THE EFFECT OF n-HEXANE FRACTION OF ETHANOLIC EXTRACT OF *Phyllanthus amarus* ON 1, 2 DIMETHYLHYDRAZINE INDUCED COLON CARCINOGENESIS IN SWISS ALBINO MICE

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BENIN CITY,

SEPTEMBER, 2023.

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A PROJECT WRITTEN AND SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN BIOCHEMISTRY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, EDO STATE, BENIN CITY, NIGERIA.

SEPTEMBER, 2022.

CERTIFICATION

This is to certify that this work carried out by **OLUSEGUN TOVIA OFUJE** with the matriculation number **LSC1802989** in the department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Edo state in partial fulfillment of the requirements for the award of Bachelor in Science in Biochemistry.

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DEDICATION

I am dedicating this work to my late mum Mrs. Itohan Olusegun whom I lost half way into this journey. It would have been a thing of joy to see you witness the end of this phase. I made it.

I am also dedicating this to my lovely Dad for being my strength and support.

I am also dedicating this to myself because it hasn't been an easy ride and I am thankful to God for giving me the strength to complete this program. You alone is worthy to be praised!

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ABSTRACT

Since prehistoric times, medicinal herbs have been used due to their therapeutic and pharmaceutical value due to the presence of some bioactive compounds which have been written and proven to show effectiveness against ailments such as jaundice, genital infections, fever, wounds amongst others. *Phyllanthus Amarus* is traditionally used for various infections, inflammation and cancer. 1,2 Dimethylhydrazine is a potent colon cancer inducer in animals. The present study investigated the effects of ethanolic extract of n-hexane fraction of *Phyllanthus Amarus* on 1, 2 Dimethylhydrazine induced colon cancer in swiss albino mice. 15 male swiss albino mice of weight 14g-26g were acclimatized for a week and randomized into 3 groups (5 per group). Group A (-DMH), Group B (DMH+ 450mg/kg body weight of ethanolic extract of n-hexane fraction of P. amarus) Group C (DMH+). 20 mg/kg body weight of DMH was administered orally for 24doses (3 times a week for 2 months). The plant extracts were administered daily for 2 weeks (14days) with the aid of a dolphin gauge immediately after colon cancer induction. In the present study, the antioxidant parameters e,g, SOD($\times 10^3$)(u/mg wet tissue) and CAT($\times 10^2$)(u/mg wet tussue) of control were significantly different from the treated group as seen in the result (2.156±0.64 and 0.42± 0.28)for SOD,(11.66 ± 0.78 and 2.93± 0.75) for CAT. For MDA($\times 10^{-4}$)(m/mg wet tissue) GPX(u/mg wet tissue) and GSH(g/wet tissue)there was no significant difference when the control group (0.209±0.015) for MDA, (0.037±0.014)for GPX and (1.23±0.087) for GSH was compared to treated group (0.17±0.02) for MDA, (0.08± 0.015) for GPX and (1.93± 0.19)for GSH. For liver function test, there was significant difference between the control (168.9 ±24.57), treated (219.42±54.8) and untreated group (156.56±11.68) for ALT activity(u/L) in the liver homogenate, but there was no significant difference at $p<0.05$ for AST(u/L) across all groups Control(25.6± 0.58), treated (26.77±1.16) and untreated(29.1±0.579). For kidney function test, the concentration of sodium ion(mEq/L)was significantly increased for treated group (205.55±3.87) when compared to control (114±5.79) and DMH group (208.0±25.24)while potassium ion(mEq/L) concentration in control group (2.656±0.158) was significant difference in treated (3.59±0.7) and also for the untreated (3.24±0.504). For Bicarbonate ion(mmol/L), there is significant difference in control group (4.117±0.08), the treated group (3.59± 0.7) and for the untreated (3.62± 0.937) . For creatinine concentration(mmol/dL) there was significant difference in the control group (2.13± 0.36) and treated(1.92±0.36) and insignificant difference in the untreated (1.883± 0.2) and for urea concentration(mg/dL) there is significant difference between the control group(0.717± 0.037) and the treated(1.083±0.134) and insignificant difference in the untreated (1.75±0.05). In addition, the effect of the plant extract was seen in the weights of the animals. There was significant decrease in the weight of untreated(initial wt. 23±83, final wt. 21.00±1.16) when compared to control(initial wt. 25.17±1.30 final wt. 27.20±1.51)and treated (20.25±1.44,finalwt.19.00±1.87). For Caspase9(×102)(ng/L), there is significant difference between the positive control(2.765±0.633) and the mice treated with n-hexane fraction of ethanolic extract of *Phyllanthus Amarus*(2.159±0.451) and that of the negative control(2.586±0.128). For interleukin-6(pg/M), there is a significant difference between the positive control(7.434±1.353) and the treated group(9.9185±2.995) and also the untreated(6.335±0.549).In conclusion, the results obtained showed that administration of 450mg/kg bwt of n-hexane fraction of ethanolic extract of plant *Phyllanthus Amarus* has some ameliorating potentials against the carcinogenic effects of DMH induced colon carcinogenesis in swisss albino mice.

CHAPTER ONE

1.1 INTRODUCTION

Since prehistoric times, various plants have been discovered with certain components which have been used for curative and therapeutic purposes. Medicinal plants, which is also known as medicinal herbs, includes a variety of plants that have been discovered and used in herbalism since prehistoric times and some of these plants possess medicinal properties. Medicinal plants have always played crucial role as sources of drug lead compounds. These medicinal plants are suggested for their therapeutic usefulness because they are thought to be abundant sources of phytochemicals and bioactive components that can be exploited in medication research and synthesis(Rasol.,2012). The most dominant natural medicine source is plants, due to their chemical and structural diversity and the biodiversity of their components. The presence of various life sustaining constituents in plants mad scientist to investigate these plants for their uses in treating certain infectious diseases and management of chronic wounds, an impressive number of modern drugs have been isolated from natural sources. Over 75 percent of the world total population depends exclusively on plants for their health and healing.

The Genus Phyllanthus is the largest in the plant family Phyllanthaceae. It is widely distributed in tropical and subtropical zones like tropical Africa, tropical America, Asia and Oceania. This genus, consists of more than 700 species and can be classified into 11 subgenuses (Uander et al., 2000). *Phyllanthus Amarus* is a leafy herbal plant and is one of the most important specie in the genus Phyllanthus pharmacologically. It is known for its ethnobotanical value in various part of the world. This plant has been found in Phillippine, Cuba, Nigeria and among others. In India, *Phyllanthus Amarus* is widely distributed as weed in cultivated and waste lands(Joseph et al.,2005). It is an important plant that is used in the problems of the stomach, genitourinary

system, liver, kidney and spleen. It has found its traditional usefulness in several health problems such diarrhoea, dysentery, dropsy, jaundice, interminent fevers, uriogenital disorders, scabies and wounds. The powdered leaves of *phyllanthus* amarus (Bahupatra) were used in clinical studies evaluating its usefulness in patients suffering from chronic damage to the liver due to protracted hepatitis B virus infection. Based on its very useful medicinal properties, *Phyllanthus Amarus* is very frequently used in traditional medicine(Raj et al., 2007).

1.2 AIM OF STUDY

The aim of this study is to examine the therapeutic effect of n-hexane fraction of ethanolic extract of *Phyllanthus amarus* extract on 1,2−Dimethylhydrazine induced colon carcinogenesis on swiss albino mice

The objectives are;

- To acquire and acclimatize the mice for one week.
- To obtain ethanolic extracts of *Phyllanthus amarus* from dried leaves of the plant.
- To fractionate the ethanolic extract with n-hexane .
- To induce carcinogenesis in swiss albino mice using dimethylhydrazine.
- To determine if n-hexane fraction of ethanolic *Phyllanthus amarus* extract can improve 1,2-dimethylhydrazine induced carcinogenesis.

1.3 LITERATURE REVIEW

1.3.1. PHYLLANTHUS FAMILY

The plants of the genus *Phyllanthus* (Euphorbiaceae) have been used as traditional medicinal materials for a long time in China, India, Brazil, and the Southeast Asian countries. This genus, consisting of more than 700 species, can be classified into 11 subgenuses (Uander et al., 1995) The 24 most popular species are primarily found in the subgenera Kirganelia, Cicca, and *Phyllanthus*, and they are employed by various ethnicities in custom.

1.3.2. PHYLLANTHUS AMARUS

Phyllanthus amarus is a small, annual plant that grows to a height of 30-60 cm. Its thin branches spread out, and each branch has two rows of small, elliptic-oblong leaves of 5-10mm long that are arranged alternately. Its radial flowers are star-shaped and of about 2mm in size (Karitika et al.,2011). It grows well in soil of high moisture with light shade, and reaches maturity in 2-3 months.

Various civilizations, particularly Amazonian tribes, have employed P. amarus in traditional medicine to treat renal and gallstones as well as in Malay traditional medicine for diarrhea, kidney problems, and gonorrhea; in Ayurvedic medicine for bronchitis, anaemia, and diabetes (Samy et al., 2005).Preclinical and clinical research investigating the plant's purported liver-protective properties have recently been conducted. It exhibits hepatic protection against paracetamol hepatoxicity in rats, according to animal testing (Wongnawa *et al*., 2005).

Figure 1: *Phyllanthus Amarus* (Roddy et al., 2008).

1.3.3 BOTANICAL CLASSIFICATION.

Kingdom - Plantae

Sub-kingdom - Trachebionte

Superdivison - Spermatophyta

Division - Magnoliophyta

Class - Magnoliopsida

Subclass - Rosidae

Order - Euphorobiales

Family - Euphorbiaceae

Genus - Phyllanthus

Species - Amarus

1.3.4. HABITAT AND ECOLOGY.

The genus *Phyllanthus* (Euphorbiaceae) is comprised of about 1000 species (Omoregie *et al*, 2020) within 300 genera and about 200 of these are native to America, 100 to Africa, 70 to Madagascar, and the remaining to Asia and Australia (Unander, 1994). '*Phyllanthus*' as a name means "leaf and flower" –this meaning probably came from the physical appearance where the flowers of the plant, as well as the fruit, become one with the leaf (Cabieses, 1993).

Webster's taxonomic revision on the *Phyllanthus* genus included the closely related genera *P. amarus* within the sub-section Swartiziani of the section *Phyllanthus*. The taxonomic distinctness, nomenclature and close relatives of *P. amarus* were fully addressed based on the morphology and general distribution (Chowdhury and Rao, 2002). *P. amarus* is thought to be closely related to *P. abnormis* which is often found in the sandy areas of Texas and Florida, USA. There is the likelihood the plant *P. amarus* originated in the Caribbean area, and spread over the tropics by trading vessels.

1.3.5. PHYTOCHEMICAL SCREENING

The leaves of *P. amarus* have been shown to possess anticarcinogenic, antitumour, antioxidant, antibacterial, antidiabetic, antifungal, and antiviral activities (Gupta and Vaghela. 2019). These medicinal properties in *P. amarus* can be attributed to the presence of its phytochemicals.

TABLE 1.1 ; This shows the result of the qualitative phytochemical analysis of the ethanol extract of *Phyllanthus amarus* leaves (Frank *et a*l., 2020) and the phytochemicals present in n-hexane extract.

S/N	SECONDARY METABOLITES		PHYTO-CONSTITUENTS
1	Lignans		Phyllanthin, hypophyllanthin, niranthin, phyltetralin, nirtetralin, isonirtetralin, hinokinin Lintetralin, isolintetralin, demethylenedioxy-niranthin, 5-demethoxy-niranthin (3-(3,4-dimethoxy-benzyl)-4-(7-methoxy-benzo[1,3]dioxol-5-yl-methyl)-dihydrofuran-2-one, 4-(3,4-dimethoxy-phenyl)-1-(7-methoxy-benzo[1,3]dioxol-5-yl)-2,3-bis-methoxymethyl-butan-1-ol
2	Flavonoids		Rutin, astragalin, kaempferol, quercetin-3-O-glucoside, quercetin, quercitrin
3	Hydrolysable tannin (Ellagitannins)	Tannin precursors	Gallic acid, ellagic acid, gallocatechin
		Simple tannins	1,6-digalloylglucopyranose, 4-O-galloylquinic acid
		Complex tannins	Geraniin, amariin, furosin, geraniinic acid B, amariinic acid, amarulone, repandusinic acid A, corilagin, isocorilagin, elaeocarpusin, *phyllanthus*iin A, B, C and D, melatonin
4	Alkaloids		Securinine, dihydrosecurinine, tetrahydrosecurinine, securinol, phyllanthine,

			allo-securine, nor-securinine, epibubbialine, isobubbialine 4-methoxy-nor-securinine, 4-methoxy dihydrosecurinine, 4-methoxytetrahydrosecurinine, 4 hydrosecurinine Phenazine and phenazine derivatives
5	Triterpenes		2Z, 6Z, 10Z, 14E, 18E, 22E-farnesylfarnesol Lupeol, phyllanthenol, phyllanthenone, phyllantheol, Oleanolic acid, ursolic acid
6	Sterols		Amarosterol A, amarosterol B
7	Volatile oil		Linalool, phytol

Table 1.1 (Omoregie *et al*, 2021; Jay *et al.*, 2011)

1.3.6. USES OF PHYLLANTHUS AMARUS

Phyllanthus amarus is a broad-spectrum medicinal plant that has received world- wide recognition (Priem et al., 2001). This plant has various applications in traditional medicine throughout the world. In the Ayurvedic medicine, decoctions of the whole plant are used to treat malaria (Mazumder et al., 2006). An aqueous extract of the aerial parts is used by some traditional healers in Tanzania, in the management of diabetes mellitus; while in other areas thesame extract is drunk and the leaves are chewed against persistent coughs and stomachaches (Chhabra and Mahunnah, 2004). The entire plant is used in India to treat hepatitis, dysectery, irritating sores (Reddy et al., 2013) and jaundice (Raja Reddy, 2008). It is also used in the West

Indies to prevent intestinal worms in children (Morton, 2007) and in Rarotonga (Cook Islands) totreat earache (Holdsworth, 2001). The anticancer effects of P. amarus protects the liver from hepatocarcinogenesis and the rootextract of P. acuminatus exerts growth inhibition in murine P-388 lymphocytic leukemia and B-16 melanoma cell lines. Thus, it is believed that the plant genus **Phyllanthus** might possess anticancer properties against prostate cancer as well (Pettit, 2006; Pows and Moore, 2005).

