UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

ANTIOXIDANT AND SELECTIVE CYTOTOXIC ACTIVITIES ON CANCER CELL LINES BY BIOACTIVATABLE COMPOUNDS IN EXTRACTS OF *TERMITOMYCES SCHIMPERI* (LYOPHYLLACEAE)

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DECLARATION

I hereby declare that this is the outcome of my research undertaken under supervision. To the best of my knowledge, it contains no information published by another author nor information which has been presented for an award of another degree elsewhere, except where due acknowledgment has been made.

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ABSTRACT

BACKGROUND: *Termitomyces schimperi* (TS) is an edible mushroom that is known to possess certain therapeutic properties. Anecdotal report from La Cote d’Ivoire suggests that the mushroom is traditionally used in combination with kaolin, to manage a number of cancers. There is, however, a dearth of information on its antioxidant and anticancer properties with or without kaolin.

AIMS: The aims of this study were to determine the antioxidant and cytotoxic potential of aqueous and ethanolic extracts of TS, and elucidate the effect of microsome-dependent bioactivation of these extracts in combination with kaolin on cancer cell lines.

METHODOLOGY: Aqueous and ethanolic (20:80, v/v) extracts of TS were prepared from the dried mushroom in combination with kaolin (TSK), and extracts of kaolin alone (K) was also prepared, giving 6 extracts in all. The six extracts were screened for secondary metabolites. Total phenolic and flavonoid content of the extracts were determined by Folin Ciocalteu and Aluminum Chloride methods, respectively. The antioxidant properties of all extracts (0 - 10 mg/ml) were evaluated, using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power assays. Cytotoxic activity of increasing concentrations of the extracts (0, 62.5, 125, 250, 500 and 1000 µg/ml) were ascertained, using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on a panel of six human cancer cell lines Jurkat (blood), PC-3, LNCaP (prostate), MCF-7 (breast), HMV II (vaginal malignant epithelial cells), and Hep G2 (liver), and a non-cancerous Chang liver cell line. Rat liver microsomes were prepared and used in bioactivation studies.
RESULTS:

*Secondary metabolites screening*: Both aqueous and ethanolic extracts of TS contained phenols, terpenoids, saponins and cardiac glycosides. Flavonoids were present in the ethanolic but not the aqueous extract of TS. Similar results were obtained for the aqueous and ethanolic extracts of the mixture of the TS and kaolin (TSK). None of the secondary metabolites were found in the extracts of kaolin only. Alkaloids were found to be absent in all the extracts.

*Antioxidant activity*: The aqueous and ethanolic extracts of TS and TSK exhibited concentration-dependent scavenging activities. The ethanolic extract of TS exhibited a higher free radical scavenging potential than the aqueous extract, as shown by EC$\text{50}$ values; 1.285 ± 0.038 and 1.971 ± 0.231 mg/ml, respectively (p<0.001). Kaolin extracts did not exhibit any scavenging activity. The ethanolic extract of TSK also showed a higher scavenging potential than aqueous extract; EC$\text{50}$ of 1.964 ± 0.124 and 4.659 ± 0.050 mg/ml, respectively, (p<0.001). When compared to the aqueous and ethanolic extracts of TS, TSK showed a reduced scavenging potential; 2.36 and 1.53-fold reduction, respectively, (p<0.001). The standard, butylated hydroxytoluene (BHT), had an EC$\text{50}$ of 0.047 ± 0.008 mg/ml (p<0.001). Additionally, there was no significant difference between the total phenol content of both aqueous and ethanolic extracts of TS. The aqueous extract of TSK showed a higher total phenol content than its ethanolic extract (3.418 ± 0.047 g and 1.682 ± 0.436g GAE, respectively, p<0.001). Both aqueous and ethanolic extract of all samples (TS, TSK and K) showed little or no flavonoid content.

*Cytotoxic activity*: The aqueous and ethanolic extracts of TS, and the aqueous extract of the TSK were found to exhibit concentration-dependent cytotoxic activity on PC-3 and Jurkat. Aqueous extract of TS demonstrated significant selectivity for PC-3 (selective index = 2.65). Cytotoxic
activity of aqueous extract of TS on PC-3 was significantly higher than its ethanolic extract as shown by their IC$_{50}$ values of 377.42 ± 32.06 µg/ml and 862.17 ± 23.82 µg/ml, respectively, (p<0.01). The IC$_{50}$ value of aqueous extract of TSK on PC-3 was found to be 1.98-fold higher than aqueous extract of TS. The IC$_{50}$ value of the ethanolic extract of TS on Jurkat was 545.23 ± 3.67 µg/ml. Aqueous and ethanolic extracts of TS and the aqueous extract of TSK did not show any cytotoxic activity on normal Chang liver cell line. Increasing concentration of curcumin (0 to 36.838 µg/ml) demonstrated drastic concentration-dependent cytotoxic activity on all cancer and normal cell lines with IC$_{50}$ values ranging from 3.89 ± 0.76 to 16.86 ± 0.95 µg/ml (p<0.001).

Liver microsome-dependent bioactivation: In the presence of liver microsomes, the aqueous extract of TS exhibited a lower IC$_{50}$ value; from >1000 µg/ml to 28.24 ± 9.53 µg/ml on Jurkat cells, representing a potentiation index (PI) of 35.41. Also, the IC$_{50}$ of the ethanolic extract of TS was found to be lower after bioactivation of extract; IC$_{50}$ value from 545.23 ± 3.67 µg/ml to 31.56 ± 2.98 µg/ml on Jurkat cells (PI = 17.28). This potentiation index was significantly higher than the one obtained by curcumin (PI = 3.64). There was no potentiation of the cytotoxic effect of the aqueous extract of TSK on Jurkat cell lines after bioactivation. Potentiation of the cytotoxic activity of the aqueous and ethanolic extracts of TS and curcumin were observed on PC-3 cell line, as shown by their potentiation indices of 1.83, 2.87 and 48.08, respectively.

CONCLUSION: The current study shows that TS possesses antioxidant and selective cytotoxic activities on PC-3 and Jurkat cell lines. This could be attributed to its appreciable free radical scavenging potential and phenolic content. Also, kaolin did not show any potentiating effect on the antioxidant and cytotoxic activities of the extracts of TS, however, after liver microsome-dependent bioactivation, there appeared to be a significant potentiation of the cytotoxic activity of TS.
DEDICATION

This thesis is dedicated to my parents, Mr. and Mrs Owusu Boadi and my wife, Mrs Victoria Owusu Boadi for their encouragement and support.
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LIST OF ABBREVIATION

