressed as mean ± SD (n = 3)

APPENDIX XI

Cell Index Values of MCF-7 Treated Cells

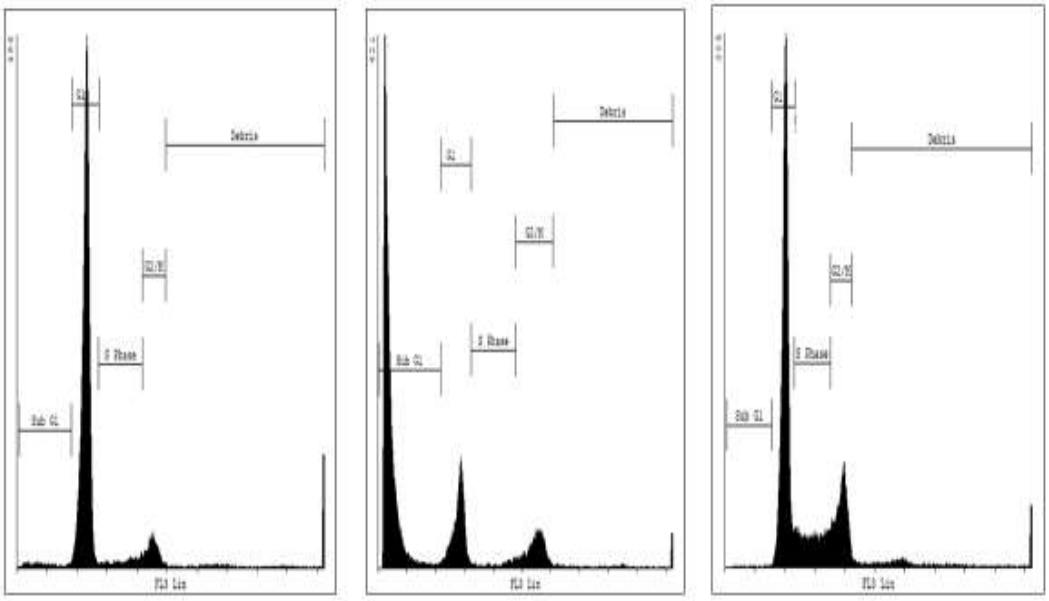
Treatment	Cell Index values
V. doniana (1µg/ml)	5.43 ± 0.26^{d}
V. doniana (10µg/ml)	3.95 ± 0.19^{c}
V. doniana (50µg/ml)	2.87 ± 0.12^{b}
F. apodanthera (1µg/ml)	6.88 ± 0.09^{e}
F. apodanthera (10µg/ml)	7.01 ± 0.12^{e}
F. apodanthera (50µg/ml)	4.29 ± 0.11^{c}
Vinblastine	1.49 ± 0.21^{a}
DMSO (Control)	7.62 ± 0.35^{f}

Keys:

Values are means of three determinations

Means with different superscripts differ significantly ($p < 0.05$)

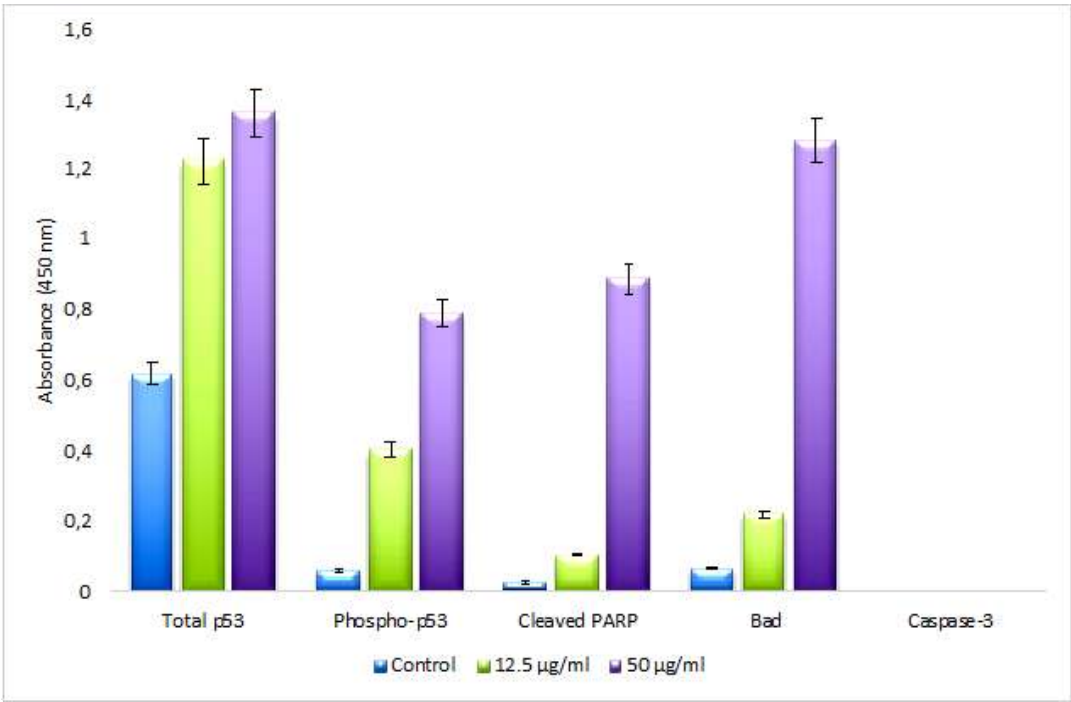
APPENDIX XII



2/24/2019

Spectrum of MCF-7 Cells from Fluorescence activated cell sorting (FACS)

