Studies on Anti-Tumour Activities of Leaf Extract of *Diodia sarmentosa* on Albino Rats

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CERTIFICATION

This is to certify that this work, "Studies on Anti-tumour Activities of Leaf Extract of *Diodia sarmentosa* on Albino Rats", was carried out by CHINEDU HENRY OKOROAFOR (20154986478) in partial fulfilment for the award of the degree of M.Sc. in Biotechnology in the Department of Biotechnology of the Federal University of Technology, Owerri.

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DEDICATION

This work is dedicated to the academic world for continuity in relevant scientific research geared towards enhancing healthy living.

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ABSTRACT

This research aimed at studying the biochemical, antioxidant and anti-tumorigenic potentials of the ethanol extract of *Diodia sarmentosa* on Uterine Leiomyoma, Benign Prostatic Hyperplasia and Hepatocellular carcinoma in adult abino wistar rats. Uterine leiomyoma was induced by daily oral administration of 200 mg/kg body weight of monosodium glutamate (MSG) for 30 days. Benign Prostatic Hyperplasia was induced by daily subcutaneous injection of 10 mg/kg body weight of testosterone propionate for 28 days and Hepatocellular carcinoma was induced by oral administration of 20 mg/kg body weight of diethyl nitrosamine (DEN) dissolved in normal saline (0.9%) and given 5 times a week for 6 weeks. Sixty three (63) adult albino rats were used for this study and they were divided into nine groups, three groups for each of the tumours and classified as normal control (NC), positive control (PC), and the treated group (TG). *In vitro* studies such as 2,2-diphenyl-1-picrylhydrazyl radical scavenging method ($DPPH^0$), Thiobabituric acid assay (TBARS) and Total Antioxidant Capacity (TAC) were done on the plant's leaves. The ethanol and aqueous extracts exhibited significant inhibition in DPPH free radical formation with IC_{50} values of 10.994 µg/ml and 10.121 µg/ml respectively compared to the ascorbic acid standard (IC_{50} value = 17.916 µg/ml). It has been reported that a lower IC_{50} value indicates a better antioxidant activity. The aqueous extract exhibited more inhibitory effect on TBARS with IC_{50} values of 2.657 µg/ml while the ethanol had an IC_{50} value of 8.53 µg/ml compared to BHT standard (IC_{50} = 2.142 µg/ml). For the total antioxidant capacity assay, the aqueous extract had higher ascorbic acid equivalent values compared to the ethanol extract. However, the two solvent extracts showed antioxidant capacity. In vivo studies were also carried out by assessing some biochemical and antioxidant parameters as well as histopathological findings. The results of the study showed an impaired antioxidant system in the positive control (untreated group) of the three study groups. Some biochemical parameters were also altered in the positive control of the three study groups mostly revealing a relationship between the various tumours and renal impairment/kidney damage. Treatment with the ethanol extract of *Diodia sarmentosa* significantly ($P<0.05$) improved the altered biochemical parameters and also significantly ($p<0.05$) increased the serum antioxidant levels. These findings were confirmed with a histopathology result which revealed the extent of cancer in the affected tissues and the efficacy of the ethanol extract of *Diodia sarmentosa* in ameliorating the damage. Conclusively, from the study, it was discovered that the ethanol extract of *Diodia sarmentosa* (Sw) leaves has biochemical, antioxidant and anti-tumorigenic potentials and therefore may be useful in the treatment of tumours.

Key words: *Diodia sarmentosa*, biochemical, anti-tumour, anti-oxidant, cancer.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Over the years and in recent times, several studies have been carried out on tumours, ranging from benign to malignant tumours. The focus has always been to improve cancer patients' care through natural therapeutic interventions in the bid to find lasting solutions to the epidemic disease. In this study, *Diodia sarmentosa* (Sw) was the plant of interest and the leaves were the site of exploration. *Diodia sarmentosa* (Sw) commonly known as Zimbabwe flora, is a straggling or procumbent perennial herb. Stems up to 4m long, distinctly 4-angled, hairy on the angles. Leaves are opposite and elliptic, up to 6.5cm long, green to yellowish-green, with rough, tubercle based hairs. Stipules frilled with 5-7 setae, up to 7mm long. Flowers in clusters at the nodes. It grows in evergreen forest particularly fringing 'mushitu' edges, open riverine vegetation, bushland and also on rocky places near river. It is widely distributed in tropical Africa, Asia, America and the Mascarene Islands. It is a dicot which belongs to the family Rubiaceae and the genus Diodia.

In some parts of Nigeria, the leaf extract is used to treat bruises, swellings, pain and minor cuts (Umoh *et al*. 2016). The whole plant is taken with pepper and salt for the treatment of dysentery in South Western Nigeria (Hemans *et al*. 2004; Soladoye *et al*. 2010). The antiulcer potential of *Diodia sarmentosa* (Sw) (whole plant) has been reported (Akah *et al*. 1998). Umoh *et al*. (2016) also demonstrated the anti-inflammatory and analgesic activities of *Diodia sarmentosa* (Sw). Presently, there is no data showing a comprehensive phytochemical and proximate analysis of the plant. Hence, the need to analyze its phytochemical components, proximate analysis and explore other medicinal properties of the plant, such as its biochemical, antioxidant and antitumor potentials. The focus is to evaluate the antitumour

activities of *Diodia sarmentosa* (Sw) on Uterine leiomyoma (a benign tumour), Benign Prostatic Hyperplasia (BPH) which is also a benign tumour and Hepatocellular Carcinoma (commonly known as liver cancer; a malignant tumour).

Rollins *et al*. (2000) has defined tumour as an abnormal growth which emanate due to a bypass of normal growth-controlling mechanisms. There are three categories of tumours namely: Benign tumours, *in situ* tumours and malignant tumours (Stojan *et al*. 2004). The benign tumours are often localized (confined in a particular space and do not have the ability to mestasize). Some examples include: warts, moles, uterine leiomyoma and benign prostatic hyperplasia (BPH). In situ tumors commonly grow in epithelium and are usually, but not invariably, infinitesimal. The cells have a close morphology with cancer cells but remain enclosed in the epithelial layer. It is usually treated because of its high tendency of developing into cancer. Examples of *in situ* tumours include: squamous cell carcinoma *in situ* and carcinoma *in situ* of the skin. Malignant tumours are fully formed cancer cells with the potency to penetrate and destroy the underlying mesenchyme local invasion. The tumour cells get their nutrients through the blood stream in normal tissues. Some tumour cells produce a range of proteins that enhances the growth of blood vessels into the tumour, thereby allowing continuous growth to occur (Stojan *et al*. 2004). Malignant tumours can emanate in any part of the body. A tumour is malignant (cancerous) when it proliferates into nearby tissues and has cells that can break away and travel through the blood or lymphatic system and spread to lymph nodes and distant parts of the body. Some examples of malignant tumours include: Breast, Liver, Lung and Pancreatic cancer.

There are also cells referred to as precancerous cells which may become fully blown cancers if not treated. Some of them have mild changes which might disappear without treatment. Although some transfer genetic changes, thereby becoming more and more abnormal as they

divide until they form cancer. Some precancerous conditions include (Cancer Research UK, 2014):

a. Atypia: These are cells that are a little bit abnormal (atypical). Atypia can be as a result of healing and inflammation but some types of atypia are precancerous.
b. Metaplasia: This implies that there is an alteration in the types of cells that are usually found in this area of the body. The cells appear normal but they are not the type of cells that are normally found in that tissue or that area. Majority of metaplasia are not precancerous but some are.
c. Dysplasia: This implies that the cells are growing faster than normal, there are more cells than normal and they are not arranged like normal cells.
d. Hyperplasia: This implies that abnormal cells are dividing and multiplying faster than normal cells. The cells appear normal under the microscope but there are more abnormal cells than normal. Some types of hyperplasia are precancerous but most are not.

The DNA is considered as the most likely site of tumor initiation, although there are several other likelihood. Carcinogens destroy specific genes probably in the stem cell of the tissue involved (Franks, 2001). Exogenous exposures to carcinogenic chemicals facilitate initiation and promotion which are the earliest events of carcinogenesis (Stojan *et al*. 2004). Initiation and promotion produce single clone of initiated cells which are activated by promoting agents. These promoting agents are not carcinogens but can induce the proliferation of initiated cells. Promoters have the sole responsibility of inducing tumor formation, although there are many other agents which can do same.

Genetic and enzymatic disorders reportedly have been caused by low intensity microwave radiation (Djindjic *et al*. 2003). Hydrocarbon carcinogens present in coal tar and a series of chemicals used in the rubber industry are common chemical carcinogens. Experiments with

laboratory animals reveals that there is a probability that viruses may initiatiate some human cancers, mainly the leukemic group (Stojan *et al*. 2004). It is also possible that interactions between chemical carcinogens and viruses lead to the formation of tumour. This is evidenced in the association between hepatitis B virus and environmental chemicals in the development of liver cancer (Guo *et al*. 2003) and there is suggestive evidence in other tumors, especially in cancer of the cervix (Stanley, 2001).

Age is another dependent factor when it comes to the type of tumour formed. Some cellular transformations are influenced by age, and this raises the chances of a neo-plastic transformation (Stojan *et al*. 2004).

1.2 STATEMENT OF PROBLEM

Cancer is considered to be the leading cause of mortality and has accounted for about 8.8 million deaths in 2015, and there are projections of a continued rising of mortality from cancer with an estimate of 11.5 million deaths in 2030 (WHO, 2017). Over the years, several therapies such as surgery, chemotherapy, targeted therapy, radiotherapy etc. have been prescribed for cancer treatment but these have met several setbacks ranging from adverse effects to second cancers. It is therefore a global health challenge with unsatisfactory treatment options.

1.3 AIM AND OBJECTIVES

1.3.1 AIM

To determine the biochemical, anti-oxidant and anti-tumour activities of ethanolic extracts of *Diodia sarmentosa* (sw) on selected tumours using albino rats.

1.3.2 OBJECTIVES OF THE STUDY

i. To determine the qualitative and quantitative phytochemical contents of leaves extract of *Diodia sarmentosa* (Sw).

ii. Evaluation of the *in vitro* antioxidant activities of *Diodia sarmentosa* (Sw) leaves.

iii. To determine the biochemical activities of the ethanolic extracts of *Diodia sarmentosa* (Sw) leaves on the study animals.

iv. To determine the *in vivo* antioxidant activities of ethanolic extracts of *Diodia sarmentosa* (Sw) leaves on the study animals.

v. To determine the antitumour potentials of ethanolic extracts of *Diodia sarmentosa*(Sw) leaves on the study animals.

1.4 JUSTIFICATION OF STUDY

As a result of an age long search for viable treatment options for cancer, so many therapies as well as drugs have been proposed for cancer treatment. The advent of modern drug therapies has undeniably improved cancer patients' cares. However, most of these drugs have adverse effects on the patients, as most of them are cytotoxic. Using Benign Prostatic Hyperplasia (BPH) as an example, it has been reported that 5α-reductase inhibitors and α1-adrenergic receptor antagonists (the two major medications currently being used for the treatment of BPH) have adverse effects (Eleazu *et al*. 2017). Some adverse effects associated with these drugs include impotence, abnormal ejaculation, orthostatic hypotension, gynaecomastia etc (Eleazu *et al*. 2017). Surgery, another option for the treatment of BPH has also been excluded as a routine treatment because of the costs and risks associated with it.

Long term use of Gonadotropin-releasing hormone analogues (GnRHa) agonists and antagonists for the treatment of uterine leiomyoma increases the chances of side effects such as osteoporosis, vaginal dryness, impotence, reduced breast size, emotional instability, depression, hair loss, and musculoskeletal stiffness (Parsanezhad *et al.* 2012). Additionally, the withdrawal of GnRH agonist monthly treatment causes menses to return after 8-12 weeks, leading to a rapid increase of the uterus and fibroid size. It has also been reported that long term use of aromatase inhibitors, another drug for the treatment of uterine leiomyoma results

in loss of bone mineralization and an increased fracture risk (Taylor & Leppert, 2012). Hence, there has been an increasing search for alternative means of managing cancer using natural remedies such as medicinal plants (Kalu *et al*. 2016) and this study is part of this much needed search for pharmacognostic solution. Our focus is on *Diodia sarmentosa* (Sw).

1.5 RESEARCH QUESTIONS

i. What are the phytochemicals present in *Diodia sarmentosa* (Sw) leaves extract?

ii. What are the biochemical activities associated with ethanolic extracts of *Diodia sarmentosa* (Sw) leaves?

iii. Do *Diodia sarmentosa* (Sw) ethanol extracts have antioxidatant potential?

iv. Do *Diodia sarmentosa* (Sw) ethanol extracts have antitumour potential?

1.6 STATEMENT OF HYPOTHESIS

H_0 = *Diodia sarmentosa* (Sw) does not have any biochemical, antioxidant and antitumour effect.

H_1 = *Diodia sarmentosa* (Sw) has biochemical, antioxidant and antitumour effect.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global Cancer Burden

According to the International Agency for Research on Cancer (IARC), the global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million cancer deaths in 2018 (IARC, 2018). One in 5 men and one in 6 women around the globe come down with cancer during their lifetime, and one in 8 men and one in 11 women die from the disease.

Everyone is susceptible to cancer development. However, according to American Cancer Society (ACS), as people get older, their chances of being diagnosed with cancer increases (ACS, 2011). Several factors have been associated with the differences in cancer incidence and mortality across the globe and this include: differences in age structure, early detection tests, availability and use of preventive services and prevalence of risk factors (ACS, 2018). There is also an age related asociation of cancer risk with an estimate of 80% of all cancers in the world diagnosed in people 50 years of age or older (Ferlay, 2018).

Many experimental findings suggest a relationship between excess body weight and risks of developing many types of cancers including; breast (postmenopausal), colorectum, corpus uteri (endometrium), esophagus (adenocarcinoma), gallbladder, kidney, liver, meningioma (cancer in the tissue covering the brain and spinal cord), multiple myeloma (cancer of plasma cells), ovary, pancreas, prostate (advanced stage), stomach, cardiac, thyroid, oral cavity, pharynx, and larynx (World Cancer Research Fund/American Institute for Cancer Research, 2018; Lauby-Secretan *et al*. 2016). The global cancer burden associated with overweight and obesity was estimated at 544,300 cases in 2012 (Pearson-Stuttard *et al*. 2018).

Globally, lung and breast cancer are the most common types of cancer worldwide in terms of the number of new cases; for each of these types, about 2.1 million diagnoses are estimated in 2018, accounting for about 11.6% of the total cancer incidence burden (IARC, 2018). Colorectal cancer (1.8 million cases, 10.2% of the total) is the third most commonly diagnosed cancer, prostate cancer is the fourth (1.3 million cases, 7.1%), and stomach cancer is the fifth (1.0 million cases, 5.7%). Lung cancer accounts for majority of the mortality (1.8 million deaths, 18.4% of the total), as a result of poor prognosis for this cancer worldwide, followed by colorectal cancer (881 000 deaths, 9.2%), stomach cancer (783 000 deaths, 8.2%), and hepatocellular carcinoma (782 000 deaths, 8.2%) (IARC, 2018). Female breast cancer ranks as the fifth leading cause of mortality (627 000 deaths, 6.6%) due to a relatively favourable prognosis, especially in more developed countries.

2.2 Cancer incidence in Africa

According to African Organization for Research and Training in Cancer (AORTIC), Africa has an enormous population of over 1 billion people and is considered the world's second-largest and second-most populous continent (AORTIC, 2013). Additionally, Africa carries a disproportionate burden of communicable and non-communicable diseases.

According to Jennifer *et al*. (2017), about 60% more Africans die from cancer than malaria and the number of mortality from cancer is rising at a geometric rate. This can be attributed to the fact that about 20% of African countries have no access to cancer treatment while for some, they have restrained access. In 2012, 14 million persons were estimated to have been diagnosed with cancer and there was a mortality of about 8 million (Abdel-Wahab *et al*. 2013). Majority of the cases and about 70% of deaths occurred in Africa and other low-and middle-income countries. Majority of Africa’s cancer burden is borne by women whose 5-year cancer prevalence is double that of men. Cancer is becoming more problematic in

Africa, and this can be attributed to ageing and growth of the population as well as increased prevalence of smoking, alcohol, physical inactivity, and reproductive behaviours, and of certain infectious agents of importance in cancer etiology (Parkin *et al*. 2014).

The five most frequent cancers in men are prostate, liver, Kaposi sarcoma, Non-Hodgkin's lymphoma (NHL) and lung cancer (AORTIC, 2013). Prostate cancer is commonly associated with genetic factors. Common risk factors for liver cancer include both alcohol use and untreated hepatitis B infection (Olsen, 2015). Kaposi's sarcoma in sub-Saharan Africa has largely been associated with later-stage HIV infection, and treatment generally requires availability of chemotherapy, surgery, or radiation options (ACS, 2011).

In women, the most common cancers are breast, cervix, liver, colorectal and NHL (AORTIC, 2013). Cervical cancer can be prevented cost-effectively by providing HPV vaccinations to young women, and early detection programs using tested "see and treat" methods are important tools to reduce mortality in settings where treatment is available (Sahasrabuddhe *et al*. 2012). Breast cancer is associated with genetic factors, reproductive patterns, and lifestyle risk factors and can be difficult to treat at later stages, but like cervical cancer, treatment options such as surgery, radiation, and chemotherapy can be effective when cases are detected early (Institute of Medicine, 2007).

The increasing rates of cancer can be explained to be a consequence of epidemiological transition currently taking place in these regions (Olsen, 2015). Globalization and changing lifestyles are one of the major reasons for increasing disease burden (Sasco, 2008). Outdoor air pollution and increased exposure to carcinogenic contaminants from occupational risks and increased industrial production in urban settings are also expected to be major risk factors in rising cancer rates throughout the African continent (NCD Alliance, 2012). Infectious diseases accounts for many of the most common cancers in low-income populations,

resulting in cervical cancer diagnoses due to human papilloma virus (HPV) exposure, bladder cancer due to complications of schistosomiasis, liver cancer due to untreated hepatitis B infection, and Kaposi's sarcoma and various lymphomas associated with HIV (ACS, 2011). Other factors such as poorly ventilated indoor cooking stoves account for an increased risk of lung, nasopharyngeal, and esophageal cancers in populations with otherwise low tobacco smoking rates (Gordon *et al*. 2014).

2.2.1 Challenges facing Cancer diagnosis, Treatment and Control in Africa

Cancer control, though a big challenge for many African countries, is never an impossible situation. Recognizing the scope of the problem has been a challenge in most African countries because of poor quality data. As a result of scarcity of pathology services in many African countries, the diagnosis of cancer is not carried out before death, nor are the causes of death recognized and centrally maintained (AORTIC, 2013). Other challenges include the following:

i. Absence of population-based cancer registries with accurate notification as regards the cause of mortality;
ii. Inadequately trained personnel. Scheffler *et al*. (2009) estimated that in 2015, the shortage of health care professionals (doctors, nurses and midwives) will be 792, 000 in 31 SSA countries with wage bill of over $2.2 billion annually.
iii. Lack of access to treatment (approximately 22% of the 54 countries in Africa have no access to any form of anti-cancer therapies which include surgical oncology, chemotherapy and radiation);
iv. Radiology and radiation therapy facilities are too few to serve the population in need. For instance, there is one radiation machine in Ethiopia for a population of 60 million, one in Democratic Republic of the Congo for a population of 73 million, one in

Malawi for a population of 16 million, one in Mozambique for a population of 23 million, and one in Senegal for a population of 13 million. There are 10 radiation facilities in Nigeria for a population of 162 million, compared to 3,331 facilities in the USA for a population of over 300 million and 86 facilities in the UK for a population of 62 million;

v. Significant out of pocket expenses incurred by people in Africa. For instance, In Nigeria, 62% of all health expenditure is out of pocket, compared to 10% in the UK. Out of pocket health expenditure is estimated to push over 100 million people globally into abject poverty (WHO, 2007);

vi. The migration of highly skilled and qualified African health care personnel to more attractive settings with better salaries, working conditions, career paths and support.

vii. Lack of palliative care, resulting in large numbers of people dying unnecessarily agonizing deaths.

Cancer efforts in low- and middle-income countries to date have arguably been majorly geared towards prevention (Olsen, 2015). Tobacco and alcohol use can be reduced through national, profit-generating policies such as sales and import taxes (Maher & Nathan, 2011). For cervical cancer, "see and treat" programs that incorporate visual inspection methods using vinegar and cryogenic removal of cancer cells is another cost-effective strategy that has become a standard of care in many low- and middle-income countries (Sahasrabuddhe *et al*. 2012). In breast cancer, early detection and screening programs have been the major strategy for improving outcomes for patients with early-stage diagnoses (Brown *et al*. 2006). For some cancer types such as lung, prostate, colorectal, and breast palliative care remains the best option especially for patients presenting with late stage diagnoses (Vento, 2013).

2.3 Cancer incidence in Nigeria

The earliest study from Nigeria on cancer was from the Ibadan cancer registry 1960-1969, there was higher rate of cancer in females with age standardized rates of 105.1 per 100,000 females and 78 per 100,000 male (Njaka, 2016).

In Nigeria, above 10,000 cancer deaths and an estimated 250,000 incidences are recorded yearly (Ferlay *et al*. 2010). Nigeria contributed 15% of the estimated 681,000 new cases of cancer that occurred in Africa in 2008 (Sylla & Wild, 2012). In 2011, 200,000 new cases of cancer in Nigeria were revealed (Njaka, 2016). Similar to the situation in the rest of the developing world, a significant proportion of the increase in incidence of cancer in Nigeria is as a result of increasing life expectancy, reduced risk of death from infectious diseases, increasing prevalence of smoking, physical inactivity as well as changing dietary and lifestyle patterns (Sylla & Wild, 2012).

According to Jedy-Agba *et al*. (2012), the commonest cancers in Nigeria in 2009-2010 were breast and cervical cancer among women and prostate cancer among men. Possible explanations for the higher proportion of cancers occurring in women include differences in incidence pattern of cancers that occur commonly in both sexes compared to the age structure of the population. Other reasons are: relative ease of diagnosis and more specific symptomatology of common female cancers compared with those in men (prostate and liver), more frequent contact with the health care system by women due to uptake of maternal/child health care services, greater population awareness of breast and cervical cancers, better health-seeking behavior by women compared to men (Mills *et al*. 2011).

2.3.1 Cancer diagnosis and treatment in Nigeria

Standard screening techniques are available for detecting a wide range of cancers. These techniques include mammography for breast cancer, fecal occult blood testing and sigmoidoscopy/colonoscopy for colorectal cancer, and Pap smear for cervical cancer (Lambert *et al*. 2009). Although, pap smear-based screening program was unsuccessful in Africa, other techniques such as on visual inspection using Lugol's iodine or acetic acid, and low-cost DNA tests to detect HPV infections, have been shown to be feasible and effective in many parts of Africa, including Kenya and South Africa (Louie *et al*. 2009; Denny *et al*. 2005). It is assumed that screening one or two times in life time between the ages of 35-55 years would reduce cancer by about 30% (Goldie *et al*. 2005). Increasing public awareness of early signs and symptoms of cancers of the breast, cervix, oral cavity, urinary bladder, colorectal, and prostate should increase the detection of these diseases at earlier stages when there are more effective options for treatment leading to better prognosis (Curado *et al*. 2007).

Different treatment options are available (Morounke *et al*. 2017). Four common types are:

1. Chemotherapy: The use of a combination of drugs to destroy, cure or control cancer cells.
2. Radiation therapy: This entails the use of diverse forms of radiation to effectively treat and manage cancer and other diseases. Radiation therapy remains an essential component of cancer treatment with about 50% of all cancer patients receiving radiation therapy during their course of illness; it contributes towards 40% of curative

treatment for cancer (Baskar *et al*. 2012). The main aim of radiation therapy is to inhibit cancer cells from multiplying.

3. Removal of the tumor and the area surrounding the tumour.
4. Antologous/allogenic Bone Marrow Transplant: Used to treat diseases that damage or destroy the bone marrow. According to International Agency for Research on Cancer (IARC) it is also used to restore bone marrow that has been damaged during cancer treatment (IARC, 2003).

2.3.2 Challenges facing cancer treatment in Nigeria

Cancer treatment is plagued with serious challenges in Nigeria, especially because of inadequate or unavailable treatment facilities. Majority of the few ones in Nigeria are in bad conditions. This has contributed to high cancer deaths recorded in Nigeria. Other challenges include: lack of screening centers (Njaka, 2016), shortage of health professional (WHO, 2006), inaccessibility of cancer drugs by most cancer patients (UICC, 2008) and poor health system. The few health facilities in low and middle income countries are usually inaccessible to poor and rural populations and this results in most people receiving little or no medical attention.

2.4 OXIDATIVE STRESS

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites and their elimination by protective mechanisms, referred to as antioxidants (Reuter *et al*. 2010). This imbalance leads to destruction of important biomolecules and cells, with potential impact on the whole organism (Durackova, 2009). The generation of reactive oxygen species (ROS) and an altered redox status are common biochemical aspects in cancer cells (Barrera, 2012). Most ROS are generated in cells by the mitochondrial respiratory chain (MRC) (Poyton *et al*. 2009). When the mitochondrial respiratory chain (MRC) becomes highly reduced, the excess electrons from complex I or complex III may increase

substantially, passing directly to O_2 to generate high amounts of superoxide anion ($O_2^{\bullet-}$) (Manuel & Mario, 2012). Superoxide is transformed to hydrogen peroxide (H_2O_2) by the detoxification enzymes manganese superoxide dismutase (MnSOD) or copper/zinc superoxide dismutase (Cu/Zn SOD), and then to water by catalase, glutathione peroxidase (GPx) or peroxiredoxin III (PRX III). However, when these enzymes cannot convert ROS such as the superoxide radical to water fast enough, oxidative damage occurs and accumulates in the mitochondria. Additionally, nitric oxide (NO) is produced within the mitochondria by mitochondrial nitric oxide synthase (mtNOS) and also freely diffuses into the mitochondria from the cytosol. NO reacts with $O_2^{\bullet-}$ to produce peroxynitrite ($ONOO^-$). These two radicals as well as others can do great damage to mitochondria and other cellular components (Turrens, 2003).

2.4.1 Sources of Oxidative Stress

Cells from all organisms are exposed to several oxidizing and harmful agents which can be classified as exogenous and endogenous. Exogenous sources are associated with the environment, medical, diagnostic ionizing and non-ionizing radiations (X- or γ-rays, α-particles from radon decay, UV radiation) or chemical agents. Endogenous (intracelullar) sources of reactive species are products of O_2 metabolism, immune responses and inflammation. These processes may result in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that react with DNA and produce several lesions and indirect effects. Ionizing radiations can destroy DNA also by direct energy deposition and ionizations (Kryston *et al*. 2011). Cancerous cells are metabolically very active and require a great supply of ATP in order to maintain proliferation and cell growth under control. This high energy demand in the mitochondrial respiratory chain contributes to the generation of ROS.

Immune response and inflamation are other sources of ROS. It is known that oxidative stress activates inflammatory pathways leading to transformation of a normal cell to tumor cell, tumor cell survival, proliferation, insensitivity to anti-growth signaling, invasion, sustained angiogenesis, and stem cell survival (Reuter *et al*. 2010). Chronic inflammation is triggered by environmental (extrinsic) factors (eg, infection, tobacco, asbestos) and host mutations (intrinsic) factors (eg, Ras, Myc, p53). Activation of Ras, Myc, and p53 cause mitochondrial dysfunction, resulting in mitochondrial ROS production and downstream signaling (eg, NFkappaB, STAT3, etc.) that promote inflammation-associated cancer (Kamp *et al*. 2011). Another source of free radical generation is the chronic exposure to viral infections; as in the case of hepatitis viruses, where there is a connection between chronic infection and induction of oxidative stress. Kryston *et al*. (2011),

2.4.2 Role of Oxidatitive Stress in Metastasis

At the begininning of the carcinogenic process, tumor cells accumulate mutations that allow them to mestasize uncontrollably (Manuel & Mario, 2012). Moreover, these alterations contribute to increase the susceptibility to accumulate additional genetic modifications, facilitating tumor progression and cancer development. An increase of DNA synthesis and mitosis triggered by non-genotoxic agents could induce mutations in new cells. These mutations could spread through new cell divisions, evolving from an initial pre-neoplastic state into a neoplastic state. If DNA damage is too high, there are important mechanisms, such as apoptosis, by which the altered cells are selectively eliminated. Protein p53 plays a fundamental role in this process, as it initiates mechanisms that eliminate, for example, those oxidized DNA bases that could cause mutations (Manuel & Mario, 2012).

Initiation involves a DNA mutation that is not lethal, but it produces a cell alteration followed by at least one round of DNA synthesis that allows fixing the damage done. Several studies

have revealed an interesting correlation between tumor size and the amount of 8-OHdG (8-hydroxy-2'-deoxyguanosine; also known as 8-oxo-deoxyguanosine, 8-oxo-dG), a nucleotide modified by the activity of free radicals (Kennedy *et al*. 1998; Yano *et al*. 2009). The promotion stage is characterized by the expansion of initiated cells, stimulating cell proliferation and/or apoptosis inhibition. As a result of this process, an identifiable lesion is formed, thus requiring the constant presence of an agent that stimulates promotion. However, it is a reversible process. Many promoter agents have a strong inhibitory capacity against antioxidants like catalases, glutathione, SOD, etc. While a high level of oxidative stress is cytotoxic for cells and stops proliferation inducing apoptosis or even necrosis, moderate levels of oxidative stress may stimulate cell division and, therefore, stimulate tumor growth and promotion (Dreher & Junod, 1996). Progression is the third and last stage of the carcinogenic process and it involves cellular and molecular changes that occur from a pre-neoplastic state to a neoplastic state. This stage is irreversible and it is characterized by the accumulation of genetic damage that allows the cell evolving from benign to malignant. ROS are considered as carcinogenic potentials that facilitate cancer promotion and progression (Pelicano *et al*. 2004).

2.4.3 Role of Oxidative Stress in Lipid Peroxidation

The generation of reactive oxygen species (ROS) and an altered redox status are common biochemical aspects in cancer cells (Barrera, 2012). ROS can react with the polyunsaturated fatty acids of lipid membranes and induce lipid peroxidation. The reactive intermediates, produced by oxidative stress, can alter the membrane by-layers and cause the lipid peroxidation of polyunsaturated fatty acids (PUFA) leading to the formation of lipoperoxyl radical ($LOO^{\bullet}$), which, in turn, reacts with a lipid to yield a lipid radical and a lipid hydroperoxide (LOOH). LOOHs are unstable: they generate new peroxyl and alkoxy radicals and decompose to secondary products (Halliwell *et al*. 1993, Gardner, 1989, Spiteller *et al*.

2001). Such free radicals produced during lipid peroxidation have some very local effects, because of their short life, but the breakdown products of lipid peroxides (such as malonaldehyde, hexanal, 4-hydroxynonenal, or acrolein) may serve as "oxidative stress second messengers," due to their prolonged half-life and their ability to diffuse from their site of formation, compared to free radicals.

2.5 ANTIOXIDANTS

Antioxidants are molecules that have an extra electron to share with the roaming free radicals, come to rescue when the body is affected by the damage caused due to excess of free radicals (Krishna, 2014). Antioxidants prevent free radicals from stealing electrons from important regions in the body. There are several types of antioxidants such as phytochemicals, anthocyanins, carotenoids and trace minerals which can be found in foods. Many *in vitro* studies have shown that dietary antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), β carotene and flavonoids, act as effective antioxidants in biological systems such as plasma, lipoproteins and cultured cells. Vitamin C effectively inhibits lipid and protein oxidation in human plasma exposed to various physiologically relevant types of oxidative stress, such as activated polymorphonuclear leukocytes, reagent or myeloperoxidase-derived hypochlorous acid, cigarette smoke, redox-active iron, or copper. Vitamin E, the most abundant lipid soluble antioxidant in human lipoproteins and tissues acts as a chain-breaking antioxidant against lipid peroxidation (Packer *et al*. 2002). β-carotene, lycopene, lutein and other carotenoids and oxy-carotenoids are efficient singlet oxygen quenchers and thus, important in protecting the eye and skin against UV-induced oxidative

damage (Mayne, 2003). Polyphenols are powerful metal chelators and scavengers of free radicals and also act as anti-inflammatory, anti ulcer, anti tumour and anti cancer agents. They interact with cellular signal pathways that control cell cycle, differentiation and apoptosis (Krishna, 2014). They bind with transition metals particularly iron and copper and thus inhibit transition metal catalysed free radical formation (Andjelkoric *et al*. 2006; Kondratyuk & Pezzuto, 2004). *In vitro* studies are able to demonstrate for flavonoids, flavonols, flavones, and most recently also for anthocyanins, a considerable antioxidative activity, mainly based on scavenging of oxygen radicals (Duthie & Dobson, 1999). Theoretical underpinnings for the efficacy of flavonoids as antioxidants *in vitro* come from the inhibition of low density lipoprotein (LDL) oxidation, likely due to their reductase capacity and protein-binding properties (Wang & Goodman, 1999).

2.5.1 Classification of Antioxidants

2.5.1.1 Enzymatic Antioxidants

The major enzymatic antioxidants directly involved in the neutralization of ROS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx) (Pacher *et al*. 2007; Halliwel, 2007). SOD, the first line of defence against free radicals, catalyzes the dismutation of superoxide anion radical ($O^{2\bullet-}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water (H_2O) and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein of GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH) (Halliwel, 2007).

2.5.1.2 Non-Enzymatic Antioxidants

The non-enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants, belonging to endogenous antioxidants, are produced by metabolism in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc (Willcox *et al*. 2004); while, nutrient antioxidants, belonging to exogenous antioxidants, are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc.

Nutrient antioxidants have been shown to be involved in detoxification of the reactive oxygen species (ROS) (Gupta & Singh, 2013) and play an important role in helping endogenous antioxidants for the neutralization of oxidative stress (Donaldson, 2004). The nutrient antioxidant deficiency is one of the causes of numerous chronic and degenerative pathologies and cancer. Each nutrient is unique in terms of its structure and antioxidant function (Willcox *et al*. 2004).

2.5.2 Sources of Antioxidants

According to American Dietetic Association (ADE), antioxidants exist as vitamins, minerals and other compounds in foods (ADE, 2010). Diets rich in antioxidants can be very beneficial. Some common sources of antioxidants include:

I. Natural Sources:

a. Fruits

Fruits contain several vitamins and mineral salts deciding about their nutritional value, and also dietary fiber (Sikora *et al*. 2008). Majority of fruits are rich source of vitamin C, carotenoids and polyphenolic compounds. Especially berry fruits are precious in this regard. Fruits of blackcurrant also contain huge amounts of vitamin C, in range of 120-215 mg/100 g (Hägg *et al*. 1995, Benvenuti *et al*. 2004), and also significant amounts of

carotenoids, mainly lutein and β-carotene (Olsson *et al*. 2004). Citrus fruits – grapefruits, lemons, oranges – are a rich source of antioxidants, because of big content of vitamin C (40-50 mg/100 g) and phenolic compounds, among which flavanones (hesperitin, naringenin, eriodictyol) dominate.

b. Vegetables

Among vegetables the best sources of antioxidants are tomatoes, red pepper, Brassica vegetables, onion, garlic and red beet (Sikora *et al*. 2008). Lycopene is present in tomatoes peel in amount of 3025 μ g/100 g, however, considerably higher amounts, and better absorbed form is supplied by tomatoes pre-serves, for example ketchup (9900 μ g/100 g) (Horbowicz & Saniewski, 2000, Lugasi *et al*. 2003). Considering vitamin C or β-carotene content, parsley roots, kale, carrot and pumpkin are listed among vegetables (Holden *et al*. 1999, Kopsell & Kopsell, 2006).

c. Animal Derived Food

Important group of natural antioxidants in animal-derived food products are amino-compounds: amino acids, peptides and proteins. Antioxidant activity of proteins from animal-derived products can be associated with addition (in food technology) of concentrates and isolates gained from high-protein plants origin (legumes seeds) and animal origin (milk, eggs) raw materials. Isolated soybean proteins, because of their good functional properties, are widly used in meat industry and they can inhibit reaction of lipid oxidation (Ulu, 2004).