ACS………………American Cancer Society
ALL……………….Acute Lymphoblastic Leukemia
AML………………Acute Myeloid Leukemia
Aqueous K……….Aqueous extract of Kaolin
Aqueous TS……...Aqueous extract of Termitomyces schimperi
Aqueous TSK…..Aqueous extract of mixture of Termitomyces schimperi and Kaolin (40:60)
B-CLL……………B-Cell Chronic Lymphocytic Leukemia
CAT………………Catalase
CGL…………….Chronic Granulocytic Leukemia
Chang Liver Cells….A derivative of Human Cervix Carcinoma
CML…………….Chronic Myelogenous Leukemia
DISC……………..Death-Inducing Signaling Complex
DMEM…………Dulbecco Modified Eagle's Culture Media
DMSO…………..Dimethyl Sulfoxide
DPPH……………2,2-diphenyl-1-picryl hydrazyl
ETOH…………..Ethanolic (20:80 v/v) Extracts
ETOH…………..K Ethanolic (20:80 v/v) extract of Kaolin
ETOH TS……….Ethanolic (20:80 v/v) extract of Termitomyces schimperi
ETOH TSK……..Ethanolic (20:80 v/v) extract of mixture of Termitomyces schimperi and Kaolin (40:60)
FBS……………..Fetal Bovine Serum
GLOBOCAN……..Global Burden of Cancer Study
GPx.....................Glutathione peroxidase
Hep G2.................Human Liver Cancer Cell Line
HMVII..................Human Malignant Melanoma Cell Line
IC50.....................Concentration that inhibits cell growth by 50%
Jurkat..................Human T Lymphocyte Cells
LNCaP..................Androgen-sensitive Human Prostate Adenocarcinoma Cells
MCF-7..................Michigan Cancer Foundation-7. A human breast adenocarcinoma cell line
MMDX.................Methoxymorpholinyl Doxorubicin
MOH....................Ministry of Health
MRA.....................Morpholino derivative of Doxorubicin
MTT.....................3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH..................Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NCI.....................National Cancer Institute
NMIMR..................Noguchi Memorial Institute for Medical Research
PBS.....................Phosphate Buffered Saline
PC-3....................Human Prostate Cancer Cell Line
PSA.....................Prostate Specific Antigen
ROS.....................Reactive Oxygen Species
RPNI-1640..............Rose Park Memorial Institute Medium
SEM.....................Standard Error of Mean
SOD.....................Superoxide dismutase
TFC.....................Total Flavonoids Content
TPC.....................Total Phenol Content
TS....................*Termitomyces schimperi*

WCRFI................World Cancer Research Fund International

WHO....................World Health Organization
CHAPTER ONE

1.0 BACKGROUND

1.1 INTRODUCTION

Cancer is a disease characterized by abnormal and uncontrollable cell division that has the potential to infiltrate and destroy normal body tissues. Cancer of the cervix, breast and liver are the commonest among women, whereas prostate, stomach, liver, Non-Hodgkin’s Lymphoma and colorectal cancers are common in men. The commonest cancer among children between the ages of 5 and 14 years is Burkitt’s lymphoma (MOH, 2011).

In 2012, new cancer cases and mortality resulting from cancers worldwide were 14.1 million and 8.2 million, respectively. Out of the new cases, 456,000 deaths occurred in Africa (WHO, 2014). By 2030, the number of morbidities and mortalities associated with cancer worldwide may reach 1.28 million and 970,000, respectively (ACS, 2011). These higher number of cases and deaths will occur as a result of ageing, population growth and lifestyle modifications associated with economic development such as smoking, unhealthy diet and physical inactivity (ACS, 2011). In Ghana, cancer morbidity is 16,600 cases annually, and that translates into 109.5 cases per 100,000 individuals (MOH, 2011).

There are multistage processes involved in the conversion of a non-cancerous cell into a cancerous cell. These transformations are the result of interaction between exogenous agents (carcinogens) and a person’s genes. The carcinogens may be physical (e.g. x-rays), biological (e.g. viral infections) or chemicals (e.g. pesticides). Also, the likelihood of one getting cancer increases with ageing. As on ages the repair mechanisms to limit cancer growth are probably less efficient (WHO, 2013).
Generally, cancer encompasses formation of malignant mass during or after changes at the epigenetic, genetic and cellular levels. Dysregulation of genes that control cell growth leads to uncontrollable cell division (Vogelstein and Kinzler, 2004). Classically, before normal cells turn cancerous, various genes including tumour suppressor genes (decrease or suppress cell growth) become mutated. Gene mutations serve as indicators for the cells to begin unregulated division. Two daughter cells are produced by duplication of the cellular component of the dividing cell (Brand and Hermfisse, 1997; Lopez-Lazaro, 2010).

Clinically, cancer management includes the use of evidence-based strategies. One of such strategies is early detection of cancer. There is a greater chance of cure if the disease is noticed early and appropriate treatment commenced (Crandal, 2014). Orthodox chemotherapy is one of the main management options. Mechanism of these drugs include, but not limited to, scavenging of free radicals or reactive oxygen species (ROS), induction of apoptosis, NF-κB inhibition, and cell-cycle inhibition (Liou and Storz, 2010; Elmore, 2007; Hans-Jurgen, 2008; Nakano, 2006). Targeting reactive oxygen species has shown lots of promise since elevated levels of intracellular ROS is implicated in the pathogenesis of cancer, sickle cell disease, Alzheimer’s disease, atherosclerosis and depression. The ROS aid proliferation, invasiveness and repress apoptosis (Barry, 2007; Valko, 2007).

Most antioxidants are known to suppress free radical formation, decrease localized oxygen concentrations, chelate metal ions and/or inhibit peroxide formation (Nawar 1996; Brewer, 2011). The body’s endogenous antioxidants such as glutathione, superoxide dismutase and catalase are often overwhelmed during oxidative stress. As such, the body’s innate antioxidant or defense system can be augmented with exogenous antioxidants through medicinal supplements and food. Exogenous antioxidants may be naturally occurring or synthetic. Commonly used
synthetic antioxidants include butylated hydroxytoluene and butylated hydroxy anisole. Natural products which contain phenols, flavonoids, carotenoids and tocopherols are good antioxidants. Several artificial antioxidants can efficiently boost defense mechanisms, but toxic effects such as renal and hepatic damage limit their use. As a result, natural compounds or products are usually preferred to synthetic agents (Brewer, 2011; Lanigan et al., 2002).

Currently, the chemotherapeutic agents used in the management of cancer include but not limited to cyclophosphamide, busulfan, carmustine, cisplatin, methotrexate, cytarabine, doxorubicin hydrochloride, paclitaxel, etoposide and tamoxifen citrate (NCI, 2017). In spite of the numerous anticancer agents available, there is still lack of a drug that possesses all the qualities of an ideal anticancer agent such as higher selectivity against cancer cells, leaving normal cells intact, no or less adverse effects to enhance compliance, prevention of disease relapse, and higher efficacy in metastatic cancers.

This underscores the need to discover new molecules with excellent efficacy and safety to complement the already existing drugs. It is worth mentioning that sixty percent of commonly used anticancer and antioxidant drugs are obtained from natural products such as plants, microbes and marine organisms (Newman et al., 2003). Moreover, mushrooms are one of the promising natural sources of anticancer and antioxidant agents (Patel and Goyal, 2012). On mammary cancer, some mushrooms have been shown to scavenge reactive oxygen species; inhibit topo-isomerase, angiogenesis, mitosis and mitotic kinase (Patel and Goyal, 2012). The mushroom *Termitomyces schimperi* (commonly called “Ahemmire” in the Akan language of Ghana) is a popular delicacy. Traditionally, ‘Ahemmire’ is used in La Cote d’Ivoire and Kenya to manage cancers. Thus, research focused on the cytotoxic and antioxidant effects of
Termitomyces schimperi will contribute to current literature on the potential of mushrooms in cancer management.

1.2 PROBLEM STATEMENT

The total number of cancer deaths worldwide is more than the combined deaths of malaria, HIV/AIDS and Tuberculosis (MOH, 2011). Cancer is responsible for 8.8 million deaths globally (WHO, 2017), and is associated with high number of hospital admissions, surgeries, laboratory and radiologic examinations. The socio-economic impact and psychological ramifications of this disease cannot be overemphasized. There are several anticancer drugs in use; however, none of these medicines possesses all the qualities of an ideal anticancer agent, hence, the need to identify potential agents with these properties. In addition, cancers have the ability to develop resistance to drug therapies, and the increasing prevalence of such cancers necessitate further research into the discovery of novel anticancer agents.