APPENDIX XIII



Levels of Apoptotic Proteins (p53, Poly(ADP-ribose) polymerase and Bad) in MCF-7 Treated Cells

Results are mean values ± SD.

* indicates$P<0.05$compared to the control

APPENDIX XIV

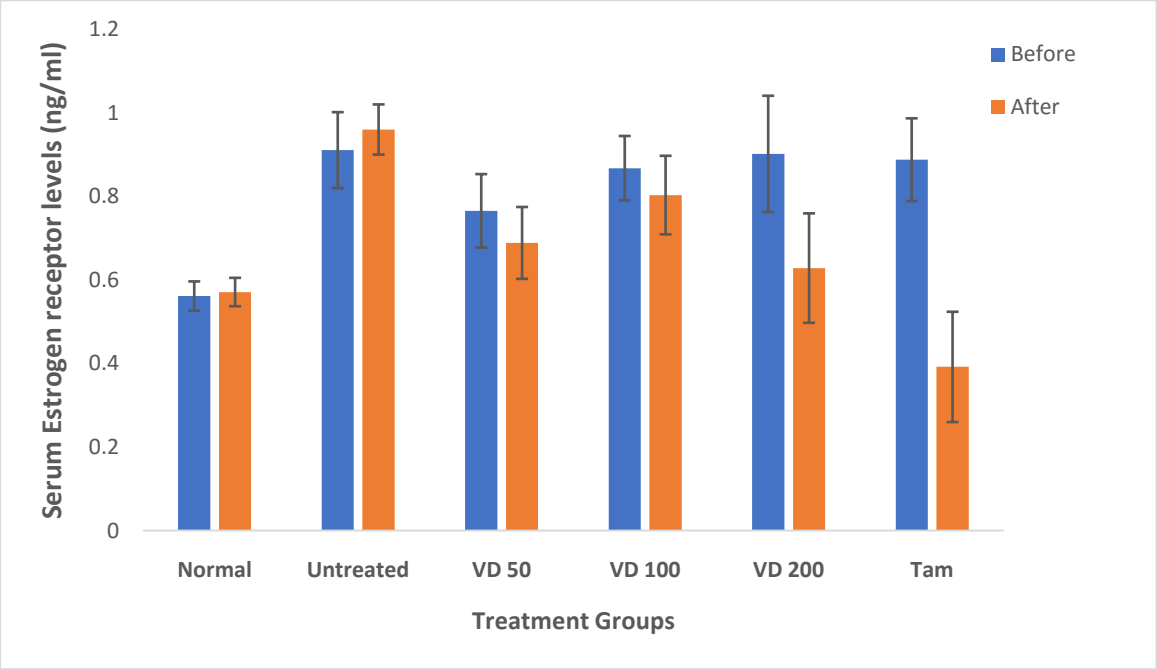
	Estrogen receptor– α levels (ng/ml)
DMBA-induced	0.87 ± 0.11^{b}
Normal (control)	0.56 ± 0.03^{a}

Serum Estrogen Receptor Levels in ExperimentalRats

Results are mean values ± SD.

Values with different superscripts indicates P<0.05

APPENDIX XV



Serum estrogen receptor levels in pretreatment and treated groups

APPENDIX XVI

Preparation of Reagents for Malondialdehyde (TBARS) Assay

Reagents

1. 30% Trichloroacetic acid (TCA)

9g of TCA (CCl_3COOH) was dissolved in distilled water and made up to 30ml with same.

2. 0.75% Thiobarbituric acid (TBA)

This was prepared by dissolving 0.23g of TBA in 0.1M HCl and made up to 30ml with same.

3. 0.15M Tris-KCl buffer (pH 7.4)

1.12g of KCl and 2.36g of Tris base were dissolved separately in distilled water and made up to 100ml with same. The pH was then adjusted to 7.4

APPENDIX XVII

Preparation of Reagents for Glutathione (Reduced) Assay

Reagents

1. GSH working standard

40mg GSH (Sigma Chemical Co., London, Mol. Weight 307.3g) was dissolved in 100ml of 0.1M phosphate buffer, pH 7.4, and then stored in the refrigerator.