Main non-protein thiol compound of animal tissues is glutathione (GSH). Its basic function in organism is protection of thiol protein groups from oxidation. Tocopherols, among which α-tocopherol is dominant compound, are located mainly in 2-layer phospholipid structure of cellular membranes, and decide (with cholesterol) about their integrity. They posses ability to scavenge off radicals such as hydroxyl,

alcoxyl, peroxyl as well as hydroxyperoxide anion radical, and ability to quench of singlet oxygen (Sikora *et al*. 2008). Animals and humans cannot synthesize carotenoids, but have only the ability to metabolise them; classic example is reaction of carotene conversion to retinol (vitamin A). In animal organism, carotenoids are located mainly in the liver, they are also present in lipid tissue and blood lipoproteides (LDL and HDL) (Surai, 2003).

d. Legumes, nuts and oil seeds

A variety of studies have reported the antioxidant activities of many legumes, such as yellow and green peas, chickpea, lentils, common beans (pinto, great northern, navy, black, dark red kidney, light red kidney, red Mexican, pink and alubia bean), fava beans, beach bean, and yellow and black soybeans (Wang *et al*. 2011). Peanuts, peas, and edible beans will be highlighted with a focus on polyphenolics and phenolic acids. Red peanut skins were found to contain 7–9% procyanidins which 50 % of these are low molecular weight phenolic oligomers (Akbarirad *et al*. 2016). The water soluble 2,3-cis-procyanidins were found to be the predominant procyanidins and are characterised by a bond linked with a β (4→8) or β (4→6) arrangement that terminates by a 2,3-trans-flavan-3-ol catechin (Yanishlieva-Maslarova & Heinonen, 2001).

Polyphenolics in walnut seed include the monomers ellagic acid, gallic acid and methyl gallate, when present as polymers and bound to sugars are known as hydrolyzable tannins, and comprise the majority of the polyphenolics present. The major lipid-soluble antioxidants found in peanut and in other oilseeds are tocopherols (Maestri *et al*. 2006). Flaxseed, sunflowers, soybean, cottonseed, and canola

antioxidants typify the antioxidants from oilseeds. An important group of antioxidants includes the sterols. These compounds have been shown to prevent thermal oxidative degradation of oils. The antioxidants of confectionery and oil sunflowers include phenolic acids, tocopherols and sterols while purple hulled varieties contain significant concentrations of anthocyanins. Tocopherol homologues are phenolic antioxidants that occur naturally in vegetable oils and provide some protection against oxidation by terminating free radicals (Gohari *et al*. 2010).

e. **Cereal**

Cereals are among the most common food components, and can be added to many food products (Yanishlieva-Maslarova & Heinonen, 2001). They are a good source of catechins; the higher amounts of these compounds were found in seeds of buckwheat, next – in oats and rye, and at least in wheat (Peterson *et al*. 2001; Holasova *et al*. 2002). Beside this compound, vanillic and p-coumaric acids play important role, even though they are present in smaller amounts. In the case of oats, the presence of other polyphenols called avertramidin has been reported, while rutin is the main polyphenol of buckwheat (Vollmannova *et al*. 2013). The antioxidants of corn are unique in the fact that carotenoids make up part of the antioxidants, which is not true in the case of most cereals.

II. Synthetic Sources

a. Pharmaceutical and Food Industry

Nowadays, most food & pharmaceutical products contain synthetic antioxidants. These compounds are added to food in order to prolong product shelf life, mainly by preventing the oxidation of unsaturated double bonds of fatty acids (Shebis *et al*.

2013). In pharmaceutical products too, antioxidants are added to enhance the stability of therapeutic agents that are susceptible to chemical degradation by oxidation. The two most common synthetic antioxidants used today are butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT). Unfortunately, new data indicating that the synthetic antioxidants used in the industry could have carcinogenic effects on human cells resurface every year, thus fueling an intense search for new, natural and efficient antioxidants (Shebis *et al*. 2013).

b. Supplements

The most common antioxidants given as food supplements are vitamins C and E (Shebis *et al*. 2013). Vitamin C is a cofactor in enzymatic reactions, including several collagen syntheses and an electron donor, which makes it a potent water-soluble antioxidant in humans. If these reactions are disrupted or damaged, they can cause severe health problems, such as scurvy (Padayatty *et al*. 2003).

Furthermore, studies showed that some gene expression and protein assimilation functions are dependent on dietary vitamin C (Lucock *et al*. 2013). Vitamin E is a fat-soluble antioxidant whose function is to stop the production of ROS formed due to fat oxidation. Among other functions, it is involved in cell signaling, gene expression regulation, immune function, and other metabolic processes (Salinthone *et al*. 2013). It has been discovered that the naturally produced vitamins have a much higher percentage of absorbance in the human body.

2.5.3 Importance of Antioxidants

Antioxidants apply as inhibitor of the oxidation process (Maestri *et al*. 2006; Mandal *et al*. 2009), even at relatively small concentration and thus have diverse physiological role in the

body (Mandal *et al*. 2009). These compounds quench dreaded free radicals and stop oxidation chains *in-vivo* as well, so they have become viewed by many as nature's answer to environmental and physiological stress, aging, atherosclerosis, and cancer (Maestri *et al*. 2006).

Consumers believe that foods rich in antioxidants may afford a degree of protection against free radical damage not only in foods, but also in the human body, protecting against cardiovascular diseases, damage of nucleic acids, and other deteriorative processes (Yanishlieva-Maslarova & Heinonen, 2001).

2.5.4 Role of Antioxidants in Oxidative Stress and Cancer

Antioxidant defense against cancer is broadly divided into two namely (Krishna, 2014):

1. Antioxidant defense system against oxidative stress
2. Antioxidant system against the cancer cell cycle

2.5.4.1 Antioxidant defense against oxidative stress

In antioxidant defense against oxidative stress, they work as:

- Preventive antioxidants which suppress formation of free radicals
- Radical scavenging antioxidants which suppress chain initiation and breaking chain propagation reactions.
- Repair and de novo antioxidants

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized (Gupta *et al*., 2014). Thus, while in one particular system, an antioxidant is effective against free radicals; in other systems, the same antioxidant could become ineffective. Also, in certain circumstances, an antioxidant may even act as a pro-oxidant, for example, it can generate toxic ROS (Young & Woodside, 2001).

Antioxidants or agents metabolised to become antioxidants may function by variety of mechanisms: 1) quenching the formation of singlet oxygen, e.g. b-carotene, retinol; 2) scavenging ROS, e.g. polyphenolics; 3) scavenging or reducing lipid free radicals, e.g. α-tocopherol; 4) scavenging prooxidant metals, e.g. polyphenolics, flavonoids; 5) oxidizing ferrous iron, e.g. caeruloplasmin, apoferritin; 6) inhibiting prooxidant enzymes, e.g. allopurinol; 7) inducing or enhancing protective enzymatic defense against oxygen or oxydants, e.g. butylated hydroxyanisole; 8) sparing or renewing intracellular antioxiants, e.g. ascorbate, N-acetylcysteine; 9) stabilising membranes against lipid peroxidation, e.g. cholesterol, 17-beta-estradiol, tamoxifen; 10) reducing oxidatively stressed cells, e.g. ethanol, sorbitol, xylitol (NADH generators); 11) inhibiting enzymes that mediate gene expression as a result of oxidative stress, e.g. tamoxifen, methoxybenzamide (Gašparović *et al*. 2010).

2.5.4.2 Antioxidant defense system against cancer cell cycle

In antioxidant defense system against cancer cell cycle, they work as:

- A stimulant for TNF-α
- Inhibit cell proliferation
- Activate intrinsic pathway
- Inhibits oxidative stresses by-products
- Arrest cell cycle

Initiation of apoptosis pathway is the major mechanism through which the above mechanisms are carried out by any antioxidant in food (Krishna, 2014). Cancer cell death involving the degradation of cellular constituents by a group of cysteine proteases called caspase which are activated either through intrinsic pathway or extrinsic pathway (Fernandez *et al*. 2013).

In intrinsic pathway, the antioxidants help in permeabilisation of mitochondria and release of cytochrome C into cytoplasm which forms a multi protein complex known as apoptosome that initiates activation of caspase cascade through caspase 9 (Von *et al*., 2007).

Extrinsic pathway is stimulated by the initiation of death receptors on the plasma membrane such as tumour necrosis factor I and when ligands bind to these receptors, the death inducing signaling complex is formed leading to activation of caspase cascade through caspase 8 (Jin *et al*. 2010). Certain antioxidants (flavonoids) in high amounts block cell cycle in the G1 phase (Indran *et al*., 2010).

2.6 CARCINOGENES

American Cancer Society (ACS, 2016) defines carcinogen as substances and exposures that can lead to cancer. Sometimes they do not affect DNA directly, but they cause cancer through several other routes. One of such instance is when they initiate an uncontrolled proliferation in cells which increases the susceptibility of DNA to mutations. Carcinogens or substances suspected to cause cancer have different levels of carcinogenicity. Some of them have an immediate effect while some cause cancer after a very long time of exposure. Some examples of known human carcinogens include: acetaldehyde (from consuming alcoholic beverages), aflatoxins, alcoholic beverages, aluminum production, 4-Aminobiphenyl, aristolochic acid (and plants containing it), arsenic and inorganic arsenic compounds, asbestos (all forms) and mineral substances (such as talc orvermiculite) that contain asbestos, azathioprine, benzene, epstein-Barr virus (infection with), estrogen postmenopausal therapy, ethanol in alcoholic beverages, *Helicobacter pylori* (infection with), hepatitis B virus (chronic infection with), hepatitis C virus (chronic infection with), Human immunodeficiency virus type 1 (HIV-1)

(infection with), Human papilloma virus (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 (infection with), Human T-cell lymphotropic virus type I (HTLV-1) (infection with), ionizing radiation (all types), Iron and steel founding (workplace exposure), Kaposi sarcoma herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8) (Infection with) and N-nitrosodiethylamine.

2.6.1 Diethylnitrosamine (DEN)

Diethylnitrosamine (DEN), a hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in deoxyribonucleic acid (DNA) repair/replication and is normally used as a carcinogen to induce liver cancer in animal models (Bhosale *et al*. 2002). DEN has been shown to be metabolized in its active ethyl radical metabolite, and the reactive product interacts with DNA causing mutation which would lead to carcinogenesis (Anis *et al*. 2001; Chakraborty *et al*. 2007). Experimental, clinical and epidemiological studies have provided evidence supporting the role of reactive oxygen species in the etiology of cancer. Diethlynitrosamine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals (Ramakrishnan *et al*. 2006; Valko *et al*. 2006).

Diethylnitrosamine (DEN) is present in tobacco smoke, water, cured and fried meals, agricultural chemicals, cosmetics and pharmaceutical agents (El-Shahat *et al*. 2011) and is commercially available for experimental use. DEN is an established powerful hepatocarcinogen in rats, which possibly works by altering the DNA structure, forming alkyl DNA adducts, and inducing chromosomal aberrations and micronuclei in the liver (Al-Rejaie *et al*. 2009; Verna *et al*. 1996).

It has also been reported that oxidative stress plays a pivotal role during carcinogenesis (Jayakumar *et al*. 2011). Although a single injection of DEN followed by partial hepatectomy coupled with 2-acetyl-aminofluorene (2-AAF) is an established procedure for developing

HCC in rodents (Solt *et al*. 1983), the sequential administration of DEN for a number of weeks has also been employed for inducing HCC (Chuang *et al*. 2000; Shiota *et al*. 1999).

2.6.2 Monosodium Glutamate (MSG)

Monosodium Glutamate (MSG) is a salt of glutamate, synthesized from L-glutamic acids and used as a flavour enhancer in foods; binder and filler for nutritional supplements, in prescription drugs, intravenous fluids given in hospitals and in the chicken pox vaccines (Ikonomidou & Turski, 1995; Rodriguez *et al*. 1998; Eskes, 1998). Glutamate is commonly found in all foods, including meat, fish, poultry, breast milk and vegetables, with vegetables containing very high amounts of free glutamate (as MSG). Various processed and prepared foods such as traditional seasonings sauce and certain restaurant foods contain significant levels of free glutamate (as MSG), both from natural sources and from added monosodium glutamate (Rodriguez *et al*. 1998; Eskes, 1998). Monosodium Glutamate (MSG) causes reduction in the secretion of growth hormones, leading to stunted growth and irreversibility in obesity, excessive weight, essentially due to accumulation of excess fats in adipose tissue (Ikonomidou and Turski, 1995; Eskes, 1998; Rodriguez *et al*. 1998; Person & Warring, 1998).

2.7 UTERINE LEIOMYOMA

According to the National Cancer Institute (NCI), the uterus is plagued with various diseases which are categorized as: Malignant, Benign and Inflammatory (NCI, 2010). A good number of the benign lesions of the uterus emanate from inflammations which are believed to have resulted from microbial infection. Additionally, the female hormones exert influence occasionally on the composite tissue and this also is believed to promote genetic diseases (Chalas *et al*. 2005). Uterine leiomyoma is a benign tumour of the muscle tissue that grows in the wall of the uterus (Adedokun *et al*. 2016). It is considered as the most common pelvic

tumor and the most common noncancerous tumors in women over the age of 30 (Lurie *et al*. 2005). Uterine leiomyoma, commonly known as fibroid, is a common gynecological problem and also the most common benign genital tract tumor, associated with pregnancy loss and sub-fertility in women of reproductive age (Ezeama *et al*. 2012; Okeke *et al*. 2011; Akinyemi *et al*. 2004). It comprises of two basic tissue types and they include: the endometrial and myometrial. Endometrial hyperplasia is the most common uterine hyperplasia (Ndubuka *et al*. 2017). There are also predictions that at about 20% of these women may have fibroid during their reproductive years. Most of the women with uterine fibroids are generally asymptomatic at early stage and consequently get less clinical attention due to undiagnosed disease (Ezeama *et al*. 2012; Jindabanjerd & Taneepanichskul, 2006). Asymptomatic tumors cause heavy and painful menstruation, painful and post-coital bleeding, and urinary frequency and urgency, infection and delayed pregnancy (Wallach & Vlahos, 2004). Symptomatic women typically complain of abdominopelvic mass with or without abnormal uterine bleeding-mainly menorrhagia (Ezeama *et al*. 2012; Okeke *et al*. 2011; Okogbo *et al*. 2011).

2.7.1 Pathogenesis of Uterine Leiomyoma

Uterine leiomyoma arises from the over growth of smooth muscle tissue layer and connective tissue in the uterus (Miettinen, 2014; Nivethithai *et al*. 2010). Myoma development comprises first, the transformation of normal myocytes and their growth into clinically apparent tumors. Apart from their tumorigenic potential, they are morphologically similar at the cellular level to normal myometrial smooth-muscle cells (MSMCs) (Medikare *et al*. 2011). Microscopic determinations reveal they have interlacing bundles of spindle-shaped or stellate smooth-muscle cells with little cellular pleomorphism or mitotic activity (Blake, 2007). Global gene expression profiling of uterine leiomyomas (ULMs) revealed that hundreds of genes were dysregulated including those with functional roles in cell

proliferation, differentiation and extracellular matrix production and so far, only a few specific genes or cytogenetic aberrations have been identified to be associated with ULMs (Medikare *et al*. 2011). While many of the dysregulated genes may function as either effectors or promoters of ULMs growth, they are likely to be secondarily induced and indirectly responsible for tumor growth into morbid and symptomatic ULMs (Zavadil *et al*. 2010). Progression of leiomyoma to malignant leiomyosarcoma (LMS) is very rare (Ansari *et al*. 2012; Sandberg, 2005). Hormonal factors which set in during pre-menopause could be essential modulators for the formation of fibroids during late reproductive years. The other factors involved are menopause, obesity, diet, exercise, racial differences, geographic differences, oral contraceptives, hormone replacement therapy, etc. (Flake *et al*. 2003). The steroid hormones, estrogen and progesterone, are also considered the most important regulators of leiomyoma growth (Medikare *et al*. 2011).

2.7.2 Drugs currently used for fibroid treatment

The primary objective of therapeutic measures is the relief of the symptoms associated with fibroid. However, the treatment options for fibroid depend on factors such as the symptoms, size, location of the lessions as well the maintainance of the patient's fertility. Some of the chemotherapeutic drugs currently used are:

i. GnRHa agonists and antagonists

   Gonadotropin-releasing hormone analogues (GnRHa) aim at reducing the production or inhibition of estrogen action on the smooth muscle cells of the uterus. Pituitary down-regulation suppresses the reproductive endocrine axis and can be exploited therapeutically to reduce circulating sex steroid levels (Taylor & Leppert, 2012). Base on this mechanism, several GnRH agonist peptides are commercially available in

long-acting injectable depot formulations to inhibit estrogen, thereby creating a menopause-like stage. One of the major remaining limitations to the wide use of the GnRH antagonists in leiomyoma treatment is the short half-life of these agents and the non-availability of depot formulations (necessitating repetitive dosing) (Sabry & Al-Hendy, 2012). Long-term uses increases risks of side effects including osteoporosis, vaginal dryness, impotence, reduced breast size, emotional instability, depression, hair loss, and musculoskeletal stiffness (Parsanezhad *et al.* 2012). Additionally, when GnRH agonist treatment by monthly depots is suspended, menses return after 8-12 weeks leading to a rapid increase of the uterus and fibroid size.

ii. Aromatase inhibitors

Aromatase inhibitors (AIs) offer reversibility, total blockage of aromatase (CYP19), and possibly reduce side effects (Taylor & Leppert, 2012). AI medicines are clasified into steroidal or non-steroidal aromatase inhibitors (NSAIs) based on their structural similarity with steroids, or as 1^{st} and 2^{nd} generations based on their evolution with time. Prolonged use with the consequent hypo-estrogenaemia could lead to loss of bone mineralization and an increased suceptiblity to fracture.

iii. Anastrozole and Letrozole

This drug is associated with a reduction in fibroid size, thinning of endometrium and cessation of bleeding. Anastrozole has a half-life of about 48h and is effective with daily oral administration. Some of the side effects associated with its usage include: hot flashes, vaginal dryness and musculoskeletal pain.

iv. Selective estrogen receptor modulators (SERMs)

SERMs, like estrogen, are agents that elicit tissue-specific responses by intensely interacting with two kinds of estrogen receptors (ERs), ERα and ERβ, inhomogenously distributed throughout the body. They are characterized by their

diverse range of agonist/antagonist actions on ER-mediated processes. Two of the best characterized SERMs are tamoxifen and raloxifene, which inhibit the effects of estrogen. Raloxifene is a more complete uterine antagonist than tamoxifen, significantly reducing fibroid size in postmenopausal women yet is less efficacious at reducing tumor volume in premenopausal women (Dai & Wu, 2011). Some of the adverse effects associated with the use of tamoxifen include: thromboembolic events, vasomotor symptoms and an increased susceptibility to endometrial cancer and cataracts.

2.8 BENIGN PROSTATIC HYPERPLASIA

Benign prostatic hyperplasia (BPH) has been identified as the most common urological condition among elderly men (Eleazu *et al*. 2017). Clinical BPH is prostate adenoma/adenomata (PA) causing a varying degree of bladder outlet obstruction with or without symptoms (Vasanwala *et al*. 2017). PA comprises an important cause of male lower urinary tract symptoms (Luo *et al*. 2013). PA is a widespread problem that increases with age. According to Australia and New Zealand Urological Nurses Society Inc. (2010), almost one in four men with prostate problems aged 40-49 years receives treatment, and this increases to three in every four men aged 70 years and older.

Benign prostatic hyperplasia emanates as a simple micronodular hyperplasia with a subsequent macroscopic nodular enlargement that may result in bladder outlet obstruction and the development of lower urinary tract symptoms (LUTS) (Aleksandra *et al*. 2015). Enlargement of the prostate enlarges the urethra and induces various symptoms such as incomplete bladder emptying, nocturia, dysuria, weak urinary stream and bladder outlet obstruction (In *et al*. 2012). Although the pathogenesis of this metabolic disorder has not been fully understood, several factors such as ageing, inflammatory mediators, hormonal,

metabolic syndrome, oxidative stress and suppression of apoptosis has been associated with its etiology (Minciullo *et al*. 2015; Eleazu *et al*. 2017).

2.8.1 Pathogenesis of Benign Prostatic Hyperplasia (BPH)

In ageing males, a significant tissue-remodelling process takes place within the prostate, especially in the transition zone (TZ) (Briganti *et al*. 2009). Interference in the delicate balance of interacting growth factor signalling pathways occurs, and stromal– epithelial interactions generate an increase in prostate volume. Specifically, the most significant modifications take place in the basal cells, which change their intracellular metabolism and become enlarged and hypertrophic (Briganti *et al*. 2009). The development of BPH is also accompanied by the occurrence of corpora amylacea and prostatic calculi. These elements typically contain phosphate salts of calcium, magnesium, potassium, calcium carbonate, or calcium oxalate (Geramoutsos *et al*. 2004). Subsequently, the altered secretions of luminal cells and the presence of corpora amylacea and prostatic calculi lead to further calcification, and clogged ducts become visible (Bostwick *et al*. 1992). All of this tissue remodelling leads to alterations of highly specialised cell types responsible for tissue homeostasis and function.

The growth of the prostate gland is dependent on the circulating androgens and intracellular steroid signaling pathways mediated through the androgen receptor (AR), a ligand-activated nuclear transcription factor (Rahman, 2016). The binding of androgens to AR stimulates transcription of a cascade of androgen-responsive genes such as prostate-specific antigen (PSA) and genes involved in cell-cycle control. The transactivation of AR that is important for the normal growth and function of the prostate is found in trans activation domain encoded by exon I of the AR gene (Xq11-12) which contains polymorphidc CAG and GGN (also GGC) repeats encoding polyglutamine and polyglycine tracts respectively (Konwar *et al*. 2008; Zeegers *et al*. 2004; Schauer & Madersbacher, 2015).

Genetic polymorphism studies of several genes involved in steroid metabolizing pathway has been shown to be associated with increased BPH risk (Bartsch *et al*. 2002; Park & Choi, 2014).

The role of α-1 adrenergic receptors (α-1 adernoceptors) in BPH has recently been studied (Rahman, 2016). The receptors seem not only to increase the tone of prostatic smooth muscle but also modify prostatic growth (Habuchi *et al*. 2000) and contribute to LUTS by affecting the bladder and the spinal cord.

Pro-inflammatory cytokines induce inflammatory mediators such as cycloxgynase -2 (COX-2) and inducible nitric oxide (INOS) that contribute to prostate growth (Sciarra *et al*. 2008). It is hypothesized that inflammation of the prostate, through the generation of reactive oxygen species (ROS), causes repeated tissue damage and post-translation DNA modifications, thereby inducing neoplasia in the prostate (Naber & Weidner, 2000). The major sources of ROS are the mitochondrial respiratory chain, an uncontrolled arachidonic acid cascade and NADPH oxidase (Dobrian *et al*. 2001).

2.8.2 Current treatment/management of Benign Prostatic Hyperplasia

Treatment options for BPH include watchful waiting, medical therapy with α-blockers or 5 α-reductase inhibitors, hormone therapy, surgery and phytotherapy (Watson *et al*. 2004; Kaplan, 2006). 5α-reductase inhibitors and α1-adrenergic receptor antagonists (terazosin, doxazosin and tamsulosin) are the two major medications currently being used in the treatment of benign prostatic hyperplasia (Eleazu *et al*. 2017). The α1-adrenergic receptor antagonists alleviate LUTS by relaxation of smooth muscle in the prostate and the neck of the bladder while the 5α-reductase inhibitors (5-ARIs) inhibit the development of BPH through a

reduction in dihydrotestosterone (DHT) production (Kim *et al*. 2017) thereby causing involution of the prostatic epithelium and slowing the progression of BPH (Marks *et al*. 1999). Finasteride and dutasteride are examples of 5α-reductase inhibitors.

The enzyme 5-alpha reductase converts testosterone to DHT which makes it possible for it to inhibit DHT production. However, the direct effects of 5-ARI in reducing serum DHT levels also affect other laboratory values. Additionally, given the intended effect of 5-ARI causing the involution of prostatic epithelial tissue, which is the main source of intraprostatic as well as serum PSA, the inhibition of DHT by 5-ARI indirectly results in a decrease in PSA (Kim *et al*. 2017). For example, the use of finasteride for 12 months duration has been found to lower serum PSA by approximately 50% (Guess *et al*. 1996). However, the use of these drugs is limited because of their side effects, including decreased libido, ejaculatory or erectile dysfunction and nasal congestion (Bullock & Andriole, 2006; Traish *et al*. 2011).

The use of complementary and alternative medicine (CAM) for the treatment of BPH is also gaining popularity. It is estimated that 30% of men diagnosed with prostate disease in North America use some CAM products (Nickel *et al*. 2008), while such products constitute approximately 50% of all medicines prescribed for BPH in Italy (Di Silverio *et al*. 1993) and almost 60% of such prescriptions in Germany and Austria (Buck, 1996).

2.9 HEPATOCELULAR CARCINOMA

Hepatocellular carcinoma commonly known as liver cancer is considered the second most common cause of cancer death worldwide, claiming about 750,000 lives annually (GLOBOCAN, 2012). Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Patients diagnosed with hepatitis B and C, alcohol abuse, metabolic syndrome, and aflatoxin toxicity are more susceptible to HCC (Zhang *et al*. 2016).

2.9.1 Pathogenesis of Hepatocellular Carcinoma

The key mechanisms involved in HCC genesis and evolution (Zhang *et al*. 2016) include: altered cell cycle regulation, aberrant angiogenesis, evasion of apoptosis and loss of intrinsic mechanisms to limit cell proliferation.

HCC is considered to have increased invasiveness with malignant transformation and metastatic potential (Koike *et al*. 2003; Hsu *et al*. 1985). HCC cells themselves express various growth factors such as vascular endothelial growth factor (VEGF) (Li *et al*. 2006), platelet-derived growth factor (PDGF) (Stock *et al*. 2007), epidermal growth factor (EGF) (Schiffer *et al*. 2005), fibroblast growth factor (FGF) (El-Assal *et al*. 2001), and insulin-like growth factor (IGF) (Feitelson *et al*. 2004), which induce cell proliferation in an autocrine fashion (Săftoiu *et al*. 2004). The receptors of these growth factors activate intracellular signals such as the RAF/MEK/ERK pathway (Huynh *et al*. 2003) and the PI3K/AKT/Mtor pathway (Chen *et al*. 2005), which induce proliferation of both cancer and endothelial cells.

A specific pathological feature of HCC is high vascularity of the tumor (Meguro *et al*. 2011). It is necessary to increase vascularity for cancer cell proliferation. VEGF, PDGF, EGF, FGF, and IGF, growth factors that facilitate high vascularity and cancer cell proliferation are expressed not only in cancer cells but also in other surrounding cells. The high expression of the growth factors is also associated with tumor invasion and portal thrombosis (Li *et al*. 2006; Stock *et al*. 2007; El-Assal *et al.* 2001). Among the growth factors, high expression of EGF is related to differentiation and invasion by the cancer cells (Schiffer *et al*. 2005). On the other hand, PDGF is related to metastatic behavior of HCC cells (Stock *et al*. 2007). The uncontrolled division of endothelial cells is essential for metastasis and invasion of cancer cells. Therefore, growth factors play an important role in proliferation of cancer cells not only in an autocrine fashion but also in a paracrine fashion through surrounding cells (Săftoiu *et*

al. 2004). In addition, antivascular factors decrease in the serum and tissue of HCC patients (Dhar *et al*. 2002). These reports indicated that specific growth factors can be targets for HCC treatment. Based on the high vascularity of HCC, endothelial cells could be a target for HCC treatment (Meguro *et al*. 2011).

2.9.2 Current Trends in Treatment and Management of Hepatocellular Carcinoma

Sorafenib, the only US FDA approved treatment for advanced HCC, extends survival by about 3 months. Sorafenib is an inhibitor of RAF that is activated by VEGF and PDGF (Takezawa *et al*. 2009; Zhu, 2008 & Hŏpfner *et al*. 2008). It has been tested in phase III clinical trials, such as the SHARP trial (Llovet *et al*. 2008) and Asia-Pacific trial (Cheng *et al*. 2009). It improves overall survival in patients with advanced HCC compared to patients administered a placebo in the BSC setting (Meguro *et al*. 2011).

Brivanib, erotinib, and TSU-68, which are inhibitors of growth factor receptors, have been clinically tested for advanced HCC patients as well. The response rates to single doses of sorafenib (Llovet *et al*. 2008; Cheng *et al*. 2009), sunitinib (Faivre *et al*. 2009; Zhu *et al*. 2009), brivanib (Raoul *et al*. 2009), erlotinib (Philip *et al*. 2005), and TSU-68 (Kanai *et al*. 2011) were 2.3–3.3%, 2.7– 2.9%, 5.0%, 9.0%, and 8.6% respectively. Phase II clinical trials using bevacizumab (Siegel *et al*. 2008), a VEGFR inhibitor, and cetuximab (Zhu *et al*. 2007), an EGFR inhibitor, had 13% and 0% RRs, respectively.

In 2014, nivolumab was revealed to play a role in the treatment of metastatic HCC (EL-Khoveiry *et al*. 2015). It was also discovered from the same study that nivolumab lead to a significant shrinkage of tumour in about 20% of patients treated with the drug. Two patients were completely treated of tumors and the responses lasted for more than 9 months in almost all patients who responded. At 1 year, about 70% of patients who received nivolumab were still alive compared with the historical tumor response rate for sorafenib of only 2% to 3%

and 1-year survival rate of 30%. Of note, severe adverse effects of nivolumab included elevated ALT, AST, and lipase levels (ASCO, 2016).

2.10 Cancer Diagnosis and Screening

Cancer diagnosis can be defined as various techniques and procedures used to detect or confirm the presence of cancer in patients who have symptoms of the disease. It entails evaluation of the patient's history, clinical examinations, review of laboratory test results and microscopic examination of tissue samples obtained by biopsy or fine-needle aspiration (WHO, 2008).

While cancer screening entails identifying unrecognized cancer or pre-cancerous lesions in an apparently healthy target population by means of tests such as HPV assay, examinations (such as VIA visual inspection with acetic acid), imaging (e.g. mammography) or other procedures that can be widely applied and are also accessible to the entire target population.

George Papanicolaou developed the first cancer screening test in 1943 through his monograph which provided a method of identifying both precancerous and malignant cervical cells (Waddle *et al*. 2015). It was also during that period that x-rays gained value in the diagnosing early breast cancers using screening mammography once lower dose x-ray machines were available.

Cancer screening differs from early diagnosis in that an entire target population is examined for unrecognized cancer or pre-cancer (WHO, 2017). It includes (Taplin *et al*. 2006):

- A system of informing and inviting the target population to participate,
- Following-up with tests results and referral for further testing among those with abnormal test results and ensuring timely pathologic diagnosis,

- Staging and access to effective treatment with routine evaluation to improve the process.

Screening programmes entail the process from invitation to treatment and involves planning, coordination, monitoring and evaluation. Early diagnosis and screening programmes use similar resources and building blocks and effective early diagnosis provides the basis for a thorough cancer control. Some of the techniques used in cancer diagnoses include:

2.10.1 Molecular Techniques

This entails the detection of molecular makers in neoplastic tissue samples which provides accurate diagnosis, prognosis and prediction of response, resistance or toxicity to therapy. Cytogenetic procedures aim at examining the chromosomes in the tissue sample in order to identify any chromosomal mutation peculiar to known cancer types. Fluorescence in situ hybridization (FISH) technique is an example of a molecular cytogenetic technique which uses probes to confirm presence or absence of specific DNA sequences or chromosomes. It is used to diagnose blood disorders or cancer which are as a result of genetic mutations on the chromosomes. PCR is a quantitative technique that allows the amplification and analysis of target DNA regions in tumor samples. Electron microscopy is used in situations where specific cellular or intracellular structures need to be examined and it helps in a more accurate tumor classification.

Immunocytochemistry identifies antigens or protein expression on a fixed tissue section through an antibody that is specific for the antigen/protein. It detects estrogen and progesterone receptors on breast tissues, oncogenes and tumor suppressor gene products on tumor samples and it is also used to characterize leukemias and lymphomas.

2.10.2 Biomarkers

These are proteins produced by cancer cells and their detection in the serum may confirm the presence of certain cancers. Biomarkers help in determining which tumors will respond to which treatments (Chatterjee & Zetter, 2005). Some of the biomarkers currently in clinical use include: BTA (bladder tumor antigen) for bladder cancer, PSA (Prostate Specific Antigen) for prostate cancers, Thyroglobulin for thyroid cancers, Estrogen receptor for breast cancer, CA125 for ovarian cancers, CA19-9 for pancreatic cancers, Carcinoembryonic antigen (CEA) for colon cancer, HCG-β for testicular cancer. An ideal biomarker should be highly specific and sensitive especially when used for staging (Collins & Workman, 2006). It should also be easily detected in the patient's blood or urine but not in a healthy person. However, despite the detection of biomarkers in a patient, a histological examination is necessary to confirm the presence of cancer.

2.10.3 Morphological Examinations

They are the easiest techniques of cancer diagnosis (Bernerd &Wittwer, 2002). It is used to microscopically examin a tissue sample inorder to detect cancer. Some sampling methods used in pathological examination include: needle aspiration (It allows the clinician to obtain a core of tissue from a mass for cytological examination. It is commonly used for tumors in which there is a noticeable or readily palpable mass such as lymph nodes, breast or thyroid); incisional biopsy (It entails the removal of a small part of a large tumor for the purpose of laboratory diagnosis and excisional biopsy (It allows a more complete pathological examination of the lesion, thereby making it the most appropriate collection method for small tumors).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Collection of Plant Material

Fresh samples of *Diodia sarmentosa* (Sw) leaves were collected from farmlands and from natural vegetation within FUTO premises. It was identified by Prof. I.I. Iloegbulam of the Department of Crop Science. The picture of a portion of *Diodia sarmentosa* plant is displayed on Fig 3.1.

3.2 Animal Models

Sixty-three (63) disease free adult albino rats were obtained from the Department of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike. This comprise 21 (9 weeks old) adult female wistar albino rats and 42 (14 weeks old) adult male Wistar albino rats. The animals were housed in standard plastic cages (7 per cage), and were exposed to approximately 12 h light/dark cycles under humid tropical conditions, and given tap water and rat chew *ad libitum*.

Figure 3.1 A portion of *Diodia sarmentosa* (Sw) plant leaves

3.3 METHOD

3.3.1 Preparation of plant extract and extraction

Fresh leaves of *Diodia sarmentosa* (Sw) were washed with distilled water and dried at room temperature and then in a laboratory oven at 40^0C. The dried plant material was ground into fine powder using laboratory mortar and pestle. Each of the ground plant materials (800 g) was soaked separately in ethanol for 48 hours on an orbital shaker. The extracts were filtered using a Buckner funnel and Whatman No.1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40^0C using a rotary evaporator.

3.3.2 Tumor induction

- Uterine fibroid was induced with 200 mg monosodium glutamate (MSG)/kg daily for 30 days through an oral gavage tube as described by Olowofolahan *et al*. (2017) with slight modifications.
- Benign Prostatic hyperplasia was induced by daily subcutaneous injection of 10 mg/kg testosterone propionate for 4 weeks (Park *et al*. 2016).
- Liver cancer was induced through an oral gavage of 20 mg/kg Diethyl nitrosamine (DEN) dissolved in normal saline (0.9%) and administered five times a week for six weeks, according to the modified method of Darwish & El-Boghdady (2011).

3.3.3 Preparation of monosodium glutamate

Synthetic monosodium glutamate (Ajinomoto co., inc. Tokyo, Japan) was prepared by dissolving 25 g MSG granules in 250 mL distilled water as described by Olowofolahan *et al*. (2017) with slight modification.

3.3.4 Experimental design

The animals were acclimatized for 10 days in an animal house before being classified into 9 groups of 7 animals each as follows:

Group 1A- **Uterine Leiomyoma Normal Control (NC-1)**: without induction, given water and normal rat chew.

Group 1B- **Uterine Leiomyoma Positive Control (PC-1)**: Uterine Leiomyoma induced in rats using MSG, but without treatment.

Group 1C- **Uterine Leiomyoma Treatment Group (TG-1)**: Uterine Leiomyoma induced in rats and treated with ethanol extracts of *Diodia sarmentosa* (Sw) leaves.

Group 2A- **Benign Prostatic Hyperplasia Normal Control (NC-2)**: without induction, given water and normal rat chew plus subcutaneous injection of olive oil.

Group 2B- **Benign Prostatic Hyperplasia Positive Control (PC-2)**: Benign Prostatic Hyperplasia induced in rats using Testosterone Propionate (TP) dissolved in olive oil, and not given treatment.

Group 2C- **Benign Prostatic Hyperplasia Treatment Group (TG-2):** Benign Prostatic Hyperplasia induced in rats using Testosterone Propionate (TP) dissolved in olive oil and then treated with ethanol extracts of *Diodia sarmentosa* (Sw) leaves.

Group 3A- **Hepatocellular Carcinoma Normal Control (NC-3):** without induction, given water and normal rat chew.

Group 3B- **Hepatocellular Carcinoma Positive Control (PC-3)**: Hepatocellular Carcinoma induced in rats using Diethylnitrosamine (DEN), but not given treatment.

Group 3C- **Hepatocellular Carcinoma Treatment Group (TG-3)**: Hepatocellular Carcinoma induced in rats using Diethylnitrosamine (DEN), and then treated with ethanol extracts of *Diodia sarmentosa* (Sw) leaves.