1.3 JUSTIFICATION

The mushroom TS is a natural product that grows in abundance and regularly taken as food in Ghana. Anecdotal reports from La Cote d’Ivoire indicate that TS is used in combination with kaolin to treat breast cancer. It has been reported that aqueous preparation of this mushroom prevents the growth of tumors in the breast and increases the survival rate of end-stage breast cancer patients (unpublished data). Following this initial research, there has been resurgence of studies to ascertain the effectiveness of this mushroom on cancer cell lines. So far, two studies have been undertaken. In the first study, preliminary in vitro studies using ethanolic (20:80, v/v) and aqueous extract of TS demonstrated cytotoxic and pro-apoptotic activities on human breast
cancer MCF-7, brain cancer U373 MG and human leukemia THP-1 cell lines (unpublished data).

In the second study, eleven (11) compounds (labelled B1 – B11) were isolated from ethanolic (20:80, v/v) extract of the mushroom. One of the isolates, B1 was characterized as Ergosta-5,7,22-trien-3β-ol (Ergosterol). The other ten isolates are yet to be characterized. PC-3, MCF-7, LNCaP and normal Chang liver cell lines were treated with all the isolates (B1 – B11). Only isolate B11 recorded IC50 values within the working concentration of 0 – 100 μg/ml. IC50 values of the other isolates were greater than 100 μg/ml. On PC-3, Normal Chang Liver and LNCaP cell lines, IC50 values of B11 were 69.55, 76.68 and 93.94 μg/ml, respectively, (Anane-Adjei et al., 2016). The selective indices of this isolate were 1.10 and 0.82 for PC-3 and LNCaP, respectively. According to the National Cancer Institute, a sample is considered to be selective if its selectivity index is greater than 2 (Badisa, 2009). Thus, it can be inferred that the isolates are weakly cytotoxic.

The current study was to delineate why the isolates had low cytotoxic activity on the cancer cells. It is worth noting, however, the difference between the first and second study; kaolin was a component of the sample (finished product) in the first study; and compounds were isolated in the second study from ethanolic extract of only TS without Kaolin suggesting that the samples from both studies were not the same.

The explicit intent of this study was to determine the cytotoxic and antioxidant activity of TS and investigate whether kaolin could potentiate the cytotoxicity of TS. Furthermore, this study was to determine the presence of bioactivatable compounds that could be responsible for possible cytotoxic activity. A typical example of anticancer agent that require bioactivation is
cyclophosphamide. Its active form (4-hydroxycyclophosphamide) is responsible for its therapeutic action. The findings of this study will provide scientific data on the traditional use of TS in the management of certain cancers.

1.4 HYPOTHESES

Kaolin may potentiate the cytotoxic and antioxidant activities of *Termitomyces schimperi*. *Termitomyces schimperi* and/or Kaolin may contain potential bioactivatable compound(s) that could have appreciable cytotoxic activity on cancer cells.

1.5 AIMS

The aims of this study were to determine the antioxidant and cytotoxic activities of the aqueous and ethanolic extracts of *Termitomyces schimperi* and elucidate the effect of microsomes-dependent bioactivation of these extracts and kaolin on cancer cells.

1.6 OBJECTIVES

1. To determine the antioxidant activities of aqueous and ethanolic extracts of *Termitomyces schimperi* alone (TS), in combination with kaolin (TSK) and kaolin alone (K).

2. To determine the cytotoxic effect of the extracts, TS, TSK and K, on Jurkat (leukemia), PC-3 and LNCaP (prostate cancer), MCF-7 (breast cancer), HMV II (melanoma), Hep G2 (liver cancer) and Chang Liver (normal) cell lines.

3. To bioactivate potential compounds of selected extract(s) of TS using rat liver microsomes.

4. To determine the cytotoxic activity of the bioactivated extract(s) on cancer cell lines.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Cancer

Uncontrolled division of abnormal cells with the potential to infiltrate and destroy normal body tissues is termed as cancer. Prostate, skin, breast, liver, bone marrow, cervix, and ovaries are common sites of cancer development. The invasion and spreading of cancer into adjoining parts or organs of the body, known as metastasis, is the main cause of cancer-related mortality (WHO, 2014).

2.1.1 Cancer Situation in Ghana, Africa and the World

Cancer is the second principal cause of mortalities globally. It is estimated that out of every six deaths, almost one may be due to cancer. Approximately, 1.16 trillion US Dollars was spent on cancer in 2010 (WHO, 2015). In 2012, cancer killed 8.2 million people, whereas 14.1 million individuals across the globe were diagnosed of cancer for the first time (WHO, 2015). Cancer deaths increased by 600,000 as the 8.2 million deaths reported in 2012 increased to 8.8 million in 2015. Over the next 20 years, new cases are projected to increase by 70% (WHO, 2017). Liver, prostate, lung, colorectum and stomach are the commonest sites of cancer development in men. Breast, cervix, lung, stomach and colorectum cancers are the commonest sites of cancer development in women (MOH, 2011).

The National Cancer Institute reported that more than 60% newly diagnosed cancer patients are Asians, Africans, South and Central Americans. Also, 70% of the global cancer deaths occur in these continents (NCI, 2016).
2.1.2 Breast Cancer Situation

Worldwide, the most predominant invasive cancer affecting women is breast cancer. Twelve percent (12%) of females worldwide are living with cancer (McGuire et al., 2015). Incidence rate of breast cancer in low to middle-income countries is rising day after day. Forty (40) women out of 100,000 are reported to be battling with cancer. Fifty percent (50%) cases of breast cancer and 58% deaths occur in developing nations (WHO, 2008). These statistics defy the earlier notion that breast cancer is a bona fide property of higher income countries. The total number of mortalities associated with the disease was 508,000 (WHO, 2013). In Ghana, it is the second most predominant cancer among women. Comparatively, the incidence-mortality ratio is far higher in high income nations than in low income nations. The incidence-mortality ratio in Ghana is estimated to be 0.68, whiles that of United States of America is 0.2 (MOH, 2011).

2.1.3 Prostate Cancer Situation

Prostate cancer mostly originates from the prostate basal cells (Goldstein et al., 2010). Both National Cancer Institute (NCI) and WHO reports indicate that only a few of prostate cancers grow faster. Majority of prostate cancers grow very slow but can metastasize into other body parts such as lymph nodes and bone. Prostate cancer is ranked sixth on the list of common cause of cancer mortality (Jemal et al., 2011).

In 2012, 1.1 million men were living with prostate cancer worldwide, with 307,000 of them dying of the disease (GLOBOCAN, 2012). Cases of prostate cancer formed 8% of all new cancer diagnoses (Stratton, 2011).

Increased availability of prostate-specific antigen (PSA) screening, even for asymptomatic men, has been the major reason for the higher number reported prostate cancer cases. In this regard,
cancers that may have gone unnoticed or are very small, which would not necessarily have become malignant, are detected with PSA screening. Comparatively, the disease is predominant in the developed countries to developing countries. 68% of cases occur in developed countries (WCRFI, 2015). However, on daily basis, the disease incidence rates are increasing in low income nations (Baade et al., 2009). In a descending order of prevalence of prostate, black men are more prone to prostate cancer, followed by white men, and then Asian men (ACS, 2006).

In Africa, especially Cameroon, Nigeria and South Africa, prostate cancer is the commonly diagnosed cancer among men (Jemal, 2012). Prostate cancer is the second most fatal male cancer in Ghana. Seventy percent of Ghanaians seek medical attention very late when the disease has metastasized (MOH, 2011).

2.1.4 Leukemia Situation

Bone marrow is mostly the site of origination of leukemia. In effect, there is over proliferation of abnormal white blood cells (NCI, 2013). Leukemia subtypes include: Chronic granulocytic or myelogenous leukemia (CGL or CML), acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and B-cell chronic lymphocytic leukemia (B-CLL or CLL). Acute lymphoblastic leukemia is characterized by over proliferation and accumulation of immature lymphocytes (lymphoblasts). The production of normal platelets, red cells and other cells are impeded by the continuous proliferation of lymphoblasts (Jameson et al., 2005).