1.3.7 PHYTOCHEMICAL AND THERAPEUTIC ACTIVITY

Anti-cancer activity

Studies by Lee, et. al. in 2011 has shown P. amarus to possess substantial anti-cancer activity against metastatic cells implicated in breast and lung carcinomas, with values ranging from 56 to 126 4g/mL and 150-240 ug/mL for methanolic and aqueous extracts respectively. In comparison, they have lower toxicity on normal cells with cell viability of 50% when treated up to 1000 Mg/mL for both aqueous and methanolic extracts. Several anti-metastasis assays also indicated effective reduction in the invasion, migration, and adhesion of the cancerous cells in a dose-dependent manner. The possible mode of cell death was shown to be via the induction of apoptosis, mediated by increased levels of reactive oxygen species and decreased mitochondrial membrane potential (Abhyankar, et al., 2010), with more than 3-fold increase of caspases-3 and7. According to another report by Kumar and Kuttan in 2004, P. amarus may also act by inhibiting unusual metabolic pathways exhibited by cancer cells, rather than directly inducing cell death. The anticancer/anti-metastatic activity of the plant is a result of its polyphenolic content.

Antioxidant activity

The aqueous extract derived from the entire P. amarus plant has demonstrated its ability to counteract free radicals and hinder lipid peroxidation across multiple research studies. In a 2011 investigation conducted by Karuna and colleagues, they assessed the antioxidant properties of the aqueous extract from the entire P. amarus plant at a dosage of 200mg per kilogram of body weight per day in male Wistar albino rats induced with streptozotocin (ST) to induce diabetes. Their findings indicated a noteworthy reduction in renal lipid peroxidation and protein oxidation, along with a significant increase in glutathione content. Furthermore, the activities of antioxidant enzymes such as glutathione reductase, glutathione peroxidase, and glutathione-transferase, as well as catalase and superoxide dismutase, showed significant improvements in comparison to ST-induced diabetic rats. While the diabetic untreated group exhibited lowered activities of catalase and superoxide dismutase, these activities were normalized in the diabetic group that received treatment.

In a separate study by Raphael et al. in 2002, it was also established that P. amarus could effectively inhibit lipid peroxidation and effectively scavenge hydroxyl and superoxide radicals in vitro. Notably, compounds such as amariin, repandusinic acid, phyllanthusiin D, phyllanthin, and various phenolic compounds isolated from P. amarus demonstrated exceptionally high levels of antioxidant activity.

Nephroprotective activity

A study conducted by Adeneye and Adokiye in 2008 investigated the protective effects of single oral doses (ranging from 100 to 400 mg/kg/day) of aqueous extracts from the leaves and seeds of P. amarus in Wistar rats over a 14-day period. This study focused on mitigating nephrotoxicity induced by acetaminophen and gentamicin. The results revealed that the extracts effectively

reduced the increase in serum creatinine and blood urea nitrogen levels in a dose-dependent manner (Adeneye and Adokiye, 2008)

Hepatoprotective activity

The protective effect of phyllanthin, a known principal constituent of P. amarus on eth.
Indu liver cell injury has been evaluated. Phyllanthin acted by reversing the effect in
by ethanol (increased alanine aminotransferase and aspartate aminotransferase activities), and restoring the antioxidant capability of rat hepatocytes including level of total glutathione, and activities of superoxide dismutase and glutathione reductase which were reduced by ethano (Chirdchupunseree and Pramyothin, 2010). In another study, Carbon tetrachloride (CCL)-induced increase in liver alanine aminotransferase, ALT, aspartate aminotransferase, AST, alkaline phosphatase, ALP and acid phosphatase, ACP, activities were mitigated by the oral administration of aqueous extract of P. amarus (Krithika and Verma, 2009a; Krithika and Verma, 2009).

Antidiabetic activity

Oral administration of aqueous extract of whole plant of P. amarus promotes glucose uptake as shown in an oral glucose tolerance test by James, et al. (2009) in which P. amarus extract induced significant reduction in serum glucose level. A significant decrease in the body weight, hyperglycemia and hyperlipidemia with P. amarus was also observed in streptozotocin (STZ)-induced diabetic rats (Karuna et al., 2011). According to Adeneye et al. (2006), the possible mode of action of P. amarus is by enhancing peripheral utilization of glucose.

Antibacterial activity

Hexane, methanol and water extracts of aerial parts of P. amarus screened for antimicrobial activities against Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus and Candida albicans were found to be actively inhibitory. Screening of the extracts for secondary metabolites indicated the presence of alkaloids, saponins, tannins and terpenoids (Alli et al., 2011).

Antiviral activity

P. amarus has been shown to also possess significant antiviral activity against a variety of viral infections, especially those caused by hepatitis C virus and B surface antigen (Ravikumar el al, 2011; Munshi, et al., 1993a; Munshi, et al., 1993b). A gallotannin containing fraction and the isolated ellagitannins, geraniin, corilagin, lignans, niranthin and himokinin isolated from P. amarus were shown to be the most potent mediators of these antiviral activities

1.4 COLORECTAL CANCER

Cancer is characterized by the uncontrolled and abnormal growth of cells. This occurs due to mutations or alterations in the DNA which is the genetic material of the cells, leading to the disruption of the normal regulatory mechanism of cell division and apotopsis(programmed cell death). It involves changes in various cellular processes such as DNA replication, repair and cells signaling pathways, which allows cancer cells to evade normal growth constraints and invade surrounding tissues. Lung cancer accounts for 11.6% of all cancer diagnoses in both sexes, followed by breast cancer in women (11.6%) and prostate cancer in men (7.9%). In terms of recognition (6.1%), colorectal cancer (CRC) is ranked third, and second in terms of mortality(9.2%).Rectal and colon cancer-related fatalities are anticipated to rise by 60% and

71.5%, respectively, by the year 2035(Douaiher et al.,2017). The aging population as well as the fact that the vast majority of people have poor food habits are factors contributing to this high occurrence. Smoking, obesity, and low levels of physical activity are other causes in some of these western nations. Some familial cancer syndromes also exhibit the high prevalence of the disease. It appears that colorectal cancer (or colon cancer) has become a more common presentation of Lynch syndrome (a non-polyposis type of cancer that is also hereditary), even though previously, carriers of the syndrome were more frequently affected by gastric cancer. This is because the rate of infection caused by Helicobacter pylori infection, one of the major causative factors of gastric cancer, has fallen dramatically in recent years. (Capelle *et al.,* 2010; Vasen *et al.,* 2015).

In 2012, colorectal cancer was ranked as the second most prevalent cancer in women and the third most common in males. Colon cancer accounted for 9.2% of cancer cases recorded in women and 10% in men worldwide in 2012, according to Globocan figures. Together, these cancers account for 9.7% of all cancer cases and are the third most common disease in the world (excluding non-melanoma skin cancer). Not surprisingly, more than half of the cases reported worldwide occur in more developed countries (Vasen *et al.*, 2015).

1.4.1 PROGRESSION OF COLORECTAL CANCER

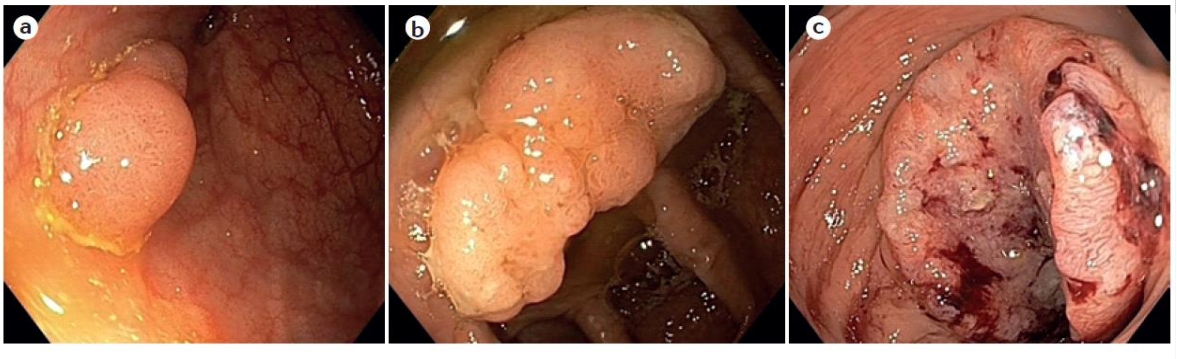


Fig 1.2: Colorectal cancer at different stages. a (a small sessile adenoma). b (an advanced, larger sessile adenoma). c (a large, dish-shaped, ulcerating sigmoid carcinoma). The tumor covers most of the circumference, but has not yet led to a substantial obstruction of the lumen (Vasen et al., 2015).

Pre-cancerous lesions or adenomatous polyps are thought to be the precursors of colorectal cancer, according to evidence accumulated from numerous sources over time. CRC develops in stages, starting with the early dysplastic lesion known as aberrant crypt focus and progressing via adenomatous polyps and invasive malignancy. It is hypothesized that CRC develops as a result of a slow accumulation of mutations that are helpful to tumor growth over time, eventually resulting in an invasive malignant tumor. The adenoma-carcinoma sequence describes this. The APC gene is often first truncated, then the KRAS gene, and finally the TP53 gene later in the course of events. (Fearon and Vogelstein, 1990). It is important to note that the loss of epigenomic and genomic stability promotes the accumulation of epigenetic changes and mutations in the aforementioned tumor suppressor genes, as well as oncogenes that drive the malignant transformation of colorectal cancer through multiple rounds of clonal expansion,

tending to select for the cells with the most aggressive and malignant behavior. (Fearon and Vogelstein, 1990; Lengauer *et al.,* 1998; Kinzler and Vogelstein, 1996).

In addition to the adenoma-carcinoma sequence, the Chromosomal Instability Pathway/APC Pathway is also involved in the progression of colorectal cancer. In this pathway, chromosomal instability (CIN) and microsatellite instability (MSI), two types of genomic instability, can be linked to mutations that cause colon cancer. In 70% of cases of colorectal cancer, the chromosomal instability is visible. (Walther *et al.,* 2009). APC gene mutations are indicative of chromosomal instability, which includes various chromosomal aberrations (chromosome 18 is the most frequent), sub-chromosomal aberrations, and loss of heterozygosity, in the conventional adenoma to carcinoma scenario.(Ogino and Goel, 2008). The fact that chromosomal instability results in the loss of tumor suppressor genes is a key feature of the condition. PI3KCA, APC, KRAS, TP53, and other crucial genes are involved in chromosomal instability (CIN), for example. (Walther *et al.,* 2009). The results of colorectal malignancies caused by CIN are worse than those caused by microsatellite instability. (Walther, Houlston and Tomlinson, 2008).

1.4.2 COLORECTAL CANCER ONCOGENES

Oncogenes with functional mutations go on to activate constitutively and proliferate uncontrollably, resulting in cancer. Certain oncogenes have been linked to the development of colorectal cancer.

1.4.2.1 TP53 Gene

The tumor protein 53 (TP53) gene, sometimes known as the "gatekeeper of the genome, holds a crucial position in the progression of numerous malignancies, including colorectal cancer. The tumor protein 53, a gene translation (or protein) product, performs a variety of tasks, including

starting the apoptosis cycle (programmed cell death). The TP53 protein may also detect DNA damage and halt the cell cycle to enable the repair of the damaged DNA. Alternatively, if the DNA damage or injury cannot be repaired, the TP53 protein starts the above-mentioned process of cell apoptosis. (Nakayama and Oshima, 2019).