3.3.5 Acute toxicity test of plant extract using LD_{50}

In screening drugs, determination of LD_{50} is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance as well as determining the dose that kills half of the animals. The timing of lethality following acute chemical exposure, as well as observing the onset, nature, severity, and reversibility of toxicity (Hodgson, 2010). The LD_{50} of the plant extract was determined using Lorke's method (Lorke, 1983). Nine (9) rats (100-110 g) were divided into 3 treatment groups of 1600 mg/kg, 2900 mg/kg and 5000 mg/kg of the extract. For each rat, the observation was made for 24 h and symptoms of toxicity and rate of mortality in each treatment group was noted. Two mortalities were recorded in the highest (5000 mg/kg) and medium (2900 mg/kg) doses after 24 hours but none was recorded in the least (1600 mg/kg) dose. From this, a safe dose of 400 mg/kg of the plant extract was chosen for the treatment.

3.3.6 Plant extract administration/treatment

- Group 1C (Uterine Leiomyoma treatment group) and Group 2C (Benign Prostatic Hyperplasia treatment group) were treated with 400 mg/kg of *Diodia sarmentosa* (Sw) once daily from the 5^{th} to the 8^{th} week after tumour induction.
- Group 3C (Hepatocellular Carcinoma treatment group) received 400 mg/kg *Diodia sarmentosa* (Sw) once daily from the 7^{th} week to the 9^{th} week after tumour induction.

3.3.7 Experimental site

The animals were bred in the animal house of Department of Biochemistry, Michael Okpara University of Agriculture, Umudike under room temperature (25-27^{0}C) and relative humidity of 40-65% with a 12 h natural light-dark cycle and were fed with commercially available rat pellets and given water *ad libitum* in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiment.

3.3.8 Qualitative Phytochemical Screening

The test sample (0.2 g) was placed in separate test tubes to assay for phytochemicals as follows:

3.3.8.1 Saponin

The test sample (0.2 g) was placed in a test tube and then 10ml of H_20. 1 ml of the solution was then put into another test tube and then 4 ml of H_20 was added. It was then shaked vigorously for 5 min. It was then allowed to stand for 1 min. A persistent foaming confirmed the presence of saponin.

3.3.8.2 Flavonoid (Ammonium test method)

Th test sample (0.2 g) was added to 15 ml Ethyl acetate. It was heated for 3 min and then filtered. To 2 ml of the filterate, 500 µl $AlCl_3$ + 500 µl NH_3 (aq) was added. A yellowish colouration confirmed the presence of flavonoid.

3.3.8.3 Carbohydrate (Molisch's test)

The sample (0.2 g) was added to 10 ml H_20 and boiled for 5 min, then filtered. To 1 ml of filterate, 100 µl Molish solution + 1 ml conc. H_2SO_4 was added. A brown ring formation at the interface confirmed the presence of carbohydrate.

3.3.8.4 Phenol

The test sample (0.2 g) was added to 10 ml of dist. H_20 plus few drops of dilute ferric chloride solution and then heated in a boiling water bath for 3 min. The filterate (2 ml) was placed in another test tube, and then diluted with dist. H_20 in the ratio 1: 4. A greenish colouration was observed and this confirmed the presence of phenol.

3.3.8.5 Reducing Sugar

The test sample (0.2 g) was added to 10 ml of dist. Water and boiled for 10 min, then filtered. The filterate (1 ml) was added to 200 µl Fehling solution A + 200 µl of Fehling solution B. It was then boiled for 5 min. A brick red colouration was seen which confirmed the presence of glycoside.

3.3.8.6 Glycoside (Fehling's Test)

The sample (0.2 g) was added to 10 ml of dist. H_20 and then boiled for 5 min. Dil. NH_3 (aq) (2 ml) was added to 2 ml of the filterate. Then 400 µl of Fehling solutions A & B was added and the mixture was boiled for 5 min. A brick red coluration was seen which confirmed the presence of glycoside.

3.3.8.7 Tannins (Ferric Chloride Method)

The sample (0.2 g) was added to 10 ml 45 % ethanol and then boiled for 5 min. The filterate (1 ml) was added to 200 µl $FeCl_3$. A blue colouration was observed which confirmed the presence of tannins.

3.3.8.8 Alkaloid

10 ml of 2% HCL was added to 0.2 g sample which was boiled for 5 min and filtered. The filterate (1 ml) was then put into two test tubes containing 1 ml of Wagner's reagent and Dragendorff's reagent respectively. No precipitate was formed.

3.3.8.9 Steroids

Acetic anhydride (2 ml) was added to 0.2 g ethanol extract of sample and then 2 ml H_2SO_4 was also added. A colour change from violet to green indicated the presence of steroids.

3.3.8.10 Terpenoid

The test sample (0.2 g) was dissolved in ethanol and then 1 ml of acetic anhydride was added followed by conc. H_2SO_4. A colour change from pink to violet indicated the presence of terpenoids.

3.3.9 Quantitative Phytochemical screening

3.3.9.1 Determination of total phenol

The Follin-Ciocalteau reagent method was used for the estimation of total phenolic extract quantities. The sample extract dilution was oxidized with Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. Different concentrations of sample extracts of the plant were prepared and then 100 µl was taken from each concentration and mixed with 0.5 ml of Folin-Ciocalteau reagent (1/10 dilution) and 1.5 ml of NA_2CO_3 2% (w/v). The blend was incubated in the dark at room temperature for 15min. The absorbance of blue-coloured solution of all samples was measured at 765 nm. The results were expressed in mg of gallic acid equivalent (GAE) per gram of dry weight of plant powders.

3.3.9.2 Determination of Total Tannins

The sample extract (1 ml) was mixed with Folin-Ciocalteau's reagent (0.5 ml), followed by the addition of saturated Na_2CO_3 solution (1 ml) and distilled water (8 ml). The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm, using UV-visible spectrophotometer. Increasing concentrations was plotted for a standard graph. The tannin content was expressed as mg tannic acid per 100 g of sample.

3.3.9.3 Determination of Total Flavonoid Content

The flavonoid content was determined by the use of a modified colorimetric method, described by Zhishen *et al*. (1999). A 0.5 ml aliquot of appropriately (2 mg/2 ml) diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2 ml of 4% NAOH solution was added to bring the final volume to 5 ml,

and then the mixture was thoroughly mixed and allowed to stand for further 15 min. Absorbance of the mixture was determined at 510 nm versus water blank.

3.3.9.4 Determination of Terpenoids

The sample (1 g) was extracted with 50 ml of ethanol and filtered. To the filtrate (2.5 ml), 2.5 ml of 5% aqueous phosphomolybdic acid solution was added and 2.5 ml of conc. H_2SO_4 was gradually added and mixed. The mixture was left to stand for 30 min and then made up to 12.5 ml with ethanol. The absorbance was taken at 700 nm.

3.3.9.5 Determination of Steroids

The sample (1 g) was extracted with 20 ml of ethanol and filtered. To the filtrate (2 ml), 2 ml of chromagen solution was added and the solution left to stand for 30min. The absorbance was read at 550 nm.

3.3.9.6 Determination of Saponins

The sample (1 g) was extracted with 10 ml of petroleum ether and decanted into a beaker. Another 10 ml of the petroleum ether was added into the beaker and the filtrate evaporated to dryness. The residue was dissolved in 6 ml of ethanol. The solution (2 ml) was put in a test tube and 2 ml of chromagen solution added into it. It was left to stand for 30 min and the absorbance was read at 550 nm.

3.3.9.7 Determination of Glycosides

The sample (1 g) was extracted with 50 ml of distilled water and filtered. To the filtrate (1 ml), 4 ml of alkaline pirate solution was added. The mixture was boiled for 5 min and allowed to cool. The absorbance was read at 490 nm.

3.3.9.8 Reducing Sugar

The sample (1 g) was extracted with 20 ml of distilled water and filtered. To 1 ml of the filtrate, 1 ml of alkaline copper reagent was added. The mixture was boiled for 5 min and allowed to cool. Then 1 ml of phosphomolybdic acid reagent and 2 ml of distilled H_2O was added and the absorbance read at 420 nm.

3.3.9.9 Determination of Soluble Carbohydrates

The sample (1 g) was extracted with 50 ml of distilled H_2O and filtered. To the 1 ml of the filtrate, saturate aqueous solution of picric acid was added and absorbance read at 580 nm.

3.3.10 Proximate Analysis of Sample

The moisture content was determined by drying at 105^0C in an oven, until a constant weight was attained. For total ash determination, the plant samples were weighed and converted to dry ash in a muffle furnace at 450 and 550^0C for incineration. The crude fat content was determined by extraction with hexane, using a Soxhlet apparatus. All these determinations were carried out according to AOAC (1990). Kjeldahl method was used for crude protein determination. Carbohydrate content was determined by calculating the difference between the sums of all the proximate compositions from 100%.

3.3.11 *In Vitro* Screening for Antioxidant Activities of Various Solvent Extracts of *Diodia sarmentosa* (Sw) Leaves using 2, 2-diphenyl-1-picrylhydrazyl Radical ($DPPH^o$) Scavenging Method.

Extracts of the plant's leaves were made using various solvents (such as hexane, methanol, ethylacetate, ethanol and water). These extracts were then screened for antioxidant activities using $DPPH^o$ method (Gyamfi *et al*. 1999). The five extracts were chosen based on the polarity index of solvents (Table 3.1).

Table 3.1: Solvent polarities for various liquids

Solvent	**Solvent Polarity Index, P**
Hexane	0.1
Carbon tetrachloride	1.56
Isopropyl ether	1.83
Toluene	2.4
Methyl-t-butyl ether	2.4
Chloroform	2.7
Diethyl ether	2.8
Dichloromethane	3.1
Isopropanol	3.92
Tetrahydrofuran	4.0
Ethyl Acetate	4.4
Methanol	5.1
Acetone	5.1
Dioxane	5.27
Acetonitrile	5.8
Water	10.2

Source: (Harris, 2015)

3.3.11.1 Procedure

The test sample (0.5g) was weighed using a sensitive weighing balance and then put into five different test tubes representing the five solvents screened.

Test tube A	Test tube B	Test tube C	Test tube D	Test tube E
Ethyl acetate	Methanol	n-hexane	Water	Ethanol
0.5 g of test sample + 10 ml of Ethyl Acetate	0.5 g of test sample + 10 ml of Methanol	0.5 g of test sample + 10 ml of n-hexane	0.5 g of test sample + 10 ml of water	0.5 g of test sample + 10 ml of Ethanol

They were left to stand at room temperature away from light for one hour and centrifuged for 5 min. The supernatant was pipetted into 3 separate test tubes of volumes 100 µl, 200 µl and 400 µl. A total of 45 test tubes were used for the five solvents, 9 for each solvent (because they were done in triplicates of 100 µl, 200 µl, and 400 µl). Each of the 36 test tubes was made up to 1ml using their respective solvents in order to have an equal volume in all the test tube (i.e. 1 ml). Then 0.5ml of 0.076 mM DPPH^{o} was used as the control and the absorbance recorded as A_0.

Thereafter, the absorbance of the assay mixture was measured at 517 nm. The respective solvents (n-hexane, methanol, Ethyl acetate and water) were used as blank before taking absorbance of the mixtures respectively; using labscience Vis spectrophotometer. Lower absorbance of the reaction mixture indicated higher radical scavenging activity.

3.3.11.2 Quantitative DPPH^{o} radical scavenging assay using the water (H_20) extract

Seven concentrations of the aqueous extract of the test sample were prepared in concentrations of 1000 µl, 500 µl, 125 µl, 62.5 µl, 31.25 µl, and 15.63 µl. Twenty-one (21) test tubes were used for this and the concentrations were in triplicate each. This was determined using the method of Gyamfi *et al*. (1999) with slight modifications. 1ml of the extract at different concentrations as stated above were diluted 2-fold (1.25-160 µg/ml) in 10ml of water and was mixed with 0.5ml of 0.076Mm DPPH^{o} in H_20. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 minutes. The negative control was 1ml of 0.076mM DPPH^{o} in H_20. L-Ascorbic acid was used as the positive control. Thereafter, the absorbance of the assay mixture was measured at 517nm. H_20 was used to blank using Labscience vis spectrophotometer. Lower absorbance of the

reaction mixture indicated higher radical scavenging activity. Percentage (%) inhibition was calculated using $(A_0 - As)/ A_0 \times 100$.

3.3.11.3 Quantitative DPPHº radical scavenging assay using the ethanol extract

Seven concentrations of the ethanol extract of the test sample was prepared in concentrations of 1000 µl, 500 µl, 125 µl, 62.5 µl, 31.25 µl, and 15.63 µl. 21 test tubes were used for this and the concentrations were in triplicate each. This was determined using the method of Gyanfi *et al*. (1999) with slight modifications. 1 ml of the extract at different concentration as stated above were diluted 2-fold (1.25-160 µg/ml) in 10 ml of ethanol and was mixed with 0.5 ml of 0.076 mM DPPHº in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 25 min. The negative control was 1ml of 0.076 mM DPPHº in H_20. L-Ascorbic acid was used as the positive control. Thereafter, the absorbance of the assay mixture was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher radical scavenging activity. Percentage (%) inhibition was calculated using $(A_0 - As)/ A_0 \times 100$. Where A_0 = Absorbance control and As = Absorbance of test sample.

3.3.11.4 Lipid Peroxidation Assay- TBARS (Thiobarbituric acid-reactive species)

A modified thiobarbituric acid-reactive species (TBARS) assay (Banerjee *et al*. 2005) was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid-rich media (Ruberto *et al*. 2000). Egg homogenate (500 µl of 10%, v/v in phosphate-buffered saline, pH 7.4) and 100 µl of sample (10-250 µg/ml) were added to a test tube and made up to 1.0 ml with distilled water. Then 50 µl of $FeS0_4$ (0.075 M) and 20 µl of L-Ascorbic acid (0.1 M) were added and incubated for 1hour at 37ºC to induce lipid peroxidation. Thereafter, 0.2 ml EDTA (0.1 M) and 1.5 ml of TBA reagent (3g TBA, 120g TCA and 10.4 ml 70% $HCL0_4$ in 800 mL of distilled H_20) were added in each sample and heated for 15 min at 100 ºC. After

cooling the samples were centrifuged for 10 min at 3000 rpm. Inhibition percentage (%) of lipid peroxidation was calculated using the equation:

% Inhibition = $(A_0 – As)/ A_0 \times 100\%$.

Where A_0 is absorbance control and As is the absorbance of test sample. Butylated hydroxytoluene (BHT) was used as standard.

3.3.11.5 Total Antioxidant Capacity (TAC) Assay

The total antioxidant capacity assay of the extract was carried out by the phosphomolybdate method. A 0.1 ml aliquot of different concentrations (15.63, 31.25, 62.5, 125, 250, 500, 1000 µl/ml) of the extract and ascorbic acid was mixed with 1ml of reagent solution (600mM sulphuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate 1:1:1). The test tubes were covered with aluminum foil and incubated in a water bath at 95ºC for 90 minutes. After the extracts were cooled to room temperature, the absorbance of the mixture was determined at 765 nm against a blank containing 1 ml of the reagent solution. Ascorbic acid was used as standard. The assay was carried out in triplicates. The total antioxidant capacity (TAC) is expressed as equivalent of ascorbic acid. The TAC was estimated using:

Conc. of Sample = (Abs sample/ Abs std) × conc. of std. Expressed as AAE mg/g.

Abs of std (Ascorbic acid) = 1.442.

Where Abs sample = Absorbance of sample, Abs std = Absorbance of standard

3.3.12 BIOCHEMICAL PARAMETERS

3.3.12.1 DETERMINATION OF PSA CONCENTRATION

The prostate specific antigen (PSA) was determined using enzyme immunoassay test kit (Pointe Scientific, Inc.).

Assay procedure:

The desired number of coated wells of microtiter was secured in the holder. Fifty microlitre (50 µl) of the serum, standard and control were dispensed into appropriate wells. Again 50 µl of zero buffer reagent was dispensed into each well. It was thoroughly mixed for 30 s, and incubated at room temperature (18-25^{0}C) for 60 min.

The incubation mixture was removed by flicking plate contents into a waste container. The microtitre wells were rinsed and flicked 5 times with distilled water. The wells were struck sharply onto absorbent paper to remove all residual water droplets. Next, 100 µl of enzyme conjugate reagent was dispensed into each well and incubated for 60 min at room temperature. At the end of the incubation period, the mixture was removed by flicking plate contents into a waste container. The microtitre wells were rinsed and flicked 5 times with distilled water. The wells were struck sharply onto absorbent paper to remove all residual water droplets. TMB reagent was dispensed into each well, gently mixed for 5 s, and incubated at room temperature for 20 min. The reaction was stopped by adding 100 µl of stop solution to each well, and then gently mixed for 30 s to ensure that all the blue colour changes to yellow completely.

The absorbance was read at 450 nm within 15 min using microplate reader (MR-9620A).

3.3.12.2 Testosterone

Testosterone was assayed using the Abnova serum testosterone kit.

Principle:

The Testosterone ELISA is based on the principle of competitive binding between testosterone in the test specimen and testosterone-horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-Testosterone.

Procedure:

Ten (10 µl) of standards, specimen and controls were dispensed into appropriate wells and 100 µl of Testosterone-HRP Conjugate Reagent into each well. Fifty (50 µl) of rabbit anti-Testosterone reagent was then dispensed into each well and mixed thouroughly for 30 s. The mixture was then incubated at 37^{O}C for 90 min. The incubation mixture was removed by flicking plate contents into a waste container. Microtiter wells were rinsed 5 times with distilled water. The wells were striked onto the absorbent paper to remove all residual water droplets. A 100 µl of TMB Reagent was dispensed into each well and mixed gently for 5 s. It was incubated at room temperature for 20min. The reaction was stopped by adding 100 µl of stop solution to each well which was gently mixed for 30 s. The absorbance was read at 450 nm with a microtiter well reader with 15min.

3.3.12.3 Progesterone

Progesterone was assayed using the NoviWell™ progesterone assay kit (HySkill Diagnostics, Bahlingen, Germany) which employs the sandwich enzyme immunoassay (SIA) microtiter method. It is a competitive enzyme immunoassay for the performance of quantitative determination of progesterone in human plasma and serum (Ratcliffe *et al*. 1988). Assays were carried out as described by the manufacturer. The assay is based on simultaneous binding of hormone to two monoclonal antibodies; one is immobilized on the microplate, the other is soluble and conjugated with horseradish peroxidase (HRP).

The reagents and samples were brought to room temperature before 50 µl standards and samples were dispensed into their respective wells. One well was left for substrate blank. After that, 50 µl of progesterone- HRP conjugate were added to each well with the exception of the one meant for substrate blank. The wells were then covered with a foil and incubated for one hour at 37^{0}C. When incubation had been completed, the foil was removed, and the

content of the wells aspirated and each well washed twice with 300 µl distilled water. Overflows from the reaction wells were avoided. The soak time between each cycle was greater than 5 s. At the end of the reaction, the remaining fluid was removed by tapping the strips on tissue paper. Afterwards, 100 µl of tetramethylbenzidine (TMB) substrate solution were dispensed into the wells and incubated for exactly 15 min at room temperature in the dark. To stop the reaction, 100 µl stop solution were dispensed into all wells in the same order and at the same rate as for the TMB substrate solution. Any blue colour developed during the incubation turned into yellow. The absorbance of the specimen was measured at 450 nm within 30 min after addition of the stop solution. The values of the samples were obtained from a graph constructed using the standards.

3.3.12.4 17β – OESTRADIOL

17β – oestradiol was assayed using the NoviWell™ 17β – Oestradiol assay kit (HySkill Diagnostics, Bahlingen, Germany) which employs the sandwich enzyme immunoassay (SIA) microtiter method. It is a competitive enzyme immunoassay for the performance of quantitative determination of 17β – Oestradiol in human plasma and serum (Ratcliffe *et al*. 1988). Assays were carried out as described by the manufacturer. The assay is based on simultaneous binding of hormone to two monoclonal antibodies; one is immobilized on the microplate, the other is soluble and conjugated with horseradish peroxidase (HRP).

Having brought the samples and reagents to room temperature, 25µl of standards and samples were dispensed into their respective wells. Following this, 100 µl oestradiol - HRP conjugate were added to each well. One well was left for substrate blank. The wells were covered with a foil and incubated for 2 hours at 37^0C. When incubation had been completed, the foil was removed, the content of wells aspirated and each well washed twice with 300 µl distilled water. Overflows from the reaction wells were avoided. The soak time between each cycle was greater than 5 s. At the end of the reaction, remaining fluid was carefully removed by

tapping strips on tissue paper. After this 100 µl of TMB substrate solution were dispensed into wells and incubate for exactly 30 min at room temperature in the dark. To stop the reaction, 100 µl stop solution was added into all wells in the same order and at the same rate as for the TMB substrate solution. Any blue colour developed during the incubation turned into yellow. The absorbance of the specimen was measured at 450 nm within 30 min after addition of the stop solution. The values of the samples were obtained from a graph constructed using the standards.

3.3.12.2 ESTIMATION OF ELECTROLYTES

DETERMINATION OF SERUM SODIUM ION (Na^+) CONCENTRATION

Serum sodium concentration was estimated using colorimetric method based on modified Maruna and Trinders method as described by Trinder (1951).

Principle: Sodium and proteins are precipitated together by Magnesium uranyl acetate as uranyl magnesium sodium acetate salt. Excess of uranyl salt reacts with potassium ferrocynide to produce a brownish color. The intensity of the color is inversely proportional to the sodium concentration in the specimen and is measured photometrically at 530 nm.

Uranyl ions + Mg^+ + Na → uranyl Mg Na precipitate

Free uranyl ions + $K_4 Fe(CN)_6$ → brown coloured complex

Procedure: Test tubes: standard and test were labeled; 1000 µl of precipitating reagent was added to each tube followed by the addition of sodium standard (10 µl) into the standard-labeled tube and 10 µl of serum into the test-labeled tube, the tubes were shaken vigorously and incubated for 5min at room temperature, then centrifuged at 2000 rpm for 2 min to obtain a clear supernatant. New test tubes: blank, standard and test were labeled; 1000 µl of sodium color reagent was added to each of the tubes, followed by the addition of standard supernatant

(20 µl) into the standard-labeled tube and 20 µl of test supernatant obtained into the test-labeled tube, 20 µl of the precipitating reagent was added into the blank, the tubes were mixed and allowed to stand for 5 min at room temperature. The absorbance of standard and test against reagent blank was read 530 nm and recorded.

$$\text{Serum sodium concentration(mmol/l)} = \frac{\text{Absorbance of blank} - \text{Absorbance of test}}{\text{Absorbance of blank} - \text{Absorbance of standard}} \text{ x Concentration of standard}$$

DETERMINATION OF SERUM POTASSIUM ION (K^+) CONCENTRATION

The concentration of serum potassium ion (K^+) was determined using the turbidometric method as described by Henry *et al*. (1974).

Principle: Na-Tetraphenylborate + $k^+ \Longrightarrow$ k-Tetraphenylborate + Na^+

The extent of turbidity is proportional to the potassium concentration and is measured photometrically at 578 nm.

Procedure: Test tubes; standard and test were labeled, 1000 µl of potassium reagent was added to each test tube followed by the addition of standard (25 µl) into the standard-labeled tube and 25 µl of serum into the test-labeled tube; the tubes were shaken and incubated for 5 min at room temperature. The spectrophotometer was zeroed using distilled water at 578 nm and the absorbance of the tubes (standard and test) were read and recorded within 10min.

$$\text{Potassium concentration(mmol/l)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Concentration of standard}$$

ESTIMATION OF CHLORIDE (Cl^-)

The chloride was estimated according to the method described by Tietz (1976).

Principle:

$Hg(SCN)_2 + 2Cl^{2-}$ —>$HgCl_2$ + 2SCN-3SCN- + Fe_2 + —> $4Fe(SCN)_3$ red complex.

Procedure

Into different tests tubes, 10 µl of sample and standard chloride calibrator were pipetted. Again 1 ml chloride reagent was added into each test tube, mixed and then incubated at 25°C for 5 min. The absorbance was taken at 500 nm.

Calculations

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of std}} \times \frac{\text{Concentration of std}}{1} = \text{Chloride concentration (mmol/l)}$$

ESTIMATION OF BICARBONATE (HCO_3)

Bicarbonate was estimated according to the method described by Tietz *et al*. (1986).

Principle

Phosphoenol pyruvate carboxylase (PEPC) catalyzes the reaction between phosphoenol pyruvate and carbon dioxide (bicarbonate) to form oxaloacetate and phosphate ion. Oxaloacetate is reduced to malate with simultaneous oxidation of an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) to NAD; the reaction is catalyzed by malate dehydrogenase (MDH). This results in a decrease in absorbance at 340 nm that is directly proportional to bicarbonate concentration in the sample

Procedure

Tubes were labeled Blank, Standard and Test as 1.0 mL of bicarbonate reagent was pipetted into each test tube and then incubated for 3 min at 37^0C. The absorbance was taken at 340 nm.

Calculation

$$\frac{\text{Abs of Blank-Abs of sample}}{\text{Abs of standard}} \times \frac{\text{concentration of standard}}{1} = \text{Bicarbonate conc. (mmol)}$$

3.3.12.3 DETERMINATION OF UREA

Urea level was determined using Urease Berthelot according to Fawcett & Scott, (1960) as described in Randox commercial kit.

Principle

Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot reaction.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

NH_3 + hypochlorite + phenol → Indophenol (blue compound)

Procedure

Test tubes were labeled as blank, standard and sample, and pipetted into test tubes as follows:

	Blank	Standard	Sample
Sample	----	----	10µl
Standard (CAL)	----	10µl	----
Distilled water	10µl	----	----
Reagent 1	100µl	100µl	100µl

The above was mixed and incubated at 37^{o}C for 10 minutes

Reagent 2	2.50ml	2.50ml	2.50ml	
Reagent 3	2.50ml	2.50ml	2.50ml	

The above was immediately mixed and incubated at 37^{o}C for 15 minutes.

The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) were read against the blank.

Calculation

$$\text{Urea Concentration} = \frac{A_{sample}}{A_{standard}} \times \text{Standard concentration (mmol/l)}$$

3.3.12.4 DETERMINATION OF CREATININE

Creatinine level was determined using Direct Endpoint according to Henry *et al*. (1974) as described in Randox commercial kit.

Principle

Creatinine reacts with picric acid in alkaline conditions to form a colour complex, which absorbs at 510nm. The rate of formation of colour is proportional to the creatinine

concentration in the sample. In the endpoint method the difference in absorbance measurements after colour formation yields a creatinine value corrected for interfering substances.

Procedure

The test vial, reagent blank, standard, control, and sample test tubes were first labelled and 3.0ml of working reagent was pipetted into test tubes. Furthermore, 0.1 ml (100 µl) of sample was transferred to their respective tubes, distilled water to reagent blank and mix. All tubes were now placed in 37^0C heating bath for 15 min. The spectrophotometer wavelength was set at 510 nm and zeroed using the reagent blank.

Calculations

The Creatinine value of unknown (sample) was determined by comparing its absorbance change with that of a known standard.

$$\text{Mg/dl} = \frac{A_{sample}}{A_{standard}} \times \text{Concentration of Standard}$$

3.3.12.5 URIC ACID (UA)

Serum uric acid was assayed using a Cromatest^{TM} (Barcelona, Spain) uric acid test kit. This is an enzymatic colorimetric method (Barham & Trinder, 1972a; Tamaoku *et al*. 1982). The reagents and samples were brought to room temperature; 1ml of monoreagent was pipetted into test tubes labelled blank, samples and standard and 25 ul of samples and standard added into respective test tubes and mixed well and incubated for 5 min at 37^0C. The absorbance of the standard and the samples were read at 550 nm against the reagent blank. The uric acid concentrations of the samples were calculated as follows:

(Absorbance of Sample/Absorbance of Standard) x Conc. of Standard = Uric acid Conc. of sample

3.3.12.6 Gamma Glutamyl Transferase (GGT)

Principle

GGT was assayed using a kit from JASTH (JAS Diagnostics Incorporated, MiamiFlorida). The JAS™ method is based on the kinetic photometric test, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Shaw *et al*. 1983). GGT in the sample catalyses the transfer of the glutamyl group from L-gamma-glutamyl-3-carboxy-4-nitroanilide to glycylglycine. The amount of 5-amino-2-nitrobenzoate formed is proportional to GGT activity and may be measured kinetically at 405 nm by the increasing intensity of the yellow color formed.

L- γ-glutamyl-3-carboxy-4-nitroanilide + Glycylglycine $\xrightarrow{\text{Gamma-GT}}$ L-γ-glutamyl-glyclglycine + 5-amino -2-nitrobenzoate

Procedure

The reagents, standards and samples were brought to room temperature by placing them on the work bench at room temperature for 30 min before 1000 µl of working reagent was pipetted into test tubes labelled blank and sample respectively. Into the test tube labelled blank, 100 µl of distilled water was added and 100 µl of sample pipetted into the test tube labelled sample and mixed thoroughly and the initial absorbance at 405 nm wavelength and at 25^0C read against reagent blank. The absorbance was read again after 1, 2, and 3 min and the change in absorbance per minute (ΔAbs) over the course of the reaction calculated. The concentration of alkaline phosphatase in the sample was calculated as follows:

GGT Conc. = ΔAbs x 2737 U/L

3.3.12.7 Aspartate amino transferase (AST) or Serum Glutamyl Oxaloacetic Transaminase (SGOT)

Principle

AST analysis was done according to the methodology recommended by International Federation of Clinical Chemistry (IFCC, 1986). The principle of test involves AST catalyzing the transfer of the amino group from aspartate to 2- oxoglutarate, forming oxalacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the malate dehydrogenase (MDH) coupled reaction. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

L-Aspartate + 2-oxoglutarate $\xrightarrow{\text{AST}}$ Oxaloacetate + L-Glutamate

Oxaloacetate + NADH $\xrightarrow{\text{MDH}}$ L-Malate + NAD^+

Procedure

The reagents and samples were brought to room temperature by placing them on the work bench at room temperature for 30 min before 1000 µl of the working reagent was pipetted into test tubes labelled; blank and sample respectively. Into the test tube labelled blank, 100 µl of distilled water was added and 100 µl of the sample was pipetted into the test tube labelled sample and mixed thoroughly and the initial absorbance read at 340 nm wavelength and at 30^0C. The absorbance was read again after 1, 2, and 3 min and the change in absorbance per

minute (ΔAbs) over the course of the reaction calculated. The concentration of AST in the sample was calculated as follows:

AST Conc. = ΔAbs x 1746 U/L

3.3.12.8 Alanine amino transferase (ALT)

Principle

ALT analysis was done using a reagent kit from JAS^{TM} diagnostics. The procedure is based on the methodology of International Federation of Clinical Chemistry as described by (Bergmeyer & Horden, 1980). The principle involves ALT catalysing the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the lactate dehydrogenase (LDH) coupled reaction. Endogenous sample pyruvate is rapidly and completely reduced by LDH during the initial incubation period so that it does not interfere with the assay.

L-Alaninine + 2-Oxoglutarate $\xrightarrow{ALT}$ Pyruvate + L-Glutamate

Pyruvate + NADH $\xrightarrow{LDH}$ L-Lactate + NAD^+

Procedure

The reagents and samples were brought to room temperature by placing them on the work bench at room temperature for 30 min after which 1000 µl working reagent was pipetted into test tubes labelled blank and sample respectively. Into the test tube labelled blank, 100 µl of distilled water was added and 100 µl of sample was pipetted into the test tube labelled sample and mixed thoroughly and the initial absorbance read at 340 nm wavelength and at 30^0C. The absorbance was read again after 1, 2 and 3 min and the change in absorbance per minute (ΔAbs) over the course of the reaction calculated. The concentration of ALT in the sample was calculated as follows:

ALT Conc. = ΔAbs x 1746 U/L

3.3.12.9 Alpha feto protein

Alpha-fetoprotein (AFP) was assayed using AccupBind ELISA Mircowells immunoenzymometric assay (Type 3) (Monobind Inc. Lake Forest, CA 92630, USA). In this method, AFP calibrator, patient serum or control was first added to a streptavidin coated well. Biotinylated and enzyme labelled monoclonal antibodies (directed against distinct and different epitopes of AFP) were added and the reactants mixed. Reaction between the various AFP antibodies and native AFP formed a sandwich complex that binded with the streptavidin coated to the well. After the completion of the required incubation period, the enzyme-AFP conjugated by aspiration. The activity of the enzyme present on the surface of the well is quantified by reaction with a suitable substrate to produce colour. The absorbance was read at 450 nm (using a refrernce wavelength of 620-630 nm to minimize well imperfections) in a microplate reader. The results were read within 30 min of adding the stop solution (one bottle containing a strong acid (1N HCL)).

3.3.12.10 Total Protein

Principle

Protein was assayed using the JAS^{TM} diagnostic protein test kit. This is a colorimetric method based on the Biuret reaction. The method is based on the modification of (Gornall *et al*. 1949). Protein in serum forms a blue coloured complex when reacted with cupric ions in an alkaline solution. The intensity of the violet colour is proportional to the amount of protein present when compared to a solution with known protein concentration.

$$\text{Protein} + Cu^{2+} \xrightarrow{\text{Akali}} \text{Coloured complex}$$

Procedure

The reagents and samples were brought to room temperature after which 1.0 ml of Biuret reagent were dispensed into test tubes labelled blank, samples and standard. Then, 20 µl of

samples and standard were added into their respective test tubes and mixed and let to stand for 10 min at room temperature. The absorbance of the samples and the standard were read at 540 nm against the reagent blank. The concentrations of the samples were calculated as follows:

(Absorbance of Sample/ absorbance of standard) × Concentration of std = Protein conc. of sample

3.3.13 *IN VIVO* ANTIOXIDANT ACTIVITY

3.3.13.1. ESTIMATION OF CATALASE

Principle

The activity of catalase was assayed by the method of Sinha (1972). Dichromate in acetic acid was reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The chromic acetate formed was measured at 570 nm. Catalase was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H_2O_2 was determined by measuring chromic acetate colorimetrically.

Procedure

To 0.9 ml of distilled water and 0.1 ml of plasma in a test tube was added 2 ml of H_2O_2 and 2 ml phosphate buffer. The reaction was initiated by adding 2 ml of dichromate acetic acid reagent to 1 ml portion of this mixture. Absorbance of the reaction was taken in 30 s interval for 2 min. The activity of catalase was expressed as U/ml of plasma (U- micromoles of H_2O_2 utilised / s).

3.3.13.2 ESTIMATION OF GLUTATHIONE PEROXIDASE (GP_X)

This was done according to the method of Paglia and Valentine (1967). Glutathione Peroxidase (GP_X) catalyses the oxidation of Glutathione (GSH) by CumeneHydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH and $NADP^{+.}$ The decrease in absorbance at 340 nm is measured.

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_20$$

$$GSSG + NADPH + H^+ \longrightarrow NADP+ + 2GSH$$

The requirements were the following:

1. Reagent 1a (Glutathione+ Glutathione Reductase+NADPH)
2. Reagent 1b (Phosphate buffer+EDTA)
3. R2 Cumene Hydroperoxide
4. R3 Diluting Agent

Procedure

A known volume, 0.05 ml of heparinised whole blood was diluted with 2 ml of diluting reagent and this was used for the assay. Measured volume (50 µl) of diluted sample was mixed with 1 ml of R1 and R2 respectively. The initial absorbance of both test and blank were read after 1 min and the timer started simultaneously. Absorbances were read again after 1 and 2 min intervals at 340 nm.

Glutathione Peroxidase activity was calculated from the formula below.

U/L of haemolysate = 8412 x ΔA 340 nm/min.

3.3.13.3 ESTIMATION OF LIPID PEROXIDATION (MALONDIALDEHYDE)

Lipid peroxidation was estimated by measuring spectrophotometrically the level of the lipid peroxidation product, malondialdehyde (MDA) as described by Wallin *et al.* (1993).

Lipid degradation occurs forming such products as malondialdehyde (from fatty acids with two or more double bonds), ethane and pentane (from the n-terminal carbons of 3 and 6 fatty acids, respectively). MDA reacts with thiobarbituric acid to form a red or pink coloured complex which in acid solution absorbs maximally at 532 nm.

Procedure

Measured volume (0.1 ml) of the serum was mixed with 0.9 ml of distilled water in a test tube. Again 0.5 ml of 25% TCA (trichloroacetic acid) and 0.5 ml of 1% TBA (thiobarbituric acid) in 0.3% NaOH were added to the mixture. The mixture was boiled for 40 minutes in water-bath and cooled in cold water and 0.1 ml of 20% sodium dodecyl sulfate (SDS) added to the cooled solution and mixed properly. The absorbance was taken at wavelength 532 nm and 600 nm against a blank.

$$\text{MDA} = \frac{\text{A532- A600 x 100}}{\text{0.5271 x 0.1}} \quad \text{(mg/dl)}$$

3.3.13.4 ESTIMATION OF SUPEROXIDE DISMUTASE

Principle

Superoxide dismutase activity was assayed by the method of Arthur and Boyne (1985) as contained in Randox kit. The method employs xanthine and xanthine oxidase to generate superoxide radicals which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-

phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of .IN.T under the conditions of the assay.