Myeloid cells mostly form platelets and red blood cells. However, when these myeloid cells become cancerous, abnormal white blood cells are produced to cause AML (Jameson et al., 2005). The presence of granulocytes (i.e. neutrophils, eosinophils and basophils), their precursors
and uncontrolled myeloid cells in blood are the major characteristics of CML. The type of leukemia where B cells are mostly affected is CLL or B-CLL.

There were reports on 352,000 new cases of leukemia and 265,000 leukemia deaths globally in 2012 (WHO, 2014). In 2015, 2.3 million individuals were reported to be living with leukemia, and as many as 353,500 people had died worldwide in 2015 (GBD, 2016).

According to reports by the National Cancer Institute and American Society of Cancer published in 2011 and 2012, respectively, a third of childhood cancers are cases of acute lymphoblastic leukemia. The prevalence of adult leukemia is only 3%. However, because cancers are more predominant in adults, 90% of persons diagnosed of leukemia are adults, with AML and CLL being the commonest (ACS, 2012; NCI, 2011).

In a period of ten years, between 2000 and 2010, the Oncology/Haematology Clinic at the Korle-Bu Teaching Hospital, recorded 830 cases of leukemia. In order of increasing frequency, the reported cases were Hodgkin’s lymphoma (4.5%), AML (6.0%), ALL (6.7%), CLL (15.8%), CGL (19.2%), Multiple myeloma (21.6%) and Non-Hodgkin’s lymphoma (26.3%) (MOH, 2011).

2.1.5 General Mechanism of Cancer

The transformation of normal cells into cancerous cells is known as oncogenesis or carcinogenesis. Malignant mass is formed in some cancers during or after changes at the epigenetic, genetic and cellular levels. Dysregulation of genes that control cell growth leads to uncontrollable cell division (Vogelstein and Kinzler, 2004). Tumour suppressor genes decreases or suppresses cell growth or division to aid in DNA repair. Proto-oncogenes rather enhance
mitosis and cell growth. Classically, before a normal cell turn cancerous, various genes, including tumour suppressor genes are mutated.

Gene mutation serves as signals for cells to begin unregulated division. Two daughter cells are produced by duplication of the cellular components of the dividing cell. Activation of anaerobic glycolysis has been shown to furnish the building constituents required for creation of the daughter cells. Hence, anaerobic glycolysis is described as essential tool for oncogenesis. Mutations in tumour suppressor genes and proto-onocogenes do not necessarily induce anaerobic glycolysis (Brand and Hermfisse, 1997; Lopez-Lazaro, 2010).

### 2.1.6 Some Targets for Antitumour Activity

These targets include scavenging of reactive oxygen species (ROS), induction of apoptosis, inhibition of NF-κB, cell-cycle inhibition, inhibition of topo-isomerase, angiogenesis, mitosis and mitotic kinase, etc. (Newsholme et al., 2012; Hans-Jurgen, 2008; Francois et al., 2005; and Shapiro and Harper, 1999).

#### 2.1.6.1 Reactive Oxygen Species Scavenging

One of the commonest product of aerobic metabolism is reactive oxygen species. Malfunction of mitochondria and very high metabolic activity has been shown to produce high ROS levels that are characteristics of tumour cells (Portakal et al., 2000). Mitochondrial oxidative respiratory chain minor products are non-radical H₂O₂, hydroxyl radicals (OH⁻), superoxide (O₂⁻) and reactive oxygen species. Superoxide oxidoreductase dismutase (SOD) catalyzes the conversion of O₂⁻ to non-radical H₂O₂ (Newsholme et al., 2012). The ROS play vital roles in intracellular signaling, protection against pathogens and other distinct cellular functions; however, they cause
diseases such as cancers, when their level are very high, especially during oxidative stress. Thus, electron free radicals transfer and redox-active metal ions chelation are made possible (Liou and Storz, 2010).

2.1.6.2 Inhibition of NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B-cells)

The ROS stimulates or activates certain key intracellular signaling cascades that promote proliferation or development of tumours. Expressions of anti-inflammatory, anti-apoptotic, cytotoxic activities are controlled by many genes. These genes are controlled by NF-κB, which is an inducible transcription factor. In malignant cells, NF-κB is mostly constitutive and highly activated. The NF-κB is one of the survival factors that activate expression of several anti-apoptotic genes, such as Bcl-XL, Mcl-1 and Bcl-2, that promotes survival of many types of tumours (Francois et al., 2005; Nakano, 2006; Reynaert et al., 2006; Suh and Rabson, 2004).

2.1.6.3 Inhibition of Cell-Cycle

Regulation of cell-cycle is one of the most critical event that affects the cell’s ability to divide and duplicate its DNA to form daughter cells. The cell’s journey through the cell-cycle is moderated by cyclins and cyclin-dependent kinases (CDKs). The latter is responsible for initiation, progression and completion of the cell-cycle. In cancerous cells, cyclins are overexpressed, whiles expression of CDK inhibitors are lost (Dickson and Schwartz, 2009; MOH, 2011). Arrest of the cell-cycle mostly happen at the gap phase 2 / mitosis (G2/M) or gap phase 1 / synthesis (G1/S) boundaries. G2/M checkpoint ensures that cells do not initiate mitosis before they have an opportunity to repair damaged DNA after replication (Cuddihy and O’Connell, 2003). At the G1/S checkpoint, the cell makes decisions to repair DNA, differentiate,
proliferate or become quiescent (enter Go) based on molecular signaling inputs or environment factors (Massague, 2004).

The CDK is a classical target for drug development. Inhibition of abherent CDKs has been shown to cause cell death (Shapiro and Harper, 1999)

2.1.7 Molecular Mechanisms of Apoptosis Induction

Intrinsic and extrinsic pathway of apoptosis induction remain the two most popular pathways mentioned in several literatures. A supplementary pathway exits which is characterized by T-cell-mediated cytotoxicity and perforin-granzyme-dependent killing of the cell (Elmore, 2007; Hans-Jurgen, 2008). Cellular stresses, such as DNA damage, activates the intrinsic mitochondrial pathway. Pro-apoptotic BH3-only proteins are activated to initiate and promote the apoptosis cascade. These proteins also deactivate inhibitors of anti-proptotic protein, Bcl-2. The Bax and Bak proteins, which are pro-apoptotic, oligomerize and cause leakage of cytochrome c by making pores in outer membrane of mitochondria. Docking sites for the binding of this cytosolic cytochrome c is provided by adaptor protein Apaf-1. The formation of apoptosome is then initiated and caspase-9 is activated.

In the case of the extrinsic pathway, death receptors are bound by their respective death ligands. Adaptor protein FADD provides docking sites for caspase-8 and caspase-10 recruitment. The caspases - 8/-10 bind to FADD to form DISC (caspase-activation death-inducing signaling complex). Both pathways do not necessarily operate independently. In addition, there are common proteins such as Bid and Caspase-3. The interconnection of both pathways is by Bid whiles the cleavage of specific target proteins is by the effector or executioner caspase (Caspase-3).
Bid is cleaved by caspase-8/-10 which results in its mitochondria translocation and subsequent release of cytochrome c (Hans-Jurgen, 2008).

2.1.8 Therapeutic Strategies in Cancer Management

There are several cancer management options. Predominantly, chemotherapy, radiation therapy, surgery, immunotherapy, targeted therapy, hormonal therapy and palliative care are used. Therapy selection hinges on the kind, location and grade of cancer, patient’s condition and inclination.

Immunotherapy

In this therapy, an individual’s own immune system is induced to combat the tumours. For example, in melanoma and renal cell carcinoma patients, immune responses can be induced by cytokines, interferons and some vaccines (Damodar et al., 2006).