2. 0.1M Phosphate buffer (pH 7.4)

0.496g of di-potassium hydrogen orthophosphate, K_2HPO_4 (Hopkins and Williams, Ltd) and 0.973g of potassium di-hydrogen orthophosphate, KH_2PO_4 (Hopkins and Williams Ltd) were dissolved in 9ml of distilled water. The pH was adjusted to 7.4 and then made up to a 100ml with distilled water.

3. Ellman's Reagent [5′, 5′-Dithiobis- (2-nitrobenzoate) DTNB]

This was prepared by dissolving 40mg (0.4g) of Ellman's reagent in 0.1M Phosphate buffer of pH 7.4 and made up to 100ml.

4. Precipitating Agent

4% Sulphursalicyclic acid ($C_7H_6S.2H_2O$ mol. wt. 254.22) was prepared by dissolving 4g of sulphursalicyclic acid in 100ml of distilled water. This is stable for approximately three weeks at 4^oC.

APPENDIX XVIII

Preparation of GSH standard curve

Stock (ml)	Phosphate buffer (ml)	Ellmans reagent (ml)	Absorbance (412nm)	GSH Concentration (g/ml)
0.02	0.48	4.50	0.040	8
0.05	0.45	4.50	0.101	20
0.10	0.40	4.50	0.194	40
0.20	0.30	4.50	0.380	80
0.30	0.20	4.50	0.572	120
0.40	0.10	4.50	0.794	60

APPENDIX XIX

Preparation of Reagents for Superoxide Dismutase (SOD) Activity Determination

Reagents

1. 0.05M Carbonate buffer (pH 10.2)

3.58g of $Na_2CO_3.10H_2O$ and 1.05g of $NaHCO_3$ were dissolved in 200ml of distilled water. The pH was adjusted to 10.2 and then made up to 250ml with distilled water.

2. 0.3mM Adrenaline

0.01g of adrenaline (epinephrine) was dissolved in 200ml-distilled water, prepared fresh when needed.

APPENDIX XX

Preparation of Reagents for Catalase Activity Determination

Reagents

1. 5% $K_2Cr_2O_7$ (Dichromate Solution)

5g of $K_2Cr_2O_7$ (Hopkins & Williams, England) was dissolved in 80ml of distilled water and made up to 100ml with same.

2. 0.2M H_2O_2 (Hydrogen peroxide)

11.50ml of 30% (w/w) H_2O_2 was diluted with distilled water in a volumetric flask and the solution made up to 500ml.

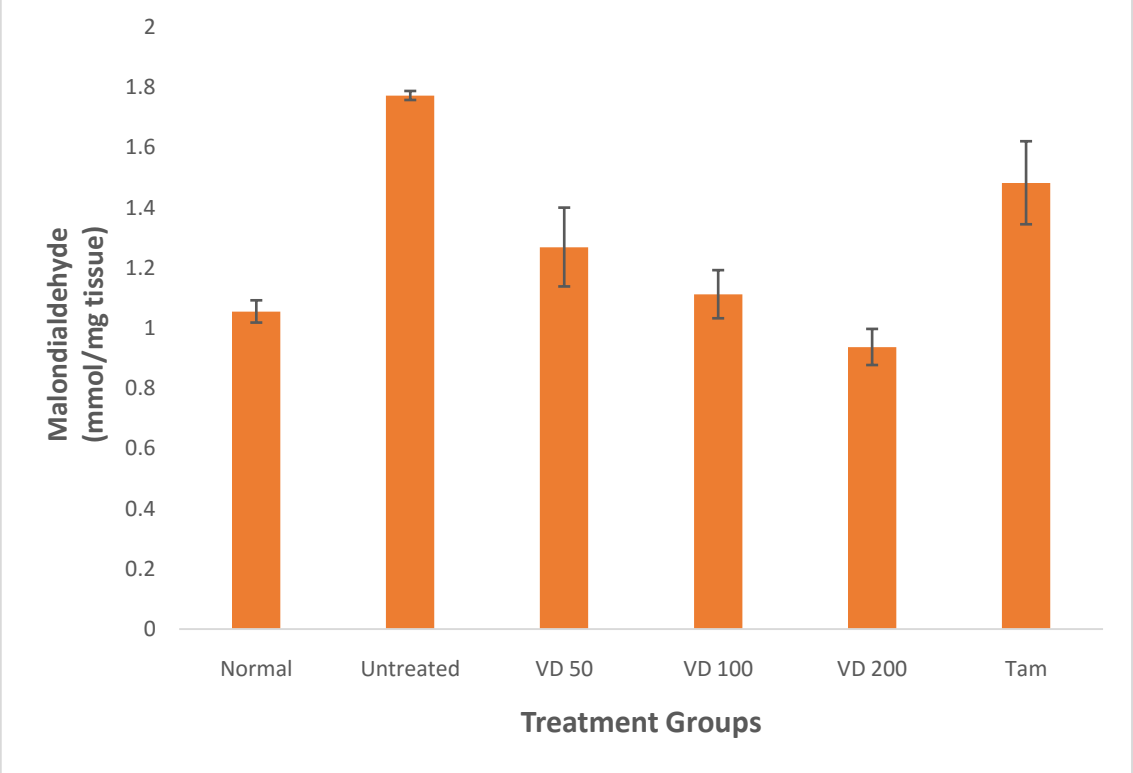
3. Dichromate/acetic acid

This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 by volume) and could be used indefinitely.