Almost 50% of colorectal cancers that are invasive possess this mutation in the TP53 gene, and it is commonly observed in cancers emanating in the rectum or distal colon (Iacopetta, 2003). The function of the TP53 in the progression of colorectal cancer may include the inactivation or loss of functionality of the TP53 gene which thus results from mutations that cause inactivation of the gene product and localized in one alleles, as well as the loss of other allele due to chromosomal instability or the occurrence of the missense kind of mutation of DNA binding domain that converts the TP53 to a pro-oncogene (Nakayama and Oshima, 2019). The outcome is thought to happen later in the adenoma-carcinoma transformation sequence as result of a comparatively low rate of occurrence in precancerous lesions, as well as increased occurrence in cases of invasive cancers (Nikolaev *et al.*, 2012). Colitis-associated carcinoma is the only known exception in which the loss of TP53 is a step that occurs earlier in pathogenesis (Schwitalla *et al.*, 2013).

1.4.3 SIGNS AND SYMPTOMS OF COLORECTAL CANCER

The most common presenting symptoms of colon cancer include change in bowel habit, abdominal pain and rectal bleeding or anaemia –although these symptoms are also common in the occurrence of other gastrointestinal conditions. Caused by a progressive narrowing of the bowel lumen, a change in bowel habit presents as one of the most common symptom for colon cancers localized on the left side of the patient's body (Anne and Clive, 2007). This is followed by diarrhoea, a stool form change, and ultimately obstruction in the intestines. Up to 10% of iron

deficiency anaemia patients are observed to have colon cancer of which most are localized on the right side. The call for concern and hence an urgent prompting for investigation colorectal cancers is iron deficiency in men and women who are not menstruating (Dunlop, 2002; Rockey and Cello, 1993; Goddard *et al.,* 2005).

Majority of patients having colon cancer in the early stage show no symptoms, and are such are diagnosed due to screening. However, the signs and presenting symptoms of colon cancer are as a result of the growth of the tumor into the lumenal or adjacent structures. This presentation is usually observable in relatively advanced colorectal cancers. It is possible to confuse early symptoms of CRC with other diseases and hence when many patients get diagnosed properly they might already have advanced diseases. It is worth of note that when detected early, potential survival rate in patients might tend to increase (John *et al.,* 2011)

1.4.4 DIAGNOSIS OF COLORECTAL CANCER

Diagnosis of colon cancer is usually as a result of assessment of the symptoms a patient has or as a result of screening conducted on the patient. A range of symptoms can be associated with CRC which include abdominal pain, blood in stools and change in bowel habits; as well as fatigue, weight loss and anaemia-related symptoms (Kuipers, *et al.,* 2015).

The advent of technology made possible the development of special techniques that include the polymerase chain reaction (PCR) that allow for easy identification of genetic biomarkers as well as mutations in specific genes related to colon cancer. The recurrence of colon cancer is closely related to the increase in serum values for carcinoembryonic antigen (CEA) (Granados-Romero, *et al.,* 2017) and the common screenings for colon cancer include; CT colonography, capsule endoscopy and colonoscopy (Kuipers, *et al.,* 2015).

1.4.5 TREATMENT OF COLORECTAL CANCER

For colorectal cancer, the recurrence rates, management and survival times are different based on the stage. For tumors in the early stage (UICC stage I), radical hemicolectomy with lymph node resection devoid of additional treatment is appropriate. In carcinomas presenting as low-risk (pT1, G1-2, L0, and R0), local procedures, such as laparoscopic segment resection or endoscopic mucosal resection may be discussed. Tumors that invade the serosa (T3) or those that spread to local lymph nodes (N+) have increased chances of recurrence, hence adjuvant treatment is often recommended (Sebastian, 2014).

Colon cancers at stage 0 are often commonly treated by removing cancer cells by the technique of colonoscopy. Stage I, II and III cancers commonly require a surgery to be performed (colon laparoscopy, rectal and stoma surgeries) (Kuipers, *et al.*, 2015); and it has been shown that laparoscopic surgery approach for colon can cancers is as safe as the commonly practiced traditional open approach (Arribas-Martin, *et al.*, 2014).

Recognized as the most used and effective cytostatic drug, **5-Fluoracil (Fluorouracil)** continues to be employed in the treatment of colorectal cancer. However, the function of the antibody variation known as Bevacizumab (Avastin) and approved by the US Food and Drug Administration (FDA) has proven to reduce vascular endothelial growth factor (VEGF) which functions as the primary angiogenesis regulator, produced by normal neoplastic cells. Preclinical trials conducted prove that a human monoclonal antibody functioning against the VEGF can retard the growth of xenografts of human tumors (Ferrara *et al.*, 2003; Kim, *et al.*, 1993).

1.4.6 SURGERY

Surgical procedure is the major from of treatment of colon cancer for patients presenting with non-metastasized colon cancer. The results of the procedure is largely dependent on the quality of the surgery (Van de Velde *et al.,* 2014a), as well as the quality of the treatment selection and preoperative staging phases of the surgery. Special attention is required at the circumferential surgical resection margins (Van de Velde *et al.,* 2014b; Quirke *et al.,* 2014). In relatively more severe and advanced occurrences of colon cancers, neoadjuvant treatment (which include chemo-radiotherapy or radiotherapy for locally advanced cancer and preoperative chemotherapy for T4 colon cancer) can lessen the tumor load as well as the tumor stage, and hence could be important to increase the chances for a successful resection procedure (Van de Velde *et al.,* 2014b). Essentially, it is important to follow strictly the procedures before treatment provided from proper staging information (Van de Velde *et al.,* 2014a; Quirke *et al.,* 2014).

1.5 1,2-DIMETHYL HYDRAZINE (DMH)

DMH, more formally known as 1,2-dimethylhydrazine is a part of the class of hydrazines which are known to be strong alkylating agents of the DNA that are naturally found in cycads. DMH is commonly employed as a carcinogen to induce colorectal cancer in animal models. The procarcinogen, DMH, when applied, following a myriad of metabolic reactions reaches the colon where it produces the final carcinogenic reactive oxygen species (ROS) (Frank et al.,2020), which alkylates the DNA and initiates the process that facilitates the development of the carcinogenesis of colon cancer (Karthikkumar *et al.,* 2020)

Figure 1.3: *Swiss albino mice induced with DMH already showing symptoms of fur loss which is an indication of cancer* (Omoregie *et al*., 2023).

1.5.1 ROLE AND HISTORY OF DMH

DMH occurs in its 1,1- dimethylhydrazine and 1,2-dimethylhydrazine isomers which are both clear and colorless compounds in liquid form (Trochimowicz, Kennedy and Krivanek, 1994). 1,1-Dimethylhydrazine is commonly used as jet and rocket fuel as well as a growth control agent in plants and in chemical synthesis as feedstock. The metabolite of DMH known as azoxymeethane (AOM) and DMH itself are procarcinogens that need to be activated metabolically to give their DNA-reactive products (Fiala *et al.*, 1984).

These DNA-alkylating agents (DMH and AOM) start their mutagen activity by methylating a guanine residue in thee DNA at the N-7 position. This alkylated guanine residue becomes paired with a thymidine residue in place of cytosine by donating a proton which ultimately produces the modification of bases. To complete the mutagenic procedure, the mismatch of guanine to thymine (G-T) and cytosine to adenine (C-A) occurs. The entire metabolic process of these procarcinogenic substances is mediated by the aid of different metabolic enzymes that include the xenobiotic-metabolizing enzymes. These enzymes mediate several N-oxidation and hydroxylation stages that include the formation of the ultimate carcinogenic compound known as methylazoxymethanol (MAM) (Fiala *et al.*, 1984).

Being the reactive metabolite of DMH and AOM, methylazoxymethanol (MAM) readily converts to methyldiazonium ion which has been observed to be able to alkylate macromolecules in the colon and liver (Fiala *et al.,* 1984), according to various studies (Notman *et al.,* 1984; Fiala *et al.*, 1987).

1.5.2 METABOLISM OF DMH

The oxidation to azomethane is the first oxidation step of DMH and this converts it to azoxymethane (AOM), which is then converted to methylazoxymethanol (MAM) by the hydroxylation of the previous compound. Hydroxylation of azoxymethane (AOM) mainly occurs on the liver through a cytochrome P450-dependent pathway, and also to a limited degree, hydroxylation occurs in the colonic mucosa (Weisburger, 1971).

Methylazoxymethanol can get to the intestine via the bile, circulation, or directly into the intestinal lumen (Weisburger, 1971), as glucosides, glucuronides, and to some degree as sulfates. Glucosides and glucuronides are cleaved by β-glucosidase and β-glucuronidase respectively, and the sulfates by enzymes which are present in erythrocytes and also in the intestinal micro flora. The chemical instability of MAM at body temperatures causes it to decompose spontaneously into nitrogen, formaldehyde and water (Fiala *et al.*, 1976).

During the decomposition phase, methyldiazonium ion (the alkylating) is formed, and it generates a reactive carbonium ion that is able to alkylate macromolecules including proteins, the DNA and RNA by enzymatic and non-enzymatic reactions localized in the colon.

Alkylating the oxygen atoms within nitrogenous bases creates the possibility of the DNA to be mispaired, and this has been suggested to be an important event in the mutagenic and carcinogenic processes (Hawks and Magee, 1974).

NAD^+-dependent dehydrogenase of the liver and colon were found to be able to act upon Methylazoxymethanol (MAM), and this suggests that the active metabolite of MAM may be the corresponding aldehyde.

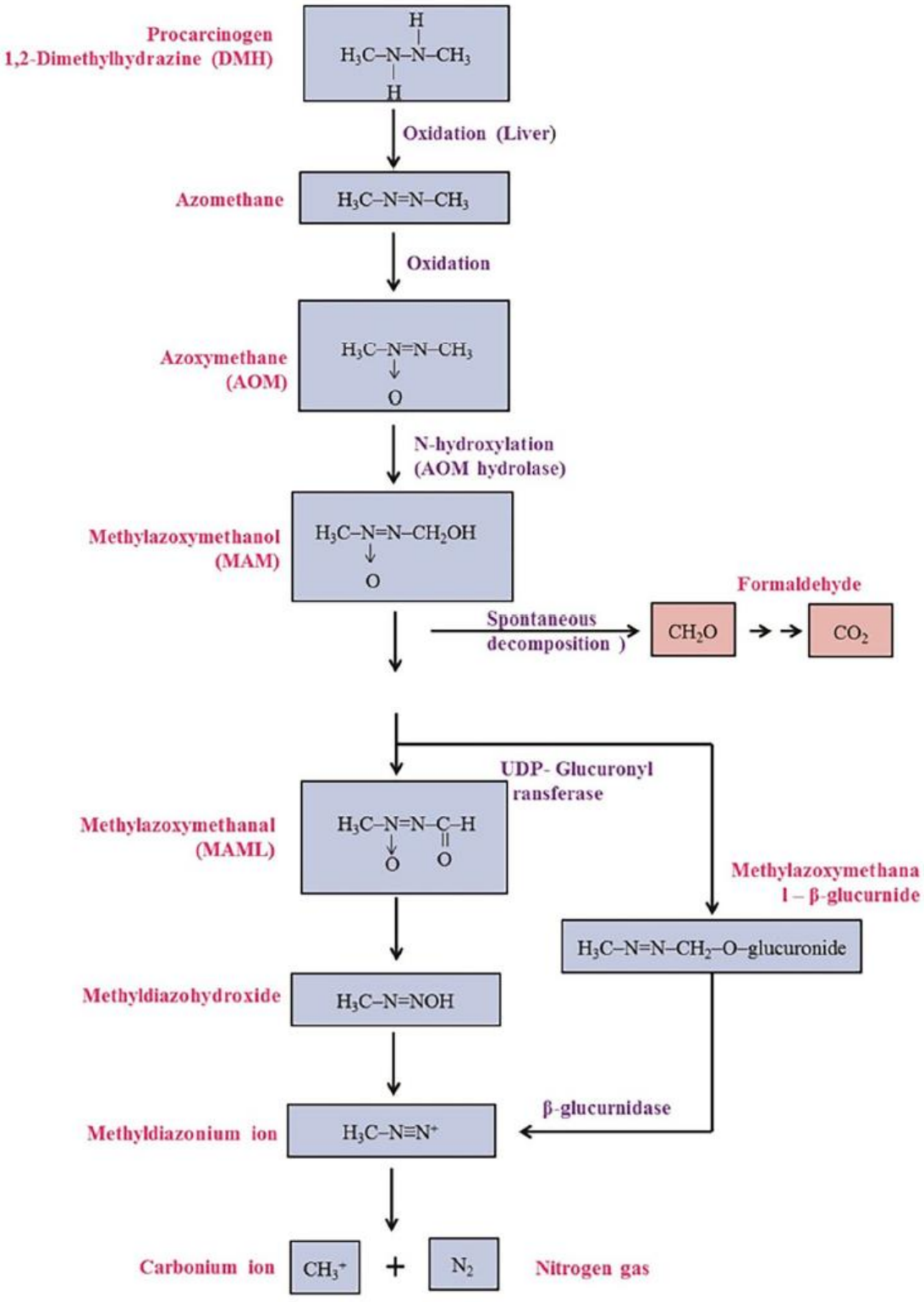


Figure 1.4: Metabolism of DMH (Grab and Zedeck, 1977).