Targeted therapy

Deregulated proteins of cancer cells can be targeted by using agents. This comprises of the use of agents specific for the deregulated proteins of cancer cells. Classical example is Imatinib, which is a tyrosine kinase inhibitor, and gefitinib. Proteins on cancer cells are also targets for antibodies to bind. Examples are Rituximab and Trastuzumab. Trastuzumab is an anti-HER2/neu antibody that is effective against breast cancer. Rituximab is effective against a variety of B-cell malignancies (Duarte, 2009).

Chemotherapy

Chemotherapy is often referred to as drugs used in cancer management. This definition is largely restrictive, since chemotherapy encompasses any drug used in treating any disease.
Chemotherapeutic agents are used with the intent to cure, control and/or palliate cancer. Most anticancer agents are administered with the intention to cure the disease (e.g. some leukemias) but they do not end up that way. In order to improve patient’s quality of life and survival rate, some medications are given with the intent to control the disease, if cure is a mirage. Such agents inhibit proliferation and metastasis and/or decrease tumour density. In advanced stages of cancer, the tumours are uncontrollable and may be causing untoward effects, such as severe pain, nausea and vomiting (ACS, 2016).

**Classification of Chemotherapy**

Chemotherapy is classified based on whether the drug targets specific phase or cycle of cell and according to the mechanism (Payne and Miles, 2013).

**Cell-Cycle-Specific and Non-Specific Chemotherapy**

The cell-cycle is organized in phases, and these are controlled by distinctive set of molecular keys. The phases are G0, G1, Synthesis (S), G2 and Mitosis (M) phase. The normal cell which is not undergoing division is in the G0 phase. The cell becomes active by going through the other phases. G1 phase is characterized by cell growth and production of new proteins. The cell then synthesizes its DNA in the S phase. There is further cell growth and preparation to divide in G2 phase. Ultimately, cell division or chromosomal separation occurs in the mitototic (M) phase. Some drugs are very cytotoxic at specific phases. For instance, vinca alkaloids inhibit formation of spindles and chromosomal arrangement, hence they are more cytotoxic in the M phase. Another anticancer drug, Methotrexate, which is an antimetabolite, inhibits DNA synthesis which makes the drug more toxic to cells when they are in S phase.
Some drugs are primarily cytotoxic only when the cell is actively dividing. They are virtually ineffective when the cells are inactive or resting. The efficacy of such drugs are maximized by increasing frequency of administration of the drug. By so doing, duration of exposure of cells to the drugs is increased.

Furthermore, other drugs are very cytotoxic irrespective of the phase the cell is in. Their dose-response relationship is linear, which implies that higher doses cause greater fractional cell deaths. Examples are platinum derivatives and alkylating agents (Payne and Miles, 2013).

**Mechanism-based classification**

Classification based on mechanism is preferred to their specificity or non-specificity in cell cycle by clinicians.

**Antimetabolites**

Antimetabolites have structures similar to biological molecules, such as nucleotides, amino acids and vitamins. As such, they compete for active sites on receptors and/or enzymes. They are classified into groups namely: purine analogues, folic acid antagonists, and pyrimidine analogues.

**Purine Analogues:** Drugs such as thioguanine and 6-mercaptopurine are derivatives of guanine and adenine, respectively. Bases such as guanine and adenine are needed for nucleotide biosynthesis. These derivatives of the purines, instead of natural purines, can be incorporated into the nucleotide, hence the DNA synthesis is interfered with.

**Pyrimidine Analogues:** These drugs are also derivatives of natural pyrimidine, namely cytosine and thymine. These derivatives can either be incorporated in DNA or inhibit key DNA synthesis enzymes such as DNA polymerase. Examples are cytarabine, 5-fluorouracil and gemcitabine.
**Folic Acid Antagonist:** Synthesis of essential molecules, such as purines and thymidylate, require the enzyme dihydrofolate reductase that converts dihydrofolate to tetrahydrofolate. Inhibition of dihydrofolate reductase by drugs such as methotrexate. Another critical point is the conversion of deoxyuridine monophosphate to thymidine monophosphate by thymidylate synthase to aid in RNA and DNA synthesis. Methotrexate inhibits also thymidylate synthase and, as such, synthesis of DNA and RNA are inhibited. Noteworthy, folinic acid, an intermediate molecule, can be supplied exogenously to be converted into tetrahydrofolate.

**Alkylating agents**

Alkylating agents interfere with DNA replication by covalently binding or linking their alkyl groups (R-CH₂) with DNA strands (either single or double). The site at which these agents crosslink differ adversely for all alkylating agents. Inability of the cell to replicate eventually leads to cell death. These drugs are very cytotoxic in S-phase of the cell-cycle. Examples of alkylating agents include, but not limited to oxazaphosphorines (e.g. ifosfamide and cyclophosphamide), nitrosureas (e.g. Lomustine), nitrogen mustards (e.g. chlorambucil), tetrazine (e.g. dacarbazine) etc.

**Cytotoxic Antibiotics**

Bleomycin, mitomycin C, anthracyclines, actinomycin D etc. are antibiotics with cytotoxic activities. Common sources of these antibiotics are fungi (e.g. Streptomyces) and bacteria. The main consequence of bleomycin and actinomycin D are causing DNA fragmentation and intercalation between cytosine and guanine base pairs, respectively. In the case of Anthracyclines, they bind to topoisomerase II enzymes and stabilize it. Topoisomerase II
enzymes are critical in winding and unwinding of DNA. Stabilizing these enzymes prevent the reconnection of strands which affect nucleic acid function and synthesis. Example of anthracyclines are daunorubicin and doxorubicin. Mitomycin C acts like an alkylating agent by forming cross linkages.

**Taxanes**

Taxanes promote microtubule assembly and prevent disassembly. Commonly used taxanes are doxetaxel and paclitaxel. The latter was isolated from a pacific yew, while the former is a semisynthetic derivative.

**Platinum-based Antineoplastics**

These agents act by cross-linking with strands of DNA. They usually act on guanine. In effect, DNA synthesis and repair are inhibited. This mechanism of action is similar to that of alkylating agents. The difference is that platinum-based antineoplastics do not have alkyl groups. Example: cisplatin, oxaliplatin and carboplatin.

**Vinca alkaloids**

Vinca alkaloids inhibit mitosis by binding to tubulins, which are fundamental units of microtubules. The formation and assembly of mitotic spindles are affected. The prevention of tubulins-forming microtubules affects cell division. Example of vinca alkaloids are vinblastine, vincristine (isolated from periwinkle), vinorelbine and vindesine (Payne and Miles, 2013)

**Shortcomings of Orthodox Chemotherapy**

There are myriad of issues with efficacy and safety of antineoplastics. Most anticancer drugs poorly target cells in the resting stage of the cell-cycle. In addition, most antineoplastics are less
cytotoxic on poorly differentiated cancers and are unable to reduce metastasis and/or invasiveness. Lastly, most of them are associated with serious adverse effects, such as nausea, vomiting, bone marrow suppression, etc (Payne and Miles, 2013).

2.2 Alternative Treatment

Research into natural products, such as mushrooms, to unravel and develop their medicinal properties is increasing at a fast pace (Patel and Goyal, 2012). Mushrooms are popular delicacies consumed in Ghana and worldwide. Beyond the nutritional value of mushrooms, they are known to have lots of medicinal benefits. Extracts of several mushrooms have shown anticancer activities against prostate, breast, bladder, liver cancers, among others. They have also shown antioxidant, anti-diabetic, immunomodulatory, anti-allergic, nephroprotective, anti-microbial and hypocholesterolemic activities (Patel and Goyal, 2012).