Procedure

To a 0.05 ml diluted sample in a test tube was added 1.7 ml mixed substrate solution and mixed. Xanthine oxidase (0.25 ml) was added and the initial absorbance taken after 30 s. The final absorbance was taken after 3 min and units of SOD per gram haemoglobin was extrapolated from a standard curve.

3.3.13.5 TOTAL ANTIOXIDANT CAPACITY (TAC)

TAC was evaluated using the method of Prieto *et al*. (1999) with slight modifications. Ascorbic acid concentrations of 0.01, 0.05, 0.10, 0.15 mg/ml were used as the standard drug. To 1 ml of homogenate in a test tube, 0.3 ml of reagent solution was added and the resulting mixture incubated at 95^0C for 90 min. After the mixture was cooled to room temperature, the absorbance of each test tube was measured against a blank at 695 nm.

3.3.13.6 ESTIMATION OF VITAMIN E (ALPHA TOCOPHEROL)

Principle

Vitamin E content was estimated by the method of Pearson & Cox (1976). This method involves the conversion of ferric ions to ferrous ionsby α-tocopherol and the formation of red coloured complex with α-dipyridyl. Absorbance of chromophore was measured at 520 nm in thespectrophotometer.

Procedure

To 0.5 ml of serum, 1.5 ml of ethanol was added and mixed. To this was added 1.0 ml of αα-dipyridyl solution and 1.0 ml of ferric chloride solution and mixed. The colour developed was

read at 520 nm in the spectrophotometer.Values were read as mg/dl of serum from a standard curve.

3.3.13.7 REDUCED GLUTATHIONE (GSH) (Moron *et al*. 1979)

Principle:

The method is based on reaction of reduced glutathione (GSH) with 5-5' dithiobis-2-nitrobenzoic acid (DTNB) to give a compound that absorbs light at 412 nm.

Procedure:

To 400 µl of homogenate, 100 µl of 25% trichloroacetic acid was added; centrifuged, and supernatant used as sample. To 2.0 ml of 0.6 mM DTNB in 0.2 M sodium phosphate (pH 8), was 0.1 ml of sample and 0.9 ml of 0.2 M phosphate buffer and the absorbance read at 412 nm against a reagent blank. The standards (0.05-5 mg/ml) were also treated in the same way.

3.3.13.8 Estimation of Ascorbic Acid

Principle

Ascorbic acid (vitamin C) was assayed using the micro techniques of clinical chemistry developed by Samuel Natelson in 1961 (Natelson, 1961). Ascorbic acid in plasma is oxidized by Cu^{+2} to form dehydroascorbic acid, which reacts with acidic 2,4- dinitrophenylhydrazine to form a red dishydrazone, which is measured at 520 nm. Ascorbic acid should be analyzed immediately or not latter than 3 h if the specimen is refrigerated.

Procedure

To start the test, 0.4 ml of serum was added rapidly to 1.6 ml of 10% trichloroacetic acid and mixed well and allowed to stand at room temperature for five minutes. The mixture was centrifuged at 2000 rpm for five minutes. One millitre of the supernatant was pipetted into the test tube for test. Then, 0.4 ml of dinitriphenyl hydrazine reagent was pipetted into three test tubes; labelled sample, standard, and blank; and 1ml of sample and standard added to the respective test tubes. One millitre of trichloroacetic acid was added to the test tube labelled

blank. The test tubes were stoppered and incubated at 37^0C for three hours. The mixture was then chilled in ice bath after which 1.6 ml of cold 65% H_2SO_4 was added and mixed well. The mixture was allowed to stand for 30 min at room temperature, and the absorbance of the standard and test read against blank at 520 nm in a spectrophotometer. The concentration of ascorbic was calculated as follows:

(Absorbance of Sample/ Absorbance of Standard) X Conc. of Standard = Vit. C conc. of sample

3.4 HISTOPATHOLOGY

Representative samples of the prostate and liver were collected from the benign prostatic hyperplasia and hepatocellular carcinoma group respectively, and then preserved in 10% neutral buffered formalin while representative samples of the uterus were collected from the Uterine Leiomyoma group and preserved in Bouin's fluid. These samples were processed using histological techniques and stained by routine H&E stain for histopathological examinations following standard protocol.

3.5 DATA ANALYSIS

Statistical Package for Social Sciences (SPSS), version 23, was used for data analysis. Results were expressed as mean ± standard deviation and tests of statistical significance were carried out using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test for multiple comparisons. Statistical significance was defined as $P < 0.05$. Correlation coefficient between analytes was calculated using Pearson's correlation coefficient at 95% confidence interval.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 *In vitro* Studies

Phytochemical analyses of *Diodia sarmentosa* leaves extract revealed the presence of phytochemicals such as saponin, flavonoid, carbohydrate, phenol, reducing sugar, glycoside, tannin, steroid and terpenoid. However, alkaloid was absent. The presence of saponin suggest that the leaves have antifungal properties. Flavonoids have been reported to possess anti-tumour properties. Carbohydrate content in the leaves reveals that the plant can be a good source of carbohydrate and may be edible. Phenols act as anti-inflammatory and can also function as hormone modulators. Glycosides are known for their ability to lower blood pressure. Presence of tannins suggest the application of the leaf extracts in wound healing and as anti-ulcers. Steroids reveal the anti-bacterial properties of the leaves extract. The results of the phytochemical analyses are presented in Table 4.1

Table 4.1: Qualitative Phytochemical Analysis of *Diodia sarmentosa* (Sw) leaves extract

Phytochemical	Observation	Bioavailability
Saponin	Persistent foaming	++
Flavonoid	A yellowish colouration	++
Carbohydrate	Brown ring formation at the interface	++
Phenol	A greenish colouration	+++
Reducing Sugar	A brick red precipitate	+++
Glycoside	A brick red colouration	+++
Tannin	A blue black colouration	+++
Steroid	Colour change from violet to green	+++
Terpenoid	Colour change from pink to violet	+++
Alkaloid	No ppt formed	ND

KEY:

+	Slightly available
++	Moderately available
+++	Very much available
ND	Not dectected

Quantitative phytochemical screening of the phytochemicals revealed a very high quantity of carbohydrate (3223±3.95) and reducing sugar (2410.87±6.15) in *Diodia sarmentosa* leaf extracts. This suggest that the leaf extracts have high energy content and may be edible. High quantities of phenol (1121.02±5.67), a potent anti-inflammatory phytochemical, was also observed. Relatively high quantitities of flavonoid (320.15±1.83) and terpenoids (149.41±3.64), potent anti-inflammators were observed. The results of the quantitative phytochemical constituents of *Diodia sarmentosa* leaves extract are shown in Table 4.2.

Table 4.2: Quantitative Phytochemical Analysis of *Diodia sarmentosa* (Sw) leaves

Phytochemical	**Mean ± std (mg/100 g)**
Total Phenol	1121.02 ± 5.67
Total Tannins	64.68 ± 1.08
Total Flavonoid Content	320.15 ± 1.83
Terpenoids	149.41 ± 3.64
Steroids	20.84 ± 0.13
Saponins	5.07 ± 0.86
Glycosides	50.38 ± 0.16
Reducing Sugar	2410.87 ± 6.15
Soluble Carbohydrates	3223 ± 3.95

The proximate analysis of *Diodia sarmentosa* (Sw) leaves evaluated the moixture content, ash content, crude fat, crude fibre, crude protein and carbohydrate content. Carbohydrate (59.07%) was noted to be the highest nutrient content, an indicator that the leaves can be a good source of carbohydrate. The moisture content (10.66%) was the second highest parameter, a determinator of the shelf life and the viability of microorganisms' growth. The crude protein (8.57%) which mediates cell responses and serves as enzymatic catalyst was the third highest parameter. The crude fibre (7.8%) was the fourth highest parameter, an indication that *Diodia sarmentosa* (Sw) leaves contain a proportion of Cellulose, Hemicellulose and Lignin. There was also a good proportion of the ash content (7.50%), which is a reflection of the mineral constituents embedded in the leaves. The crude fat (6.40%), which is the universally stored form of energy in living organisms was the least in quantity. The results of the proximate analysis are shown in Table 4.3.

Table 4.3: Proximate Analysis of *Diodia sarmentosa* (Sw) leaves

Parameter	**Quantity (%)**
Moisture Content	10.66
Ash Content	7.50
Crude Fat	6.40
Crude Fibre	7.80
Crude Protein	8.57
Carbohydrate	59.07

4.1.2 *In vitro* Antioxidant Activities of Ethanol and Aqueous Extracts of *Diodia sarmentosa* leaves (Sw)

The results of the 2, 2-diphenyl-1-picrylhydrazyl Radical ($DPPH^0$) of ethanol and aqueous extracts of *Diodia sarmentosa* (Sw) leaves are presented in figure 4.1. A graph of inhibition (%) was plotted against DPPH concentration (μg/ml). The Pearson's correlation coefficients between radical scavenging activity of the aqueous extract (DPPHa) and its concentration showed a significant negative correlation ($r = -0.955$, $p<0.05$) while a positive correlation ($r = 0.704$, $p<0.05$) was observed between the radical scavenging activity of the ethanol extract (DPPHe) and its concentration. This implies that as the DPPH concentration of the aqueous extract increased, the activity reduced while for the ethanol extract, the DPPH radical scavenging activity increased with increasing concentration.

When compared with the ascorbic acid standard (DPPHa (std)), there was a negative correlation ($r = -0.511$, $p<0.05$) between the radical scavenging activity of the aqueous extract and the standard and a positive correlation ($r = 0.257$, $p<0.05$) was observed between the ethanol extract and the standard. That is to say, as DPPH activity of the ascorbic acid increased, the DPPH activity of the aqueous extract reduced while the DPPH activity of the ethanol extract increased with increasing activity of the standard.

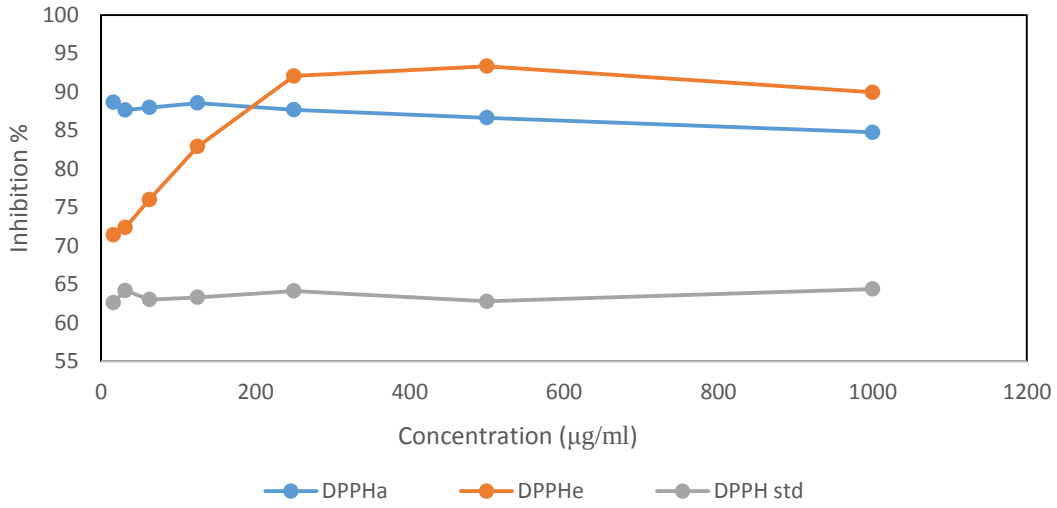

Figure 4.1: Quantitative DPPHº scavenging assay of the aqueous and ethanol extract of *Dioda sarmentosa* (Sw) leaves

DPPHa =DPPH of aqueous extract; DPPHe= DPPH of ethanol extract; DPPH std= DPPH of ascorbic acid standard

The Thiobarbituric Acid Reactive Substances (TBARS) of the aqueous and ethanol extract of *Diodia sarmentosa* (Sw) leaves extract (Figure. 4.2) was obtained by plotting a graph of inhibition (%) against concentration (μg/ml). Pearson correlation coefficient revealed a significant positive correlation ($r = 0.553$, $p<0.05$) between the lipid peroxidation activity of the aqueous extract (TBARSa) and its concentration (that is to say, the lipid peroxidation activity of the aqueous extract increased with increasing concentration). A significant negative correlation ($r = -0.834$, $p<0.05$) was observed between the lipid peroxidation activity of the ethanol extract (TBARSe) and its concentration (the lipid peroxidation activity decreased with increasing concentration). When compared with the BHT standard, there was a significant positive correlation ($r = 0.824$, $p<0.05$) between the aqueous extract and the BHT standard (this indicates similar degree of association between their activity and their respective concentrations). A negative correlation ($r = -0.526$, $p<0.05$) was observed between the ethanol extract and the BHT standard.

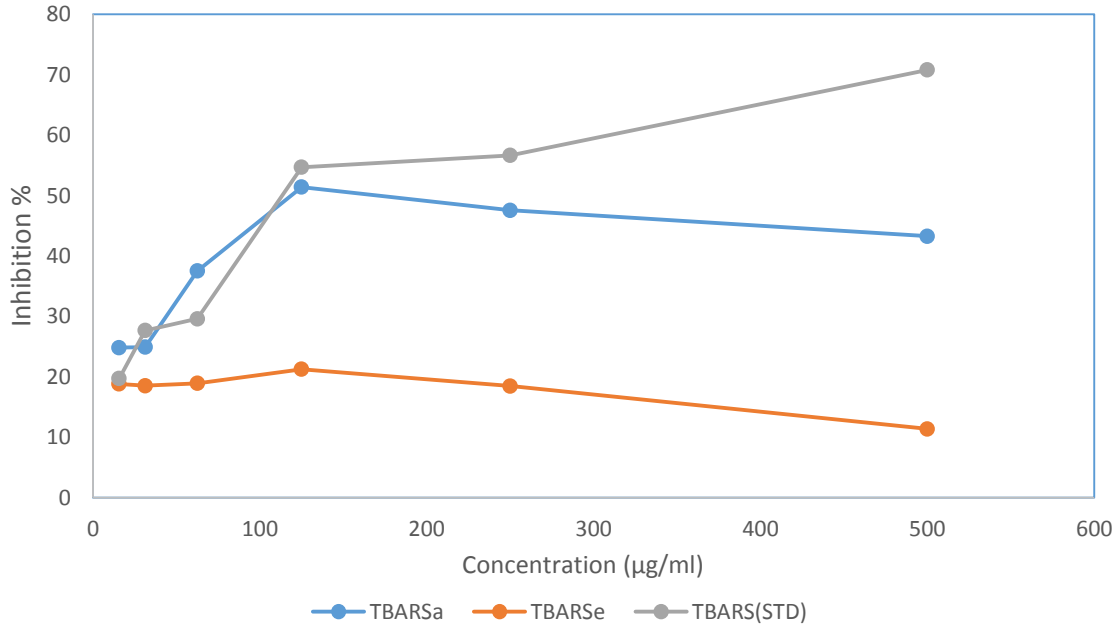

Figure 4.2: Thiobarbituric acid-reactive substances (TBARS) Assay of the aqueous and ethanol extract of *Diodia sarmentosa* (Sw) leaves

TBARSa = TBARS for aqueous extract; TBARSe= TBARS for ethanol extract; TBARS (std) = TBARS for BHT standard

The results of the Total Antioxidant Capacity (TAC) is shown in Figure 4.3. Person's correlation coefficient reveals a significant positive correlation ($r = 0.987$, $p<0.05$) between the total antioxidant capacity of the aqueous extract and its concentrations. Positive correlation ($r = 0.424$, $p<0.05$) was also observed for the ethanol extract. All ascorbic acid equivalent (AAE) values were greater than 1 showing good antioxidant capacity.

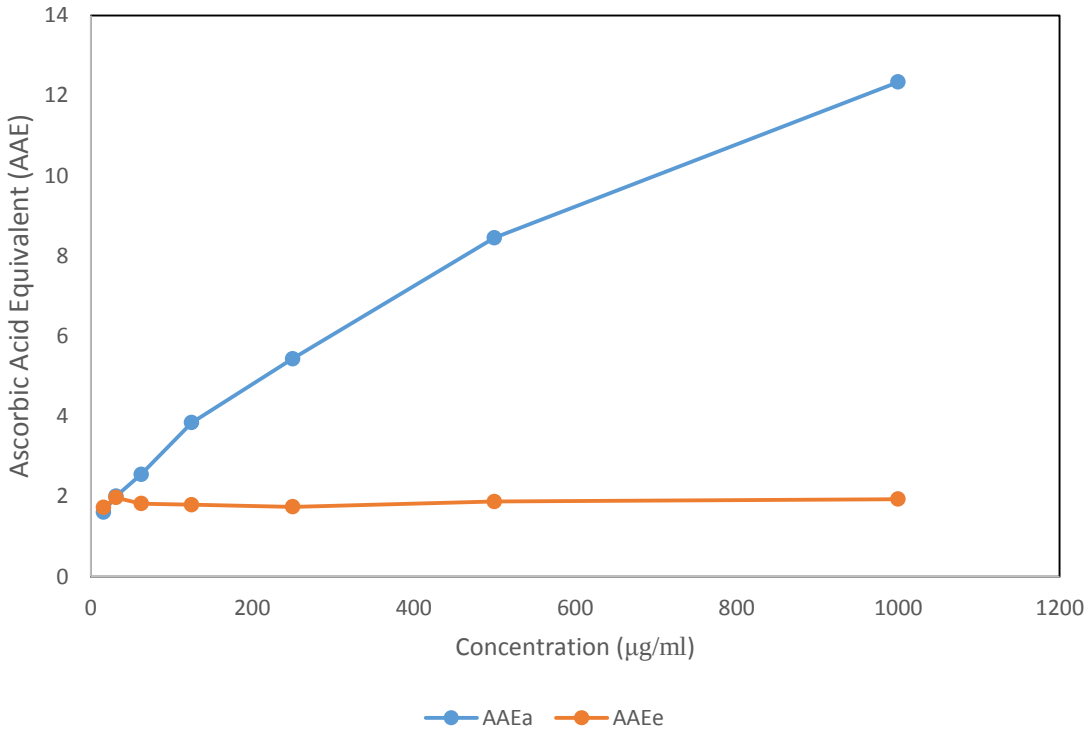

Figure 4.3: Total Antioxidant Capacity of the aqueous and ethanol extract of *Diodia sarmentosa* (Sw) leaves

AAE= Ascorbic acid equivalent; AAEa= Ascorbic Acid Equivalent of aqueous extract; AAEe= Ascorbic Acid Equivalent of ethanol extract

4.1.3 *In Vivo* Studies

4.1.3.1 Uterine Leiomyoma

The results of the biochemical parameters of the uterine leiomyoma animal study group is displayed in **Figure 4.4**. The biochemical parameters analysed include: Urea, Creatinine, Potassium (k), Chloride (Cl), Bicarbonate (HCO_3), Sodium (Na), Uric acid (UA), Total protein (TP), Estradiol and Progesterone. The values of chloride (Cl) and sodium (Na) were divided by 10 to accommodate other very low values on the graph. There are three (3) controls in this study namely: the **Normal Control** (**NC-1**) (this is the group of animal without induction, given water and normal rat chew); the **Positive Control (PC-1)** (this is the group of animals that were induced with uterine leiomyoma using monosodium glutamate (MSG) but not given treatment) and finally, the **Treated Group (TG-1)** (this is the group of animals that were induced and then treated with 400mg/kg body weight of ethanol extracts of *Diodia sarmentosa* leaves for 30 days).

Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different. Example: for the first parameter (Urea); there was a significant ($P<0.05$) increase in serum levels of the positive control (PC-1) compared to the normal control (NC-1) and there was a significant ($P<0.05$) decrease in the treated group (TG-1) compared to the positive control (PC-1), hence the different alphabets (a, b and c), while in bicarbonate ($HC0_3$), there was no significant difference in serum levels of positive control (PC-1) compared to normal control (NC-1) and there was no significant difference in serum levels of the treated group compared to the positive control (PC-1). Hence they share the same alphabet (a).

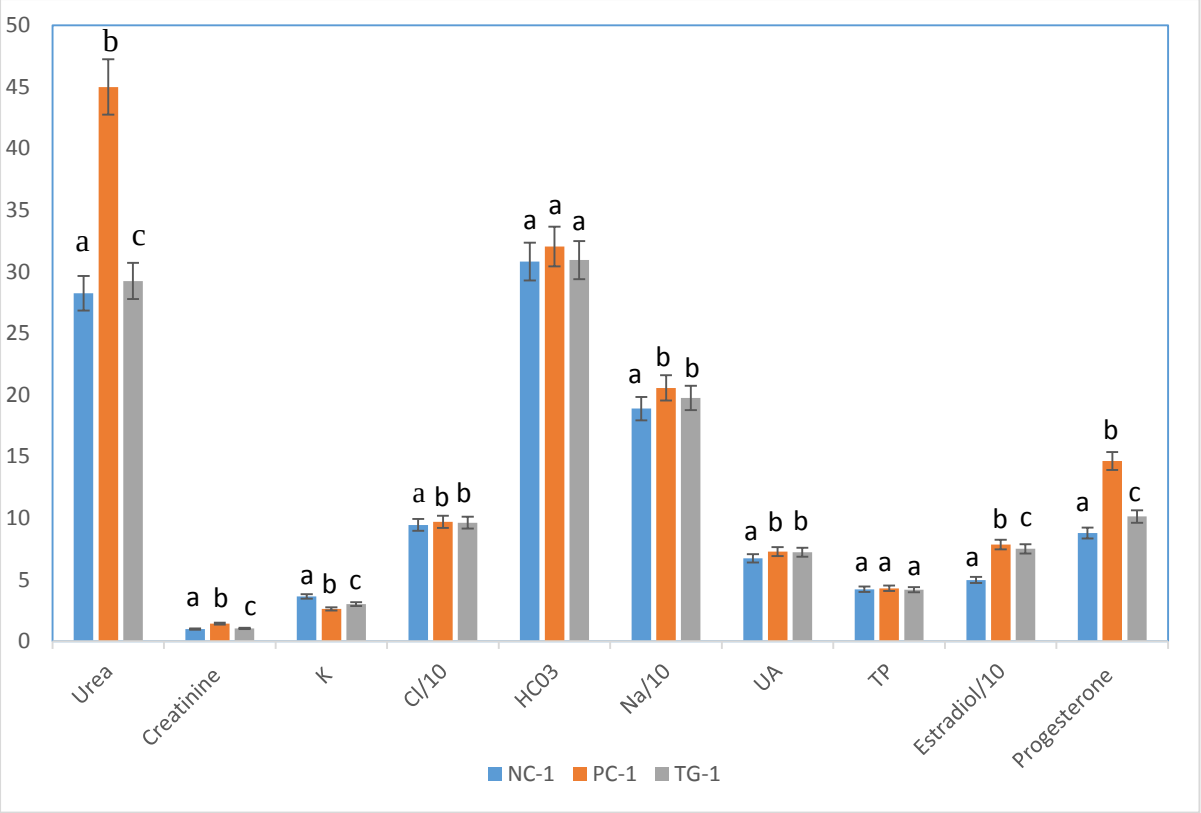

Figure 4.4: Biochemical Parameters of the Uterine Leiomyoma animal study group

Values are expressed as Mean ± Standard deviation (n=4).

K: potassium, Cl: chloride, HCO_3: bicarbonate, Na: sodium, UA: uric acid, TP: total protein

The oxidative stress and antioxidant parameters analysed for the uterine leiomyoma animal study group include: malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), vitamin E (Vit. E), catalase, glutathione (GSH), Vitamine C (Vit. C) and total antioxidant capacity (TAC). The three (3) controls in this study are the same as that used for the biochemical parameters where NC-1 (is the Normal Control), PC-1 (is Positive Control), TG-1 (is Treatment Group).

Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different. The results of the oxidative stress and antioxidant parameters assayed are displayed on **Figure 4.5.**

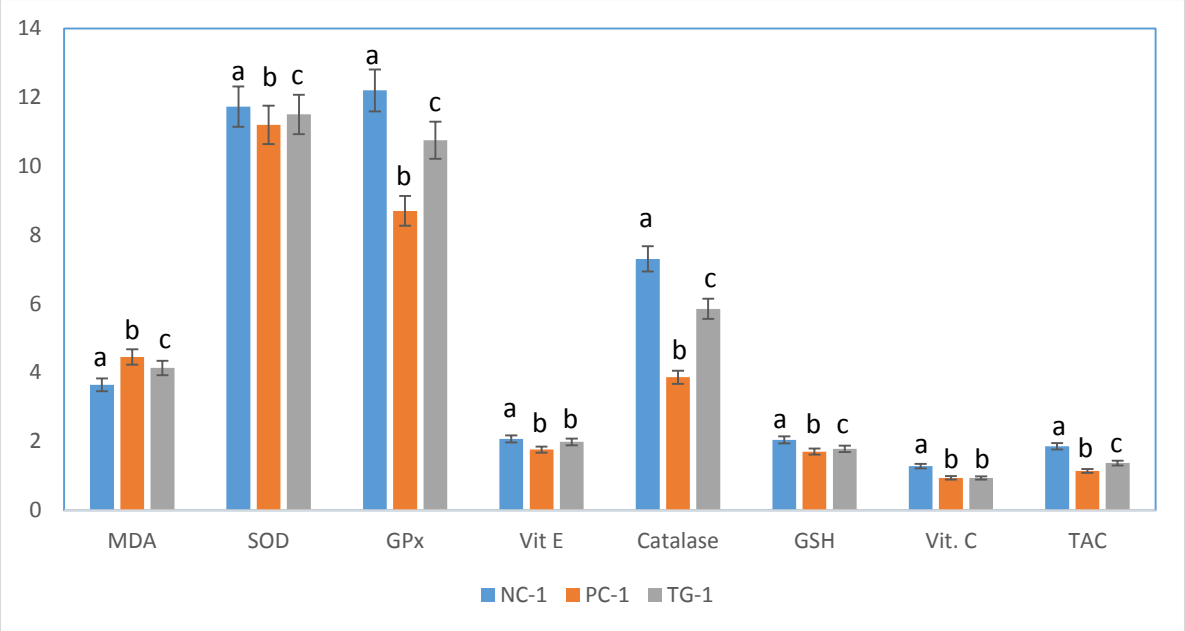

Figure 4.5: Oxidative Stress and Antioxidant Parameters of Uterine Leiomyoma

Values are expressed as Mean ± Standard deviation (n=4).

MDA: malondiadehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; Vit. E: vitamin E; GSH: glutathione; Vit. C: vitamin C; TAC: total antioxidant capacity

4.1.3.2 Histopathology of the Uterus

The photomicrograph of a portion of the uterus of the Normal Control (NC-1) for the Uterine leiomyoma animal study group is displayed on **Plate 4.1.** This shows a normal histologic architecture of the uterus revealing absence of any form of induction. The endometrium (En) with the epithelium (Ep) of the endometrial mucosa is seen resting on relatively straight basement membrane with its mucosal surface facing the uterine lumen (L) and thrown into the longitudinal folds. The extensive lamina propia bears the glands (Gl) in the normal control. The blood vessels (BV) are also indicated. Haemotoxylin and Eosin (H&E) staining was used. The photomicrograph in **Plate 4.1** is at a high maginification of X400.

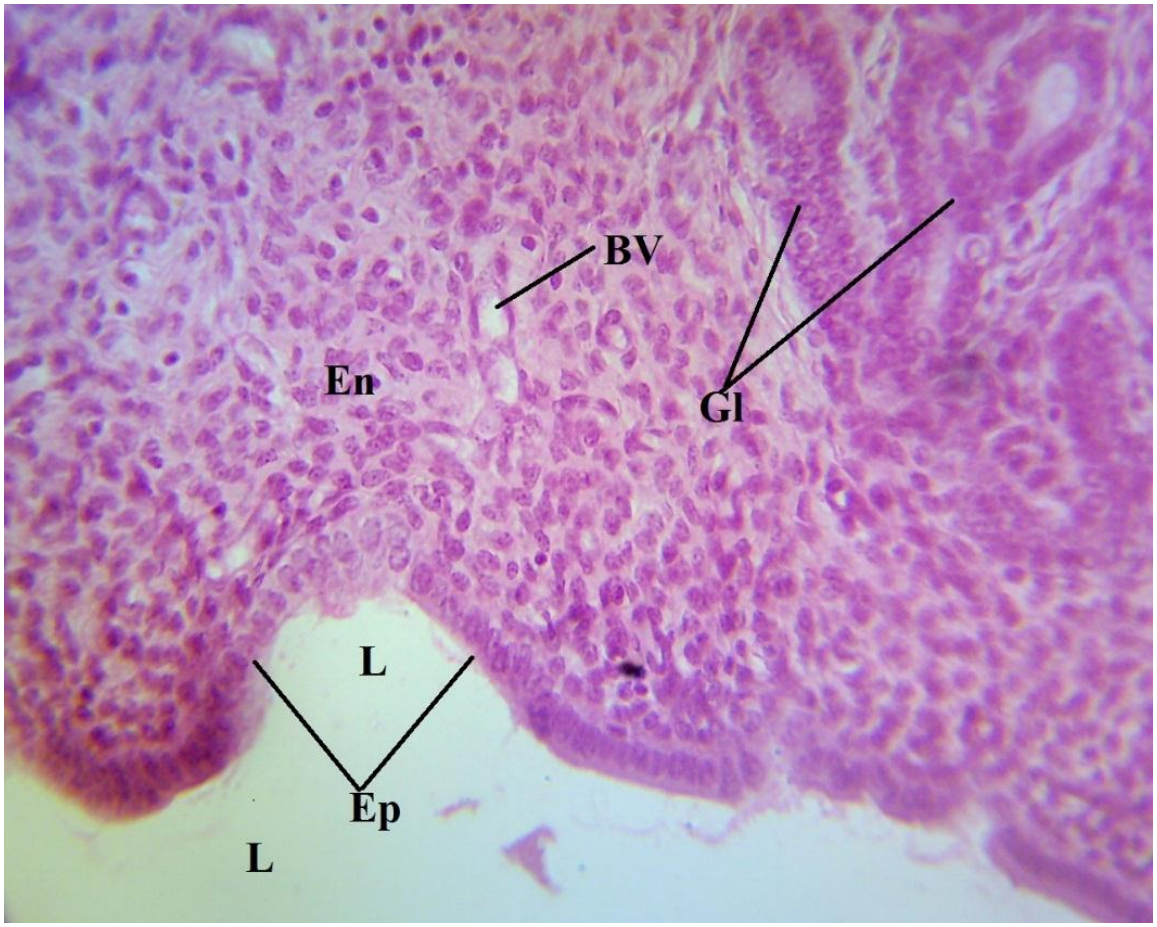

Plate 4.1: Photomicrograph of a portion of the uterus of the Normal Control (NC-1) for the Uterine Leiomyoma animal study group showing normal histologic architecture H&E X400

En: endometrium; Ep: epithelium; L: lumen; Gl: glands; Bv: blood vessels

A photomicrograph of a portion of the uterus of the positive control (PC-1) is displayed on **Plate 4.2**. This is the group induced with uterine leiomyoma using monosodium glutamate but not given treatment. The photomicrograph reveals densely packed spindle shaped fibrous tissue (F) and multifocal tumour cells (arrow) in the endometrium. This confirms a successful induction of uterine leiomyoma (uterine fibroid) in the positive control. The myometrium (My), perimetrium (arrow head) and the glands (Gl) are also shown. H&E, X400.

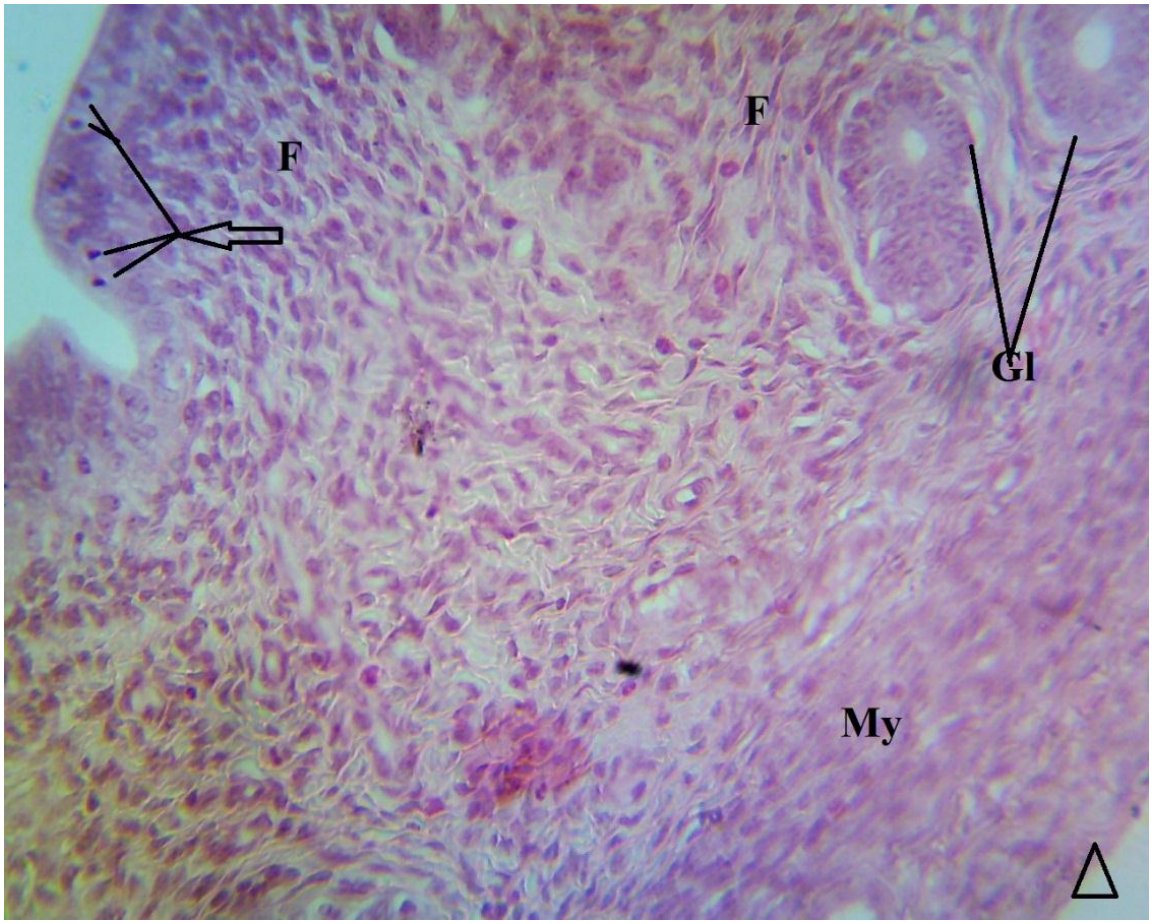

Plate 4.2: Photomicrograph of a portion of the uterus of the Positive Control (PC-1) for the Uterine Leiomyoma animal study group. H&E, X400.

F: fibrous tissue; Gl: glands; My: myometrium

Plate 4.3 is the photomicrograph of a portion of the uterus of the Treated Group (TG-1) at a high magnification (X400). It reveals a diffused atrophy of the uterus which occurs secondarily to radiotherapy and/or chemotherapy. The fibrous tissues are reduced in number and smooth muscle cells are decreased resulting in reduced endometrial and myometrial wall thickness.