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant materials

The leaves of *Phyllanthus amarus* was collected from the botanical garden of University of Benin, and were identified by an expert in the Department of Botany, University of Benin, Benin city.

2.1.2 Equipment and Apparatus.

The following are lists of equipment and apparatus that was used;

- Separating funnel.
- Beakers.
- Measuring cylinder.
- Water Bath.
- Glass stirrer.
- Mortal and Pestle.
- Whatman no. 1 filter paper.
- EDTA bottles.
- Centrifuge.
- Microscope.
- Conical flask.
- Visible spectrometer.
- Electronic weighing balance.
- Micro pipette.

- Microscopic slide.
- Syringe.
- Test tube and its rack.
- Surgical blade
- Methylated spirit.
- Guage (dolphin feeding needles).
- Volumetric flask.
- Foil paper.

2.1.3 Chemical/Reagents.

- 5-fluorouracil.
- Distilled water, H_20
- Hydrogen peroxide.
- 0.05M phosphate buffer,
- Carcinoembryonic antigen (CEA/CD66) kit, Wkea Med Supplies Corp. China.
- Tumour Necrosis Factor α(TNF-α) kit. Nanhu Dist, Jiaxing, Zhejiang, China
- Dihydrochloride (DMH), Tokyo chemical industry co., LTD.6-15-9 Toshima, Kita-ku, Tokyo,
- Japan
- Ethanol solution JHD, China.
- Epinephrine.
- Potassium dihydrogen phosphate.

- Sodium dihydrogen phosphate.
- Methylene blue.
- Calcium chloride($CaCl_2$)
- Sodium chloride(NaCl)
- Potassium chloride(KCl)
- Formalin phosphate solution.
- Phosphate buffer.
- Sodium carbonate($NaCO_2$).
- 0.4M Sodium Hydroxide(NAOH).
- 0.25M/0.05M Hydrochloric acid(HCl).
- 6M Sulphuric acid(H_2SO_4).
- 30% Hydrogen peroxide.
- EDTA disodium.
- Sodium hydrogen carbonate($NaHCO_3$).
- 250 ml DPX mountant.

2.2 METHODS

2.2.1 Extraction Procedures For Plant Materials.

The leaves of *Phyllanthus amarus* were air-dried at the department of Biochemistry, University of Benin, and pulverized to powdery form in the Pharmacognosy Laboratory in the Department of Pharmacy, University of Benin before the extraction process.

Pulverized and weighed samples of *Phyllanthus Amarus*(1650g) were submerged in ethanol solution(7.5 litres). The plant was macerated for 72 hours at intervals of 4hours with a glass stirrer. The plant extracts(filtrates) were separated into clean sterile glass jars using a cheese

cloth and the residues appropriately discarded and was concentrated with the aid of a vacuum concentrator at 30Oc*. The concentrates was then weighed and used as experiment sampleThe extracts were subsequently freeze dried and stored in the refrigerator until required for analysis.

Formula for Percentage yield:

$$\mathbf{X = \frac{\text{Dry weight of extract}}{\text{Dry weight sample}} \times 100\%}$$

2.2.2 Fractionation process

1000g of the crude extract after being air dried was mixed in a little amount of water and then added to a separating funnel after which 7.5 litres of n-hexane was added. The mixture was stirred with a glass rod and allowed to settle after which two distant layers are formed(the crude layer at the buttom and the n-hexane fraction of P. amarus as the supernantant). The crude extract is collected with a beaker leaving us with the n-hexane fraction. This process is repeated until a clear solution is obtained and this is stored as the n-hexane fraction.

2.2.3 Animal Study.

15 male swiss albino mice of weight 14g-26g were purchased from Kene-Gold venture, at the Department of AEB, University of Benin, Benin City, Edo state. Thy were maintained and acclimatized to diet and environment 1 week after arrival. They were housed in a density of 5 animals per rack mounted plastic with detachable steel aerated cover cages and were fed qwith growers mash in regular pellets and were also given tap wate. The temeperature and lightening(12hours light/dark cycle)were constantly controlled. The animals were grouped as follow ;

GROUPS	CATEGORY
GROUP A	POSITIVE CONTROL
GROUP B	450mg/kg BODY WEIGHT OF n-HEXANE FRACTION OF ET. EXTRACT OF P.AMARUS
GROUP C	NEGATIVE CONTROL

2.2.3.1 Administration of 1,2-dimethylhydrazine

The chemical used was obtained from Tokyo Chemical Industry Co. LTD. Tokyo, Japan. 1,2 – Dimethylhydrazine (1,2 – DMH), has a molecular weight of 133.02, melting point;168^{o}C, it was dissolved in freshly made physiologic saline. The drug was administered to the mice orally with a gavage into their throats according to their individual weights. Administration of 1,2- DMH to the mice was at an interval of 2 days, a period of 2 months which totaled to 24 doses administered to the mice. During the administration certain changes and activities were observed which include: loss of weight, loss of fur, loss of appetite, tumor growth, and weakness

2.2.3.2 Administration of Plant extract.

Upon completion of the doses of carcinogen, the DMH induced mice were randomized in 3 groups, Group A, Group B and Goup C with 5 mice each. The powdered form of the ethanol extract was weighed to know the weight of the extract. The extract to be administered was prepared with the individual body weights of the mice to ensure the right amount was

administered. The extract was administered to the mice orally with a gavage for a period of 14 days.

GROUPS	CATEGORY	ADMINISTRATION
GROUP A	POSITIVE CONTROL	NO ADMINISTRATION
GROUP B	NEGATIVE CONTROL	DMH ONLY
GROUP C	450mg/kg BODY WEIGHT OF n-HEXANE FRACTION OF ET. EXTRACT OF P.AMARUS	DMH+450mg/kg BODY WEIGHT OF n-HEXANE FRACTION OF ET. EXTRACT OF P.AMARUS

- **Measurement used for calculating amount of extact to be administered;**

$$X = \frac{\text{Mass} \times 450}{1000}$$

2.2.3.4 Animal sacrifice and sample collection

The animals were carefully handled following the guidelines established for the treatment of laboratory animals. They were sacrificed at the end of the 14 day administration period of 450mg/kgbwt of n-hexane fration of ethanolic extract of P.Amarus after an overnight fast. The animals were sacrificed by cervical puncture and the blood samples were collected into Eppendorf tubes which were labelled according to the animals. The kidney and liver were harvested and weighed and collected in an organ bag with formalin for histopathology analysis. A section of the colon was placed in an organ bag containing phosphate buffer of pH 7.4 and

placed on This was later homogenized for antioxidant assay. The other part of the colon was used for ACF (Aberrant Cryptic Foci).

2.2.3.5 Preparation of Plasma Samples

The blood samples which were placed in Eppendorf tubes were spun in a centrifuge at 3000rpm for 5 minutes. The clear serum (plasma) was collected using a Pasteur pipette, the serum was collected into newly labelled bottles and stored at a temperature of 7^{o} until required for analysis.

2.2.3.6 Tissue Homogenate Preparation

The excised weighed organs (liver and kidney) were homogenized with mortar and pestle in 10ml of normal saline solution. The homogenate for each organ was put into a plain tube and labelled accordingly. The labeled tubes containing the homogenates were spun in a centrifuge at 3000rpm for 10 mins to obtain the clear supernatant, which was transferred to plain containers labelled accordingly and was used for liver and kidney function tests.

2.3 GENE EXPRESSION

2.3.1 Caspase 9

Caspase-9 is important in destruction of cells by aiding the apoptotic cell death process in early stages of development as it is very important to control proliferation of diseases through the continual removal of dysfunctional grossly cells in the lifecycle. Failure to activate Caspase-9 has detrimental pathophysiological and physiological outcomes that ultimately lead to developmental disorders, degenerative disease conditions, and cancers also (Li et al, 2017).

The presence of variants of nucleic acid sequence of the Caspase-9 gene and following corruption of the apoptosis pathway has been shown to be involved in the susceptibility of tumors in lung, gastric, pancreatic, bladder and colorectal cancers (Theodoropoulos et al, 2011).

Principle

Caspase 9 exista as a proenzyme in the native state, having no activity. It becomes active in the apoptotic stage and contributes to the apoptotic process. Caspase-9 is conjugated with sequence-specific peptides acetyl-Leu-Glu-His-Asp p-nitroanilide (Ac-LEHD-pNA) to yellow p-nitroaniline (pNA) group. Substrate being cut by caspase-9 leads to dissociation of the yellow pNA group. The pNA absorbs maximally at 405nm. The measure of optical density gives indication of caspase-9 activity.

Procedure

Microwells were stripped twice with the wash buffer and aspirated. Standard dilutions were added to the microwell plates and 100 µl of sample diluent in all standard wells. 50 µl of Sample Diluent was added to sample wells.

The detection antibody was prepared and 50 µl added to all wells. The wells were covered and incubated at room temperature for 22 hours.

Anti-rabbit-IgG-HRP was preapared. On completion of incubation time, mircowells were washed with wash buffer 3 times and aspirated. 100 µl diluted anti-rabbit-IgG-HRP was added to al wells, then covered and incubated for 1 hour at room temperature.

Microwells were washed 3 times with wash buffer. 100 µl of TMB Substrate Solution as added to all wells, and incubated for about 10 minutes at room temperature. Stop solution was added and optical density measured at 450 nm.

2.3 BIOCHEMICAL ASSAYS

2.3.1 Oxidative Stress Markers

2.3.1.1 Superoxide Dismutase, SOD

Superoxide dismutase, (SOD) is a copper-containing antioxidant that scavenges superoxide oxide radicals and mops them up. Its activity is considered a measure of cellular oxidative stress levels.

Principle.

Epinephrine (adrenaline) auto-oxidises rapidly in aqueous solution. The auto-oxidation of epinephrine depends on the presence of superoxide anion. The superoxide dismutase inhibits the auto-oxidation of adrenaline by catalyzing the breakdown of superoxide anions. The degree of inhibition is thus a reflection of the superoxide dismutase activity, and is determined at one unit of the enzyme activity.

Procedure

Labeled test tubes for the standard/blank and samples were set up. 0.2mL of the appropriate

enzyme extracts were added to each labeled test tube. Then 2.5mL of carbonate buffer was added to the labeled test tubes, followed by equilibration at room temperature. 3.0mL. of epinephrine solution was added to each of the test tubes, and after mixing absorbance was taken at 420nm (Bannister and Calabrese. 1987).

Calculation

$$\% \text{ Inhibition} = \frac{O.D_{test} - O.D_{reference}}{O.Dtest} \times \frac{100}{1}$$

$$\text{Enzyme Activity (units/mg protein)} = \frac{\% \text{ inhibition}}{50 \times Y}$$

Where Y = mg of protein in the volume of sample.

A unit of SOD activity was taken as the amount of SOD required to cause 50 % inhibition of the auto-oxidation of adrenaline to adrenochrome per minute

2.3.1.2 Malondialdehyde, MDA

Malondialdehyde, also called thiobarbituric acid reactive substances (BARS), is one of the most prevalent byproducts of lipid peroxidation during oxidative stress.

Principle

Malondialdehyde was estimated in this study using the method of Buege and Aust (1975). Malondialdehyde which is formed from the breakdown of polyunsaturated fatty acids (PUFAs) serves as à convenient index for the determination of the extent of the peroxidation reaction. Malondialdehyde reacts with thiobarbituric acid to give a red coloured complex which absorbs at 535nm.

Determination Of MDA Concentration

The concentration of MDA was determined according to the method of Guttridge and Wilkins (1982). a modification of the procedure used by Hunter, et al., (1963). The principle that underlies this assay is that MDA - a product of lipid peroxidation when heated with thiobarbituric acid (TBA), in the presence of an acid, forms a pink or reddish complex that is measured spectrophotometrically at 532nm.

Assay Procedure

Aliquot of the colon homogenate was added to 3.0 mL of TCA - TBA - HCI reagent and mixed thoroughly by swirling. The solution was heated for 15 min in a boiling water bath. After

cooling, the flocculent precipitate was removed via centrifugation at 1000 g for 10 min. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

Calculation

The MDA concentration of each sample was calculated as shown in the equation below;

$$\frac{\mathbf{OD \times V_t \times 1000}}{\boldsymbol{\alpha \times V \times L \times}}$$

where;

O.D = Absorbance of sample test at 535 nm

V_t = Total volume of the reaction mixture = 3.6 mL

α = Molar extinction coefficient of product = $1.56 \times 10^5 M^{-1}cm^{-1}$

L = Light path = 1.0 cm

V = Volume of sample homogenate used = 0.6 mL.