Mushroom genera with anticancer activity include the following; Cordyceps, Fomes, Agaricus, Ganoderma, Pleurotus, Inonotus, Trametes, Calvatia, Schizophyllum, Clitocybe and Inocybe. Mushroom polysaccharides have gained considerable interest in the search for the discovery of new anticancer agents. Some mushrooms on mammary cancer cells have been found to scavenge reactive oxygen species, inhibit topo-isomerase, angiogenesis, mitosis and mitotic kinases. Others induce apoptosis which causes arrest of the cell-cycle and inhibition of cell proliferation (Patel S, 2012). Mushrooms that have antioxidant properties decrease DNA damage caused by reactive oxygen species (Karas et al., 2000; Kelkel et al., 2011; Teodoro et al., 2012).
2.2.1 *Termitomyces schimperi* (TS)

Over 30 species of *Termitomyces* grow worldwide. *Termitomyces schimperi*, *T. globulus*, *T. robustus*, *T. clypeatus*, *T. microcarpus* and *T. reticulatus* are the commonest species of Termitomyces in Ghana (Obodai et al., 2014). The taste, attractiveness, high protein content and medicinal value of *T. schimperi* make it very popular. Traditionally, it is used to manage kwashiorkor (a condition of protein deficiency), high blood pressure, obesity, cough, sore throat, tonsillitis, rheumatism and diarrhea (Obodai et al., 2014; Patel S, 2012).

2.2.1.1 Botany of *Termitomyces schimperi*

*Termitomyces schimperi* belongs to the kingdom Fungi, division Basidiomycota, class Agaricomycetes, order Agaricales, family Lyophyllaceae, genus Termitomyces and species *T. schimperi*. TS grows naturally and has symbiotic association with the termite specie *Macrotermes michelseni*. In Ghana, TS is commonly known as *Ahemmire* (royal mushroom) among the Akans. In Namibia, the mushroom is called *Omajowa*. The fruity body of TS is like the "size of a man’s fist" on emergence. The usual diameter of the fruity body ranges from 15 to 28 cm, however, it may up to reach 40 cm. The mound’s soil can stain white and thick caps into yellowish or reddish-brown. Underneath the uppermost layer are white scales. A single mound can have about 50 sporocarps. The mushrooms usually grows after drenching rains of 12 mm or throughout rainy seasons. The fruity bodies grow up to 50 cm above soil level and in 5’s or 10’s around the lower part of the mound. The pseudorhiza which resembles a plant’s root is about 90 cm, and grow towards its origin in the termite nest. The pseudorhiza above the ground has less dense consistency than the lower, narrower part. The hyphae, if left in the mound, can grow for
years. The partial veil is consumed by termites when the mushroom matures (Westhuizen and Ecker, 1991)

Figure 1. *Termitomyces schimperi*

(a) Cultivated *Termitomyces schimperi* and (b) *Termitomyces schimperi* growing on mound
Some researches done on *Termitomyces schimperi*

Generally, much work has not been done on this mushroom in the area of antioxidant and anticancer activities. An ethnobotanical survey conducted in Abidjan (La Cote d’Ivoire) showed that *T. schimperi* is used to manage breast cancer. Preliminary studies on various cancer cell lines (MCF-7, U373-MG and THP-1) have demonstrated that the ethanolic (20:80, v/v) and the aqueous extracts of *T. schimperi* possesses anti-proliferative and pro-apoptotic properties (unpublished data). Phytosterols, such as ergosterol and ergosterol derivatives, have been isolated, characterized and purified from *Termitomyces schimperi*. The fungi species and their growth conditions determine the level of ergosterol. Ergosterol levels are not constant throughout the growth stages of the mushroom (Pasanen *et al.*, 1999). In the University of Ghana, ergosta-5,7,22-trien-3β-ol (ergosterol) has been characterized from ethanolic extracts (20:80, v/v) of *Termitomyces schimperi* (Anane-Adjei *et al.*, 2016). Laccase enzyme with optimum activity at 70°C has been isolated and purified from *Termitomyces schimperi* (Haileka, 2015).

### 2.2.1.2 Antioxidant Compounds in Mushrooms

Many edible mushrooms and their isolated compounds have inhibited or stopped the process of oxidation. The fruity bodies, broth and mycelium of these fungi, are rich in antioxidant compounds such as carotenoid, tocopherols, phenolics, polysacharrides, flavonoids, ergothiomerine, glycosides, and ascorbic acids. Various methods are employed in assessing the antioxidant activities of mushrooms.

Principles behind some methods include, transfer of hydrogen atoms and electrons, chelation of cupric (Cu²⁺) and ferrous (Fe²⁺) ions, measuring superoxide, glutathione peroxidase, and catalase activities. The protective properties of these antioxidant compounds are demonstrated at different
stages of the oxidation process. There are two major groups of antioxidant mechanisms, namely primary and secondary (preventive). The primary mechanisms include scavenging of free radicals, chain breaking, inhibition of lipid hydroperoxides, consequence of metal deactivation and quenching of singlet oxygen ($^1$O$_2$), regeneration of primary antioxidants, etc. Some mushroom compounds induce cell signals that lead to changes in gene expression and activation of enzymes that remove reactive oxygen species (Emad and Sanaa, 2013).

**Polyphenols**

The commonest antioxidants found in diet are polyphenols. These polyphenols are involved in several biological activities in the body. The number of phenol rings present and structural elements joining these rings together form the basis of classifying polyphenols. In effect, there is distinction between flavonoids, stilbenes, phenolic acids and lignans. In diets, majority of these polyphenols are in the form of glycosides, polymers and esters. These substances are hydrolyzed by intestinal enzymes or colonic microflora, since in their native form, they are non-absorbable. The most pre-dominant polyphenols are phenolic acids. These phenolic acids are classified into two main types, namely hydroxycinnamic and hydroxybenzoic acids. The former is obtained from non-phenolic molecule cinnamic acid, while the latter is from benzoic acid. The commonest benzoic acid derivatives present in mushrooms include gallic acid, $p$-hydroxybenzoic, vanillic, protocatechuic, gentisic acid, etc. The cinnamic acid derivatives include tannic acid, $p$-coumaric and ellagic acid. Mechanisms employed by many polyphenols include elimination of free radical species and/or promotion of endogenous antioxidant capacity and stimulating endogenous antioxidant synthesis. Polyphenols have been shown to modulate activities of NF-$\kappa$B, Sirutin1 (SIRT1) (Wang et al., 2014). Cells damaged by free radicals are repaired and/or prevented from premature deaths by rescue genes such as SIRT1. This gene also
causes the mitochondria to process greater amounts of energy to help in metabolism of younger cells. Notwithstanding this, polyphenols are also known to have pro-oxidative properties. Curcumin, quercetin and resveratrol, all polyphenolic compounds, have also shown pro-oxidant effect by acting as photosensitizers in the production of singlet oxygen (Lagunes and Trigos, 2015). Epigallocatechin-3-gallate (EGCG) induces apoptosis by activating c-Jun N-terminal kinases (JNKs) and caspase-3 which are part of MAPKs signaling cascade (Emad and Sanaa, 2013).

**Flavonoids**

It is well known that in France, the prevalence of cardiovascular diseases are low despite the high smoking habit and consumption of saturated fat (Ferrieres, 2004). This phenomenon was interconnected with modest and consistent intake of flavonoid-enriched red wine. The flavonoid structure consist of an oxygen-containing pyran ring (C) which joins a flavan nucleus comprising of two benzene rings. Examples of flavonoids are flavonols, isoflavones, flavones, anthocyanidins etc. The commonest flavonoid present in food is flavonol. Quercetin, resveratrol, myricetin, chrystin, among other flavonoids, are found in some mushrooms (Ferreria et al., 2009; Kozarski et al., 2015). Mechanisms employed by flavonoids include donation of hydrogen to scavenge reactive oxygen species, modulation of gene expression and signaling pathways, regeneration of α-tocopherol and other membrane-bound antioxidants, inhibition of xanthine oxidases and lipoxygenases (Chahar et al., 2011; Emad and Sanaa, 2013).