4. Phosphate buffer (0.01M, pH 7.0)

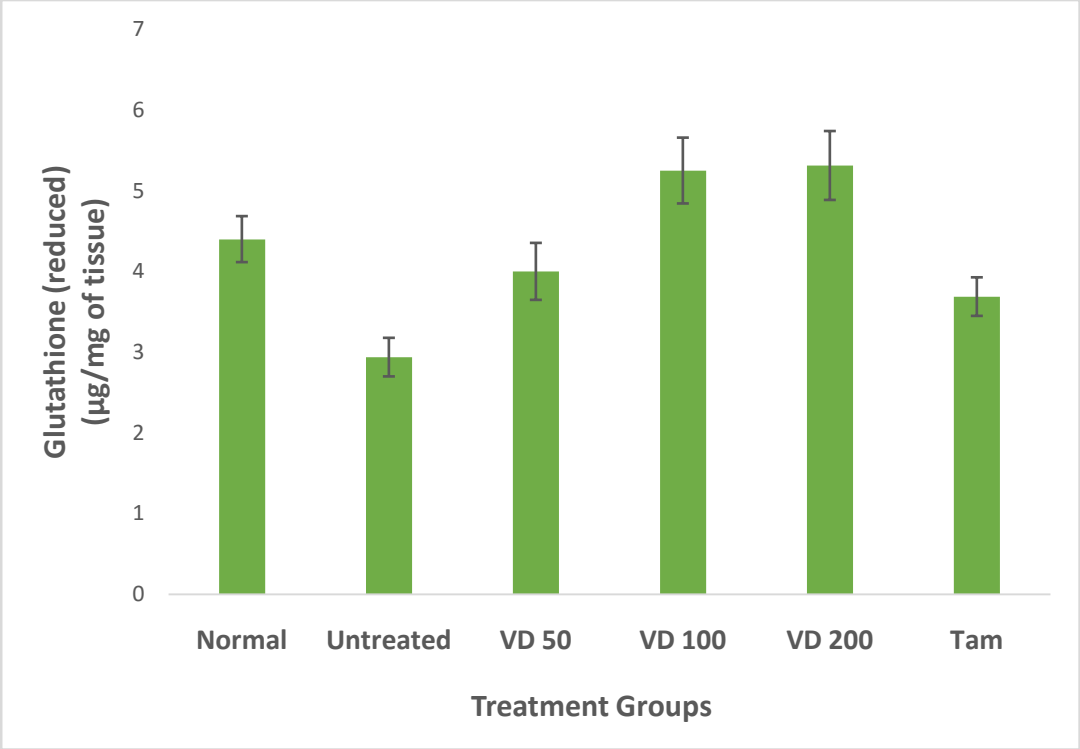
3.58g of Na_2HPO_4 $12H_2O$ and 1.19g NaH_2PO_4 $2H_2O$ dissolved in 900ml of distilled water. The pH adjusted to 7.0 and distilled water added to make up to 1 litre.

APPENDIX XXI



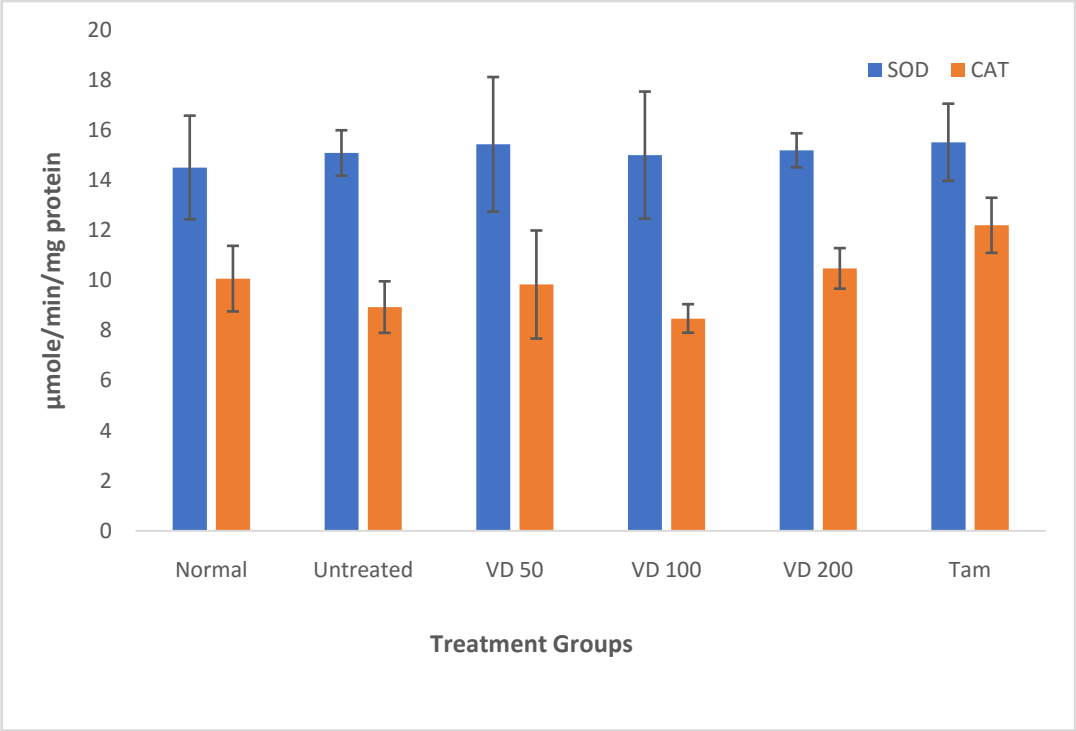
Effect of *V. doniana* administration on Malondialdehyde levels of DMBA-induced rats