Y = mg of tissue in the sample used

The unit of MDA is moles/mg wet tissue.

2.3.1.3 Catalase, CAT

Catalase degrades and detoxifies cellular hydrogen peroxides, H202 (an agent of oxidative damage) to yield water and molecular oxygen. Special microbodies called peroxisomes house this enzyme.

Principle

The catalase assay is a two-way procedure. The rate of dismutation of hydrogen peroxide, H_20_2 to water and molecular oxygen is proportional to the concentration/activity of catalase in cells.

$H_2O_2 \xrightarrow{\text{catalase}} 2H_2O+O_2$

The sample containing catalase is incubated in the presence of a known concentration of H_2O_2 After incubation for exactly a minute, the reaction is stopped by sodium azide. The amount H_2O_2 remaining in the reaction is then determined by an oxidation coupling reaction of 4-aminopyrene, APP (4-aminophexazone) and 3,5-dichloro-2-hydroxybenzenesulphonic acid, DHBS in the presence of H2O2 and catalysed by horse radish peroxidase (HRP).

Procedure

2.5mL. of 30mM phosphate buffered H:O; was added to sample and blank test tubes. 250uL of distilled water was added to the blank tube, while 2.75ml of 0.05M phosphate buffer (pH 7.4) was added to the standard tube, and 250uL of each sample d to labeled test tubes. The content of each test tube was mixed and allowed to stand for 3 minutes. 500uL of 6M sulphuric acid was added to all the test tubes, followed by mixing. Then, 3.5mL Of 0.01M $KMnO_4$, was added one by one to each test tube, placed in a cuvette and absorbance read at 480nm.

Calculation

The activity of catalase in each sample is calculated thus:

$$\frac{O.D/min \times V_t \times 1000}{M \times V \times L \times Y}$$

where,

O.D = Absorbance of sample test at 480 nm

V_t = Total volume of the reaction mixture = 6.75mL

M = Molar extinction coefficient of H:02= 43.6 M^{-1} cm^{-1}

L = Light path = 1.0 cm

V = Volume of sample homogenate used = 2.5 mL.

Y = mg of organ in homogenate

2.3.1.4 Determination of Glutathione Peroxidase Activity.

Glutathione peroxidase (GPx) activity was measured according to the method described by Nyman (1959).

Principle

This is based on the oxidation of pyrogallol to purpuragallin by peroxidase, resulting to a deep brown coloration, which is read at 430 nm.

Procedure

To an aliquot of homogenate (0.2 ml.), 5mL of phosphate-buffered H¿O2, and 1.5 mL of pyrogallol

were added. The reaction mixture was allowed to stand for 30 min at room temperature. A deep colour was formed, which was read at 430 nm.

Calculation

$$\text{Enzyme Activity} = \frac{OD/min \times V_t \times Df}{E \times Vs \times Y}$$

Where;

OD = Absorbance of test

V_t = Total volume of reaction mixture

Df = Dilution factor

E = Molar extinction coefficient (12/M/cm)

Vs= Volume of sample

Y= mg of organ in homogenate used

2.3.1.5 Determination of Plasma Concentration of Reduced Glutathione

The plasma concentration of reduced glutathione (GSH) was determined using the method described by Ellman (1959).

Reagents

5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), sodium citrate, and trichloroacetic acid (TCA)

Procedure

To 1.0 mL of plasma, 2.5 mL of 10 % TCA was added and centrifuged at 3000 g for 10 min. Then, 1.0 mL of the supernatant was treated with 0.5 mL. of Ellman's reagent (0.0189 % DTNB and -1 % sodium citrate) and 3.0 mL of 0.3 M phosphate buffer (pH 8.0). The yellow colour developed was read immediately at 412 nm and expressed as uM GSH/g plasma.

Calculation

$$\text{Concentration of GSH} = \frac{A_{test} \times \text{Conc. Standard}}{A_{standard}}$$

$$\% \text{ Glutathione Reduced} = \frac{(A_0 - A_1) \times 100}{A_0}$$

Where;

A_0 = Absorbance of reference sample

A_1 = Absorbance of sample

2.3.2 LIVER FUNCTION TESTS

2.3.2.1 Determination of ALT Activity

Principle

α-ketoglutarate + L-Alanine $\xrightarrow{\text{ALT}}$ L-glutamaic + pyruvale

Pyruvate1 2. 4-dinitrophenylhydrazine $\longrightarrow$ pyruvate hydrazone + 2. 4-dinitrophenol

The activity of ALT was assayed by monitoring the concentration of pyruvate hydrazone formed 4-dinitrophenylhydrazine.

Assay Procedure

Plasma sample (0.1 ml.) and 0.5 mL. of reagent I were pipetted into a test tube. The blank contained 0.I ml of distilled water and 0.5 ml: of reagent I. Each tube was mixed and incubated for 30 min at 37 °C. Portions of reagent 2 (0.5 ml.) were added to each tube, and the comments were mixed and incubated for another 20 min at 25°C. Then, 5.0 ml. of 0.4 mol/t. NaOH solution was added to each tube. The tubes were mixed and absorbance read at 540nm against reagent blank after 5 min. The activities of ALT corresponding to the absorbance values obtained were extrapolated from ALT standard calibration curve.

2.3.2.2 Determination of Activity of AST

Principle

α-ketoglutarate + L-aspartate $\xrightarrow{\text{AST}}$ L-glutamate + Oxaloacetate

Oxaloacetate + 2, 4-dinitrophenyIhydrazine $\longrightarrow$ Oxaloacetate hydrazone + 2, 4-dinitrophenol

The activity of AST was assayed by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine.

Assay Procedure

Two tubes were arranged in duplicate on rack and labelled "sample blank" and "sample",

respectively. Plasma (0.1 mL.) and 0.5 mL of reagent 1 were added to each tube. The contents of the tubes were mixed and allowed to stand for 20 min at 25 °C. Then, 5.0 mL NaOH solution was added to each tube and the contents were mixed and absorbance read at 540 nm against the blank after 5 min. The activities of ALT corresponding to the absorbance values obtained were extrapolated from ALT standard calibration curve.

2.3.3 KIDNEY FUNCTION TESTS.

2.3.3.1 Determination of Homogenate Creatinine Concentration

Principle

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Assay Procedure

Exactly 2.0 mL. of working reagent was added to two tubes labelled "standard" and "sample". The standard solution (0.2 mL) was added to the tube labelled "standard", while 0.2 ml. of plasia was added to the tube labelled "sample". After 30 sec absorbance A was read at 492 nm and exactly 2 min later, absorbance A_2 was read.

Calculation

$$\text{Conc. of Creatinine (mg/dl)} = \frac{A_{sample} \times \text{Conc. of Standard}}{A_{standard}}$$

2.3.3.2 Determination of Plasma Urea Concentration

Principle

Urea in plasma is hydrolysed to Ammonia in the presence of Urease. The Ammonia is then

measured photometrically (Berthelot's reaction).

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

NH, + Hypochlorite + Phenol $\longrightarrow$ Indolephenol (blue compound)

Assay Procedure

Aliquots of plasma, standard and distilled water (10 uL each) were added to tubes labelled "sample", "standard" and "blank". Exactly 0.1 mL of R1 was added to the tubes and mixed thoroughly. The tubes were incubated for 10 min at 37 °C, after which 2.5 mL of R2 was added. Exactly 2.5 mL. of R3 was also added to the tubes, and incubated for another 15 min at 37 °C. Absorbance of each tube was read at 546 nm against the blank.

Calculation

$$\text{Urea Concentration (mg/dL)} = \frac{A_{sample} \times \text{Conc. of Standard}}{A_{standard}}$$

$$\text{Potassium ion Conc.} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard(mEq/L)}$$

2.2.9.3 Determination of Plasma Bicarbonate Ion (HCO_3^-)Concentration

Principle

The bicarbonate reagent utilizes the enzymatic method developed by Forester et al. In this procedure bicarbonate (HCO_3^-) and phosphocnolpyruvate (PEP) are converted to oxaloacetate and phosphate in the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC). Malate dehydrogenase (MD) catalyzes the reduction of oxaloacetate to malate with the concomitant oxidation of reduced nicotinamide adenine dinucleotide (NADH). This oxidation of NADH

results in a decrease in absorbance of the reaction mixture measured bichromatically at 380/410 nm proportional to the Bicarbonate content of the sample.

$$PEP + HCO_3^- \xrightarrow[Mg^{2+}]{PEPC} Oxaloacetate + H_2PO_4$$

$$Oxaloacetate + NADH + H^+ \xrightarrow{MD} Malate + NAD^+$$

Assay Procedure

To each of the test tubes labelled test, standard, and blank, 1.0 mL. of carbon dioxide reagent was added. All tubes were incubated for 3 min at 37 °C. Then, 50 uL of sample and standard were added to tubes labelled test and standard, respectively, while distilled water was added to the blank. The solution was mixed and allowed to stand at room temperature for 5 min after which the absorbance was read at 340 nm against the reagent blank. The concentration of CO_2 was calculated as follows:

$$\text{Conc. of } CO_2(\text{mmol/L}) = \frac{\text{Abs of blank - Abs of Sample}}{\text{Abs of blank- Abs of Standard}} \times \text{Concentration of Standard}$$

2.2.9.4 Determination of Plasma Sodium Ion (Na^+) Concentration

Principle

Sodium is precipitated as the triple salt, sodium magnesium uranyl acetate, with the excess uranium then being reacted with ferrocyanide, producing a chromophore whose absorbance varies inversely with the concentration of sodium in the test specimen.

Assay Procedure

To each of the labelled test tubes, sample, standard, and blank, 1.0 mL of filtrate reagent was dispensed. Then, 50 uL of plasma and standard were added to their respective tubes, while distilled water was added to the blank. The tubes were mixed and vigorously shaken for 3 min and was then centrifuged at 1,500 g for 10 min, Subsequently, labelled test tubes corresponding to the above filtrate tubes were arranged in rack. Then, 1.0 mL. of acid reagent (diluted acetic acid) was added to all the tubes after which 50 uL of supernatant was added to the respective tubes and mixed thoroughly. Exactly 50 uL of colour reagent was added to the tubes and mixed and the absorbance was read at 550 nm.

$$\text{Sodium on Conc.} = \frac{\text{Abs of blank - Abs of Sample}}{\text{Abs of blank - Abs of Standard}} \times \text{Conc. of Standard (mEq/L)}$$

2.2.9.5 Determination of Plasma Potassium Ion (K^+) Concentration

Principle

Under alkaline condition, sodium tetraphenylborate reacts with potassium ion in a sample to form the potassium tetraphenylborate which is white and small particles with low solubility Potassium tetraphenylborate particles are in a stable suspension state in the solution. The turbidity is proportional to the potassium ion concentration in the sample.

Assay Procedure

Plasma (50uL) was mixed with 0.2mL of color reagents, while the blank contained 50uL of reagent blank and 0.2mLof color reagent. The contents of the tubes were thoroughly mixed. After 5 min of incubation at 25^{o}C, the absorbance was read at 450nm against blank. The concentration of Potassium ion was calculated as shown in the equation below;

$$\text{Potassium ion Conc} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard (mEq/L)}$$

2.2.10. Histological Examination of the Tissues

Portions of the liver, kidney and colon were serially sectioned and fixed in 10 % formalin for 48h. The specimen was then dehydrated through a graded series of alcohol and cleared in three changes of xylene before being embedded in paraffin. Serial sections, each of 4 um thickness, were made and stained with hematoxylin and coin according to standard method. Histological assessment was performed under light microscopy. In every H and E section a minimum of 25 circular tubule were measured in two axes drawn perpendicular to each other using an image analyser (Image Proplus 3.0).

CHAPTER THREE

3.0 RESULTS

STASTICAL ANALYSIS

The results are presented as mean ± SEM. They were analyzed statistically via one-way Analysis of Variance (ANOVA) using Microsoft excel (2010) worksheet, with significant difference set at $p \leq 0.05$.

3.1. ANTIOXIDANT PARAMETERS

Table 3.1.1 Effect of n-hexane fraction of ethanolic extract of Phyllanthus amarus on some oxidative stress markers of DMH induced colorectal cancer in Mice

GROUPS	SOD ($x10^3$)(u/mg wet tissue)	CAT ($x10^2$)(u/mg wet tissue)	MDA ($x10^{-4}$)(m/mg wet tissue)	GPX (u/mg wet tissue)	GSH (u/mg wet tissue)
Normal Control (-DMH)	2.156 ± 0.64^a	11.66 ± 0.78^a	0.209 ± 0.015^a	0.037 ± 0.014^a	1.23 ± 0.087^a
DMH+450mg/kg bw of n-hexane fraction of Et. *P. amarus*)	0.42 ± 0.28^b	2.93 ± 0.75^b	0.17 ± 0.02^a	0.08 ± 0.015^a	1.93 ± 0.19^a
Negative control (+DMH only)	0.50 ± 0.092^b	1.19 ± 0.268^c	1.6 ± 0.142^b	0.005 ± 0.0028^b	2.41 ± 1.343^b

Table 3.1: results from antioxidant assay

All values are expressed as mean ± SEM(n=5) . Values with different lowercase, superscript represent significance difference at $p<0.05$

DMH = 1,2- DimethyHydrazine , MDA = Malondialdehyde, CAT = Catalase, SOD = Superoxide Dismutase, GSH = Reduced Glutathione, GPX = Glutathione Peroxidase.