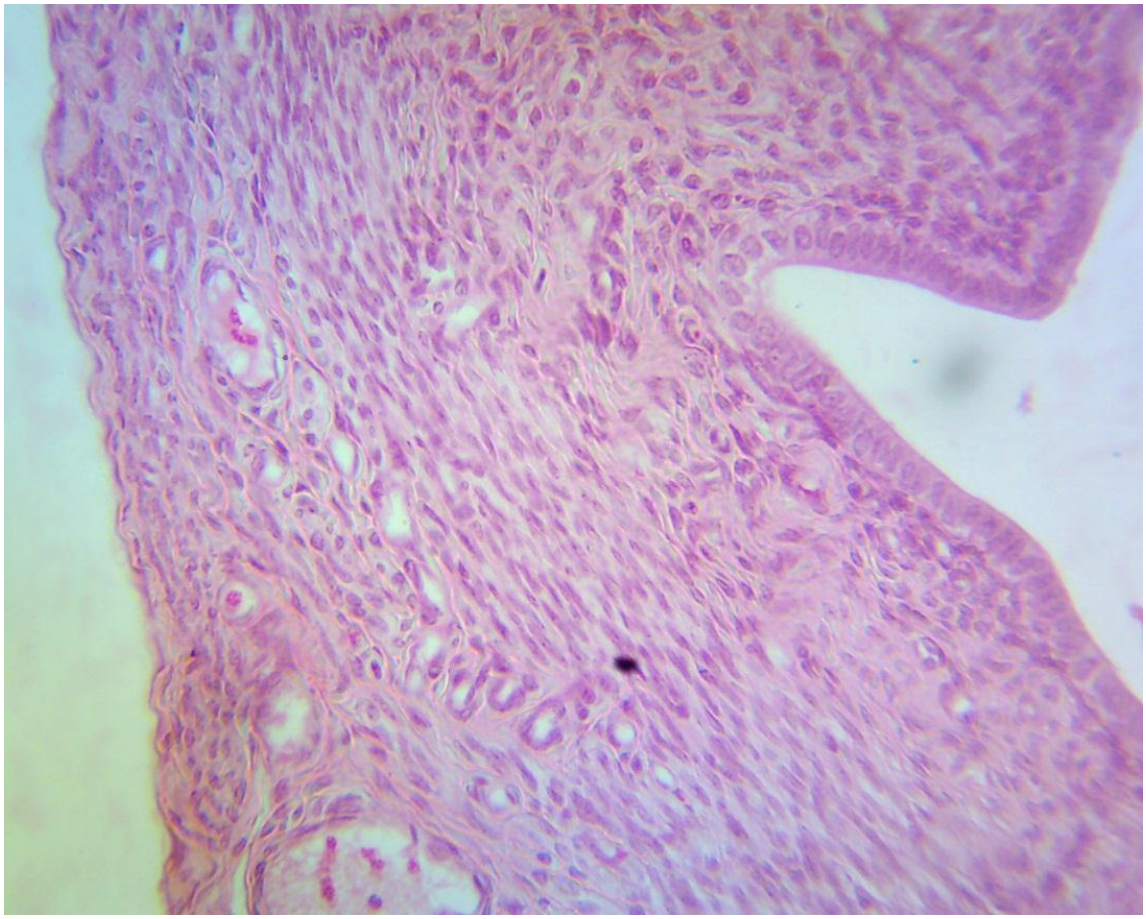

Plate 4.3: Photomicrograph of a portion of the uterus of the Treated Group (TG-1) for the Uterine Leiomyoma animal study group. H&E, X400.

4.1.3.3 Benign Prostatic Hyperplasia

The biochemical parameters analysed for Benign Prostatic Hyperplasia (BPH) animal study group include: Urea, Creatinine, Potassium (K), Chloride (Cl), Sodium (Na), Bicarbonate (HCO_3), Uric Acid (UA), Testosterone and Prostate Specific Antigen (PSA). The values of chloride (Cl) and sodium (Na) were divided by 10 to accommodate other very low values on the graph. There are three (3) controls in this study namely: **Normal Control (NC-2)** (this is the group of animal without induction, given water and normal rat chew); **Positive Control (PC-2)** (this is the group induced with benign prostatic hyperplasia using Testosterone Propionate (TP) but not given treatment) and **Treated Group (TG-2)** (this is the group of animal induced with benign prostatic hyperplasia, then treated with 400mg/kg body weight of ethanol extracts of *Diodia sarmentosa* (Sw)).

The results of the biochemical parameters analysed for benign prostatic hyperplasia are displayed in **Figure 4.6**. Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different.

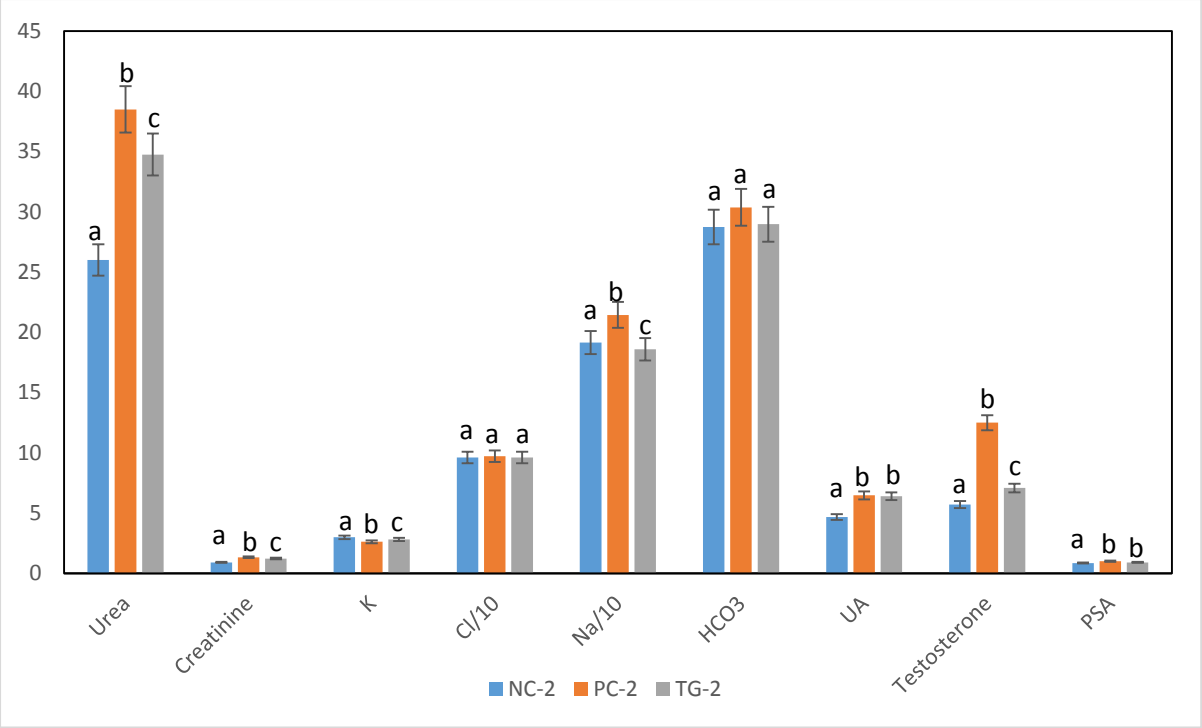

Figure 4.6: Biochemical Parameters for Benign Prostatic Hyperplasia animal study group

Values are expressed as Mean ± Standard deviation (n=4).

K: potassium, Cl: chloride, Na: sodium, HCO_3: bicarbonate, UA: uric acid, PSA: prostate specific antigen

The results of the oxidative stress and antioxidant parameters are displayed in **Figure 4.7**. Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different.

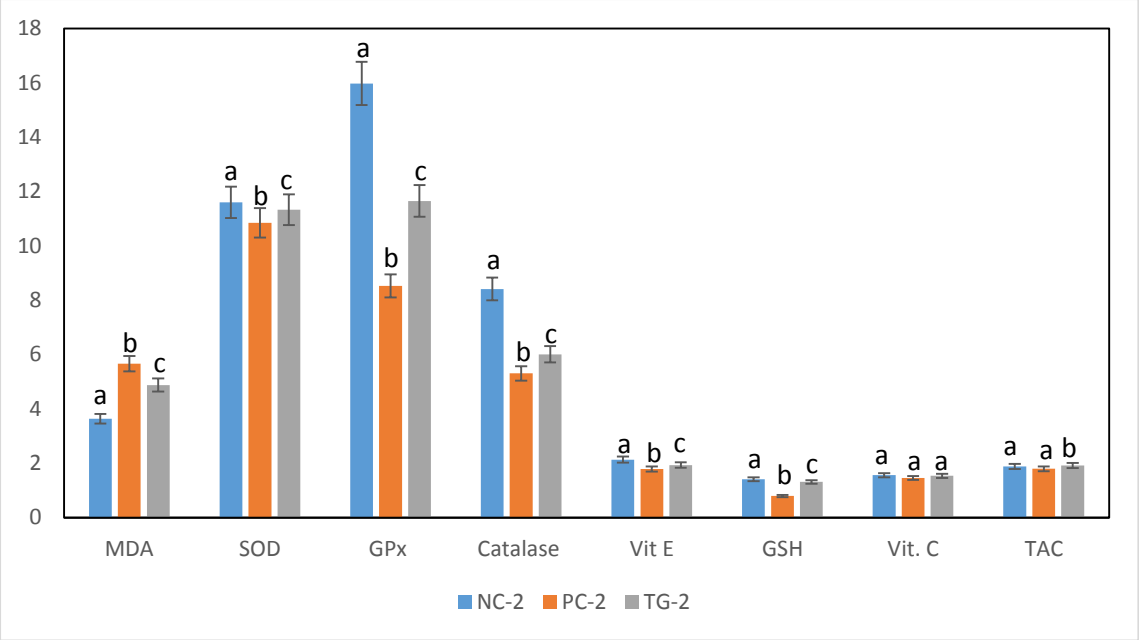

Figure 4.7: Oxidative Stress and Antioxidant Parameters of Benign Prostatic Hyperplasia animal study group

Values are expressed as Mean ± Standard deviation (n=4).

MDA: malondiadehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; Vit. E: vitamin E; GSH: glutathione; Vit. C: vitamin C; TAC: total antioxidant capacity

The harvested prostate of the various controls (normal control, positive control and treated group) for Benign Prostatic Hyperplasia were weighed before fixing them in formalin and the results are displayed in **Figure 4.8**. The relative prostate weight (g) was also evaluated by normalization to the body weight of the animals (that is organ weight/body weight). The result of the relative prostate weight is displayed on **Figure 4.9**.

Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different.

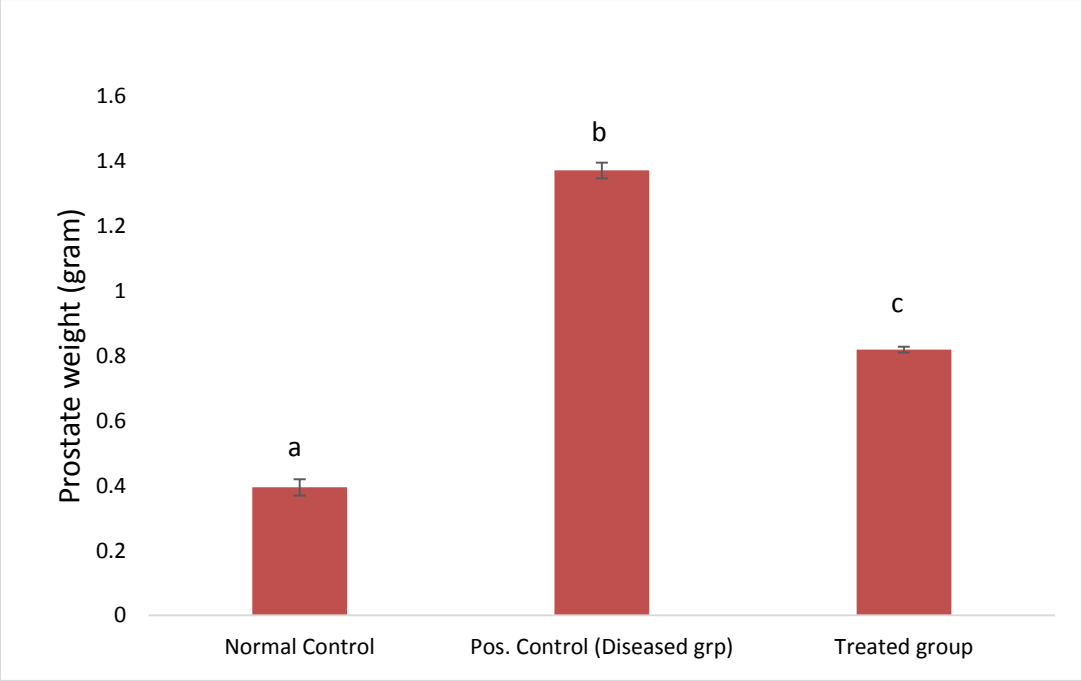

Figure 4.8: Prostate weight (gram) of the Benign Prostatic Hyperplasia animal study group (in grams)

Values are expressed as Mean ± Standard deviation (n=4).

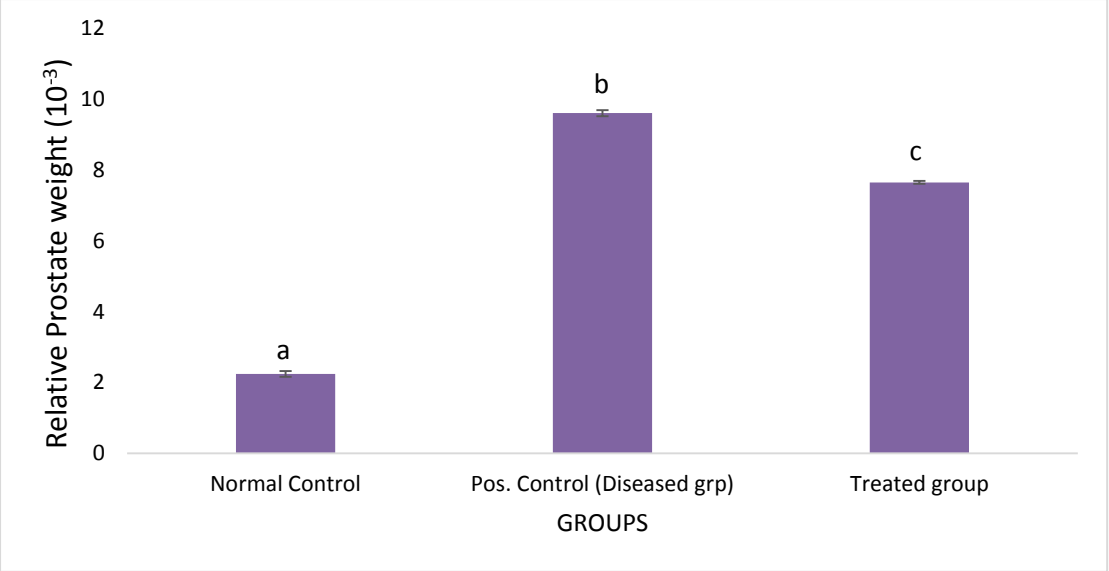

Figure 4.9: Relative Prostate weight (g) of the Benign Prostatic Hyperplasia animal study group

Values are expressed as Mean ± Standard deviation (n=4).

4.1.3.4 Histology of the Prostate gland

Plate 4.4 shows a photomicrograph of a portion of the prostate gland of the normal control (NC-2) of the Benign Prostatic Hyperplasia (BPH) animal study group in low magnification (X100). It reveals a normal histologic architecture, indicating absence of any disease condition. It also reveals a small section of the capsule (Cap), secretory tubuloaveoli of the gland (arrow heads) which vary greatly in form. The epithelium (Ep), smooth muscle (SM) and prostatic concretions (C) are also shown. The same portion of the prostate gland of the normal control for BPH is also viewed with at a higher magnification (X400) (**Plate 4.5**). At a higher magnification, the secretory tubuloaveoli of the gland (arrow heads), epithelium (Ep), smooth muscle (SM) and prostatic concretions (C) are also revealed.

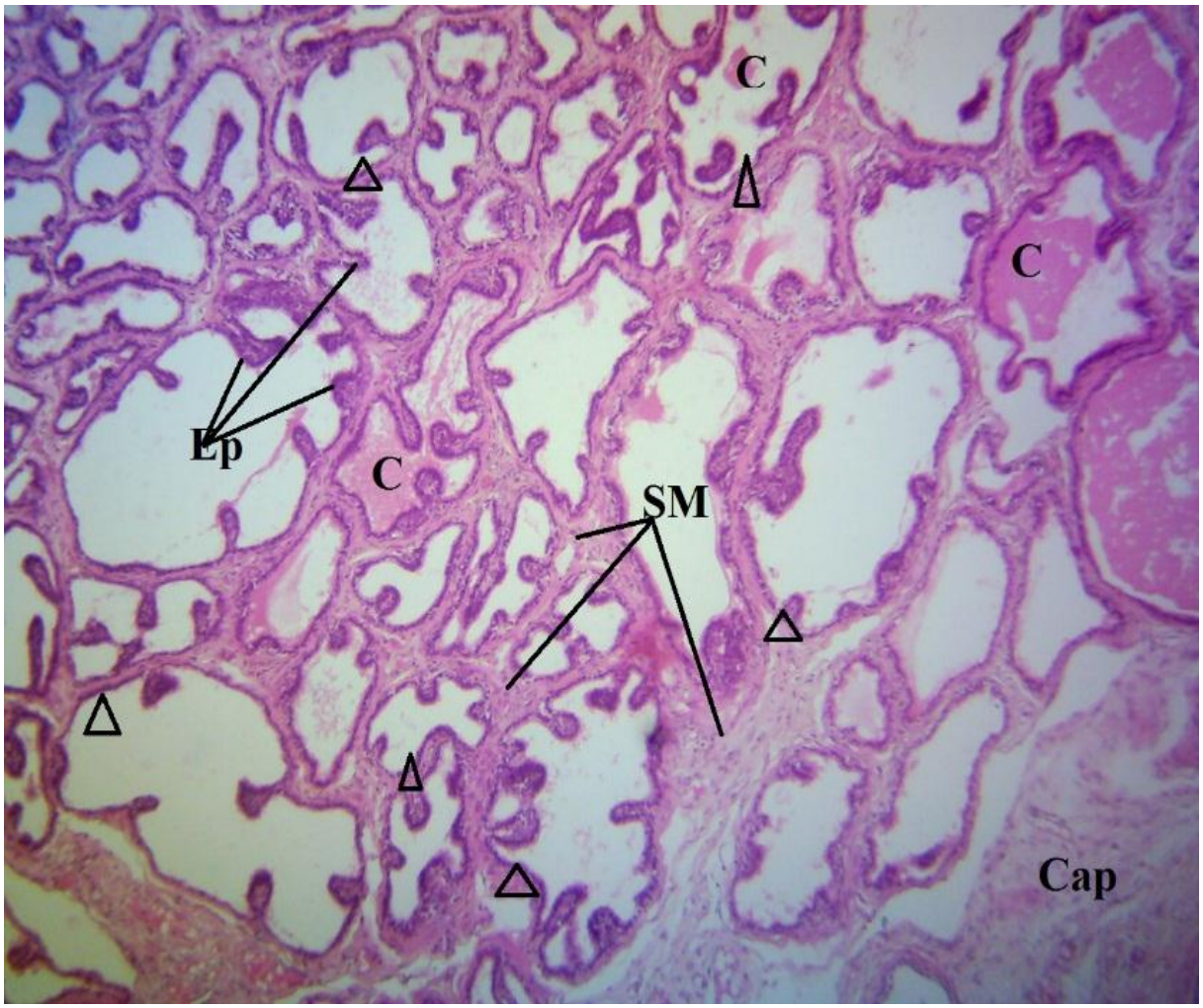

Plate 4.4: Photomicrograph of a portion of the prostate gland of the Normal Control (NC-2) for the Benign Prostatic Hyperplasia animal study group. H&E, X100.

Cap: capsule; Ep: epithelium; SM: smooth muscle; C: prostatic concretions

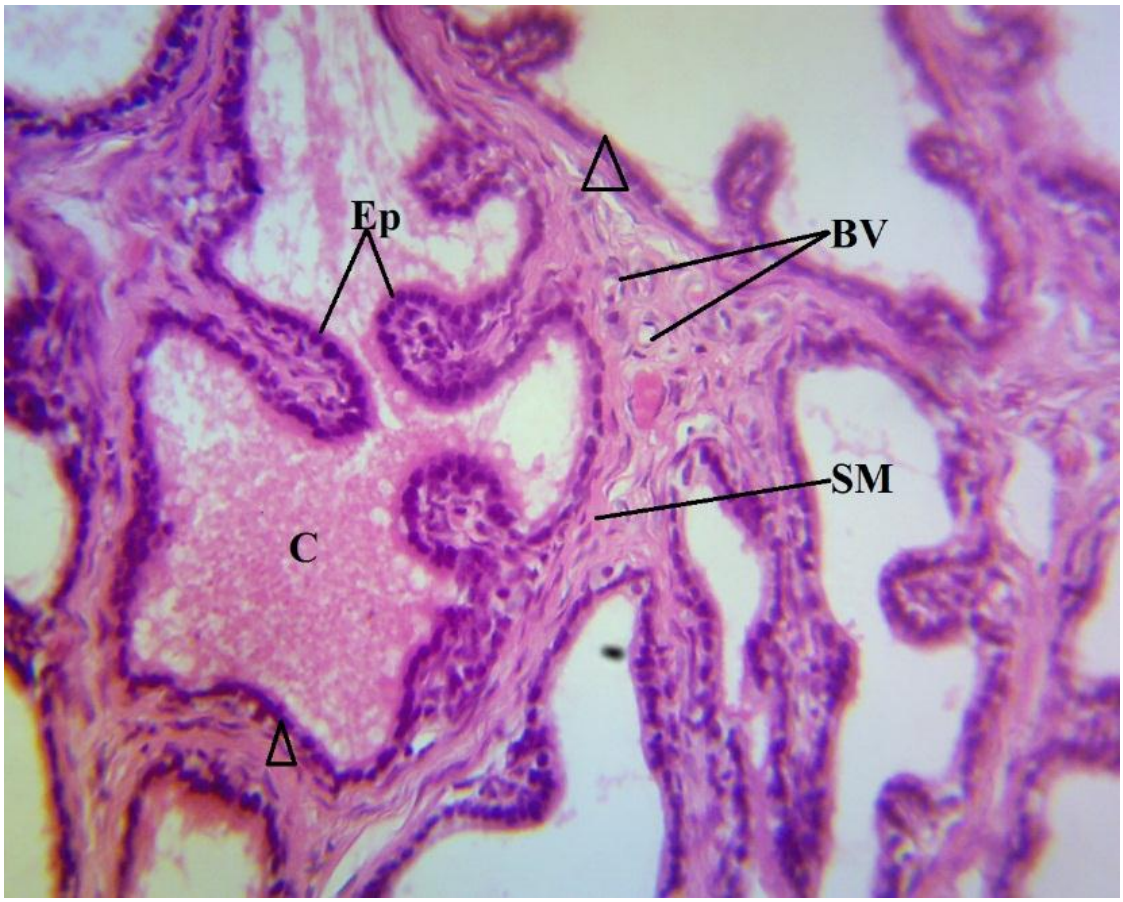

Plate 4.5: Photomicrograph of a portion of the prostate gland of the Normal Control (NC-2) for the Benign Prostatic Hyperplasia animal study group. H&E, X400.

C: prostatic concretions; Ep: epithelium; SM: smooth muscle; CAP: capsule

A photomicrograph of a portion of the prostate gland of the positive control (PC-2) for BPH animal study group at a low magnification (X100) is shown on **Plate 4.6**. It reveals hyperplasia of epithelium lining the glands. The cells form folds that protrude into the lumen of the glands. At a higher magnification (X400) view, diffused marked amounts of secretory products that form prostatic concretions (C) in the lumen can be seen (**Plate 4.7**). This confirms a successful induction of benign hyperplasia in the positive control of BPH animal study group.

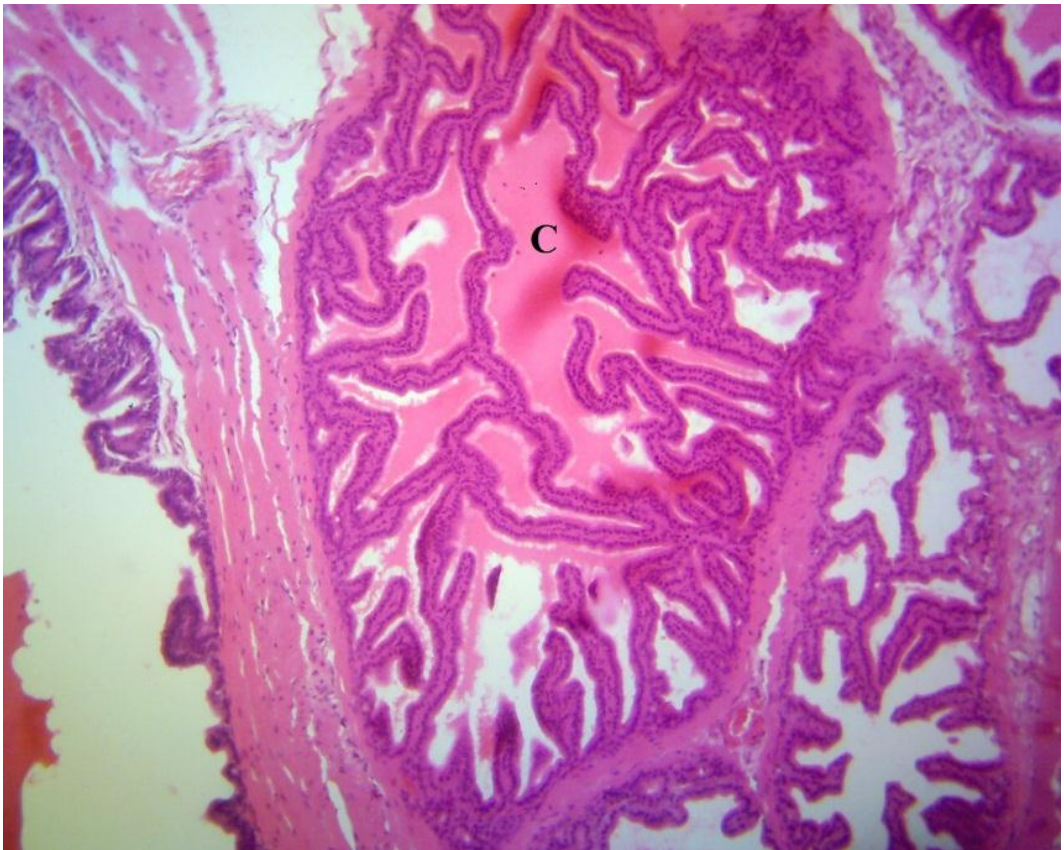

Plate 4.6: Photomicrograph of a portion of the prostate gland of the Positive Control (PC-2) for the Benign Prostatic Hyperplasia animal study group. H&E, X100.

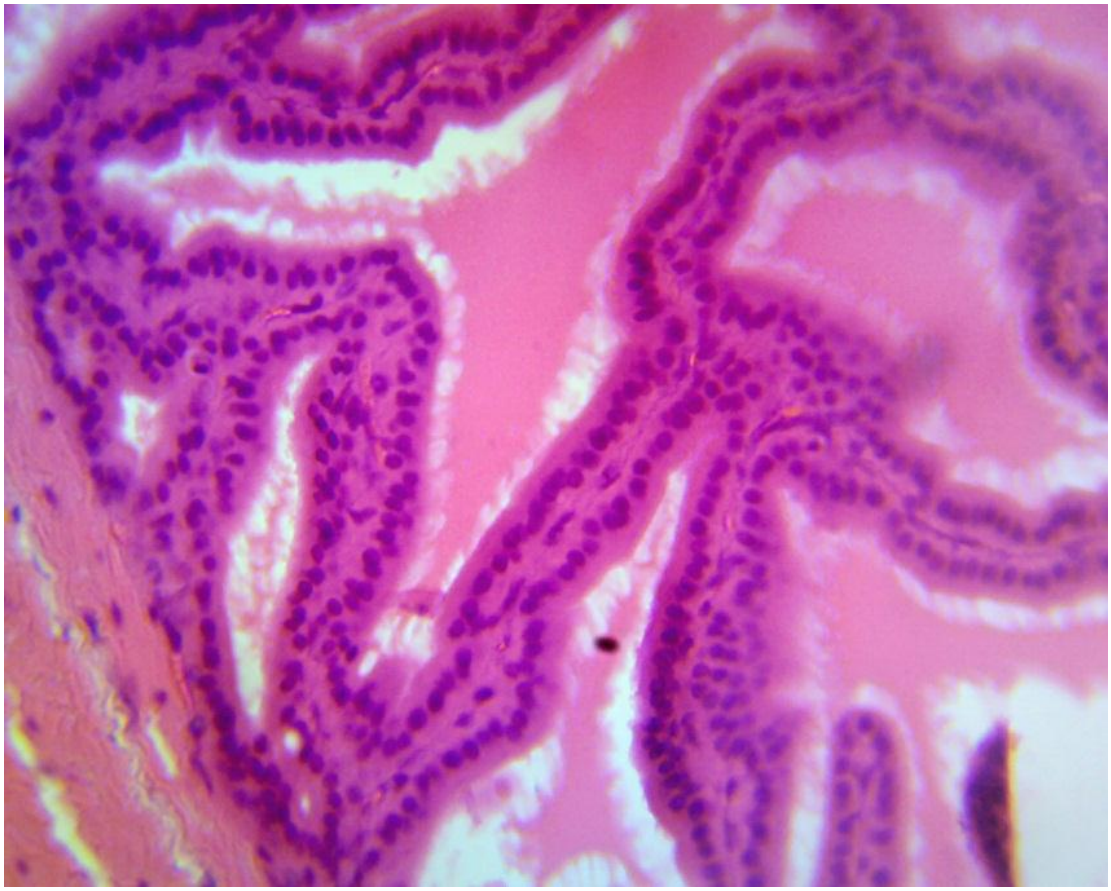

Plate 4.7: Photomicrograph of a portion of the prostate gland of the Positive Control (PC-2) for the Benign Prostatic Hyperplasia animal study group. H&E, X400.

Plate 4.8 and **Plate 4.9** are photomicrographs of a portion of the prostate gland of the treated group (TG-2) for BPH animal study group at low (X100) and high (X400) magnification view respectively. They show diffused atrophy that occurs secondarily to radiotherapy and/or endocrine therapy. The smooth muscle cells are decreased resulting in reduced smooth muscle wall thickness. The epithelium lining the glands and the folds that protrude into the lumen from section in positive control (PC-2), are reduced. There is also flattening of the mucosal folds in the treated group (TG-2).

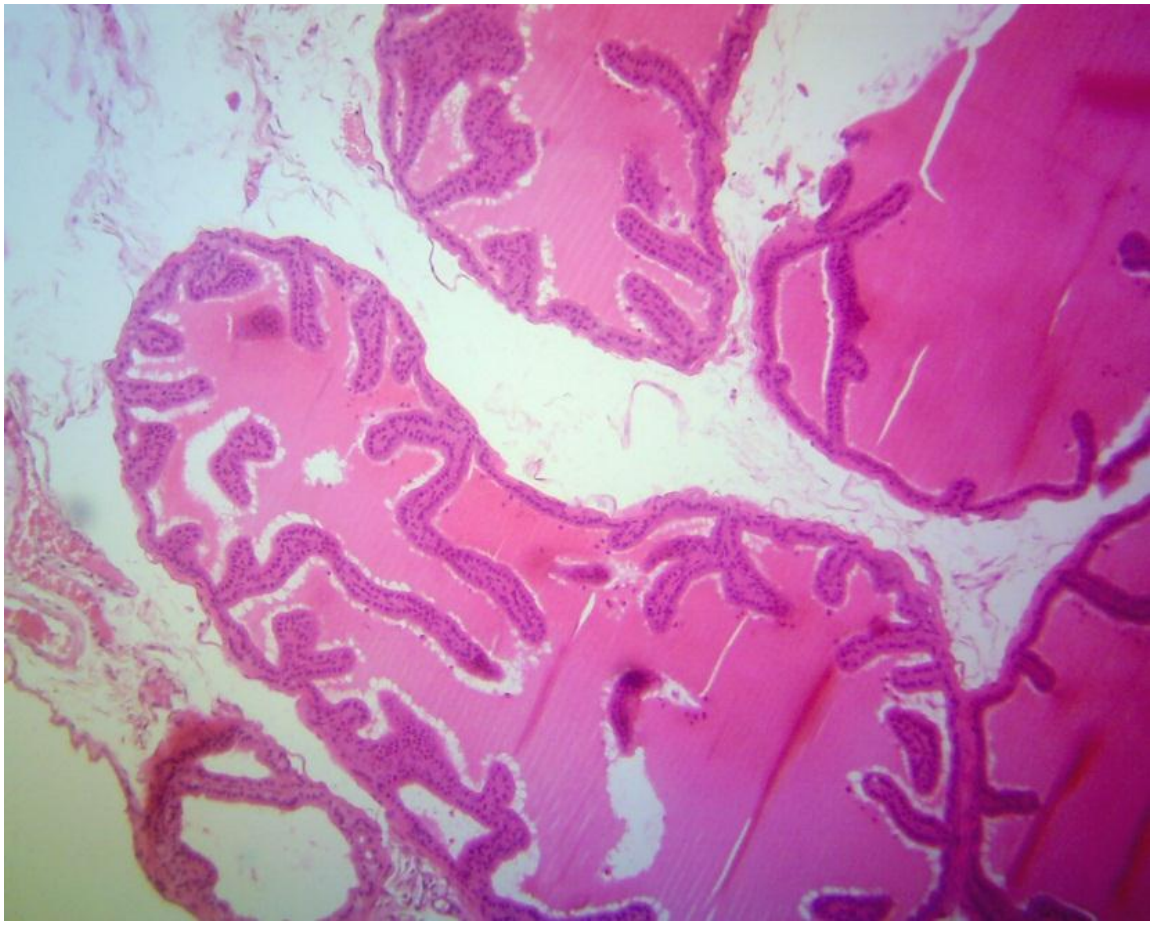

Plate 4.8: Photomicrograph of a portion of the prostate gland of the Treated Group (TG-2) for the Benign Prostatic Hyperplasia animal study group. H&E, X100.

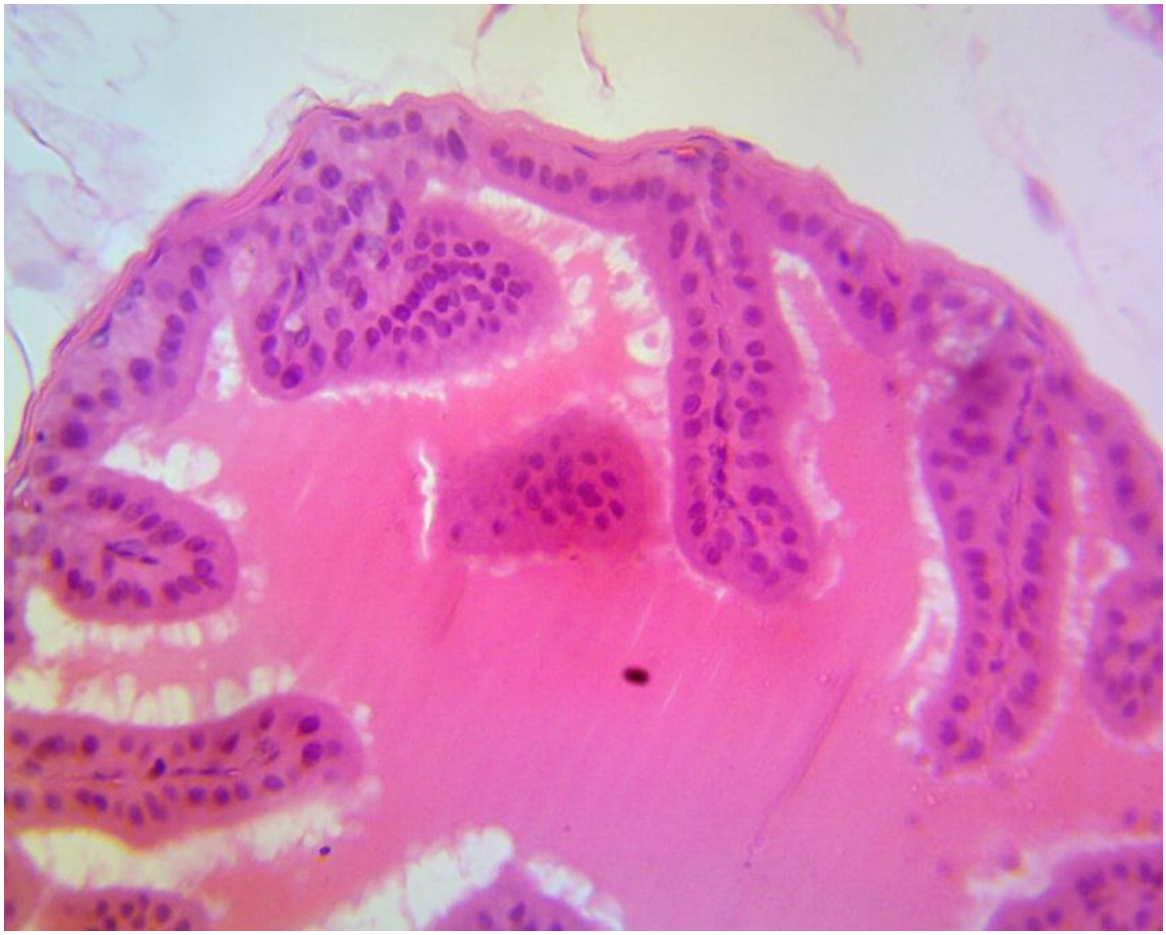

Plate 4.9: Photomicrograph of a portion of the prostate gland of the Treated Group (TG-2) for the Benign Prostatic Hyperplasia animal study group. H&E, X400.