**Polysaccharides**

Polysaccharides are also major bioactive components in edible mushrooms that act as immunostimulatory and adaptogen agents, which prevent tumour metastases (Kozarski, 2015).
The polysaccharides exert antioxidant activity by scavenging reactive oxygen species, chelate Fe$^{2+}$, inhibit lipid peroxidation, increase activity of enzymes, such as glutathione, catalase and dismutase. β-glucans are the main mushroom polysaccharides responsible for its antioxidant property. The availability of hydrogen from certain monosaccharide units aid in scavenging free radicals. This hydrogen atom transfer (HAT) reaction is akin to that of phenols. Excessive antioxidant activities of polysaccharides from edible mushrooms has been shown to be beneficial in preventing the pathogenesis of many diseases, such as gastrointestinal cancers and peptic ulcer, which is partly due to oxidative stress (Kozarski, 2015).

2.2.2 Kaolin

Kaolin is a natural commercial clay product used worldwide. Between the years 1950 to 1990, lots of research were undertaken on kaolin to unravel and authenticate its medicinal value and toxicities. Very few studies have been done on it recently. Irrespective of the rich data on kaolin published several years ago, kaolin is usually confused with bentonite, zeolite and many other clay products (Parker 1988; Rees et al., 1992; IZA, 2017). Even though these substances differ in composition and activity, they are usually used interchangeably.

According to World Health Organization’s Environmental Health Criteria 231 document, clay is referred to as finely ground minerals with particles size smaller than silt or a finely ground sedimentary deposit of clay minerals. Notwithstanding, shale and some argillaceous soils, which are mainly composed of finely-ground deposits of non-aluminosilicates or clay minerals, can also be referred to as clay (Adamis and Williams, 2005).

Kaolin, also known as China clay, is clay composed of 10–95% of the mineral kaolinite. However, the usual amount of kaolinite in most kaolins is 85 – 95%. Other minerals, such as
quartz and mica, are found in kaolin. Occasionally, montmorillonite, feldspar, illite, anastase, bauxite, ilemite, haematite, zircon, attapulgite, rutile, silliminate, halloysite and graphite can be found in kaolin (Rees et al., 1992). The name kaolin originates from the Chinese word “Kau-Ling” which means high ridge. On the other hand, a rock that is formed in situ by devitrification of volcanic ash and made of highly colloidal and plastic clay, with montmorillonite as the major mineral, is known as bentonite. Water sources, such as seawater, fresh water, or alkaline lakes are the only media where the ash is transformed into bentonite (Adamis and Williams, 2005). Zeolite is an aluminosilicate mineral, which is microporous, and composed of Al, Si, O and metals, including Zn, Sn and Ti (Tschernich, 1992). There are 232 unique zeolite frameworks. Over 40 of these zeolites are naturally occurring. Natural zeolites are formed by the reaction of alkaline groundwater with ash layers and volcanic rocks (Grace et al, 2010; IZA, 2017; Tschernich, 1992).

**Physical and chemical properties of Kaolin**

Kaolinite (the major component of kaolin) is white, greyish-white or slightly coloured. The common molecular formula of kaolinite is $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$. The structure is made up of tetrahedral silica sheets, which alternate with octahedral alumina sheets. The tips of these sheets are arranged in such a way that they form common layer. In this layer, two-thirds of the oxygen atoms are shared by aluminum and silicon, leading to the formation of O instead of OH. The structure unit’s charges are balanced. There is very little substitution in the lattice of most kaolinite. It has cation exchange capacity. Smaller molecular substances, such as proteins, viruses, bacteria, lecithin, etc. are adsorbed by kaolinite. The adsorption is very limited, since these molecular substances are not bound between layers of the particles. They are only adsorbed on edges and planes (surfaces) of the particles. Heated kaolinite loses water at 400°C, and it is
almost dry at 525°C. The crystallinity and particle size affects the dehydration of kaolinite (Adamis and Williams, 2005).

Effects of Kaolin on in vitro test systems, laboratory mammals and humans

Naturally, kaolin is part of the soil and occurs sparsely in the air we breathe. In other words, kaolin is ubiquitous, so the general population is exposed to some levels of kaolinite. Experimentally, effects of kaolin in both in vivo and in vitro test systems are dependent on the constituents of the kaolin. Some studies have reported that kaolin is cytotoxic, while others have reported that it is non-cytotoxic. In addition, kaolin that had in vitro cytotoxic effects could not produce same effects in vivo. Noteworthy, most literature on kaolin studies were done more than three decades ago.

In a study, kaolin was reported to have low cytotoxicity (Daniel and Le Bouffant, 1980). In another experiment, two kaolin samples, that were well-crystallized and disordered kaolinites, were relatively non-cytotoxic at 80 µg/ml (Gormley et al., 1983). In addition, a cytotoxic kaolin could not cause much fibrosis in rats (Le Bouffant et al., 1980). Kaolin in its pure form showed marked cytotoxicity (Robertson et al., 1982). Kaolin was cytotoxic to alveolar macrophages. The membrane-damaging mechanism was likened to that of silicates (Low et al., 1980).

The major reason attributed to these contradictory results is that kaolin samples vary markedly. Kaolin has variable amounts of ancillary minerals other than kaolinite, such as quartz, which is a major determinant of toxicity, and also, the crystallinity of kaolinite could either be high or low (Adamis and Williams, 2005).
Medical uses of Kaolin

Kaolinite has activities that are relevant to medicine. It is a very efficient adsorbent that adsorbs proteins, lipids, bacteria, viruses and other smaller molecular substances. It is also used to remove non-specific haemaglutinin inhibitors from serum, initiate coagulation of plasma by activation of factor XII, and also induce aggregation of platelets (Adamis and Williams, 2005).

2.3. Biotransformation of Extract Components Using Microsomes

The process of modifying chemical compounds by an organism is known as biotransformation. Metabolism of xenobiotics, including drugs, is an example of biotransformation. Usually, drugs are biotransformed to render them more water-soluble to avoid reabsorption in renal tubules or enhance its excretion into the urine. Notwithstanding, a compound can undergo any of these four changes after metabolism (Ashauer, 2012).

One of these changes include conversion of an inactive compound into an active metabolite. For example, cyclophosphamide, an anticancer drug, requires biotransformation to its active form (4-hydroxycyclophosphamide) for its therapeutic action.

Secondly, an active compound can be converted into a more active product. For example, morphine to morphine-6-glucuronide.

Thirdly, a non-toxic or less toxic compound can be converted to a more toxic compound. For example, isonizide to acetyl isoniazide. This process is known as biotoxification.

Lastly, an active drug can be converted into an inactive metabolite which is very common with most drugs eg. paracetamol.

There are three main pathways of drug biotransformation, namely: Phase I, Phase II and Phase III. Phase I reactions are described as non-synthetic. They involve the introduction, modification,
or unmasking of functional groups or cleavage of the parent compound. The parent compound undergoes either reduction, hydrolysis, oxidation, etc (Le, 2016). The Phase I metabolites are more water-soluble and less active. Cytochrome P450 enzymes, oxygen and NADPH play key roles in generating these metabolites (Ashauer, 2012).

Phase II reactions are described as synthetic. They involve the conjugation of products of Phase I or parent compound with the right functional groups or endogenous molecules, such as glycine, glucuronic acid, sulfate etc. to form more polar metabolites. The metabolites are easily excreted in bile or urine, compared to those formed in Phase I. Noteworthy, some drugs may undergo either Phase I or II, or both (Le, 2016).