The result showed that there was significant($p< 0.05$) change in the levels of endogenous antioxidants MDA, GPX, GSH, SOD and CAT between the control, DMH only and mice treated with 450mg/kg bwt of n-hexane fraction of Et. *P. amarus*.

3.2 LIVER FUNCTION TEST

Table 3.1.2 Effect of n-hexane fraction of ethanolic extract of Phyllanthus amarus on AST and ALT parameters of DMH induced colorectal cancer in swiss albino mice

GROUPS	AST(u/L)	ALT(u/L)
Normal Control (-DMH)	**25.6 ± 0.58^{a}**	**168.9 ±24.57^{a}**
DMH+450mg/kg bwt of n-hexane fraction of Et. *P. amarus*)	**26.77±1.16^{a}**	**219.42±54.8^{a}**
Negative control (+DMH only)	**29.1± 0.579^{b}**	**156.56±11.68^{b}**

Table 3.2: results from electrolytes and kidney parameters

All values are expressed as mean ± SEM . Values with different lowercase, superscript represent significance difference at $p<0.05$

DMH= 1,2-DimethyHydrazine, AST=Aspartate aminotransferase, ALT=Alanine aminotransferase.

The result showed that there was significant($p< 0.05$) change in the levels of Liver parameters AST and ALT between the control, DMH only and mice treated with 450mg/kg bwt of n-hexane fraction of Et. *P. amarus*.

3.3 KIDNEY FUNCTION TEST

Table 3.1.3 Effect of n-hexane fraction of ethanolic extract of Phyllanthus amarus on some kidney parameters of DMH induced colorectal cancer in Mice.

GROUPS	Na+ (mEq/L)	K+ (mEq/L)	HCO3 (mmol/L)	Urea (mg/dL)	Creatinine (mg/dL)
Normal Control (-DMH)	**114.51±5.79[a]**	**2.656±0.158[a]**	**4.117±0.08[a]**	**0.717±0.037a**	**2.13±0.36a**
DMH+450mg/kg bwt of n-hexane fraction of Et. *P. amarus*)	**205.55±3.87[b]**	**3.59±0.7[a]**	**2.518±0.27[a]**	**1.083±0.134[a]**	**1.92 ±0.13[a]**
Negative control (+DMH only)	**208.0±25.24[b]**	**3.24± 0.504[a]**	**3.62±0.937[a]**	**1.75±0.05[b]**	**1.883±0.2[b]**

Table 3.3: results from electrolytes and kidney parameters

All values are expressed as mean ± SEM . Values with different lowercase, superscript represent significance difference at $p<0.05$

DMH = 1,2- DimethyHydrazine , Na^+ = Sodium ion, k^+ = Potassium ion, HCO^{3-} = Bicarbonate ion

The result showed that there was significant($p< 0.05$) change in the levels of kidney parameters Na+, K+, HCO3-, Urea and Creatinine between the control, DMH only and mice treated with 450mg/kg bwt of n-hexane fraction of Et. *P. amarus.*

EXPRESSION

Table 3.4 Effect of n-hexane fraction of Ethanolic extract of *Phyallanthus amarus* on Some Gene Expression Parameters of DMH induced Carcinogenesis in Mice.

GROUPS	Interlukin-6(pg/M)	Caspase 9 ($x10^2$)(ng/l)
Normal Control (-DMH)	**7.434±1.353**	**2.765 ± 0.633**
(DMH + 450 mg/kg bwt of Et. extract *P. amarus*)	**9.9185±2.995**	**2.159± 0.451**
Negative control (+DMH only).	**6.335±0.549**	**2.**586± **0.128**

Table 3.4: results from gene expression assay

All values are expressed as Mean ± SEM (n = 5), with significant difference at $p \leq 0.05$

For Interlukin-6 there was significant difference between the normal control when compared with that of the treated group and negative control. For Caspase 9,there was significant change when the normal control was compared with the treated group.

3.5 BODY WEIGHT

3.5.1 Table showing body weight of animals in the control group, the group treated with 450mg/kg of body weight of n-hexane fraction of ethanolic extract of Phyllanthus amarus and the negative control group

Weights(mg)	Normal control (-DMH)	(DMH + 450 mg/kg bwt of Et. extract *P. amarus*)	Negative control (+DMH only).
Initial weight	**25.17±1.30**	**20.25±1.44**	**23.83±1.40**
Final weight	**27.20±1.51**	**19.00±1.87**	**21.00±1.66**
Weight gain	**2.03**	**-1.25**	**-2.83**

Table 3.5: results from body weight table

All values are expressed as Mean ± SEM (n = 5), with significant difference at $p \leq 0.05$ The result showed that there was statistical significant difference at p,0.05 of both the final weight and the initial weight of animals found in all three groups.

3.6 HISTOPATHOLOGICAL STUDIES

3.6.1 Effect of 350mg/ kg body weight extract of *Phyallanthus amarus* on the liver and kidney of DMH induced colon carcinogenesis in mice.

- **GROUP A (CONTROL)**

LIVER (X400 MAGNIFICATION)

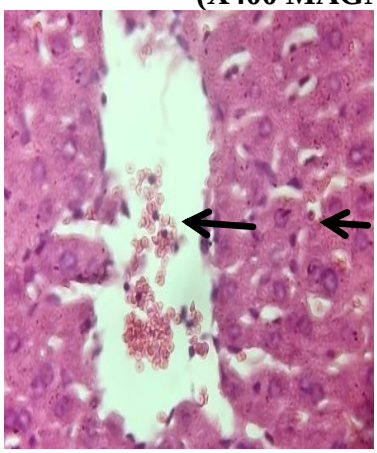


Fig 5. Liver histology reveals central vein (long arrow) well fenestrated sinusoids and hepatocytes with pyknotic nucleus (short arrow)

KIDNEY (X400 MAGNIFICATION)

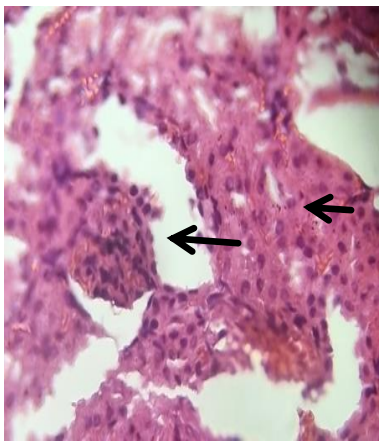


Fig 6. Kidney reveals remarkable renal corpuscle with visible glomerulus (long arrow), tubules and interstitial Liver histology reveals central vein (long arrow) well fenestrated sinusoids and hepatocytes with pyknotic nucleus (short arrow).

- **GROUP B (NEGATIVE CONTROL)**

LIVER (X400 MAGNIFICATION)

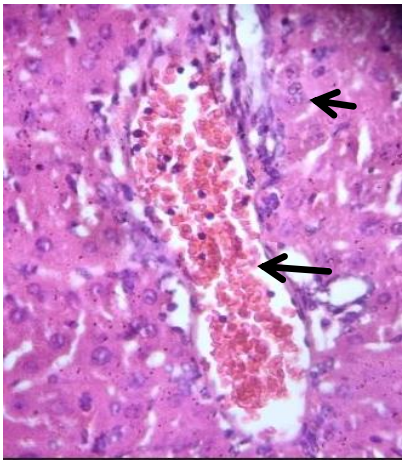


Fig 7. Liver histology reveals congested dilated central vein (long arrow) surrounded by pools of mononuclear exudate and well fenestrated sinusoids and hepatocytes with nuclear polymorphism (short arrow).

KIDNEY (X400 MAGNIFICATION)

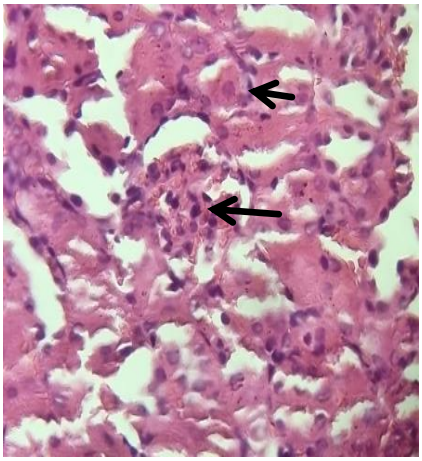
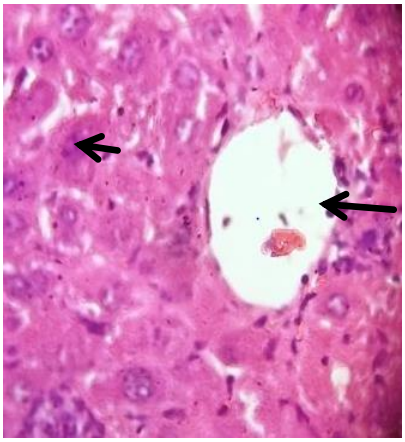


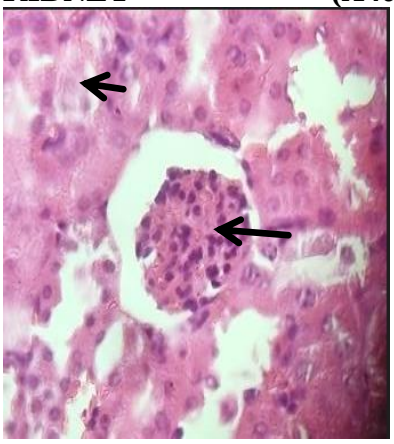
Fig 8. Kidney reveals unremarkable renal corpuscle with distorted glomerulus (long arrow) with distorted tubules (short arrow) and interstitial.

- **GROUP C n-Hexane**
  LIVER (X400 MAGNIFICATION)



Liver histology reveals prominent central vein (long arrow) surrounded by focal mononuclear exudates and well fenestrated sinusoids and hepatocytes (short arrow)

KIDNEY (X400 MAGNIFICATION)



Kidney reveals unremarkable renal corpuscle with enlarged glomerulus (long arrow) with not so prominent tubules (short arrow) and interstitial.

CHAPTER FOUR

4.1 DISCUSSION

Plants have beeen used since prehistoric times for therapeutic purposes due to the presence of bioactive components. Plants' bioactive substances have the intrinsic potential to control (and prevent) cancer. Theseplant compounds may be useful, playing crucial roles in the prevention and management of colorectal colon cancer, according to evidence from study findings. This stidy was carried out to evaluate the therapeutic effect of 450mg/kg bwt n-hexane fraction of ethanolic *Phyllanthus amarus* on 1,2-dimethylhydrazine induced colorectal .

Reactive oxygen species (ROS) and malignancies, particularly colon cancer, are linked, according to growing research. Reactive oxygen species (ROS) have a role in the development of cancer. These organisms have the capacity to oxidize DNA's purine and pyrimidine bases, which results in single strand breaks that alter the genetic makeup of a cell and genetic instability. These mutations are mainly due to base substitutions, deletions or insertions (Wars and Ashan, 2006)

From the results obtained in this experiments as shown in the table (chapter three) and charts(appendix I) for antioxidants assay, SOD(superoxide dismutase), showed significant difference($p<0.05$) between the positive control and the negative control(the diseased) which is normal but insignificant differences between the negative control amd the mice treated with 450mg/kg bwt of n-hexane fraction of Ethanolic extract of *Phyllantus amarus.*

*In MDA (malondialdehyde) concentration, there was a significant diff*erence between the positive control when compared with the negative control(DMH only) as well as that of the positive control and the mice treated with 450mg/kg bwt of n-hexane fraction of Ethanolic

extract of *Phyllanthus Amarus* which indicates that there is a limitation to the effectiveness of the extract due to a short time of treatment against colon cancer.(Chevalier, 2000).

The Catalase activity(CAT) for the positive control when compared to the negative control shows significant difference ($p<0.05$) and when the positive control is compared with the mice treated with 450mg/kg bwt of n-hexane fraction of ethanolic extract of *Phyllanthus Amarus*.

GPx (Glutathione peroxidase) and GSH (Reduced Glutathione) followed the same trend as the positive control when compared with the negative control showed significant difference and no significant difference when the mice treated with 450kmg/kg bwt of n-hexane fraction of ethanolic extract of *Phyllanthus Amarus* was compared to the positive control as this indicates the effectiveness of the extract.

SOD and catalase activities were highest in positive control, reduced in treated mice and lowest in negative control. MDA activity was highest in negative control, reduced in treated mice and lowest in positive control (Taiwo et al., 2009).