4.1.3.5 Hepatocellular Carcinoma

The chloride and sodium parameters were divided by 10 to accommodate the very low concentrations of some parameters on the graph. The study group consists of three (3) controls namely: **Normal Control (NC-3)** (this the animal group without induction, given water and normal rat chew); **Positive Control (PC-3)** (this is the animal group that was induced with hepatocellular carcinoma using diethylnitrosamine and not treated) and the **Treated Group (TG-3)** (this is the animal group which was induced with hepatocellular carcinoma, then treated with 400mg/kg body weight of ethanol extracts of *Diodia sarmentosa*). The results are presented in **Figure 4.10**. Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different.

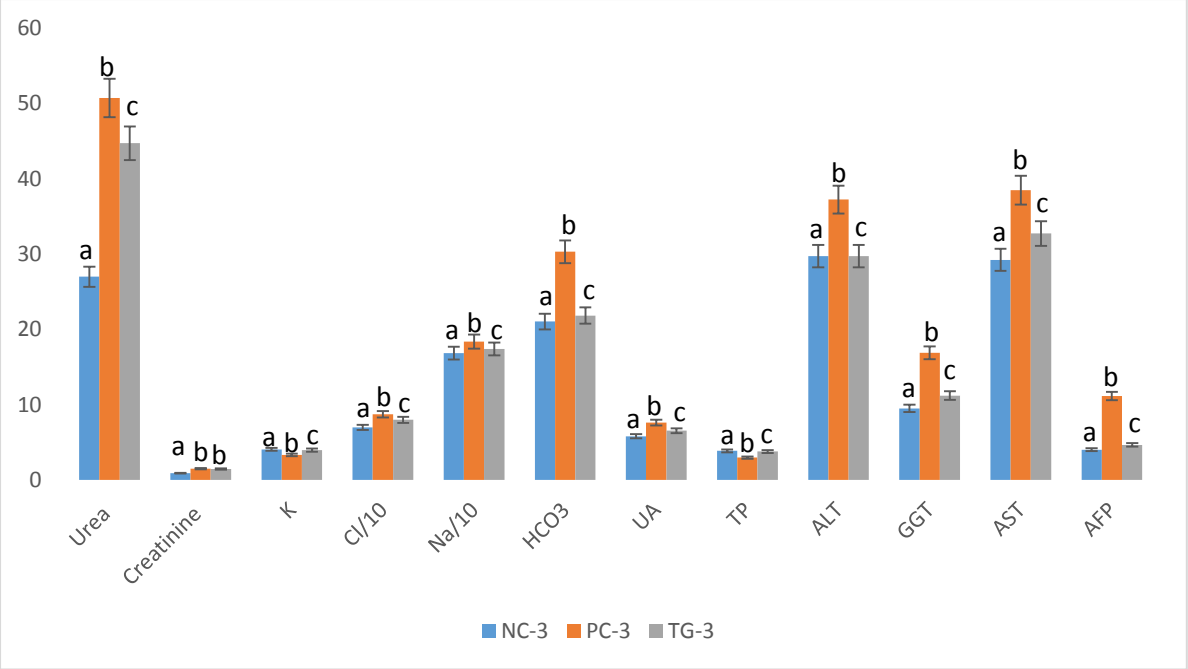

Figure 4.10: Biochemical Parameters of the Hepatocellular Carcinoma animal study group

Values are expressed as Mean ± Standard deviation (n=4).

K: potassium, Cl: chloride, Na: sodium, HCO_3: bicarbonate, UA: uric acid, TP: total protein, ALT: alanine aminotransferase, GGT: gamma glutamyltransferase, AST: aspartate aminotransferase, AFP: alfa feto protein.

Figure 4.11 is the result of the oxidative stress and antioxidant parameters analysed for hepatocellular carcinoma animal study group. The parameters analysed include: malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, vitamine E (Vit. E), glutathione (GSH), vitamin C (Vit. C) and total antioxidant capacity (TAC). The three controls: normal control (NC-3), positive control (PC-3) and treated group (TG-3) are same as that in the biochemical parameters of hepatocellular carcinoma animal study group. Groups with different alphabets are significantly ($p<0.05$) different while groups that share the same alphabet are not significantly different.

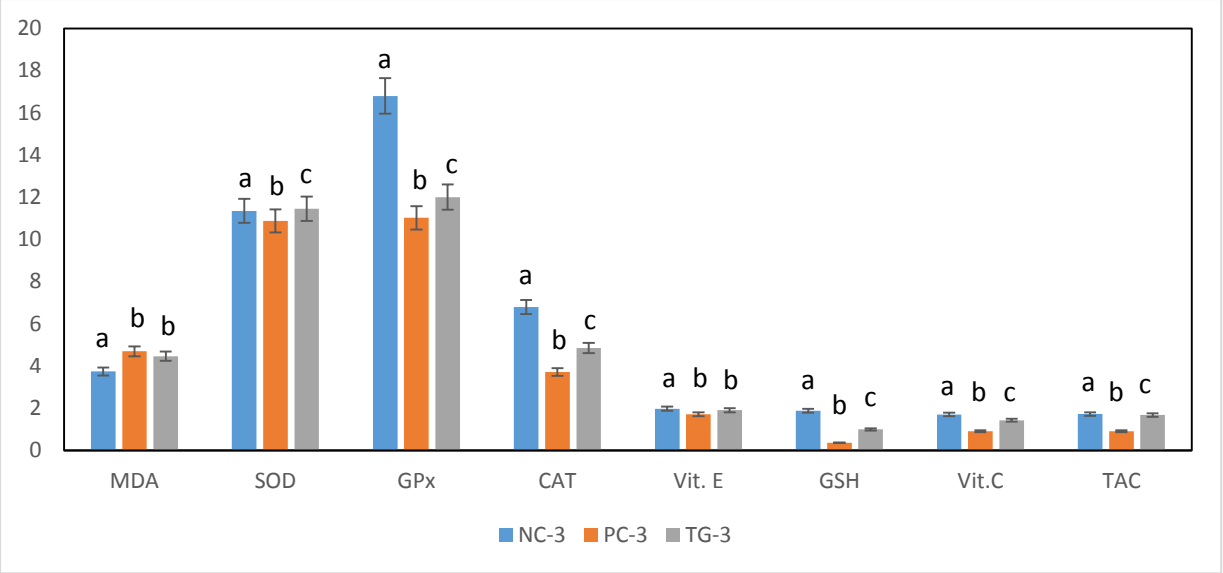

Figure 4.11: Oxidative Stress and Antioxidant Parameters of the Hepatocellular Carcinoma animal study group

Values are expressed as Mean ± Standard deviation (n=4).

MDA: malondiadehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; Vit. E: vitamin E; GSH: glutathione; Vit. C: vitamin C; TAC: total antioxidant capacity

4.1.3.6 Histopathology of the Liver

A photomicrograph of a portion of the liver of the Normal Control (NC-3) for hepatocellular animal study group at a low magnification (X100) is shown on **Plate 4.10**. This shows a normal histologic architecture exposing the central vein (CV) and hepatocytes (Hp) arranged in single cell thick plates/cords and seperated by sinusoids. A higher magnification (X400) (**Plate 4.11**), reveals a connective tissue septum that carries the branches of hepatic artery (HA) and portal vein (PV), bile duct (BD), lymphatic vessels and nerves. The artery and vein, along with the bile duct are collectively referred to as a portal triad.

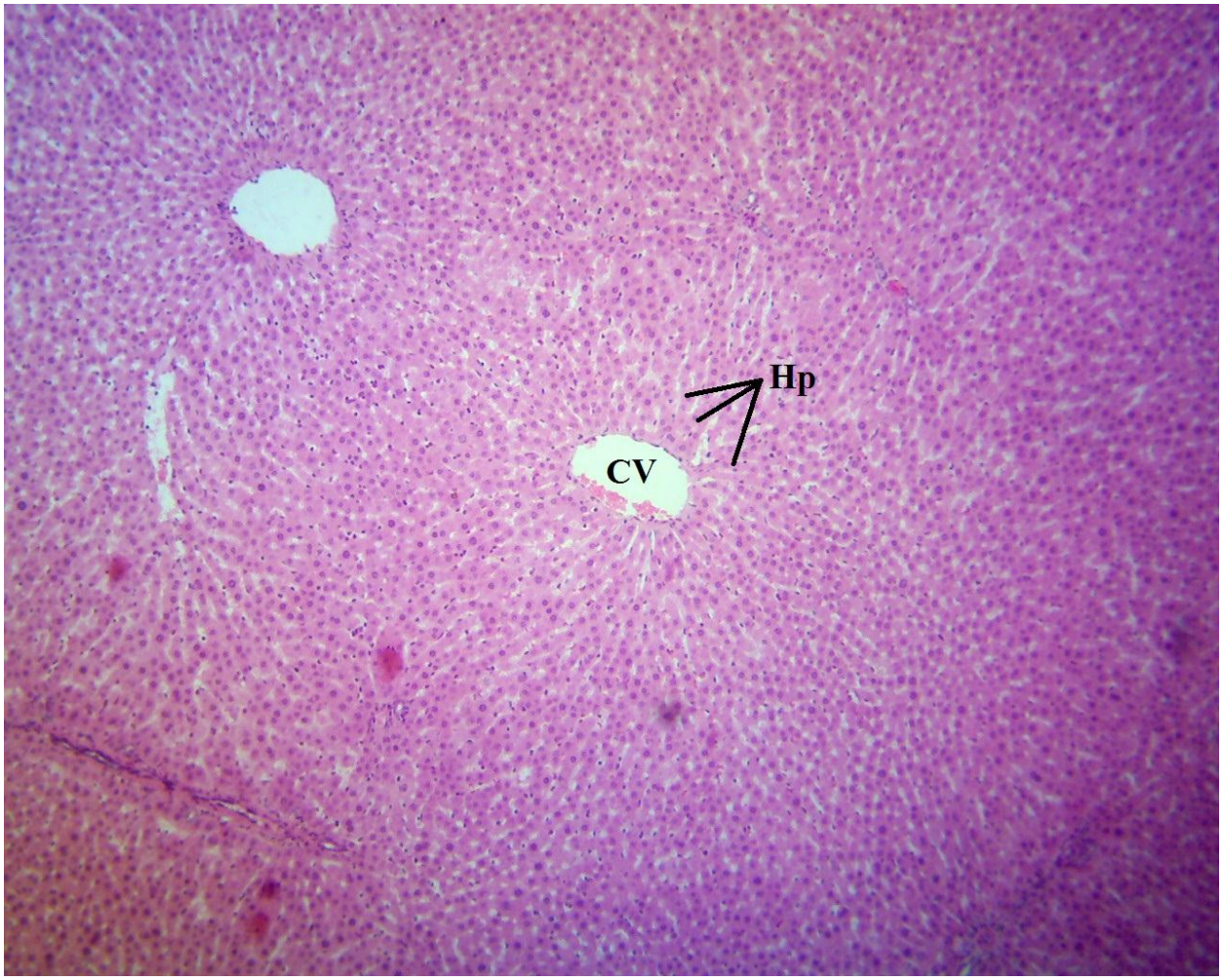

Plate 4.10: Photomicrograph of a portion of the liver of the Normal Control (NC-3) for Hepatocellular animal study group. H&E, X100

Hp: hepatocytes; CV: central vein

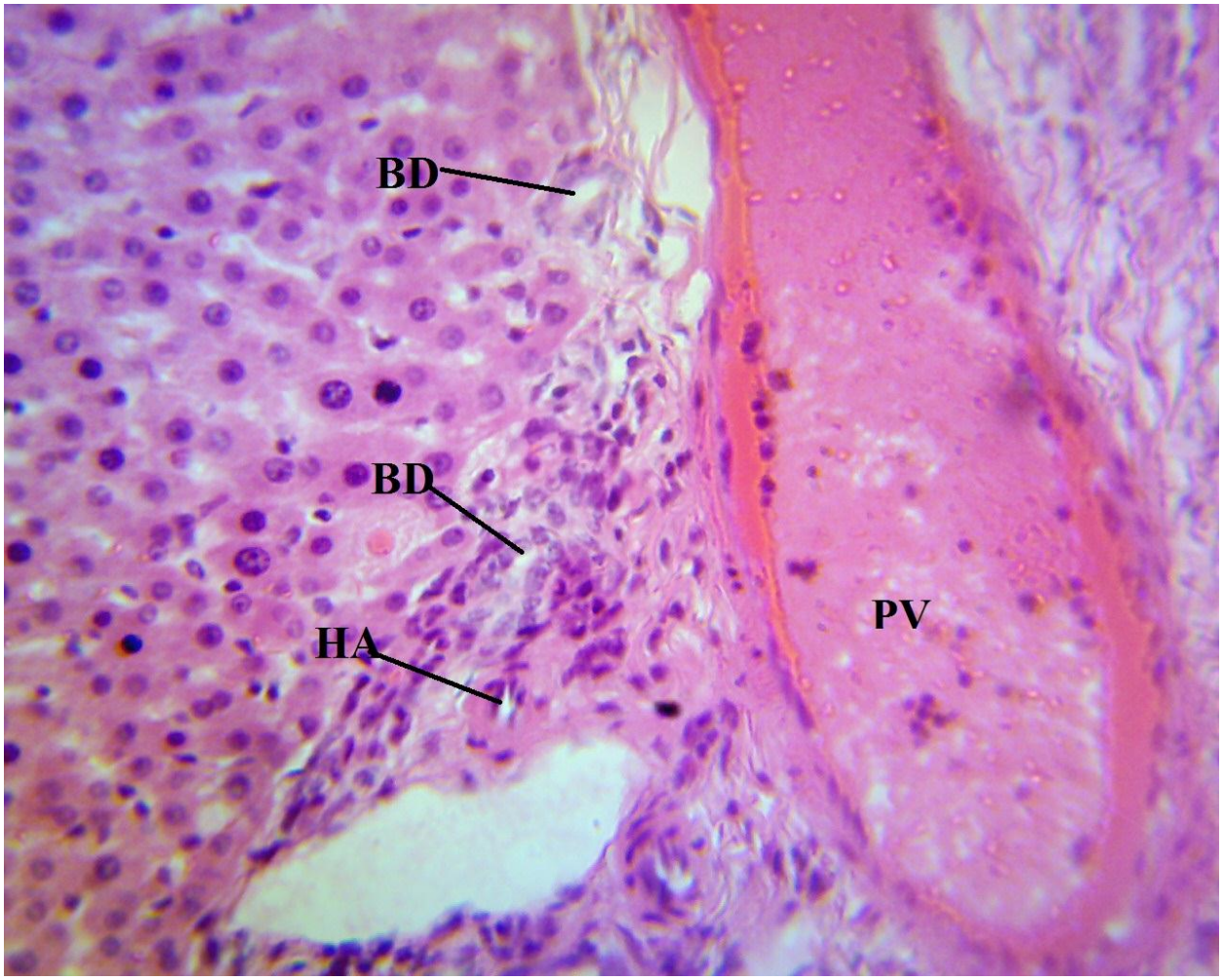

Plate 4.11: Photomicrograph of the liver portal canal of the Normal Control (NC-3) for Hepatocellular Carcinoma animal study group. H&E, X400.

BD: bile duct; HA: hepatic artery; PV: portal vein

Photomicrographs of the liver of the Positive Control (PC-3) of the hepatocellular carcinoma animal study group at low (X100) and high (X400) magnification are revealed in **Plate 4.12** and **Plate 4.13** respectively. They reveal a solid growth pattern with large tumour nodules seperated by indistinct fibrous bands (two arrow heads). This tumour is obvious by the very abnormal liver achitecture. There is also presence of more than 2-3 cell thick hepatocellular plates/cords. Note the nuclear atypia of tumour cells with obvious resemblance to hepatocytes but that show enlarged nuclei (high N/C ratio) with prominent nucleoli (arrows).

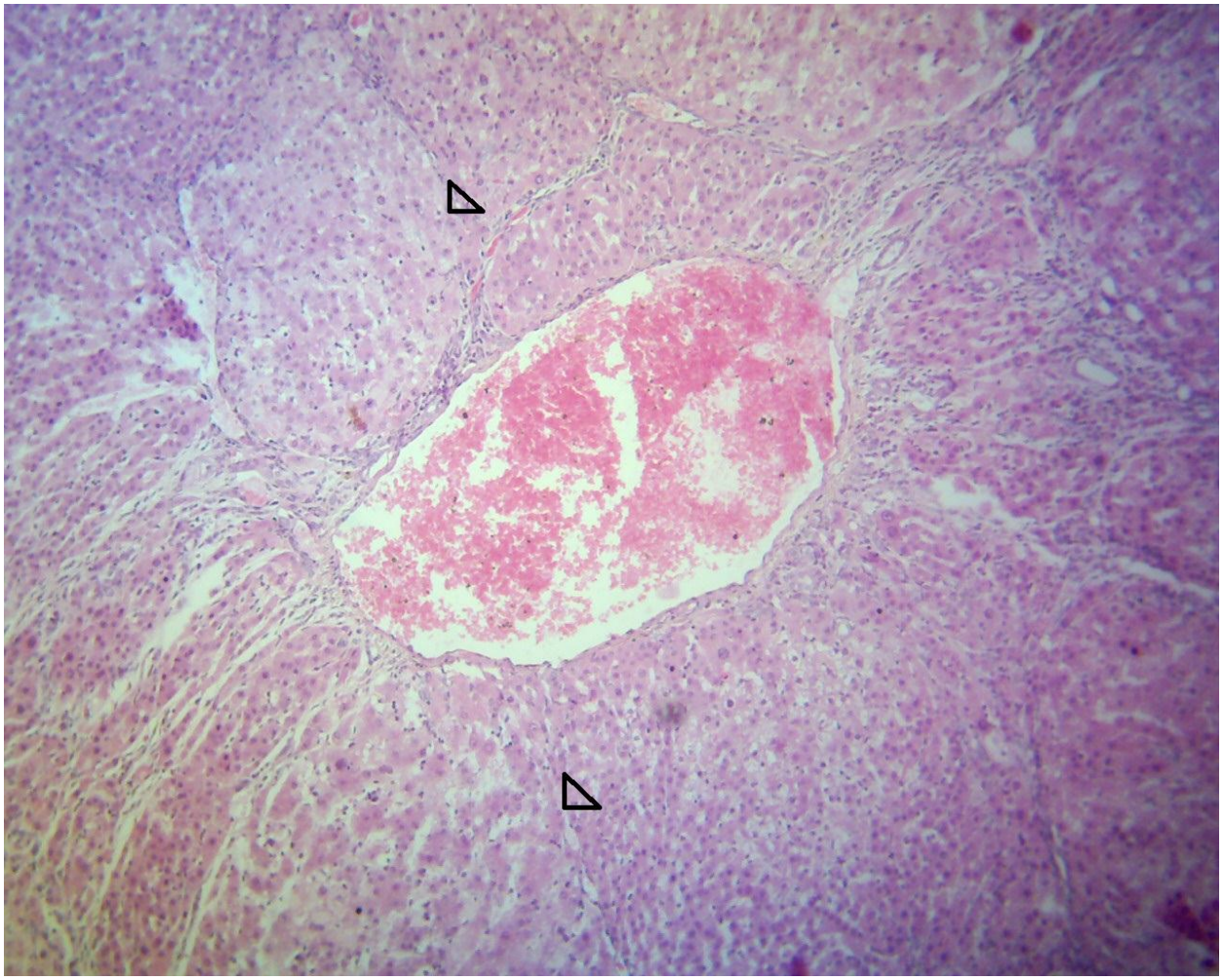

Plate 4.12: Photomicrograph of the liver of the Positive Control (PC-3) of the Hepatocellular Carcinoma study group. H&E, X100.

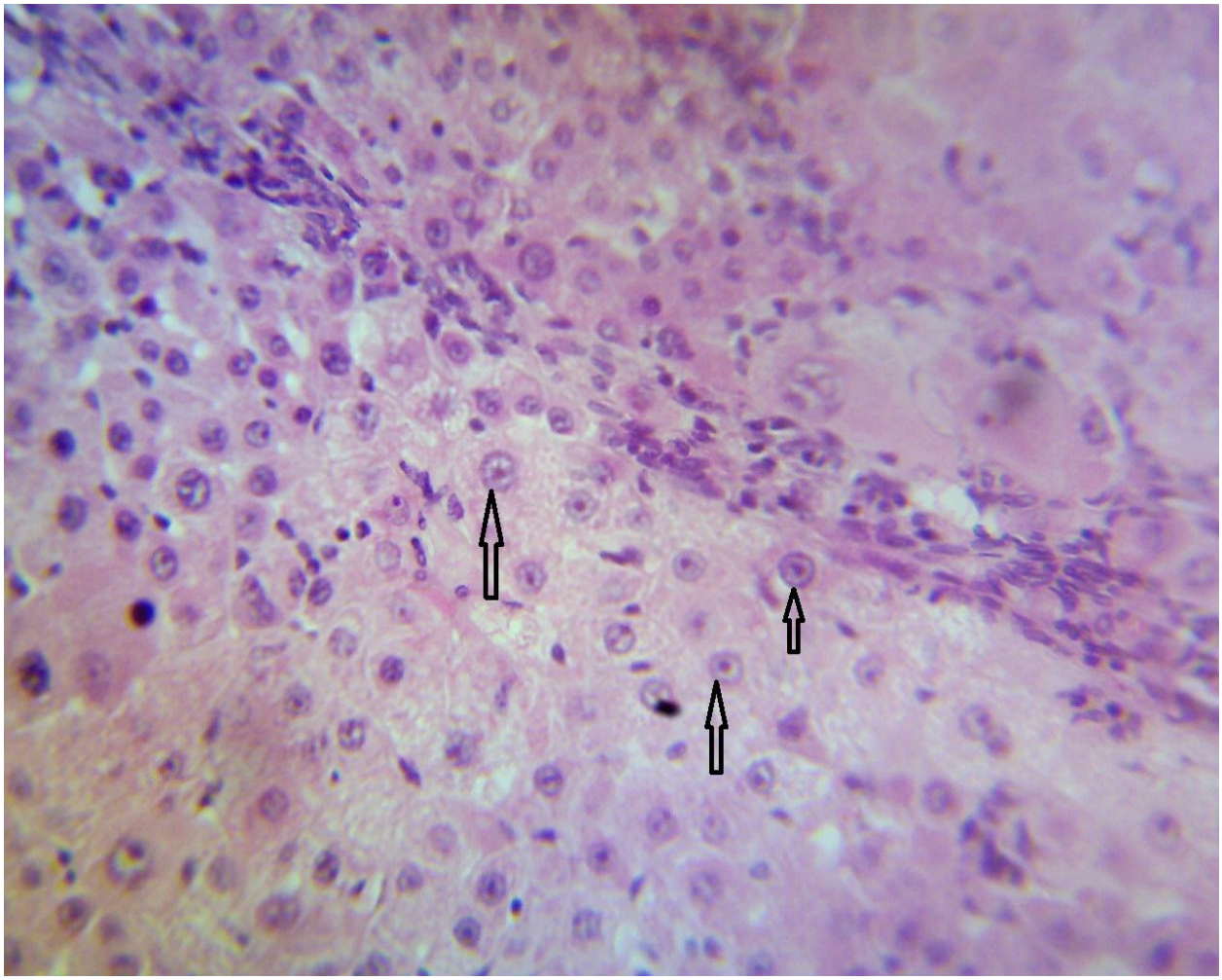

Plate 4.13: Photomicrograph of the liver of the Positive Control (PC-3) of the Hepatocellular Carcinoma animal study group. H&E, X400.

Plate 4.14 and **Plate 4.15** are photomicrographs of portions of the liver of the treated group (TG-3) for hepatocellular carcinoma animal study group at a low (X100) and high (X400) magnification respectively. They reveal diffused necrosis and atrophy (arrows) of tumour nodules and the presence of one cell thick hepatocellular plates seperated by sinusoids (Hp) with normal nucleus and cytoplasmic ratio and the presence of atypia nucleoli.

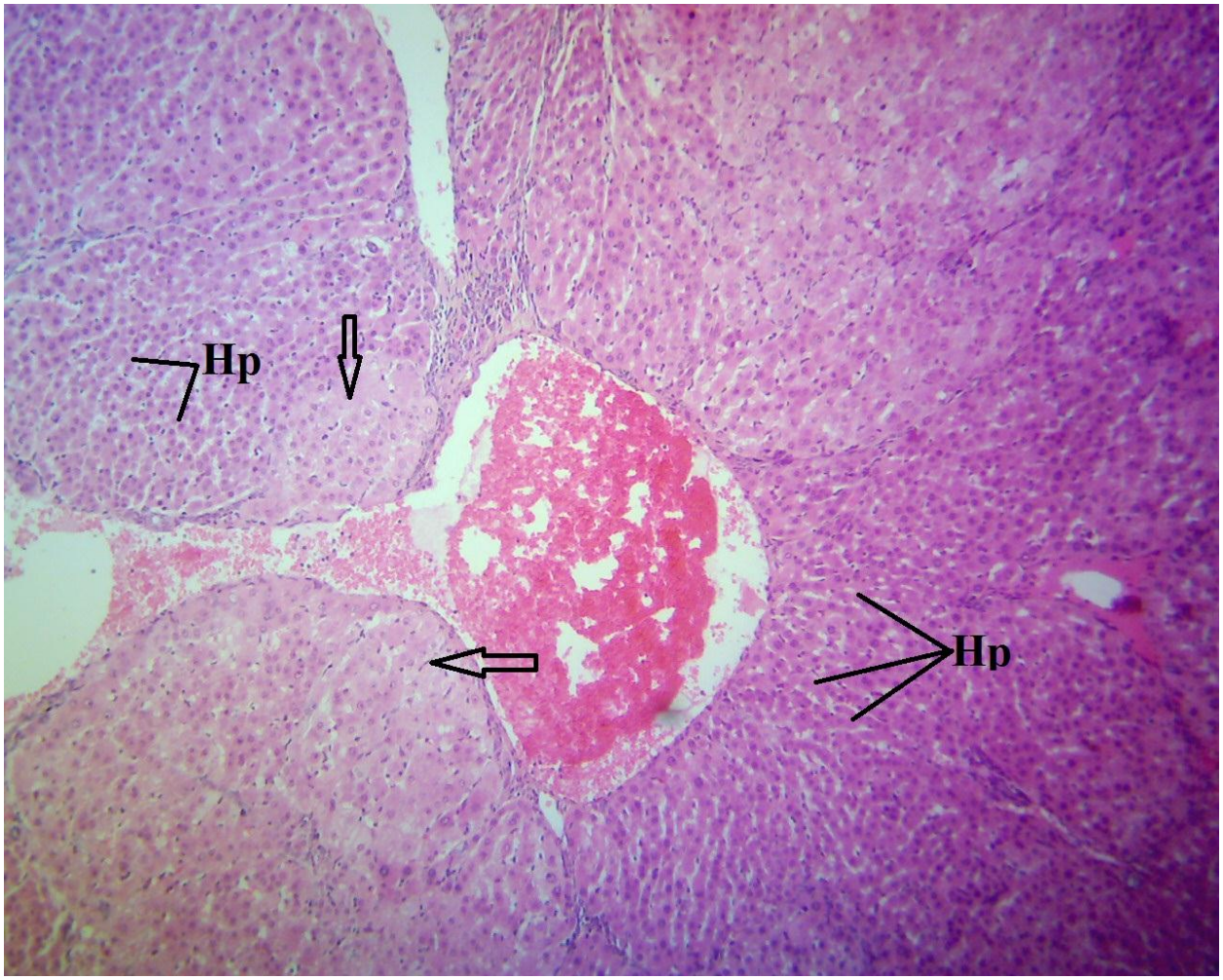

Plate 4.14: Photomicrograph of a portion of the liver of Treated Group (TG-3) for Hepatocellular Carcinoma animal study group. H&E, X100.

Hp: sinusoids

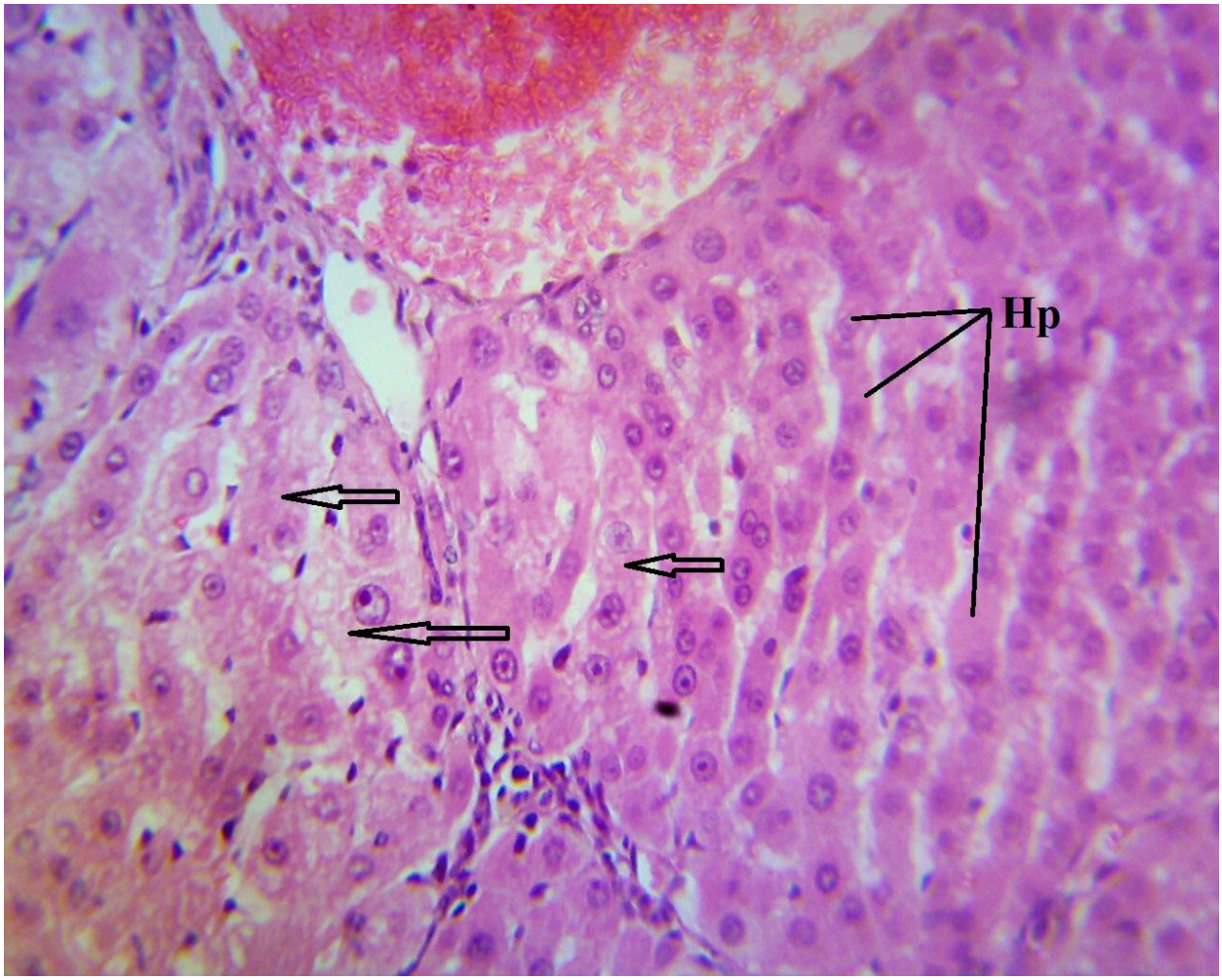

Plate 4.15: Photomicrograph of a portion of the liver of Treatment Group (TG-3) for Hepatocellular Carcinoma animal study group. H&E, X400.

4.2 DISCUSSION

4.2.1 *In vitro* Studies

Qualitative screening of the *Diodia sarmentosa* (Sw) leaves indicated the presence of all the phytochemical constituents (saponin, flavonoid, carbohydrate, phenol, reducing sugar, glycoside, tannin, steroid and terpenoid) tested except alkaloid (Table 4.1). Presence of saponin indicates that the leaves can be useful in treating yeast and fungal infections (Sheikh *et al*. 2013). Schneider and Wolfling (2004) reported that saponins inhibit sodium ions efflux by blockage of the influx of concentration in the cells activating a sodium-calcium ions anti-porter in cardiac muscle, and the increase in calcium ions influx through this anti-porter strengthens the contraction of heart muscles. Saponins can also inhibit the growth of cancer cells, boost immune system and energy, lower cholesterol, act as natural anti-inflammatory, antibiotic, and anti-oxidant, and can reduce the uptake of certain nutrients including glucose and cholesterol at the gut through intralumenal physicochemical interaction (Aberoumand, 2012; Ray-Sahelian, 2012; De Silva *et al*. 2013) and have haemolytic activity (Khalil & Eladawy, 1994). The anti-inflammatory effect of saponin concurs with the findings of Umoh *et al*. (2016) that the plant has anti-inflammatory properties.

Flavonoids are known to have antioxidant effects and have been shown to inhibit the initiation, promotion, and progression of tumors (Kim *et al*. 1994); reduction of coronary heart disease has been reported to be associated with intake of flavonoid (Hertog *et al*. 1993). Apart from the antioxidant properties of flavonoid, other biological functions it possesses include protection against platelet aggregation, microorganisms, hepatotoxins, viruses, tumors, ulcers, free radicals, inflammation, and allergies (Barakat *et al*. 1993). The presence of carbohydrate and its relatively high quantity could suggest that the leaves will be a good source of carbohydrate.

Secondary metabolites in plants such as phenolic compounds are essential for plant growth, reproduction, as protecting agents against pathogens, preventing chronic illnesses such as cardiovascular diseases, certain types of cancers, neurodegenerative diseases, and diabetes (Scalbert *et al*. 2005). Presence of phenol suggest that the leaves could be used as anti inflammatory, immune enhancers and hormone modulator (Okwu & Omodamino, 2005). Phenols are reported to also possess the ability to block specific enzymes that cause inflammation and to prevent disease (Okwu, 2004). Findings in this study also support that of Umoh *et al*. (2016) who reported the anti-inflammatory properties of the leaf. Presence of glycosides suggest that the leaves extract have the ability to lower blood pressure (Nyarko, 1990).

Tannin is one of the major active ingredients found in plant based medicines (Haslem, 1996). It has also been reported that tannins possess physiological astringent and haemostatic properties, which hasten wound healing and ameliorates inflamed mucus membrane (Ajuru *et al*. 2017). Results of this study validates that of Akah *et al*. (1998) regarding presence of tannins and of the antiulcer effect of *Diodia sarmentosa* (Sw) leaf. Tannins have important roles such as stable and potent anti-oxidants (Trease & Evans, 1983; Tyler *et al*. 1988; Awosike, 1991; Ogunleye & Ibitoye, 2003). Tannins also have been reported to form complexes with digestive enzymes, thus reducing the digestibility of proteins in foods (Amelio, 1999).

The results of the proximate analysis show that *Diodia sarmentosa* (Sw) leaves are enriched with protein, fibre, ash, moisture, fat and carbohydrate (Table 4.3). This suggests that the leaves have nutritional attributes and have potential applications in industries. The very high content of carbohydrate (59.07%), which makes energy available for physical performance and regulate nerve tissue suggest that *Diodia sarmentosa* will be a good source of carbohydrate. The moixture content (10.66%) determines the shelf life and the viability of

microorganisms' growth and has been shown to also cause caking in flour (Saldanha, 2003). The crude protein (8.57%) mediates cell responses and serves as enzymatic catalyst. The crude fibre (7.8%) content, indicates that *Diodia sarmentosa* (Sw) leaves contain a proportion of Cellulose, Hemicellulose and Lignin. There was also a good proportion of the ash content (7.50%), which is a reflection of the mineral constituents embedded in the leaves. The crude fat (6.40%), shows the percentage of stored form of energy in the leaves.

The aqueous and ethanol extracts of the plant showed antioxidant activity when assayed using $DPPH^{o}$. The IC_{50} (inhibitory concentration at 50%) value for aqueous and ethanol extracts were 10.121 µg/ml and 10.994 µg/ml respectively compared to the ascorbic acid standard which was 17.916 µg/ml. It has been reported that a lower IC_{50} value indicates a better antioxidant activity (Rezaeizadeh *et al*. 2011, Bhupendra *et al*. 2012). This shows that the two extracts have a better antioxidant activity than the standard. However, when compared with the ascorbic acid standard using Pearson's correlation coefficient, the ethanol extract was positively correlated while the aqeous extract was negatively correlated at 95% confidence level. The percentage radical scavenging activity of the ethanol extract also appeared to be dependent on its concentration as the radical scavenging activity continuously increased with increasing concentration (I% = 71.42%- 89.95%). This was not the case with the aqueous extract which decreased with increasing concentration (I% = 88.64% - 84.75%).