APPENDIX XXII



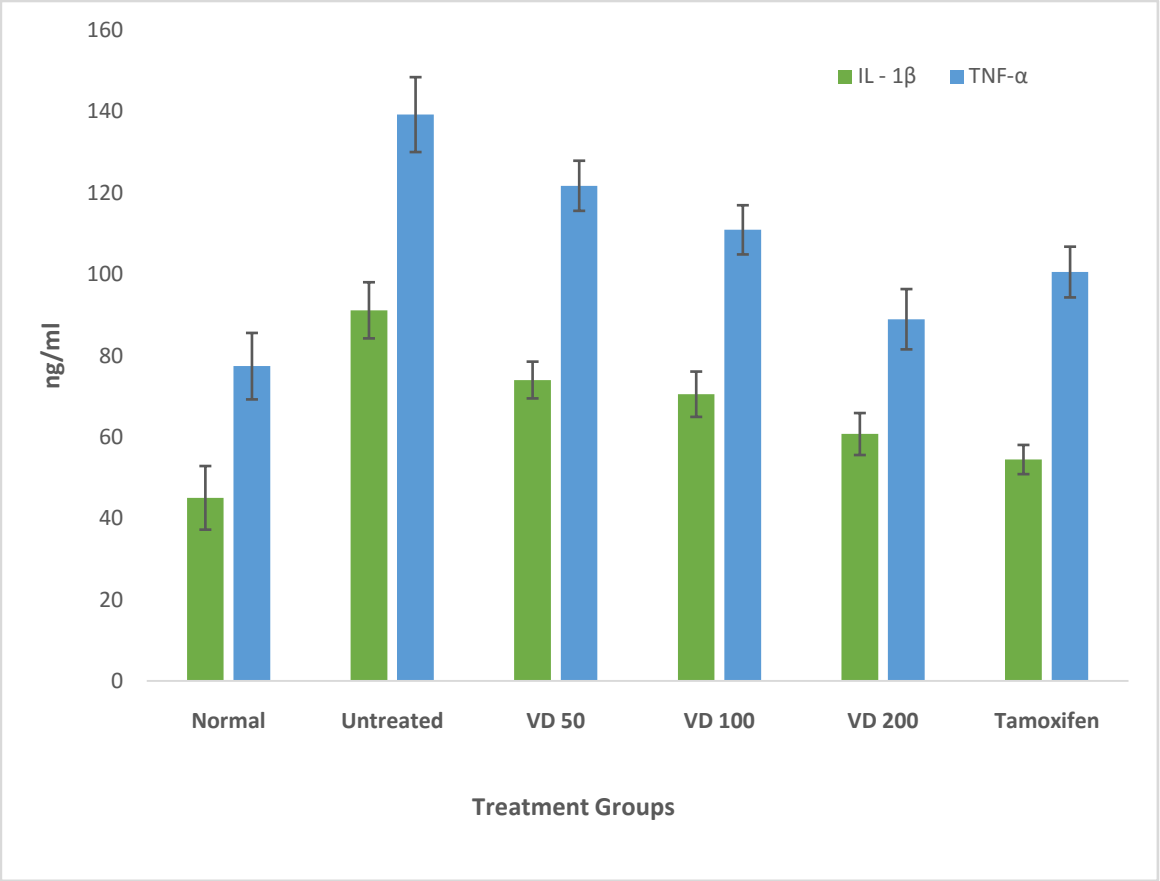
Effect of *V. doniana* administration on Glutathione (reduced) levels of DMBA-induced rats

APPENDIX XXIII



Effect of *V. doniana* administration on Catalase and Superoxide dismutase Activity of DMBA-induced rats

APPENDIX XXIV



Effect of *V. doniana* administration on Interleukin 1β and Tumor necrosis factor-α levels of DMBA-induced rats