Within cells SOD generates H_2O_2, (peroxide) from reactive oxygen species. Peroxide generated is detoxified (by conversion to $H_¿O$) by the action of the enzyme catalase. In the instance where there is minimal or no activity of SOD and catalase to perform the processes stated above, lipid peroxidation would occur and hence increase in the activity of malondialdehyde (MDA) which supports the fact that, in diseased conditions; MDA is high while SOD and CAT are low.

For the liver function tests, Alanine aminotransferase (ALT) activity showed significant difference ($p<0.05$) between the positive, negative and treated groups. The group treated with2450mg/kg bwt of n- hexane fraction of ethanolic extract of P. amarus showed the highest activity, with the negative control group showing the lowest activity. The activity of ALT

between the positive contact group and the mice treated are within the same range and this indicates the effectiveness of the n-hexane fraction of ethanolic extract of P. amarus.

Aspartate transaminase (AST) results indicates an insignificant change between the positive control and the group treaated with the extract. However, there was a significant difference between the normal control and the negative control. This indicates the effectiveness of n-hexane fraction of ethanolic extract of P. amarus on colon cancer as AST and ALT are liver marker enzymes and they are high in normal condition and low in diseased conditions as the liver homogenate was used to carry out this test.

The results from the kidney parameters(3.3), the concentration of sodium ion was significantly increased for the treated group(hypernatremia) when compared to control and the DMH group while potassium ion concentration in control group was significantly different in treated and also for the untreated. For Bicarbonate ion, there is significant difference in control group, the treated group and for the untreated. For creatinine concentration, there was significant difference in the control group and treated and insignificant difference in the untreated. For urea concentration there is significant difference between the control group and the treated and insignificant difference in the untreated.

Caspase 9 is important in the process of apoptosis in cells, ensuring the regulated cell death, including the death of cancer cells. Mutant caspase 9 protein, generated from a mutant gene, cannot regulate the proliferation of cancerous cells. Significant difference ($p>0.05$) existed between positive control mice analyzed against 450mg/kg body weight n-hexane fraction of Ethanolic extract of *P. amarus* treated mice, as well as positive control mice analyzed against negative control. Caspase 9 activity was highest in normal mice, reduced in mice treated with

extracts from *P. amarus*, and lowest in negative control mice (+DMH alone)(Sebastian, 2014). Interlukin 6 is an inflammatory marker.

From the results in table (3.4)), there is a significant change between the positive control group when analyzed against the treated group and the the negative control for Interlukin 6and the values is highest in the treated group. For caspase 9, there is no significant change between the positive control group and the negative control group and a significant change between the positive control and the group treated with 450mg/kg bwt of ethanolic extract of P. amarus.

Histopathological examinations of the liver of the untreated group reveals congested dilated central vein surrounded by pools of mononuclear exudates and well fenestrated sinusoids and hepatocytes with nuclear polymorphism while that have the positive and treated group reveals prominent central vein surrounded by focal mononuclear exudates and well fenestrated sinusoids and hepatocytes and the postive control had well fenestrated sinusoids. The kidney of the positive control shows a remarkable renal corpuscle with visible glomerulus. tubules and interstitial. The treated group and untreated group had similar results which indicates unremarkable renal corpuscle with enlarged glomerulus with not so prominent tubules and interstitial. The insignificant difference may be as a result of short period of administration of the treatment.

The weights of the mice also showed significant difference ($p>0.05$) at the beginning and end of the experiment(Table 3.5) thus the effectiveness of the treatment.

CONCLUSION

The administration of 450mg/kg body weight of n-hexane fraction of Ethanolic extract of *Phyllanthus Amarus* shows anti-cancer activities.This study also showed that n-hexane fraction extracts possess innate antioxidant abilities due to its ameliorative effects on Reactive Oxygen Species (ROS). This effectiveness of *Phyllanthus amarus* against colon cancer was also supported by Frank and colleagues (Frank *et al*, 2020). I suggest that more work should be carried out in plant to fractionate the crude extract to identify the active compounds against colon carcinogenesis.

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APPENDIX I

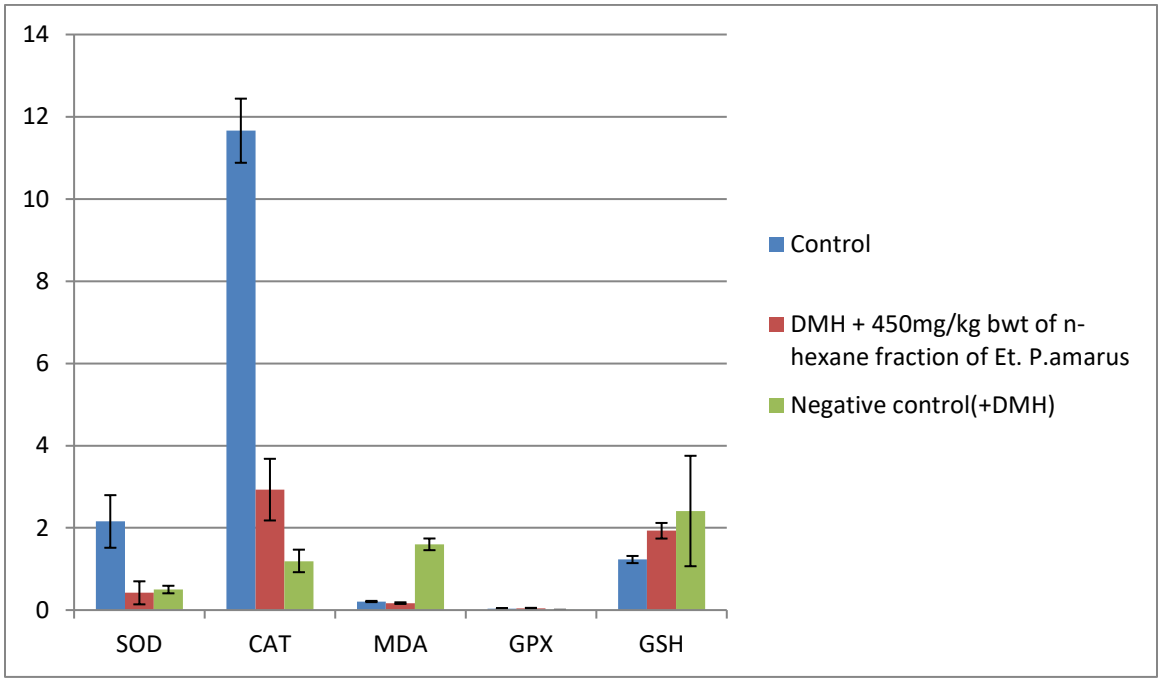

Figure 3.1: Table 3.1: results from antioxidant assay

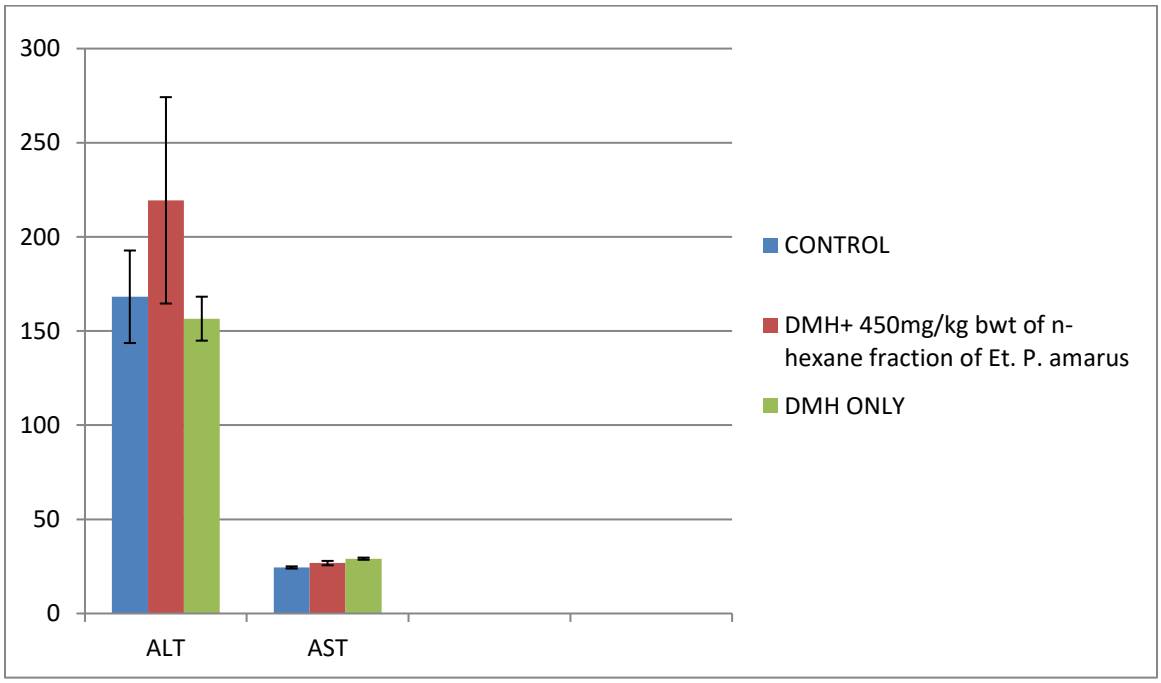

Figure 3.2: results from liver function tests

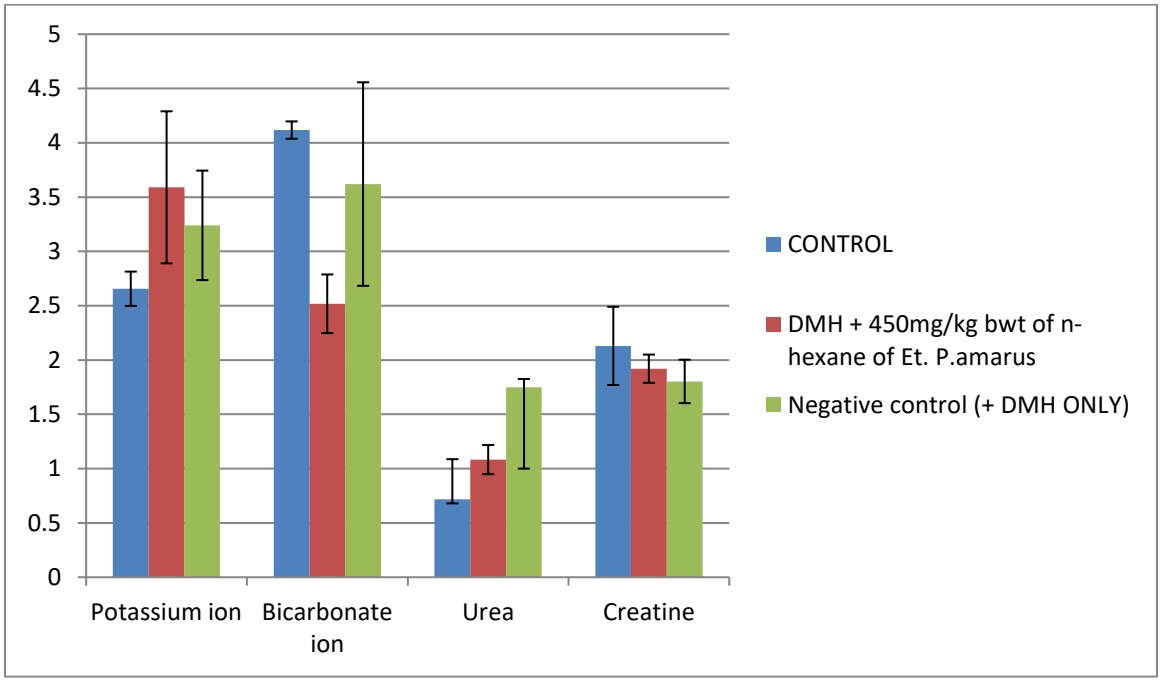

Figure 3.3: results from kidney function tests

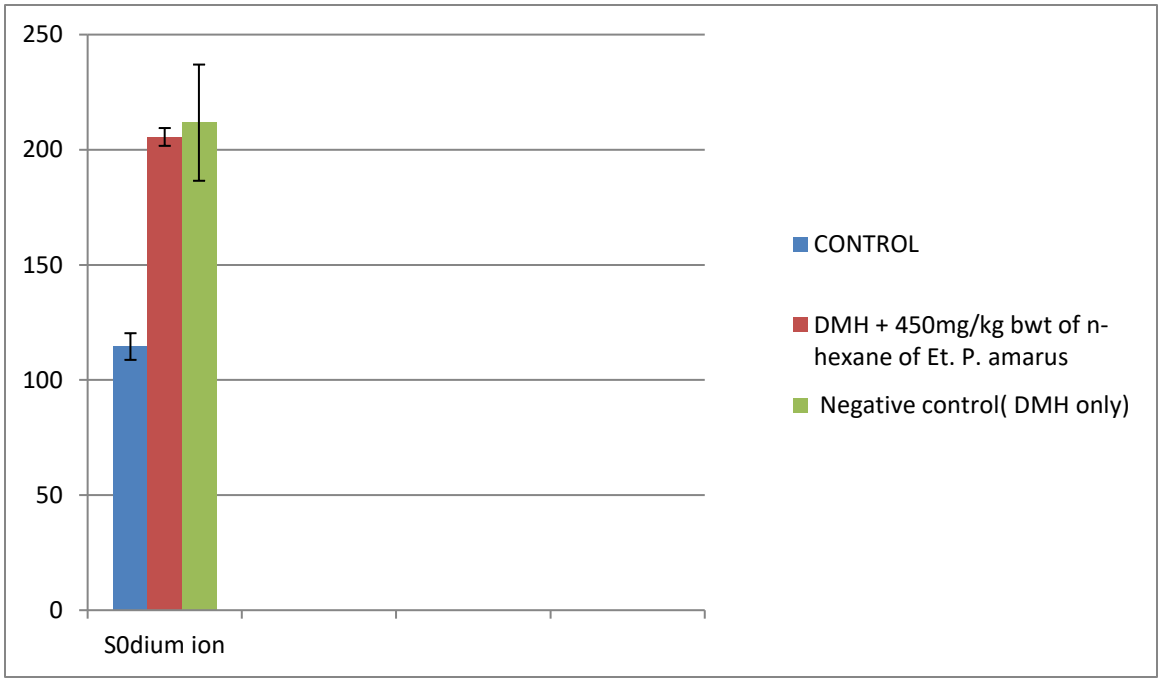

Figure 3.3: results from kidney function tests ; sodium ion concentration.