In the Thiobabituric acid assay (TBARS), the aqueous extract showed a significantly higher percentage inhibition compared to the ethanol extract. The IC_{50} values for the aqueous and ethanol extracts were 2.657 µg/ml and 8.53 µg/ml respectively compared to IC_{50} value of the BHT standard, which was 2.142 µg/ml. This suggest that the phytochemicals soluble in water possess a stronger potential to reduce MDA formation.

For the total antioxidant capacity assay, the aqueous extract had higher ascorbic acid equivalent values compared to the ethanol extract. This is quite normal since the solubility of ascorbic acid increases in the solvent of high polarity (Neto *et al*. 2010). However, the two solvent extracts showed antioxidant capacity.

4.2.2 *IN VIVO* STUDIES

4.2.2.1 Uterine Leiomyoma

4.2.2.1.1 Biochemical Parameters

A striking feature of uterine fibroids is their dependency on the ovarian steroids, estrogen and progesterone (Ishikawa *et al*. 2010). Ovarian activity is essential for fibroid growth (Olowofolahan *et al*. 2017). A large body of experimental data and circumstantial evidence suggests that estrogen stimulates the growth of uterine fibroids through estrogen receptor α (Marsh & Bulun, 2006). The primary roles of estrogen and estrogen receptor α in fibroid growth are permissive in that they enable tissue to respond to progesterone by inducing the expression of progesterone receptor (Bulun *et al*. 1994). Fibroid tissue is exposed to ovarian estrogen and to estrogen produced locally through the aromatase activity in fibroid cells (Zia *et al*. 2014).

The serum oestrogen level in the Uterine Leiomyoma Positive Control (PC-1) was significantly ($p<0.05$) increased compared with the Uterine Leiomyoma Normal Control (NC-1) (Figure 4.4). The effects of MSG on serum estradiol (estrogen) levels could be attributed to the activation of the enzyme, aromatase, which catalyzed the conversion of testosterone to β-estradiol and aromatization of ring A of β-estradiol, which increased the activity of the enzyme, resulting in increased estradiol synthesis (Olowofolahan *et al*. 2017). The serum progesterone levels were also significantly ($p<0.05$) increased in PC-1 compared to normal control (NC-1) (Figure 4.4). The reason for increase in serum progesterone levels

in the MSG-treated rats was probably due to increased levels of luteinizing hormone as a result of MSG treatment (Olowofolahan *et al*. 2017). These results concur with the findings of Zia *et al*. (2014) and Olowofolahan *et al*. (2017) who found increased serum levels of progesterone in the plasma of MSG-treated animals, which were significantly higher than those found in control animals. However, treatments with the ethanol extract of *Diodia sarmentosa* (SW) leaves mitigated the hyperplasia and the elevated oestrogen and progesterone levels that have been induced by the MSG treatment.

The significant ($p<0.05$) increase in serum levels of creatinine, urea, chloride, sodium, uric acid and the subsequent significant ($p<0.05$) decrease in serum potassium levels in positive control (PC-1) compared with normal control (NC-1) (Figure 4.4) suggest a possible impairment of the kidney. The fibroids, by mechanical obstruction/compression of the pelvic ureters, may cause impairment, with hydroureters and hydronephrosis. These results are consistent with earlier findings that patients with obstructive renal impairment caused by uterine fibroids are frequently seen by gynaecologists (Fletcher *et al*. 2013). It has also been reported that Serum uric acid levels can be elevated by reduced excretion via the kidneys (Angelopoulos *et al*. 2009). However, there was no singnificant ($p<0.05$) increase in serum levels of bicarbonate in PC-1 compared with NC-1 (Figure 4.4). There was also no significant difference in serum levels of total protein in PC-1 compared with NC-1 (Figure 4.4), suggesting that there is no relationship between the total protein levels and fibroid growth. This disagrees with the findings of Obochi *et al*. (2009) which states that there is a correlation between serum total protein levels and fibroid growth, but agrees with the findings of Dapilah (2009) that there is no significant difference in serum total protein levels of 200 women (aged between 20-40 yrs) diagnosed with fibroid and the recruited controls used for the study. Treatments with the ethanol extract of *Diodia sarmentosa* (Sw) leaves significantly decreased the serum levels of urea and creatinine. However, there was no significant difference in

serum levels of bicarbonate, sodium, uric acid and total protein in treated group (TG-1) compared with PC-1 (Figure 4.4).

4.2.2.1.2 Oxidative Stress and Antioxidant parameters

The significant ($p<0.05$) increase in serum levels of malondialdehyde (MDA), a marker of oxidative stress, in PC-1 compared with NC-1 (Figure 4.5) is an indication of the induction of oxidative stress by MSG in the rats. Oxygen radical production, which increased with clinical progression of disease involves increased lipid peroxidation, as a result of which there were cellular membrane degeneration and DNA damage (Pejic *et al*. 2006). Additionally, increase in levels of MDA indicated the upsurged lipid peroxidation as a consequence of the increase in free radical generation, these free radical may cause profound alteration in the function of the cell membrane and also structural organization of DNA, leading to mutation, therefore, it can be stated that lipid peroxidation product is one of the possible causes of uterine fibroid progression (Bilal, 2013). Moreover, oxidative stress has been shown to be a major player in common profibroitic gynecologic disorders such as fibroids (Vural *et al*. 2012, Rahman, 2007). This result is in agreement with findings of other investigations (Pejic *et al*. 2009; Mohammad *et al*. 2014; Dapilah, 2009).

The serum level of antioxidants (superoxide dismutase (SOD), glutathione peroxidase (GPx), Vit.C, Vit.E, catalase, reduced glutathione (GSH) and total antioxidant capacity (TAC)) were significantly ($p<0.05$) reduced in PC-1 compared to NC-1 (Figure 4.5). This can be explained that, in trying to counter oxidative stress induced by MSG, the system used up most of its enzymatic and non-enzymatic antioxidants resulting to a decrease in serum levels of these antioxidants. These results are also in agreement with earlier reports (Fletcher *et al*. 2013; Oyeyemi *et al*. 2016; Dapilah, 2009). Additionally, uterine fibroids have been reported to be

characterized by an impaired antioxidant cellular system (Fletcher *et al*. 2013). The ethanol extract of *Diodia sarmentosa* leaves was able to reduce the oxidative stress induced in the rats as seen in the significant ($p<0.05$) decrease in serum malondialdehyde (MDA) levels in TG-1 compared to PC-1 (Figure 4.5). It also improved the antioxidant status as seen in the significant ($p<0.05$) increase in serum levels of SOD, GPx, catalase, GSH, and total antioxidant capacity, however, there was no significant increase in serum levels of vitamin E and Vitamin C in TG-1 compared to PC-1 (Figure 4.5). The densely packed spindle shaped fibrous tissue and multifocal tumour cells in the endometrium as seen in the positive control (PC-1) was also reduced in number, resulting in reduced endometrial and myometrial wall thickness in the treated group (TG-1).

4.2.2.2 BENIGN PROSTATIC HYPERPLASIA

4.2.2.2.1 Biochemical parameters

Benign prostatic hyperplasia (BPH) is one of the most common diseases among men aged 40 yrs and above. It produces lower urinary tract symptoms which reduce the quality of daily life (Pais, 2010). Safe and effective natural interventions that reduce the symptoms and reverse or halt the progression of BPH have been the subject of considerable research interest (Hardik *et al*. 2014).

The significant ($p<0.05$) increase in serum levels of urea, creatinine, bicarbonate, sodium and uric acid and subsequent significant decrease in serum potassium levels in Benign Prostatic Hyperplasia Positive Control (PC-2) compared to Normal Control (NC-2) (Figure 4.6) suggest that BPH disorder is likely to interfere with normal renal functions that may progress to renal impairment. These results are consistent with findings of earlier investigations by Reshma *et al*. (2014) who found elevated levels of urea and creatinine in BPH patients compared to control, and also reported that BPH and cancer of prostate, the most common

types of prostatic disorders in old age, are more susceptible to the development of renal dysfunction (Reshma *et al*. 2014). In the physiological point of view, as the prostate enlarges, it compresses the urethra, preventing the outflow of urine and contributing to the common lower urinary tract symptoms (Ricardo *et al*. 2012). Treatment with ethanol extracts of *Diodia sarmentosa* (SW) was able to significantly ($p<0.05$) reduce the serum levels of urea, creatinine, sodium as well as increase the serum levels of potassium in the Benign Prostatic Hyperplasia treatment group (TG-2) compared with positive control (PC-2) (Figure 4.6), thereby improving the induced lower urinary tract abnormalities, although there was no significant difference in serum levels of uric acid in TG-2 compared to PC-2.

Testosterone also plays important role in development of BPH which is reflected by increase in prostate weight and size (Carter *et al*. 1992). The significant ($p<0.05$) increase in serum testosterone and PSA in PC-2 compared to NC-2 (Figure 4.6) confirms the above claim. Treatment with the plant extract was able to significantly ($p<0.05$) decrease the serum levels of testosterone, but did not significantly decrease serum PSA levels (Figure 4.6). A previous study has also shown that gonadectomy, which eliminates testicular testosterone during the early phase of BPH is more effective in inhibiting prostatic overgrowth than are antiandrogens or 5α-reductase inhibitors (Sikes *et al*. 1990). It is therefore assumed that the inhibitory effect of *Diodia sarmentosa* (SW) occurs at the time of the development of BPH. The insignificant difference ($p<0.05$) in serum level of chloride in positive control (PC-2) compared to normal control (NC-2) (Figure 4.6) suggests that this parameter may not have a direct effect on BPH development.

Previous studies using rat models has deduced that changes in prostate weight and histomorphology provide the main evidence for the inhibitory effects of substances on BPH development (Shin *et al*. 2012; Zheng *et al*. 2013; Cha *et al*. 2015). In the present study, ethanol extract of *Diodia sarmentosa* (Sw) reduced the prostate weight, prostate weight index

(Figure 4.8 & Figure 4.9) and histological abnormalities in testosterone induced BPH rats which supports the idea that *Diodia sarmentosa* (SW) may inhibit BPH development.

4.2.2.2.2 Oxidative Stress and Antioxidant Parameters

Oxidative stress triggers metabolic reprogramming responsible for malignant transformation and tumor development, including invasion and metastasis (Yoshida, 2015). There was a significant ($p<0.05$) increase in serum levels of malondialdehyde (MDA), an oxidative stress marker in positive control (PC-2) compared to normal control (NC-2) (Figure 4.7). MDA is an end product derived from peroxidation of polyunsaturated fatty acids and related esters. Additionally, MDA does not just reflect lipid peroxidation, but is also a by-product of cyclooxygenase activity in platelets, and persistent platelet activation is a common feature of many clinical syndromes associated with enhanced lipid peroxidation. Thus, measurement of MDA levels in plasma or serum provides a convenient in vivo index of lipid peroxidation and represents a noninvasive biomarker of oxidative stress often clinically employed to investigate radical-mediated physiological and pathological conditions (Meagher & FitzGerald, 2000). Significantly increased levels of oxidative stress and DNA damage suggest that oxidative damage plays an important role in prostate tumorigenesis and timely management of oxidative stress can be of importance in preventing the occurrence of prostatic hyperplasia (Aydin *et al*. 2006; Bostanci, 2013). This is in consonance with various studies that have reported on association between oxidative stress and the development of BPH (Park *et al*. 2016; Udensi & Tchounwou, 2016; Minciullo *et al*. 2015).

The significant ($p<0.05$) decrease in serum levels of MDA in the treatment group (TG-2) compared to the positive control (PC-2) (Figure 4.7) suggest that the ethanol extracts of *Diodia sarmentosa* (SW) leaves was able to counter the oxidative stress induced by

testosterone propionate (TP) induction. The significant ($p<0.05$) decrease in serum levels of SOD, GPx, vit. E, catalase and GSH in PC-2 compared to NC-2 (Figure 4.7) suggested a possible association between antioxidant enzyme activity and levels of DNA base lesions in BPH tissues as reported previously by Olinski *et al*. (1995). Normally, highly oxidative situations are removed by natural protective mechanisms such as the superoxide dismutase (SOD) enzyme system, glutathione peroxidase (GPX) and catalase (CAT) enzyme, as well as vitaminic antioxidants like α-tocopherol and ascorbate (Minciullo *et al*. 2015). The extent of ROS-induced oxidative damage can be exacerbated by a decreased efficiency of antioxidant defense mechanisms. Additionally, balance between OS and the antioxidant component of the cells has a role in developing prostate disease (Khandrika *et al*. 2009). Increases in oxidative stress and decreased antioxidant mechanisms in prostate diseases have been reported in several studies; however, the data are not univocal. Lower activities of SOD (Arsova-Sarafinovska *et al*. 2009; Aydin *et al*. 2006), GPX (Arsova-Sarafinovska *et al*. 2009; Merendino *et al*. 2003) and reduced GSH concentration (Srivastava & Mittal, 2005) versus corresponding controls have been repoted in BPH patients; however, one of these studies (Arsova-Sarafinovska *et al*. 2009) reported different results between patients from Macedonia and Turkey, the latter showing a decreased activity only in GPX but this study recorded a decrease in all the antioxidant enzymes with an exception to vitamin c and total antioxidant capacity.

Vitamin E (α-Tocopherol) and vitamin C (ascorbate) act in synergy in the membrane and cytosol of the cell (Minciullo *et al*. 2015). α-Tocopherol scavenges lipid peroxy free radicals and interrupts the chain reaction of lipid peroxidation, becoming oxidized itself in the process. Ascorbate present in the aqueous compartments acts as a water-soluble chain-breaking antioxidant, converts the tocopheroxyl radical back to active α-tocopherol, thereby replenishing antioxidant activity of α-tocopherol (Winkler *et al*. 1994). This might explain

why there is a significant ($p<0.05$) decrease in serum levels of vitamin E in PC-2 compared with NC-2 and there was no significant difference in serum levels of vitamin C in PC-2 compared to NC-2 (Figure 4.7), revealing the antioxidant used in the first line of defence. The insignificant difference in serum levels of total antioxidant capacity (TAC) in PC-2 compared with NC-2 (Figure 4.7) is in consonance with the findings of Jones *et al*. (2013).

The increase in serum levels of SOD, GPx, vitamin E, catalase, GSH and TAC in the Benign Prostatic Hyperplasia treated group (TG-2) compared with the positive control (PC-2) (Figure 4.7) reveals that the ethanol extract of *Diodia sarmentosa* (Sw) has the ability to mop up the free radicals, replenish depleted antioxidants in a BPH condition, thereby reversing oxidative stress induced.

4.2.2.3 HEPATOCELLULAR CARCINOMA

4.2.2.3.1 Biochemical Parameters

Significant ($p<0.05$) increase in serum levels of the transaminases (ALT, AST) & GGT in the Hepatocellular Carcinoma Positive Control (PC-3) compared to the Normal Control (NC-3) (Figure 4.10) reveal that DEN induced hepatocellular carcinoma (HCC). Several studies have reported similar elevation in the activities of serum AST and ALT during diethylnitrosamine administration (Bansal *et al*. 2000). The liver associated enzymes, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and gamma glutamyl transferase (GGT) are measures of liver homeostasis (Robert, 1999). Such elevations in the transaminases are considered as the most sensitive markers in the diagnosis of hepatocellular damage and loss of functional integrity of the membrane which subsequently break down membrane architecture of the cells and leads to their spillage into serum where their concentration is elevated (Sayed-Ahmed *et al*. 2010; Hassan *et al*. 2014). Moreover, GGT elevation reveals cholestasis and bile duct necrosis. This significant elevation of GGT in

serum may be attributed to its liberation from the plasma membrane into the circulation as it is located on the outer membrane of the hepatic cells, indicating damage of the cell membrane as a result of carcinogenesis (Hassan *et al*. 2014). This observed increase in liver function enzyme markers by DEN could be a secondary event following DEN-induced lipid peroxidation of hepatocyte membranes with the consequent increase in the leakage of enzymes from liver tissues by a production of reactive oxygen and nitrogen species. This action of DEN plays an important role in DEN-induced initiation of hepatic carcinogenesis (Sayed-Ahmed *et al*. 2010).

Alpha feto protein (AFP) is a glycoprotein which is normally produced during fetal development by the hepatocytes, yolk sac and to a lesser extent by the gastrointestinal tract. It is a biomarker indicating the cancerous state of the liver (Roy & Gadad, 2016). Significant ($p<0.05$) increase in serum AFP level in the positive control (PC-3) compared to normal control (NC-3) (Figure 4.10), might be as a result of DEN intoxication that caused necrosis of the hepatocytes (Fathy *et al*. 2017). Hepatocyte localization within the liver plate or outside it is the defining factor that regulates the activity of AFP synthesis on a cellular level (Motalleb *et al*. 2008).

Proteins and its synthesis is carried out in the liver, and it is an important phenomenon in normal as well as in neoplastic conditions. The highest rate of synthesis of tissue proteins and major protein mass is severely affected by cancer (Kumar *et al*. 2016). Significant ($p<0.05$) reduction in serum total protein in DEN treated group (Figure 4.10) could be ascribed to the development of DEN-induced hepatic lesions (Plate 4.12 and Plate 4.13) observed in the present study that affected protein synthesis, and this concurs with earlier findings (Ha *et al*. 2001, Ahmed *et al*. 2014). Ha *et al*. (2001) observed the same effect on administration of 0.01 percent DEN in drinking water *ad libitum* for 13 weeks in adult male wistar albino rats where serum protein levels were significantly decreased in the DEN group. The significant

increase in serum levels of uric acid in PC-3 compared to NC-3 (Figure 4.10) suggest that elevated serum uric acid levels may be a risk factor for the incidence of chronic liver disease. This is in consonance with the findings of Benerji *et al*. (2013) who reported novel associations between serum uric acid levels and the incidence of cirrhosis-related hospitalization or the presence of elevated serum ALT or GGT (Benerji *et al*. 2013). However, the result of this study revealed that treatment with ethanol extract of *Diodia sarmentosa* had no obvious effect on uric acid level as there was no significant diference ($p<0.05$) between the treated group compared with the positive control group. There have also been several studies investigating the association between serum uric acid and cancer in both healthy people and cancer patients (Yiwei, 2018). A large prospective study (Strasak *et al*. 2007) on more than 28,000 elderly Austrian women found an association between high serum uric acid level (>5.41 ml/dl) and fatal cancer events ($p<0.0001$). Strasak *et al*. (2007) also confirmed similar findings in a male population across a wide age range. Further, they demonstrated a dose-response to baseline serum uric acid, which was a time dependent risk factor for cancer incidence. Treatment with ethanol extract of *Diodia sarmentosa* (Sw) leaves significantly ($p<0.05$) reduced the serum levels of alaninine transaminase (ALT), gamma glutamyl transaminase (GGT), aspartate transaminase (AST), and alpha feto protein (AFP) (Figure 4.10), thus, regenerating these liver enzymes.

Significant ($p<0.05$) increases in serum levels of urea, creatinine, chloride, sodium and bicarbonate and the subsequent decrease in serum potassium levels in positive control (PC-3) compared to normal control (NC-3) (Figure 4.10), indicates a possible kidney injury in the DEN induced group. These data are in consonance with earlier findings by Bucsics & Krones (2017) which associated kidney injury with liver diseases. Treatment with *Diodia sarmentosa* (Sw) was able to significantly reduce serum levels of urea, chloride, sodium, uric acid and

bicarbonate as well improve potassium levels. However, there was no significant difference (P>0.05) in serum creatinine levels of treated group compared to positive control (PC-3).

4.2.2.3.2 Oxidative Stress and Antioxidant Parameters

Induction of diethylnitrosamine in the albino wistar rats led to the generation of reactive oxygen species and significant deleterious changes in antioxidant status. This can be seen in the significant ($p<0.05$) increase in serum levels of MDA (Figure 4.11) and significant ($P<0.05$) decrease in antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), vitamin E (alpha tocopherol), catalase, reduced glutathione (GSH), vitamin C and total antioxidant capacity in the Hepatocellular Carcinoma Positive Control (PC-3) compared with the Normal Control (NC-3) (Figure 4.11).

Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species ($O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$) generated exceeds the antioxidant capability of the cell (Sies, 1991).

The status of lipid peroxidation (MDA) as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidative stress (Khan, 2006). Free radical scavenging enzymes like superoxide dismutase (SOD) and catalase protect the biological systems from oxidative stress. The SOD dismutates superoxide radicals ($O_2.^-$) into hydrogen peroxide (H_2O_2) and O_2 (Fridovich, 1986). Catalase further detoxifies H_2O_2 into H_2O and O_2 (Murray *et al*. 2003). Glutathione peroxidase also functions in detoxifying H_2O_2 similar to catalase. Thus, SOD, catalase and glutathione peroxidase act mutually and constitute the enzymic antioxidative defense mechanism against reactive oxygen species (Bhattacharjee & Sil, 2006). The decrease in the activities of these enzymes in this study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the metabolism of diethylnitrosamine. This is further substantiated by an elevation in the levels of lipid peroxidation.

The significant ($p<0.05$) decrease in serum levels of the antioxidant vitamins (vitamin C and E) and GSH observed following diethylnitrosamine administration might be due to the excessive utilization of these vitamins in scavenging the free radicals formed during the metabolism of diethylnitrosamine (DEN). Non-enzymic antioxidants like vitamin-C and E act synergistically to scavenge the free radicals formed in the biological system (Pradeep *et al*. 2007). Reduced glutathione (GSH) acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation (Chaudiere, 1994). Vitamin-C also scavenges and detoxifies free radicals in combination with vitamin-E and glutathione (George, 2003). It plays a vital role by regenerating the reduced form of vitamin-E and preventing the formation of excessive free radicals (Das, 1994). Similar reports have shown an elevation in the status of lipid peroxidation in the liver during diethylnitrosamine treatment (Nakae *et al*. 1997; Sanchez-Parez *et al*. 2005) and the present results are in consonance with these findings. These results are also in consonance with earlier findings of impaired antioxidant system in DEN induced wistar albino rats (Pradeep *et al*. 2007).

Treatment with *Diodia sarmentosa* (Sw) was able to improve the antioxidant defense system as seen in the significant ($p<0.05$) increase in serum levels of SOD, GPx, catalase, GSH, vitamin C and total antioxidant capacity in the Hepatocellular Carcinoma treatment group (TG-3) compared to the positive control (PC-3) (Figure 4.11). It was also able to maintain the GSH homeostasis in the system as seen in the significant increase of this antioxidant in the treatment group (TG-3) compared to positive control (PC-3) (Figure 4.11). Increase in GSH levels in turn contributes to the recycling of other antioxidants such as vitamin-E and vitamin-C (Exner *et al*. 2000). This can be seen also in the increased activities of vitamin C in the treated group (TG-3) (Figure 4.11). However, there was no significant ($p<0.05$) difference in serum levels of vitamin E and MDA in the treated group (TG-3) compared to positive control (PC-3). The histological abnormalities in the positive control (PC-3) such as the

nuclear atypia of tumour cells with obvious resemblance to hepatocytes but that show enlarged nuclei were greatly reduced in the treated group (TG-3). This is evidenced by the presence of one cell-thick hepatocellular plates/cords seperated by sinusoids with normal nucleus and cytoplasmic ratio and the absence of atypia nucleoli in the treated group (TG-3).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Evidence from the qualitative and quantitative phytochemical evaluation as well as the different in vitro antioxidant assessments of the ethanol extracts of *Diodia sarmentosa* (Sw) revealed that the plant has antioxidant properties. This was also confirmed by the results of the *in vivo* evaluation of appropriate parameters for antioxidant activity. Additionally, the ethanol extracts of *Diodia sarmentosa* (Sw) revealed in the experimental rats, biochemical activities which are veritable tools in the understanding of cancer biology and relevant in the drug discovery process needed to enhance cancer treatment and survival. These findings were also well embellished with histopathological studies which also revealed the extent of oxidative stress in the tissues as well as progression of the plant extract in mitigating the abnormalities in the affected tissues. Based on the findings of this study, the null hypothesis that *Diodia sarmentosa* (Sw) does not have any biochemical, antioxidant and antitumour properties have been rejected and the alternate hypothesis which states that *Diodia sarmentosa* (Sw) has biochemical, antioxidant and antitumor properties has been accepted.

5.2 RECOMMENDATION

In this study, only ethanol extraction of *Diodia sarmentosa* (Sw) leaves was used *in vivo*, other solvents and combined effects should be looked into in further studies. More work should also be done to ascertain if the biochemical, antioxidant and anti-tumour activities revealed, are dose dependent and possibly, isolation of the relevant phytochemicals responsible for these effects should be carried out.

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APPENDICES

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1. Correlation between Concentration and Parameters

Correlation: CONC, DPPHa, DPPHe, DPPHa(Std)

	CONC	DPPHa	DPPHe
DPPHa	-0.955		
	0.001		
DPPHe	0.704	-0.583	
	0.078	0.169	
DPPHa(Std)	0.415	-0.511	0.257
	0.354	0.241	0.578

Cell Contents: Pearson correlation
P-Value

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One-way ANOVA: DPPHa(Std), DPPHe, DPPHa

```
Method

Null hypothesis         All means are equal
Alternative hypothesis  At least one mean is different
Significance level      α = 0.05

Equal variances were assumed for the analysis.


Factor Information

Factor  Levels  Values
Factor       3  DPPHa(Std), DPPHe, DPPHa


Analysis of Variance

Source  DF  Adj SS   Adj MS  F-Value  P-Value
Factor   2  2242.2  1121.12    36.90    0.000
Error   18   546.9    30.38
Total   20  2789.1


Model Summary

      S    R-sq  R-sq(adj)  R-sq(pred)
5.51191  80.39%     78.21%      73.31%


Means

Factor      N    Mean  StDev       95% CI
DPPHa(Std)  7  63.487  0.734  (59.110, 67.864)
DPPHe       7   82.59   9.42  ( 78.21,  86.97)
DPPHa       7  87.420  1.354  (83.043, 91.796)

Pooled StDev = 5.51191
```

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Correlation: CONC, AAEa, AAEe

```
       CONC   AAEa
AAEa  0.987
      0.000

AAEe  0.424  0.380
      0.344  0.401


Cell Contents: Pearson correlation
               P-Value
```

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One-way ANOVA: AAEa, AAEe

```
Method

Null hypothesis         All means are equal
Alternative hypothesis  At least one mean is different
Significance level      α = 0.05

Equal variances were assumed for the analysis.


Factor Information

Factor  Levels  Values
Factor       2  AAEa, AAEe


Analysis of Variance

Source  DF  Adj SS  Adj MS  F-Value  P-Value
Factor   1   39.01  39.013     5.00    0.045
Error   12   93.56   7.797
Total   13  132.57


Model Summary

      S    R-sq  R-sq(adj)  R-sq(pred)
2.79227  29.43%     23.55%       3.94%


Means

Factor  N    Mean   StDev        95% CI
AAEa    7    5.18    3.95  (   2.88,   7.48)
AAEe    7  1.8396  0.0937  (-0.4599, 4.1391)

Pooled StDev = 2.7922
```

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Welcome to Minitab, press F1 for help.
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Correlation: Concentration, TBARSa, TBARSe, TBARS (STD)

```
               Concentration         TBARSa         TBARSe
TBARSa                 0.553
                       0.255

TBARSe                -0.834         -0.025
                       0.039          0.963

TBARS(STD)             0.901          0.824         -0.526
                       0.014          0.044          0.284


Cell Contents: Pearson correlation
               P-Value
```

12/12/2018 3:38:58 AM

One-way ANOVA: TBARSa, TBARSe, TBARS(STD)

```
Method

Null hypothesis         All means are equal
Alternative hypothesis  At least one mean is different
Significance level      α = 0.05

Equal variances were assumed for the analysis.


Factor Information

Factor  Levels  Values
Factor       3  TBARSa, TBARSe, TBARS(STD)


Analysis of Variance

Source  DF  Adj SS  Adj MS  F-Value  P-Value
Factor   2    2157  1078.4     5.88    0.013
Error   15    2751   183.4
Total   17    4908


Model Summary

      S    R-sq  R-sq(adj)  R-sq(pred)
13.5435  43.94%     36.47%      19.28%

Means

Factor      N   Mean  StDev      95% CI
TBARSa      6  38.26  11.34  (26.48, 50.05)
TBARSe      6  17.91   3.36  ( 6.12, 29.69)
TBARS(STD)  6  43.20  20.26  (31.41, 54.98)

Pooled StDev = 13.5435
```

Uterine Leiomyoma Biochemical Descriptive

		N	Mean	Std. Deviation	Std. Error	95% Confid Lower Bound
Urea	Normal Control	4	28.2500	.95743	.47871	26.7265
	Pos. Control (Diseased grp)	4	45.0000	.81650	.40825	43.7008
	Treated group	4	29.2500	.50000	.25000	28.4544
	Total	12	34.1667	8.04344	2.32194	29.0561
Creatinine	Normal Control	4	1.0025	.01708	.00854	.9753
	Pos. Control (Diseased grp)	4	1.4300	.02582	.01291	1.3889
	Treated group	4	1.0550	.03416	.01708	1.0006
	Total	12	1.1625	.20028	.05782	1.0352
Potassium	Normal Control	4	3.6500	.12910	.06455	3.4446

	Pos. Control (Diseased grp)	4	2.6250	.05000	.02500	2.5454
	Treated group	4	3.0250	.12583	.06292	2.8248
	Total	12	3.1000	.45126	.13027	2.8133
Chloride	Normal Control	4	94.5000	.57735	.28868	93.5813
	Pos. Control (Diseased grp)	4	97.0000	1.41421	.70711	94.7497
	Treated group	4	96.2500	.50000	.25000	95.4544
	Total	12	95.9167	1.37895	.39807	95.0405
Bicarbonate	Normal Control	4	30.8250	1.29711	.64856	28.7610
	Pos. Control (Diseased grp)	4	32.0500	2.00749	1.00374	28.8556
	Treated group	4	30.9500	1.61142	.80571	28.3859
	Total	12	31.2750	1.61139	.46517	30.2512
Sodium	Normal Control	4	188.8750	2.61709	1.30855	184.7106
	Pos. Control (Diseased grp)	4	205.7000	2.23010	1.11505	202.1514
	Treated group	4	197.4500	12.46261	6.23131	177.6192
	Total	12	197.3417	9.85185	2.84399	191.0821
Uric_Acid	Normal Control	4	6.7400	.07616	.03808	6.6188
	Pos. Control (Diseased grp)	4	7.2775	.08057	.04029	7.1493
	Treated group	4	7.2400	.23480	.11740	6.8664
	Total	12	7.0858	.28962	.08361	6.9018
Total_Protein	Normal Control	4	4.2250	.09574	.04787	4.0727
	Pos. Control (Diseased grp)	4	4.3000	.21602	.10801	3.9563
	Treated group	4	4.2000	.16330	.08165	3.9402
	Total	12	4.2417	.15643	.04516	4.1423
Estradiol	Normal Control	4	49.7775	.55584	.27792	48.8930
	Pos. Control (Diseased grp)	4	78.5975	1.00540	.50270	76.9977
	Treated group	4	75.0950	.25053	.12527	74.6963
	Total	12	67.8233	13.42520	3.87552	59.2934
Progesterone	Normal Control	4	8.7800	.24913	.12457	8.3836
	Pos. Control (Diseased grp)	4	14.6375	.27861	.13931	14.1942
	Treated group	4	10.1325	.22322	.11161	9.7773
	Total	12	11.1833	2.62531	.75786	9.5153

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Urea	Between Groups	706.167	2	353.083	577.773	.000
	Within Groups	5.500	9	.611		
	Total	711.667	11			
Creatinine	Between Groups	.435	2	.217	306.953	.000
	Within Groups	.006	9	.001		

	Total	.441	11			
Potassium	Between Groups	2.135	2	1.068	91.500	.000
	Within Groups	.105	9	.012		
	Total	2.240	11			
Chloride	Between Groups	13.167	2	6.583	7.645	.011
	Within Groups	7.750	9	.861		
	Total	20.917	11			
Bicarbonate	Between Groups	3.635	2	1.818	.656	.542
	Within Groups	24.928	9	2.770		
	Total	28.563	11			
Sodium	Between Groups	566.232	2	283.116	5.082	.033
	Within Groups	501.417	9	55.713		
	Total	1067.649	11			
Uric_Acid	Between Groups	.720	2	.360	16.027	.001
	Within Groups	.202	9	.022		
	Total	.923	11			
Total_Protein	Between Groups	.022	2	.011	.394	.685
	Within Groups	.248	9	.028		
	Total	.269	11			
Estradiol	Between Groups	1978.448	2	989.224	2146.520	.000
	Within Groups	4.148	9	.461		
	Total	1982.595	11			
Progesterone	Between Groups	75.246	2	37.623	595.563	.000
	Within Groups	.569	9	.063		
	Total	75.815	11			

Multiple Comparisons

LSD

Dependent Variable	(I) UTERINE_LEIOMYOMA	(J) UTERINE_LEIOMYOMA	Mean Difference (I-J)	Std. Erro
Urea	Normal Control	Pos. Control (Diseased grp)	-16.75000*	
		Treated group	-1.00000	
	Pos. Control (Diseased grp)	Normal Control	16.75000*	
		Treated group	15.75000*	
	Treated group	Normal Control	1.00000	
		Pos. Control (Diseased grp)	-15.75000*	
Creatinine	Normal Control	Pos. Control (Diseased grp)	-.42750*	
		Treated group	-.05250*	
	Pos. Control (Diseased grp)	Normal Control	.42750*	

		Treated group	.37500*
	Treated group	Normal Control	.05250*
		Pos. Control (Diseased grp)	-.37500*
Potassium	Normal Control	Pos. Control (Diseased grp)	1.02500*
		Treated group	.62500*
	Pos. Control (Diseased grp)	Normal Control	-1.02500*
		Treated group	-.40000*
	Treated group	Normal Control	-.62500*
		Pos. Control (Diseased grp)	.40000*
Chloride	Normal Control	Pos. Control (Diseased grp)	-2.50000*
		Treated group	-1.75000*
	Pos. Control (Diseased grp)	Normal Control	2.50000*
		Treated group	.75000
	Treated group	Normal Control	1.75000*
		Pos. Control (Diseased grp)	-.75000
Bicarbonate	Normal Control	Pos. Control (Diseased grp)	-1.22500
		Treated group	-.12500
	Pos. Control (Diseased grp)	Normal Control	1.22500
		Treated group	1.10000
	Treated group	Normal Control	.12500
		Pos. Control (Diseased grp)	-1.10000
Sodium	Normal Control	Pos. Control (Diseased grp)	-16.82500*
		Treated group	-8.57500
	Pos. Control (Diseased grp)	Normal Control	16.82500*
		Treated group	8.25000
	Treated group	Normal Control	8.57500
		Pos. Control (Diseased grp)	-8.25000
Uric_Acid	Normal Control	Pos. Control (Diseased grp)	-.53750*
		Treated group	-.50000*
	Pos. Control (Diseased grp)	Normal Control	.53750*
		Treated group	.03750
	Treated group	Normal Control	.50000*
		Pos. Control (Diseased grp)	-.03750
Total_Protein	Normal Control	Pos. Control (Diseased grp)	-.07500
		Treated group	.02500
	Pos. Control (Diseased grp)	Normal Control	.07500
		Treated group	.10000
	Treated group	Normal Control	-.02500
		Pos. Control (Diseased grp)	-.10000
Estradiol	Normal Control	Pos. Control (Diseased grp)	-28.82000*
		Treated group	-25.31750*

	Pos. Control (Diseased grp)	Normal Control	28.82000*
		Treated group	3.50250*
	Treated group	Normal Control	25.31750*
		Pos. Control (Diseased grp)	-3.50250*
Progesterone	Normal Control	Pos. Control (Diseased grp)	-5.85750*
		Treated group	-1.35250*
	Pos. Control (Diseased grp)	Normal Control	5.85750*
		Treated group	4.50500*
	Treated group	Normal Control	1.35250*
		Pos. Control (Diseased grp)	-4.50500*

*. The mean difference is significant at the 0.05 level.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interva	
						Lower Bound	Up
MDA	Normal Control	4	3.6450	.17311	.08655	3.3695	
	Pos. Control (Diseased grp)	4	4.4550	.06856	.03428	4.3459	
	Treated group	4	4.1350	.01732	.00866	4.1074	
	Total	12	4.0783	.36136	.10431	3.8487	
SOD	Normal Control	4	11.7250	.12583	.06292	11.5248	
	Pos. Control (Diseased grp)	4	11.2000	.21602	.10801	10.8563	
	Treated group	4	11.5000	.08165	.04082	11.3701	
	Total	12	11.4750	.26328	.07600	11.3077	
GPx	Normal Control	4	12.2000	.14142	.07071	11.9750	
	Pos. Control (Diseased grp)	4	8.7000	.18257	.09129	8.4095	
	Treated group	4	10.7500	.26458	.13229	10.3290	
	Total	12	10.5500	1.51087	.43615	9.5900	
Vit_E	Normal Control	4	1.7650	.02380	.01190	1.7271	
	Pos. Control (Diseased grp)	4	2.0725	.07089	.03544	1.9597	