APPENDIX XXV

Histological Lesions Score of DMBA-induced and control rats

Group of rats	Inflammatory cell infiltration	Neoplastic cells	Glandular hyperplasia
Normal	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
DMBA-induced, Untreated	2.00 ± 0.00	2.33 ± 0.58	3.00 ± 0.00
DMBA-induced + 50 mg/kg *V. doniana*	1.33 ± 0.58*	1.33 ± 0.58	1.33 ± 0.58*
DMBA-induced + 100 mg/kg *V. doniana*	1.00 ± 0.00*	1.00 ± 0.00*	1.33 ± 0.58*
DMBA-induced + 200 mg/kg *V. doniana*	1.00 ± 0.00*	1.00 ± 0.00*	1.00 ± 0.00*
Tamoxifen (20 mg/kg)	1.00 ± 0.00*	1.00 ± 0.00*	1.00 ± 0.00*

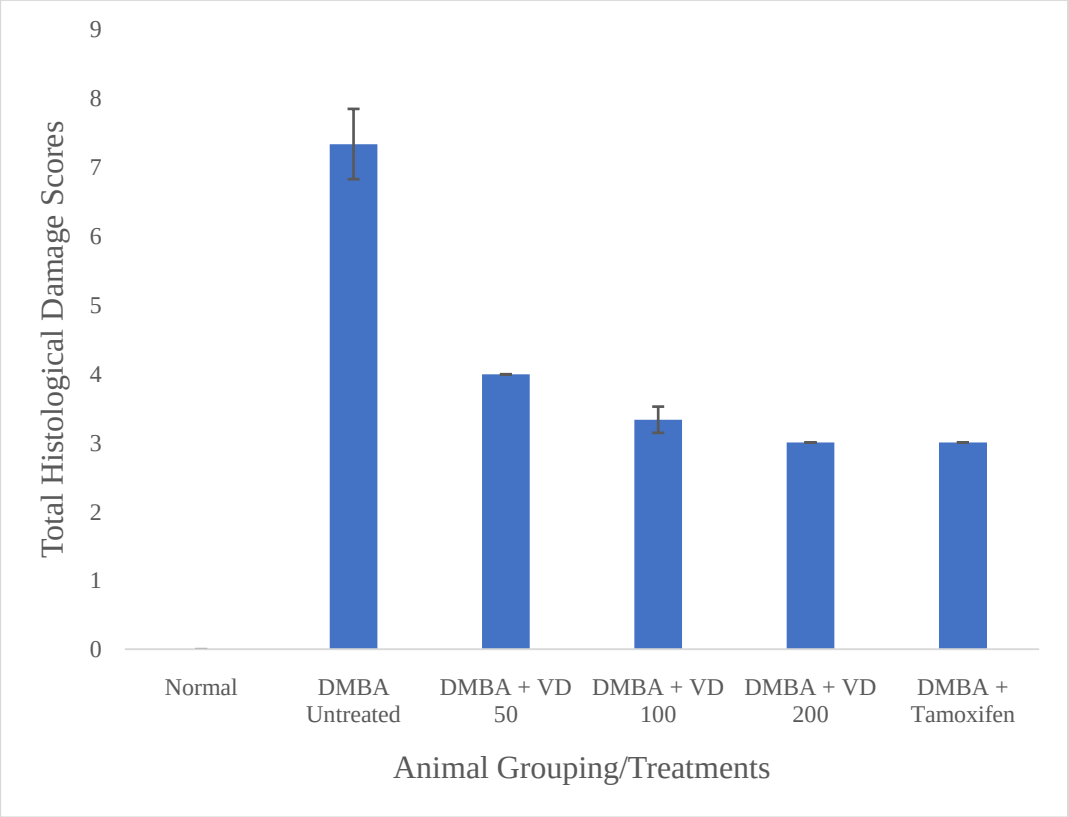
* : Values differ significantly from DMBA-induced, untreated control ($p<0.05$)