APPENDIX II

ASSAY CONCENTRATION VALUES

BIOCHEMICAL PARAMETERS

OXIDATIVE STRESS MARKERS

	Catalase		
VALUES	+ve Control	Treated	-ve Control
1	11.08	2.48	1.71
2	10.7	4.4	0.82
3	13.2	1.91	1.03
MEAN	11.663	2.93	1.187
STANDARD DEVIATION	1.3528	1.304	0.465
VARIANCE	1.8302	1.7019	0.2164
STANDARD ERROR	0.78	0.75	0.268

	SOD (Superoxide Dismutase)		
VALUES	+ve Control	Treated	-ve Control
1	0.895	0.052	0.33
2	2.58	0.98	0.508
3	2.995	0.23	0.658
MEAN	2.156	0.420	0.4986
STANDARD	1.112	0.492	0.1641

DEVIATION			
VARIANCE	1.236	0.242	0.026
STANDARD ERROR	0.64	0.28	0.092

	MDA (Malondialdehyde)		
VALUES	+ve Control	Treated	-ve Control
1	0.182	0.212	1.7
2	0.21	0.175	1.78
3	0.237	0.113	1.32
MEAN	0.209	0.173	1.6
STANDARD DEVIATION	0.027	0.039	0.245
VARIANCE	0.0007	0.001	0.0604
STANDARD ERROR	0.015	0.02	0.142

BIOCHEMICAL	**GPX (Glutathione Peroxidase)**		
VALUES	+ve Control	Treated	-ve Control
1	0.015	0.028	0.053
2	0.034	0.072	0.042
3	0.062	0.021	0.044
MEAN	0.037	0.040	0.046
STANDARD DEVIATION	0.236	0.027	0.0058

VARIANCE	0.0005	0.0007	3.43E-05
STANDARD ERROR	0.014	0.015	0.026

	GSH (Reduced Glutathione		
VALUES	+ve Control	Treated	-ve Control
1	1.18	2.14	1.03
2	1.11	1.55	5.1
3	1.14	2.1	1.11
MEAN	1.23	1.93	2.413
STANDARD DEVIATION	0.151	0.329	2.327
VARIANCE	0.0229	0.108	5.4152
STANDARD ERROR	0.087	0.19	1.343

LIVER FUNCTION TESTS

BIOCHEMICAL	**AST**		
VALUES	+ve Control	Treated	-ve Control
1	24.44	27.94	29.68
2	26.19	27.94	27.94
3	26.19	24.44	29.68
MEAN	25.60	26.77	29.1
STANDARD DEVIATION	1.010	2.020	1.004

VARIANCE	1.02	4.083	1.0092
STANDARD ERROR	0.58	1.16	0.579

	ALT		
VALUES	+ve Control	Treated	-ve Control
1	136.19	328.25	153.65
2	216.5	153.65	178.09
3	151.9	176.35	137.93
MEAN	168.19	219.41	156.55
STANDARD DEVIATION	42.56	94.93	20.23
VARIANCE	1811.6	9012.3	409.54
STANDARD ERROR	24.57	54.8	11.68

KIDNEY FUNCTION TESTS

	Sodium ion(Na+)		
VALUES	+ve Control	Treated	-ve Control
1	125.6	207.61	254.59
2	110.69	210.99	167.85
3	106.51	198.06	201.6
MEAN	25.606	205.55	208.2
STANDARD DEVIATION	10.03	6.70	43.72

VARIANCE	100.7	44.96	1911.6
STANDARD ERROR	5.79	3.87	0.280

	Potassium ion(K+)		
VALUES	+ve Control	Treated	-ve Control
1	2.904	3.32	3.69
2	2.36	4.94	2.23
3	2.704	2.53	3.79
MEAN	2.656	3.59	3.23
STANDARD DEVIATION	0.275	1.22	0.87
VARIANCE	0.075	1.509	0.762
STANDARD ERROR	0.158	0.7	0.504

	Bicarbonate ion(HCO3-)		
VALUES	+ve Control	Treated	-ve Control
1	4.144	2.576	2.016
2	4.256	2.968	5.264
3	3.951	2.012	3.572
MEAN	4.977	2.51	3.617
STANDARD DEVIATION	0.154	0.480	1.624
VARIANCE	0.023	0.2309	2.638

STANDARD ERROR	0.08	0.27	0.937

	Urea		
VALUES	+ve Control	Treated	-ve Control
1	0.787	0.846	1.706
2	0.656	1.312	1.645
3	0.71	1.09	1.895
MEAN	0.717	1.082	1.748
STANDARD DEVIATION	0.0658	0.233	0.130
VARIANCE	0.0043	0.054	0.016
STANDARD ERROR	0.037	0.134	0.075

BIOCHEMICAL	**Creatinine**		
VALUES	+ve Control	Treated	-ve Control
1	1.161	2.17	2.17
2	2.83	1.72	1.5
3	1.95	1.86	1.74
MEAN	2.13	1.916	1.803
STANDARD DEVIATION	0.629	0.230	0.339
VARIANCE	0.697	0.053	0.115
STANDARD ERROR	0.36	0.13	0.2

APPENDIX III

STATISTICAL ANALYSIS

t-Test: Two-Sample Assuming Unequal Variances				
SOD				
	CONTROL	*DMH + A*		
Mean	2.156667	0.420667		
Variance	1.236908	0.242561		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	2.47205			
P(T<=t) one-tail	0.04495			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.089901			
t Critical two-tail	3.182446			

t-Test: Two-Sample Assuming Unequal Variances				
	CONTROL	*DMH ONLY*		
Mean	2.156667	0.498667		
Variance	1.236908	0.026961		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	2.55443			
P(T<=t) one-tail	0.062565			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.125129			
t Critical two-tail	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
MDA	*Variable 1*	*Variable 2*		
Mean	0.209667	0.173333		
Variance	0.000756	0.001562		
Observations	3	3		
Hypothesized Mean Difference	0			
df	4			
t Stat	1.306913			
P(T<=t) one-tail	0.130656			
t Critical one-tail	2.131847			
P(T<=t) two-tail	0.261312			
t Critical two-tail	2.776445			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	0.209667	1.6		
Variance	0.000756	0.0604		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-9.73775			
P(T<=t) one-tail	0.005191			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.010382			

t- critical two	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
CAT	*Variable 1*	*Variable 2*		
Mean	11.66333	2.93		
Variance	1.830233	1.7019		
Observations	3	3		
Hypothesized Mean Difference	0			
df	4			
t Stat	8.048633			
P(T<=t) one-tail	0.000647			
t Critical one-tail	2.131847			
P(T<=t) two-tail	0.001294			
t Critical two-tail	2.776445			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	11.66333	1.186667		
Variance	1.830233	0.216433		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	12.68412			
P(T<=t) one-tail	0.003079			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.006158			
t Critical two-tail	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
GPX	*Variable 1*	*Variable 2*		
Mean	0.037	0.040333		
Variance	0.000559	0.000764		
Observations	3	3		
Hypothesized Mean Difference	0			
df	4			
t Stat	-0.15871			
P(T<=t) one-tail	0.440794			
t Critical one-tail	2.131847			
P(T<=t) two-tail	0.881588			
t Critical two-tail	2.776445			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	0.037	0.046333		
Variance	0.000559	3.43E-05		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-0.66366			
P(T<=t) one-tail	0.287586			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.575172			
t Critical two-tail	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
GSH	*Variable 1*	*Variable 2*		
Mean	1.23	1.93		
Variance	0.0229	0.1087		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	-3.34219			
P(T<=t) one-tail	0.022158			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.044315			
t Critical two-tail	3.182446			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	1.23	2.41333		
Variance	0.0229	5.41523		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-0.87891			
P(T<=t) one-tail	0.23607			
t Critical one-tail	2.91998			
P(T<=t) two-tail	0.47215			
t Critical two-tail	4.30265			

t-Test: Two-Sample Assuming Unequal Variances				
AST	*Variable 1*	*Variable 2*		
Mean	25.60667	26.77333		
Variance	1.020833	4.083333		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	-0.89443			
P(T<=t) one-tail	0.218499			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.436998			
t Critical two-tail	3.182446			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	25.60667	29.1		
Variance	1.020833	1.0092		
Observations	3	3		
Hypothesized Mean Difference	0			
df	4			
t Stat	-4.24668			
P(T<=t) one-tail	0.006597			
t Critical one-tail	2.131847			
P(T<=t) two-tail	0.013193			
t Critical two-tail	2.776445			

t-Test: Two-Sample Assuming Unequal Variances				
ALT	*Variable 1*	*Variable 2*		
Mean	168.1967	219.4167		
Variance	1811.61	9012.343		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	-0.85272			
P(T<=t) one-tail	0.228241			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.456482			
t Critical two-tail	3.182446			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	168.1967	156.5567		
Variance	1811.61	409.5429		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	0.427784			
P(T<=t) one-tail	0.348829			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.697659			
t Critical two-tail	3.182446			

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t-Test: Two-Sample Assuming Unequal Variances				
SODIUM ION	*Variable 1*	*Variable 2*		
Mean	114.2667	205.5533		
Variance	100.7014	44.96863		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	-13.1003			
P(T<=t) one-tail	0.00048			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.000961			
t Critical two-tail	3.182446			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	114.2667	208.02		
Variance	100.7014	1911.677		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-3.61987			
P(T<=t) one-tail	0.03428			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.06856			
t Critical two-tail	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
Potassium ion	*CONTROL*			
Mean	2.656	3.596667		
Variance	0.075712	1.509433		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-1.29408			
P(T<=t) one-tail	0.162461			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.324922			
t Critical two-tail	4.302653			

t- Test: Two-sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	2.656	3.236667		
Variance	0.075712	0.762533		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-1.0985			
P(T<=t) one-tail	0.19328			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.38656			
t Critical two-tail	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
UREA	*Variable 1*	*Variable 2*		
Mean	0.717667	1.082667		
Variance	0.004334	0.054329		
Observations	3	3		
Hypothesized Mean Difference	0			
df	2			
t Stat	-2.61017			
P(T<=t) one-tail	0.06038			
t Critical one-tail	2.919986			
P(T<=t) two-tail	0.12076			
t Critical two-tail	4.302653			

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	0.717667	1.748667		
Variance	0.004334	0.01699		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	-12.2286			
P(T<=t) one-tail	0.000589			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.001178			
t Critical two-tail	3.182446			

t-Test: Two-Sample Assuming Unequal Variances			
CREATININE	*CONTROL*	*Variable 2*	
Mean	1.980333	1.916667	
Variance	0.69708	0.053033	
Observations	3	3	
Hypothesized Mean Difference	0		
df	2		
t Stat	0.127324		
P(T<=t) one-tail	0.455166		
t Critical one-tail	2.919986		
P(T<=t) two-tail	0.910331		
t Critical two-tail	4.302653		

t-Test: Two-Sample Assuming Unequal Variances				
	Variable 1	*Variable 2*		
Mean	1.980333	1.803333		
Variance	0.69708	0.115233		
Observations	3	3		
Hypothesized Mean Difference	0			
df	3			
t Stat	0.340151			
P(T<=t) one-tail	0.378083			
t Critical one-tail	2.353363			
P(T<=t) two-tail	0.756167			
t Critical two-tail	3.182446			