	Treated group	4	1.9850	.13102	.06551	1.7765
	Total	12	1.9408	.15641	.04515	1.8415
CAT	Normal Control	4	7.3025	.06500	.03250	7.1991
	Pos. Control (Diseased grp)	4	3.8650	.11590	.05795	3.6806
	Treated group	4	5.8500	.06055	.03028	5.7536
	Total	12	5.6725	1.47358	.42539	4.7362
GSH	Normal Control	4	2.0450	.02646	.01323	2.0029
	Pos. Control (Diseased grp)	4	1.7050	.04509	.02255	1.6332
	Treated group	4	1.7850	.05260	.02630	1.7013
	Total	12	1.8450	.15647	.04517	1.7456
Vit_C	Normal Control	4	1.2850	.04796	.02398	1.2087
	Pos. Control (Diseased grp)	4	.9425	.08180	.04090	.8123
	Treated group	4	.9400	.09129	.04564	.7947
	Total	12	1.0558	.18268	.05274	.9398
TAC	Normal Control	4	1.8550	.11590	.05795	1.6706
	Pos. Control (Diseased grp)	4	1.1425	.16998	.08499	.8720
	Treated group	4	1.3725	.00957	.00479	1.3573
	Total	12	1.4567	.32823	.09475	1.2481

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
MDA	Between Groups	1.331	2	.666	57.117	.000
	Within Groups	.105	9	.012		
	Total	1.436	11			
SOD	Between Groups	.555	2	.278	12.036	.003
	Within Groups	.207	9	.023		
	Total	.763	11			
GPx	Between Groups	24.740	2	12.370	300.892	.000
	Within Groups	.370	9	.041		
	Total	25.110	11			
Vit_E	Between Groups	.201	2	.100	13.236	.002
	Within Groups	.068	9	.008		
	Total	.269	11			
CAT	Between Groups	23.822	2	11.911	1675.628	.000
	Within Groups	.064	9	.007		
	Total	23.886	11			
GSH	Between Groups	.253	2	.126	68.945	.000
	Within Groups	.017	9	.002		
	Total	.269	11			

Vit_C	Between Groups	.315	2	.158	27.283	.000
	Within Groups	.052	9	.006		
	Total	.367	11			
TAC	Between Groups	1.058	2	.529	37.408	.000
	Within Groups	.127	9	.014		
	Total	1.185	11			

Multiple Comparisons

LSD

Dependent Variable	(I) Uterine_Leiomyoma_Antioxidant	(J) Uterine_Leiomyoma_Antioxidant	Mean Difference (I-J)	Std. Error
MDA	Normal Control	Pos. Control (Diseased grp)	-.81000*	.07634
		Treated group	-.49000*	.07634
	Pos. Control (Diseased grp)	Normal Control	.81000*	.07634
		Treated group	.32000*	.07634
	Treated group	Normal Control	.49000*	.07634
		Pos. Control (Diseased grp)	-.32000*	.07634
SOD	Normal Control	Pos. Control (Diseased grp)	.52500*	.10737
		Treated group	.22500	.10737
	Pos. Control (Diseased grp)	Normal Control	-.52500*	.10737
		Treated group	-.30000*	.10737
	Treated group	Normal Control	-.22500	.10737
		Pos. Control (Diseased grp)	.30000*	.10737
GPx	Normal Control	Pos. Control (Diseased grp)	3.50000*	.14337
		Treated group	1.45000*	.14337
	Pos. Control (Diseased grp)	Normal Control	-3.50000*	.14337
		Treated group	-2.05000*	.14337
	Treated group	Normal Control	-1.45000*	.14337
		Pos. Control (Diseased grp)	2.05000*	.14337
Vit_E	Normal Control	Pos. Control (Diseased grp)	-.30750*	.06159
		Treated group	-.22000*	.06159
	Pos. Control (Diseased grp)	Normal Control	.30750*	.06159
		Treated group	.08750	.06159
	Treated group	Normal Control	.22000*	.06159
		Pos. Control (Diseased grp)	-.08750	.06159
CAT	Normal Control	Pos. Control (Diseased grp)	3.43750*	.05962
		Treated group	1.45250*	.05962
	Pos. Control (Diseased grp)	Normal Control	-3.43750*	.05962

		Treated group	-1.98500*	.05962
	Treated group	Normal Control	-1.45250*	.05962
		Pos. Control (Diseased grp)	1.98500*	.05962
GSH	Normal Control	Pos. Control (Diseased grp)	.34000*	.03028
		Treated group	.26000*	.03028
	Pos. Control (Diseased grp)	Normal Control	-.34000*	.03028
		Treated group	-.08000*	.03028
	Treated group	Normal Control	-.26000*	.03028
		Pos. Control (Diseased grp)	.08000*	.03028
Vit_C	Normal Control	Pos. Control (Diseased grp)	.34250*	.05374
		Treated group	.34500*	.05374
	Pos. Control (Diseased grp)	Normal Control	-.34250*	.05374
		Treated group	.00250	.05374
	Treated group	Normal Control	-.34500*	.05374
		Pos. Control (Diseased grp)	-.00250	.05374
TAC	Normal Control	Pos. Control (Diseased grp)	.71250*	.08408
		Treated group	.48250*	.08408
	Pos. Control (Diseased grp)	Normal Control	-.71250*	.08408
		Treated group	-.23000*	.08408
	Treated group	Normal Control	-.48250*	.08408
		Pos. Control (Diseased grp)	.23000*	.08408

*. The mean difference is significant at the 0.05 level.

BPH Biochemical Descriptives

yield

Parameter		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval Lower Bound	Upp
Urea	Normal Control	4	26.0000	0.81650	0.40825	24.7008	
	Pos. Control (Diseased grp)	4	38.5000	1.91485	0.95743	35.4530	
	Treated Group	4	34.7500	0.95743	0.47871	33.2265	
	Total	12	33.0833	5.59965	1.61648	29.5255	
Creatinine	Normal Control	4	0.9175	0.01708	0.00854	0.8903	
	Pos. Control (Diseased grp)	4	1.3425	0.01708	0.00854	1.3153	
	Treated Group	4	1.2400	0.01414	0.00707	1.2175	
	Total	12	1.1667	0.18970	0.05476	1.0461	
K	Normal Control	4	3.0000	0.08165	0.04082	2.8701	
	Pos. Control (Diseased grp)	4	2.6250	0.17078	0.08539	2.3532	
	Treated Group	4	2.8250	0.09574	0.04787	2.6727	
	Total	12	2.8167	0.19462	0.05618	2.6930	
Cl	Normal Control	4	96.2500	0.95743	0.47871	94.7265	

	Pos. Control (Diseased grp)	4	97.2500	0.95743	0.47871	95.7265	
	Treated Group	4	96.2500	0.50000	0.25000	95.4544	
	Total	12	96.5833	0.90034	0.25990	96.0113	
Na	Normal Control	4	191.5750	12.23720	6.11860	172.1029	
	Pos. Control (Diseased grp)	4	214.4750	3.55844	1.77922	208.8127	
	Treated Group	4	186.0500	3.43074	1.71537	180.5909	
	Total	12	197.3667	14.58445	4.21017	188.1002	
HCO3	Normal Control	4	28.7500	1.56098	0.78049	26.2661	
	Pos. Control (Disease grp)	4	30.3750	0.66018	0.33009	29.3245	
	Treated Group	4	28.9750	0.17078	0.08539	28.7032	
	Total	12	29.3667	1.16411	0.33605	28.6270	
Uric Acid	Normal Control	4	4.6750	0.25000	0.12500	4.2772	
	Pos. control (Disease grp)	4	6.4750	0.18930	0.09465	6.1738	
	Treated Group	4	6.4000	0.16330	0.08165	6.1402	
	Total	12	5.8500	0.88780	0.25628	5.2859	
Testosterone	Normal Control	4	5.7175	0.84728	0.42364	4.3693	
	Pos. Control (Disease grp)	4	12.5150	1.62613	0.81307	9.9275	
	Treated Group	4	7.0875	0.17231	0.08616	6.8133	
	Total	12	8.4400	3.21309	0.92754	6.3985	
PSA	Normal Control	4	0.8650	0.04359	0.02179	0.7956	
	Pos. Control (Disease grp)	4	1.0200	0.10296	0.05148	0.8562	
	Treated Group	4	0.9200	0.03162	0.01581	0.8697	
	Total	12	0.9350	0.09040	0.02610	0.8776	

ANOVA

yield

Parameter		Sum of Squares	Df	Mean Square	F	Sig.
Urea	Between Groups	329.167	2	164.583	94.048	0.000
	Within Groups	15.750	9	1.750		
	Total	344.917	11			
Creatinine	Between Groups	0.394	2	0.197	753.543	0.000
	Within Groups	0.002	9	0.000		
	Total	0.396	11			
K	Between Groups	0.282	2	0.141	9.389	0.006
	Within Groups	0.135	9	0.015		
	Total	0.417	11			
Cl	Between Groups	2.667	2	1.333	1.920	0.202
	Within Groups	6.250	9	00.694		
	Total	8.917	11			

Na	Between Groups	1817.222	2	908.611	15.649	0.001
	Within Groups	522.545	9	58.061		
	Total	2339.767	11			
HCO3	Between Groups	6.202	2	3.101	3.206	0.089
	Within Groups	8.705	9	0.967		
	Total	14.907	11			
Uric Acid	Between Groups	8.295	2	4.148	99.540	0.000
	Within Groups	0.375	9	.042		
	Total	8.670	11			
Testosterone	Between Groups	103.388	2	51.694	45.721	0.000
	Within Groups	10.176	9	1.131		
	Total	113.563	11			
PSA	Between Groups	0.049	2	0.025	5.489	0.028
	Within Groups	0.040	9	0.004		
	Total	0.090	11			

Multiple Comparisons

Dependent Variable: yield

LSD

Dependent Variable	(I) BPH	(J) BPH	Mean Difference (I-J)	Std. Error	Sig.	95% Confi Lower Bound
Urea	Normal Control	Positive Control(Diseased grp)	-12.50000*	.93541	0.000	-14.6161
		Treated Group	-8.75000*	.93541	0.000	-10.8661
	Positive Control	Normal Control	12.50000*	.93541	0.000	10.3839
		Treated Group	3.75000*	.93541	0.003	1.6339
	Treated Group	Normal Control	8.75000*	.93541	0.000	6.6339
		Positive Control(Diseased grp)	-3.75000*	.93541	0.003	-5.8661
Creatinine	Normal Control	Positive Control(Diseased grp)	-.42500*	.01143	0.000	-.4508
		Treated Group	-.32250*	.01143	0.000	-.3483
	Positive Control	Normal Control	.42500*	.01143	0.000	.3992
		Treated Group	.10250*	.01143	0.000	.0767
	Treated Group	Normal Control	.32250*	.01143	0.000	.2967
		Positive Control(Diseased grp)	-.10250*	.01143	0.000	-.1283
K	Normal Control	Positive Control(Diseased grp)	.37500*	.08660	0.002	.1791
		Treated Group	.17500	.08660	0.074	-.0209
	Positive Control	Normal Control	-.37500*	.08660	0.002	-.5709
		Treated Group	-.20000*	.08660	0.046	-.3959
	Treated Group	Normal Control	-.17500	.08660	0.074	-.3709
		Positive Control(Diseased grp)	.20000*	.08660	0.046	.0041
Cl	Normal Control	Positive Control(Diseased grp)	-1.00000	.58926	0.124	-2.3330

		Treated Group	.00000	.58926	1.000	-1.3330
	Positive Control	Normal Control	1.00000	.58926	0.124	-.3330
		Treated Group	1.00000	.58926	0.124	-.3330
	Treated Group	Normal Control	.00000	.58926	1.000	-1.3330
		Positive Control(Diseased grp)	-1.00000	.58926	.124	-2.3330
Na	Normal Control	Positive Control(Diseased grp)	-22.90000*	5.38798	0.002	-35.0884
		Treated Group	5.52500	5.38798	0.332	-6.6634
	Positive Control	Normal Control	22.90000*	5.38798	0.002	10.7116
		Treated Group	28.42500*	5.38798	0.001	16.2366
	Treated Group	Normal Control	-5.52500	5.38798	0.332	-17.7134
		Positive Control(Diseased grp)	-28.42500*	5.38798	0.001	-40.6134
HCO3	Normal Control	Positive Control(Diseased grp)	-1.62500*	0.69542	0.044	-3.1982
		Treated Group	-.22500	0.69542	0.754	-1.7982
	Positive Control	Normal Control	1.62500*	0.69542	0.044	.0518
		Treated Group	1.40000	0.69542	0.075	-.1732
	Treated Group	Normal Control	0.22500	0.69542	0.754	-1.3482
		Positive Control(Diseased grp)	-1.40000	0.69542	0.075	-2.9732
Uric Acid	Normal Control	Positive Control(Diseased grp)	-1.80000*	0.14434	0.000	-2.1265
		Treated Group	-1.72500*	0.14434	0.000	-2.0515
	Positive Control	Normal Control	1.80000*	0.14434	0.000	1.4735
		Treated Group	0.07500	0.14434	0.616	-.2515
	Treated Group	Normal Control	1.72500*	0.14434	0.000	1.3985
		Positive Control(Diseased grp)	-.07500	0.14434	0.616	-.4015
Testosterone	Normal Control	Positive Control(Diseased grp)	-6.79750*	0.75187	0.000	-8.4984
		Treated Group	-1.37000	0.75187	0.102	-3.0709
	Positive Control	Normal Control	6.79750*	0.75187	0.000	5.0966
		Treated Group	5.42750*	0.75187	0.000	3.7266
	Treated Group	Normal Control	1.37000	0.75187	0.102	-.3309
		Positive Control(Diseased grp)	-5.42750*	0.75187	0.000	-7.1284
PSA	Normal Control	Positive Control(Diseased grp)	-.15500*	0.04743	0.010	-.2623
		Treated Group	-.05500	0.04743	0.276	-.1623
	Positive Control	Normal Control	.15500*	0.04743	0.010	.0477
		Treated Group	.10000	0.04743	0.064	-.0073
	Treated Group	Normal Control	.05500	0.04743	0.276	-.0523
		Positive Control(Diseased grp)	-.10000	0.04743	0.064	-.2073

*. The mean difference is significant at the 0.05 level.

BPH Antioxidant Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidenc Mea Lower Bound	U
MDA	Normal Control	4	3.6375	.18007	.09003	3.3510	
	Pos. Control (Diseased grp)	4	5.6600	.26771	.13385	5.2340	
	Treated group	4	4.8775	.13326	.06663	4.6655	
	Total	12	4.7250	.88862	.25652	4.1604	
SOD	Normal Control	4	11.6000	.16330	.08165	11.3402	
	Pos. Control (Diseased grp)	4	10.8500	.28868	.14434	10.3907	
	Treated group	4	11.3250	.09574	.04787	11.1727	
	Total	12	11.2583	.37040	.10693	11.0230	
GPx	Normal Control	4	15.9750	.17078	.08539	15.7032	
	Pos. Control (Diseased grp)	4	8.5250	.25000	.12500	8.1272	
	Treated group	4	11.6500	.36968	.18484	11.0617	

	Total	12	12.0500	3.20014	.92380	10.0167	
Vit_E	Normal Control	4	2.1350	.06658	.05721	2.0291	
	Pos. Control (Diseased grp)	4	1.7875	.11442	.03329	1.6054	
	Treated group	4	1.9350	.12503	.06252	1.7360	
	Total	12	1.9525	.17654	.05096	1.8403	
CAT	Normal Control	4	8.4125	.30281	.15140	7.9307	
	Pos. Control (Diseased grp)	4	5.3075	.21654	.10827	4.9629	
	Treated group	4	6.0075	.13500	.06750	5.7927	
	Total	12	6.5758	1.40423	.40537	5.6836	
GSH	Normal Control	4	1.4150	.24853	.12426	1.0195	
	Pos. Control (Diseased grp)	4	.7950	.05260	.02630	.7113	
	Treated group	4	1.3150	.07416	.03708	1.1970	
	Total	12	1.1750	.31572	.09114	.9744	
Vit_C	Normal Control	4	1.5600	.08406	.04203	1.4262	
	Pos. Control (Diseased grp)	4	1.4600	.15297	.07649	1.2166	
	Treated group	4	1.5400	.46318	.23159	.8030	
	Total	12	1.5200	.26240	.07575	1.3533	
TAC	Normal Control	4	1.8800	.06683	.03342	1.7737	
	Pos. Control (Diseased grp)	4	1.7975	.04646	.02323	1.7236	
	Treated group	4	1.9175	.04349	.02175	1.8483	
	Total	12	1.8650	.07116	.02054	1.8198	

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
MDA	Between Groups	8.321	2	4.160	102.428	.000
	Within Groups	.366	9	.041		
	Total	8.686	11			
SOD	Between Groups	1.152	2	.576	14.497	.002
	Within Groups	.357	9	.040		
	Total	1.509	11			
GPx	Between Groups	111.965	2	55.983	735.536	.000
	Within Groups	.685	9	.076		
	Total	112.650	11			
Vit_E	Between Groups	.243	2	.122	11.009	.004
	Within Groups	.099	9	.011		
	Total	.343	11			
CAT	Between Groups	.220	2	10.610	202.987	.000
	Within Groups	.470	9	.052		
	Total	21.690	11			

GSH	Between Groups	.886	2	.443	18.985	.001
	Within Groups	.210	9	.023		
	Total	1.097	11			
Vit_C	Between Groups	.022	2	.011	.137	.874
	Within Groups	.735	9	.082		
	Total	.757	11			
TAC	Between Groups	.030	2	.015	5.310	.030
	Within Groups	.026	9	.003		
	Total	.056	11			

Multiple Comparisons

LSD

Dependent Variable	(I) BPH_Antioxidant	(J) BPH_Antioxidant	Mean Difference (I-J)	Std. Error
MDA	Normal Control	Pos. Control (Diseased grp)	-2.02250*	.14251
		Treated group	-1.24000*	.14251
	Pos. Control (Diseased grp)	Normal Control	2.02250*	.14251
		Treated group	.78250*	.14251
	Treated group	Normal Control	1.24000*	.14251
		Pos. Control (Diseased grp)	-.78250*	.14251
SOD	Normal Control	Pos. Control (Diseased grp)	.75000*	.14093
		Treated group	.27500	.14093
	Pos. Control (Diseased grp)	Normal Control	-.75000*	.14093
		Treated group	-.47500*	.14093
	Treated group	Normal Control	-.27500	.14093
		Pos. Control (Diseased grp)	.47500*	.14093
GPx	Normal Control	Pos. Control (Diseased grp)	7.45000*	.19508
		Treated group	4.32500*	.19508
	Pos. Control (Diseased grp)	Normal Control	-7.45000*	.19508
		Treated group	-3.12500*	.19508
	Treated group	Normal Control	-4.32500*	.19508
		Pos. Control (Diseased grp)	3.12500*	.19508
Vit_E	Normal Control	Pos. Control (Diseased grp)	-.34750*	.07434
		Treated group	-.14750	.07434
	Pos. Control (Diseased grp)	Normal Control	.34750*	.07434
		Treated group	.20000*	.07434
	Treated group	Normal Control	.14750	.07434
		Pos. Control (Diseased grp)	-.20000*	.07434
CAT	Normal Control	Pos. Control (Diseased grp)	3.10500*	.16166

		Treated group	2.40500*	.16166
	Pos. Control (Diseased grp)	Normal Control	-3.10500*	.16166
		Treated group	.70000*	.16166
	Treated group	Normal Control	-2.40500*	.16166
		Pos. Control (Diseased grp)	.70000*	.16166
GSH	Normal Control	Pos. Control (Diseased grp)	.62000*	.10804
		Treated group	.10000	.10804
	Pos. Control (Diseased grp)	Normal Control	-.62000*	.10804
		Treated group	-.52000*	.10804
	Treated group	Normal Control	-.10000	.10804
		Pos. Control (Diseased grp)	.52000*	.10804
Vit. C	Normal Control	Pos. Control (Diseased grp)	.10000	.20207
		Treated group	.02000	.20207
	Pos. Control (Diseased grp)	Normal Control	-.10000	.20207
		Treated group	-.08000	.20207
	Treated group	Normal Control	-.02000	.20207
		Pos. Control (Diseased grp)	.08000	.20207
TAC	Normal Control	Pos. Control (Diseased grp)	.08250	.03768
		Treated group	-.03750	.03768
	Pos. Control (Diseased grp)	Normal Control	-.08250	.03768
		Treated group	-.12000*	.03768
	Treated group	Normal Control	.03750	.03768
		Pos. Control (Diseased grp)	.12000*	.03768

*. The mean difference is significant at the 0.05 level.

Hepatocellular Carcinoma Biochemical Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confid Lower Bour
Urea	Normal Control	4	27.0000	2.44949	1.22474	23.
	Pos. Control (Diseased grp)	4	50.7500	2.21736	1.10868	47.
	Treated group	4	44.7500	2.50000	1.25000	40.
	Total	12	40.8333	10.75203	3.10384	34.
Creatinine	Normal Control	4	.9075	.03594	.01797	.
	Pos. Control (Diseased grp)	4	1.5075	.06397	.03198	1.
	Treated group	4	1.4625	.02062	.01031	1.
	Total	12	1.2925	.28775	.08307	1.
Potassium	Normal Control	4	4.0675	.11471	.05735	3.
	Pos. Control (Diseased grp)	4	3.3325	.06021	.03010	3.
	Treated group	4	3.9675	.10844	.05422	3.
	Total	12	3.7892	.35122	.10139	3.

Chloride	Normal Control	4	69.7500	3.09570	1.54785	64.
	Pos. Control (Diseased grp)	4	87.2500	1.25831	.62915	85.
	Treated group	4	80.0000	2.16025	1.08012	76.
	Total	12	79.0000	7.78110	2.24621	74.
Sodium	Normal Control	4	168.5750	1.82643	.91321	165.
	Pos. Control (Diseased grp)	4	183.8750	3.85519	1.92760	177.
	Treated group	4	174.0500	3.07409	1.53704	169.
	Total	12	175.5000	7.15885	2.06658	170.
Uric_Acid	Normal Control	4	5.8150	.14387	.07194	5.
	Pos. Control (Diseased grp)	4	7.6275	.10436	.05218	7.
	Treated group	4	6.5400	.07703	.03851	6.
	Total	12	6.6608	.78454	.22648	6.
Total_Protein	Normal Control	4	3.8725	.05315	.02658	3.
	Pos. Control (Diseased grp)	4	2.9850	.05802	.02901	2.
	Treated group	4	3.7850	.13478	.06739	3.
	Total	12	3.5475	.42499	.12269	3.
Bicarbonate	Normal Control	4	21.0500	.85440	.42720	19.
	Pos. Control (Diseased grp)	4	30.3250	1.60286	.80143	27.
	Treated group	4	21.8500	1.11206	.55603	20.
	Total	12	24.4083	4.52196	1.30538	21.
ALT	Normal Control	4	29.7500	1.25831	.62915	27.
	Pos. Control (Diseased grp)	4	37.2500	1.70783	.85391	34.
	Treated group	4	29.7500	1.70783	.85391	27.
	Total	12	32.2500	3.95716	1.14233	29.
GGT	Normal Control	4	9.5090	.95250	.47625	7.
	Pos. Control (Diseased grp)	4	16.9080	.29545	.14772	16.
	Treated group	4	11.2135	.52470	.26235	10.
	Total	12	12.5435	3.35631	.96888	10.
AST	Normal Control	4	29.2500	1.70783	.85391	26.
	Pos. Control (Diseased grp)	4	38.5000	2.64575	1.32288	34.
	Treated group	4	32.7500	.95743	.47871	31.
	Total	12	33.5000	4.33799	1.25227	30.
AFP	Normal Control	4	4.0250	.30957	.15478	3.
	Pos. Control (Diseased grp)	4	11.1500	.26458	.13229	10.
	Treated group	4	4.6500	.26458	.13229	4.
	Total	12	6.6083	3.37436	.97409	4.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.

Urea	Between Groups	1220.167	2	610.083	106.617	.000
	Within Groups	51.500	9	5.722		
	Total	1271.667	11			
Creatinine	Between Groups	.893	2	.447	230.720	.000
	Within Groups	.017	9	.002		
	Total	.911	11			
Potassium	Between Groups	1.271	2	.636	66.811	.000
	Within Groups	.086	9	.010		
	Total	1.357	11			
Chloride	Between Groups	618.500	2	309.250	58.595	.000
	Within Groups	47.500	9	5.278		
	Total	666.000	11			
Sodium	Between Groups	480.795	2	240.398	26.084	.000
	Within Groups	82.945	9	9.216		
	Total	563.740	11			
Uric_Acid	Between Groups	6.658	2	3.329	266.139	.000
	Within Groups	.113	9	.013		
	Total	6.770	11			
Total_Protein	Between Groups	1.914	2	.957	117.850	.000
	Within Groups	.073	9	.008		
	Total	1.987	11			
Bicarbonate	Between Groups	211.322	2	105.661	69.884	.000
	Within Groups	13.608	9	1.512		
	Total	224.929	11			
ALT	Between Groups	150.000	2	75.000	30.337	.000
	Within Groups	22.250	9	2.472		
	Total	172.250	11			
GGT	Between Groups	120.104	2	60.052	141.872	.000
	Within Groups	3.810	9	.423		
	Total	123.913	11			
AST	Between Groups	174.500	2	87.250	24.162	.000
	Within Groups	32.500	9	3.611		
	Total	207.000	11			
AFP	Between Groups	124.542	2	62.271	792.138	.000
	Within Groups	.707	9	.079		
	Total	125.249	11			

Multiple Comparisons

LSD

Dependent Variable	(I) HCC	(J) HCC	Mean Difference (I-J)	Std. Error
Urea	Normal Control	Pos. Control (Diseased grp)	-23.75000*	1.69148
		Treated group	-17.75000*	1.69148
	Pos. Control (Diseased grp)	Normal Control	23.75000*	1.69148
		Treated group	6.00000*	1.69148
	Treated group	Normal Control	17.75000*	1.69148
		Pos. Control (Diseased grp)	-6.00000*	1.69148
Creatinine	Normal Control	Pos. Control (Diseased grp)	-.60000*	.03111
		Treated group	-.55500*	.03111
	Pos. Control (Diseased grp)	Normal Control	.60000*	.03111
		Treated group	.04500	.03111
	Treated group	Normal Control	.55500*	.03111
		Pos. Control (Diseased grp)	-.04500	.03111
Potassium	Normal Control	Pos. Control (Diseased grp)	.73500*	.06897
		Treated group	.10000	.06897
	Pos. Control (Diseased grp)	Normal Control	-.73500*	.06897
		Treated group	-.63500*	.06897
	Treated group	Normal Control	-.10000	.06897
		Pos. Control (Diseased grp)	.63500*	.06897
Chloride	Normal Control	Pos. Control (Diseased grp)	-17.50000*	1.62447
		Treated group	-10.25000*	1.62447
	Pos. Control (Diseased grp)	Normal Control	17.50000*	1.62447
		Treated group	7.25000*	1.62447
	Treated group	Normal Control	10.25000*	1.62447
		Pos. Control (Diseased grp)	-7.25000*	1.62447
Sodium	Normal Control	Pos. Control (Diseased grp)	-15.30000*	2.14664
		Treated group	-5.47500*	2.14664
	Pos. Control (Diseased grp)	Normal Control	15.30000*	2.14664
		Treated group	9.82500*	2.14664
	Treated group	Normal Control	5.47500*	2.14664
		Pos. Control (Diseased grp)	-9.82500*	2.14664
Uric_Acid	Normal Control	Pos. Control (Diseased grp)	-1.81250*	.07908
		Treated group	-.72500*	.07908
	Pos. Control (Diseased grp)	Normal Control	1.81250*	.07908
		Treated group	1.08750*	.07908
	Treated group	Normal Control	.72500*	.07908
		Pos. Control (Diseased grp)	-1.08750*	.07908
Total_Protein	Normal Control	Pos. Control (Diseased grp)	-.88750*	.06372
		Treated group	-.08750	.06372

	Pos. Control (Diseased grp)	Normal Control	.88750*	.06372
		Treated group	-.80000*	.06372
	Treated group	Normal Control	-.08750	.06372
		Pos. Control (Diseased grp)	.80000*	.06372
Bicarbonate	Normal Control	Pos. Control (Diseased grp)	-9.27500*	.86947
		Treated group	-.80000	.86947
	Pos. Control (Diseased grp)	Normal Control	9.27500*	.86947
		Treated group	8.47500*	.86947
	Treated group	Normal Control	.80000	.86947
		Pos. Control (Diseased grp)	-8.47500*	.86947
ALT	Normal Control	Pos. Control (Diseased grp)	-7.50000*	1.11181
		Treated group	.00000	1.11181
	Pos. Control (Diseased grp)	Normal Control	7.50000*	1.11181
		Treated group	7.50000*	1.11181
	Treated group	Normal Control	.00000	1.11181
		Pos. Control (Diseased grp)	-7.50000*	1.11181
GGT	Normal Control	Pos. Control (Diseased grp)	-7.39900*	.46005
		Treated group	-1.70450*	.46005
	Pos. Control (Diseased grp)	Normal Control	7.39900*	.46005
		Treated group	5.69450*	.46005
	Treated group	Normal Control	1.70450*	.46005
		Pos. Control (Diseased grp)	-5.69450*	.46005
AST	Normal Control	Pos. Control (Diseased grp)	-9.25000*	1.34371
		Treated group	-3.50000*	1.34371
	Pos. Control (Diseased grp)	Normal Control	9.25000*	1.34371
		Treated group	5.75000*	1.34371
	Treated group	Normal Control	3.50000*	1.34371
		Pos. Control (Diseased grp)	-5.75000*	1.34371
AFP	Normal Control	Pos. Control (Diseased grp)	-7.12500*	.19826
		Treated group	-.62500*	.19826
	Pos. Control (Diseased grp)	Normal Control	7.12500*	.19826
		Treated group	6.50000*	.19826
	Treated group	Normal Control	.62500*	.19826
		Pos. Control (Diseased grp)	-6.50000*	.19826

*. The mean difference is significant at the 0.05 level.

Hepatocellular Carcinoma Antioxidant Descriptive

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interva	
						Lower Bound	Up
MDA	Normal Control	4	3.7400	.22136	.11068	3.3878	
	Pos. Control (Diseased grp)	4	4.7000	.21954	.10977	4.3507	
	Treated group	4	4.4650	.45317	.22659	3.7439	
	Total	12	4.3017	.51443	.14850	3.9748	
SOD	Normal Control	4	11.3500	.34157	.17078	10.8065	
	Pos. Control (Diseased grp)	4	10.8750	.34034	.17017	10.3334	
	Treated group	4	11.4500	.28868	.14434	10.9907	
	Total	12	11.2250	.39341	.11357	10.9750	
GPx	Normal Control	4	16.8000	.18257	.09129	16.5095	
	Pos. Control (Diseased grp)	4	11.0250	.40311	.20156	10.3836	
	Treated group	4	12.0000	.18257	.09129	11.7095	
	Total	12	13.2750	2.64820	.76447	11.5924	
Vit_E	Normal Control	4	1.9725	.06021	.03010	1.8767	
	Pos. Control (Diseased grp)	4	1.7125	.02017	.01109	1.6772	

	Treated group	4	1.9025	.07805	.03902	1.7783
	Total	12	1.8625	.12628	.03646	1.7823
CAT	Normal Control	4	6.7950	.03416	.01708	6.7406
	Pos. Control (Diseased grp)	4	3.7150	.12477	.06238	3.5165
	Treated group	4	4.8500	.14095	.07047	4.6257
	Total	12	5.1200	1.33212	.38455	4.2736
GSH	Normal Control	4	1.8825	.19276	.09638	1.5758
	Pos. Control (Diseased grp)	4	.3600	.01633	.00816	.3340
	Treated group	4	.9925	.11673	.05836	.8068
	Total	12	1.0783	.66287	.19135	.6572
Vit_C	Normal Control	4	1.7025	.08421	.04211	1.5685
	Pos. Control (Diseased grp)	4	.9050	.01732	.00866	.8774
	Treated group	4	1.4300	.05354	.02677	1.3448
	Total	12	1.3458	.34971	.10095	1.1236
TAC	Normal Control	4	1.7200	.20083	.10042	1.4004
	Pos. Control (Diseased grp)	4	.9025	.04272	.02136	.8345
	Treated group	4	1.6775	.14569	.07284	1.4457
	Total	12	1.4333	.41390	.11948	1.1704

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
MDA	Between Groups	2.003	2	1.002	9.931	.005
	Within Groups	.908	9	.101		
	Total	2.911	11			
SOD	Between Groups	.755	2	.377	3.586	.072
	Within Groups	.948	9	.105		
	Total	1.703	11			
GPx	Between Groups	76.455	2	38.228	500.433	.000
	Within Groups	.688	9	.076		
	Total	77.143	11			
Vit_E	Between Groups	.145	2	.072	21.277	.000
	Within Groups	.031	9	.003		
	Total	.175	11			
CAT	Between Groups	19.410	2	9.705	795.500	.000
	Within Groups	.110	9	.012		
	Total	19.520	11			
GSH	Between Groups	4.680	2	2.340	137.519	.000
	Within Groups	.153	9	.017		
	Total	4.833	11			
Vit_C	Between Groups	1.315	2	.657	192.212	.000

	Within Groups	.031	9	.003		
	Total	1.345	11			
TAC	Between Groups	1.694	2	.847	40.097	.000
	Within Groups	.190	9	.021		
	Total	1.884	11			

Multiple Comparisons

LSD

Dependent Variable	(I) Hepatocarcinoma_Antioxidant	(J) Hepatocarcinoma_Antioxidant	Mean Difference (I-J)	Std. Error
MDA	Normal Control	Pos. Control (Diseased grp)	-.96000*	.22456
		Treated group	-.72500*	.22456
	Pos. Control (Diseased grp)	Normal Control	.96000*	.22456
		Treated group	.23500	.22456
	Treated group	Normal Control	.72500*	.22456
		Pos. Control (Diseased grp)	-.23500	.22456
SOD	Normal Control	Pos. Control (Diseased grp)	.47500	.22943
		Treated group	-.10000	.22943
	Pos. Control (Diseased grp)	Normal Control	-.47500	.22943
		Treated group	-.57500*	.22943
	Treated group	Normal Control	.10000	.22943
		Pos. Control (Diseased grp)	.57500*	.22943
GPx	Normal Control	Pos. Control (Diseased grp)	5.77500*	.19543
		Treated group	4.80000*	.19543
	Pos. Control (Diseased grp)	Normal Control	-5.77500*	.19543
		Treated group	-.97500*	.19543
	Treated group	Normal Control	-4.80000*	.19543
		Pos. Control (Diseased grp)	.97500*	.19543
Vit_E	Normal Control	Pos. Control (Diseased grp)	-.26000*	.04125
		Treated group	-.19000*	.04125
	Pos. Control (Diseased grp)	Normal Control	.26000*	.04125
		Treated group	.07000	.04125
	Treated group	Normal Control	.19000*	.04125
		Pos. Control (Diseased grp)	-.07000	.04125
CAT	Normal Control	Pos. Control (Diseased grp)	3.08000*	.07810
		Treated group	1.94500*	.07810
	Pos. Control (Diseased grp)	Normal Control	-3.08000*	.07810
		Treated group	-1.13500*	.07810
	Treated group	Normal Control	-1.94500*	.07810

		Pos. Control (Diseased grp)	1.13500*	.07810
GSH	Normal Control	Pos. Control (Diseased grp)	1.52250*	.09224
		Treated group	.89000*	.09224
	Pos. Control (Diseased grp)	Normal Control	-1.52250*	.09224
		Treated group	-.63250*	.09224
	Treated group	Normal Control	-.89000*	.09224
		Pos. Control (Diseased grp)	.63250*	.09224
Vit_C	Normal Control	Pos. Control (Diseased grp)	.79750*	.04135
		Treated group	.27250*	.04135
	Pos. Control (Diseased grp)	Normal Control	-.79750*	.04135
		Treated group	-.52500*	.04135
	Treated group	Normal Control	-.27250*	.04135
		Pos. Control (Diseased grp)	.52500*	.04135
TAC	Normal Control	Pos. Control (Diseased grp)	.81750*	.10278
		Treated group	.04250	.10278
	Pos. Control (Diseased grp)	Normal Control	-.81750*	.10278
		Treated group	-.77500*	.10278
	Treated group	Normal Control	-.04250	.10278
		Pos. Control (Diseased grp)	.77500*	.10278

*. The mean difference is significant at the 0.05 level.