<1 - mild - < 5 per high power field

1 – 3 - moderate - 5 to ≤ 25 per high power field

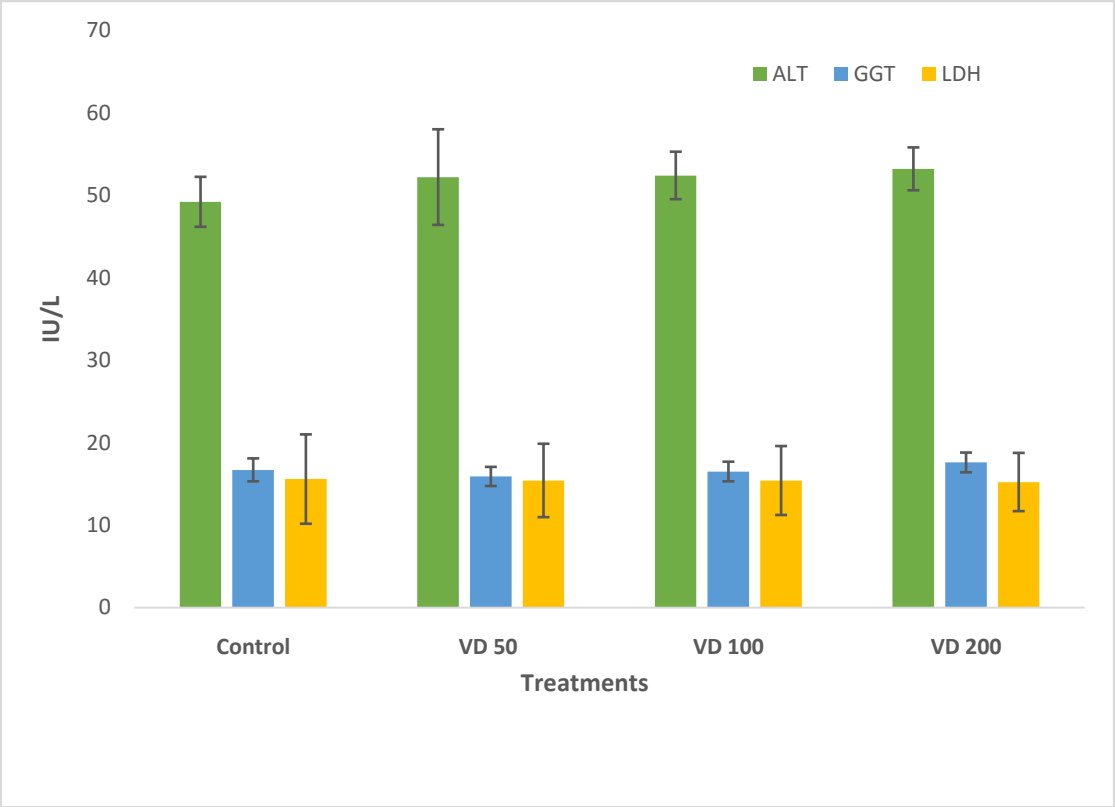
>3 - severe - > 25 per high power field

APPENDIX XXVI



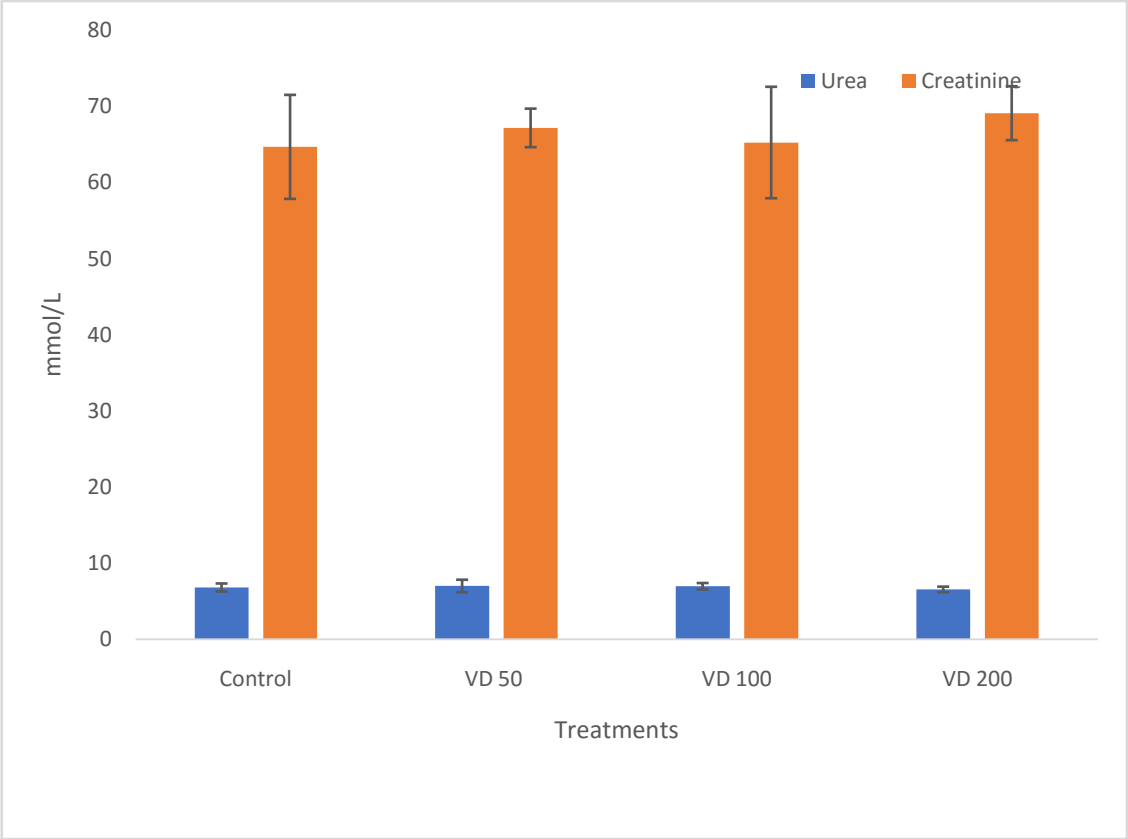
Total Histological Damage Scores of Normal and DMBA-induced and Treated Rats

APPENDIX XXVII



Effect of Sub-acute *V. doniana* administration on some Serum Markers of Tissue Damage

APPENDIX XXVIII



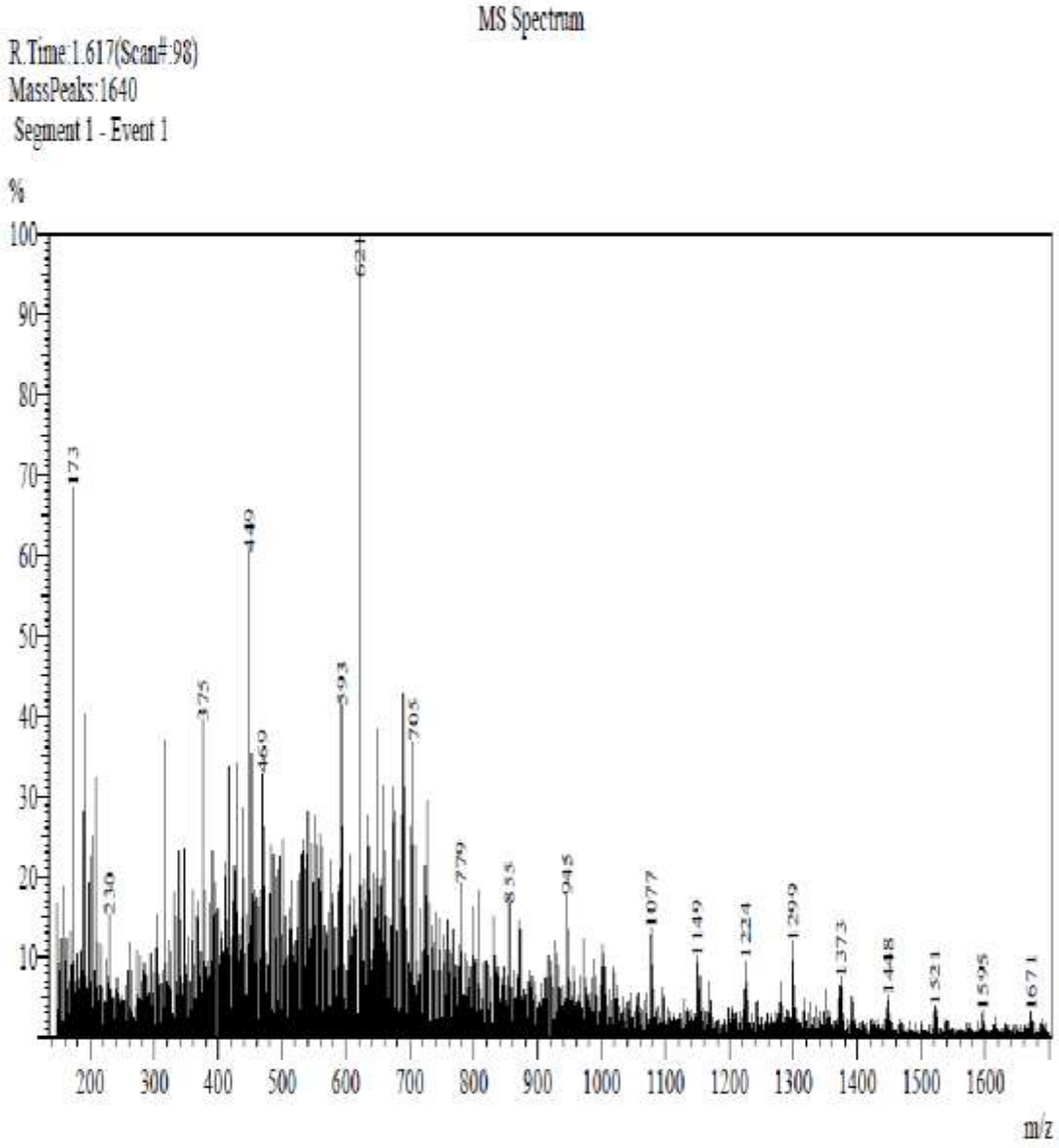
Effect of Sub-acute *V. doniana* administration on Serum Urea and Creatinine levels in Normal Rats

APPENDIX XXIX

Proximate Composition of Rat Feed (Vital Feed)

Parameter	**Amount**
Crude protein	24.00 %
Fat	15.00 %
Crude fibre	12.00 %
Moisture	7.00 %
Nitrogen free extract	29.35 %
Ash	11 %
Calcium	1.1 %
Phosphorus	0.55 %
Metabolisable energy	2850 Kcal/kg

APPENDIX XXX



LCMS Spectra of *V. doniana* extract