Phase III is a further metabolism step after Phase II. The compound conjugate may undergo further biotransformation. For instance, mercapturic acid is conjugated with glutathione in Phase II. The enzyme gamma-glutamyl transpeptidase and the dipeptidase remove γ-glutamate and glycine residues in the glutathione moiety. Thereafter, cysteine residues are acetylated. Excretion of these conjugates and their metabolites can occur (Homolya, 2003)

The liver is the main organ that biotransforms exogenous and endogenous substances. Microsomes and subcellular fractions of liver have been used extensively to study the metabolic fate of drugs. These microsomes are rich in cytochrome P-450, flavin monoxygenase (FMO) and some Phase II enzymes (e.g. uridine glucuronide transferase), whiles the S9 fraction contains all the Phase I and Phase II enzymes.

Methoxymorpholinyl doxorubicin (MMDX), when activated by liver cytochrome P450 enzymes, increases its toxicity on cultured tumour cells (breast MCF-7, brain (U251), lung (A549) and colon (LS180) cells). Incubation of MMDX with liver microsome and NADPH potentiated
cytotoxicity up to 100-fold. Nanomolar IC50 (i.e. concentration at which cell growth is inhibited by 50%) were recorded (Lu and Waxman, 2004).

Morpholino derivative of doxorubicin (MRA), when also activated by liver cytochrome P450 enzymes, increases its toxicity on cultured tumour cells. Incubation of MRA with liver microsome and NADPH potentiated cytotoxicity by 50-100 fold. This potentiation was cytochrome- P450-dependent, since it was inhibited by hypoxia or carbon monoxide. Biotransformation by microsome is substrate-specific, hence, not all prodrugs can be converted. In the same experiment, the cytotoxicity of doxorubicin and analogues of doxorubicin, such as methoxypiperidinyl, cyanomorpholinyl, A'-hydroxyethyl or the O-bridged cyanomorpholinyl doxorubicin, were not potentiated (Lewis et al. 1992).
CHAPTER THREE

3.0 METHODOLOGY

Ethic clearance was obtained from the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana. Standard Operating Procedures and Good Laboratory Practices (GLP) were followed.

3.1 Reagents

Jurkat, PC-3, LNCaP, MCF-7, Hep G2, HMVII and Chang Liver cell lines, Rat Liver Microsomes, Fetal Bovine Serum (FBS), Trypan Blue, Curcumin, Dulbecco Modified Eagle’s Culture Media (DMEM), Rose Park Memorial Institute (RPMI)-1640 medium, Butylated Hydroxytoluene (BHT), Sodium Carbonate, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Ethanol, Phosphate Buffered Saline (PBS), Distilled Water, Folin-Ciocalteau Reagent, Normal Saline, Dimethyl Sulfoxide (DMSO), and Gallic Acid were acquired from the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research (NMIMR).

3.2 Sample Collection and Preparation

3.2.1 Sample Collection

The fruity bodies of *Termitomyces schimperi* were collected in October, 2015 from Agboville (Agneby-Tiassa Region), La Cote d’Ivoire. The mushroom was identified and authenticated by the late Professor Ake-Assi Laurent. A voucher specimen (N° 18033) was deposited in the herbarium of the National Centre of Floristic of University of Cocody-Abidjan.
3.2.2 Sample Preparation

The mushrooms were washed three times with water. They were air-dried and pulverized. The kaolin was ground into fine powder.

3.2.3 Aqueous extraction: One part of mushroom was stirred with 10 parts of distilled water. Thus, 50g of *Termitomyces schimperi* (TS) was stirred with 500ml of distilled water. The mixture was magnetically stirred for 6 hours and allowed to stand for 24 hours. Thereafter, the supernatant was collected and filtered with Whatmann paper No. 1. The filtrate was frozen at -20 °C. The frozen sample was freeze-dried to get a crude aqueous extract (Aqueous TS). The kaolin extract was prepared, using the same procedure. In the case of preparing the *Termitomyces schimperi*–Kaolin extract, 40% of the mushroom was first mixed with 60% kaolin, followed by the rest of the protocol.

3.2.4 Ethanolic (80 %) extraction: Ethanol-water system in the ratio 8:2 was prepared. Thereafter, 50 g of TS was mixed with 500 ml ethanol-water system. The mixture was stirred for 6 hours and macerated for 24 hours. The supernatant was collected and filtered with Whatmann paper No.1. A rotary evaporator set at 70°C was used to concentrate the filtrate. The concentrated mixture was freeze-dried to get the crude ethanolic extract (ETOH TS).

The procedure was repeated using *Termitomyces schimperi*-Kaolin mixture and Kaolin alone to get crude extracts ETOH TSK and ETOH K.
3.3 In Vitro Studies

3.3.1 Screening of Secondary Metabolites

The method used by Ayoola et al., 2008 for screening secondary metabolites was adopted with slight modification. Tests for phenols, flavonoids, alkaloids, glycosides, saponins, tannins and triterpenoids were done (Ayoola et al., 2008).

3.3.1.2 Test for flavonoids

Aqueous solution (5 ml) of the extracts were prepared, followed by 5 ml of dilute ammonia and then 1 ml of concentrated H$_2$SO$_4$. The formation of a yellow colouration that disappears on standing indicates the presence of flavonoids in the extract.

3.3.1.1 Test for terpenoids (Salkowski test)

Chloroform (2 ml) was added to 0.2 g of the extracts followed by 3 ml of concentrated H$_2$SO$_4$ carefully added. The presence of a reddish-brown colouration at the interface indicates the presence of terpenoids in the extract.

3.3.1.3 Test for saponins

Distilled water (5 ml) was added to 0.2 g of each extract in a test tube. The solution was shaken vigorously. The presence of a stable persistent froth indicates the presence of saponins in the extract.

3.3.1.4 Test for tannins

Distilled water (10 ml) was added to 0.2 g of each extract in a test tube. It was then boiled and filtered. A few drops of 0.1% ferric chloride were added. The presence of a blue-black or brownish-green colouration indicates the presence of tannins in the extract.
3.3.1.5 Test for alkaloids

Acid alcohol (i.e. 70% ethanol and concentrated HCl) (10 ml) was used to dissolve 0.2 g of each extract. The mixture was then boiled and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia was added. The alkaloidal base was extracted by adding 5 ml of chloroform and gently shaking the mixture. Acetic acid (10 ml) was used to extract the chloroform layer. This was then split into two portions. Mayer’s reagent was added to the first, and Draggendorff’s reagent was added to the second portion. For Mayer’s test, the presence of a cream precipitate indicates the presence of alkaloids. For the Draggendorff test, the presence of a reddish-brown precipitate indicates the presence of alkaloids in the extract.

3.3.1.6 Cardiac glycosides test (Keller-Killiani test)

Water (5 ml) was used to dissolve 0.2 g of each extract. Glacial acetic acid (2 ml), containing one drop of ferric chloride solution, was added. This mixture was underlayed with 1 ml of concentrated H₂SO₄. Formation of a brown ring at the interface indicates the presence of deoxysugar typical of cardenolides. In addition, a violent ring will be formed below the brown ring. In the acetic acid layer, a greenish ring will be formed above the brown ring, which gradually spreads in the layer.
3.3.2 Antioxidant Assays

Antioxidant assays conducted on the extracts were DPPH Radical Scavenging and Reducing Power Assay. In addition, the total contents of phenols and flavonoids were also determined.

3.3.2.1 DPPH (2, 2- diphenyl-1-picryl hydrazyl) Assay

Principle

The method of Acheampong, 2015 and Brand-Williams et al., 1995 were adopted with slight modification.

The DPPH free radical becomes a stable diamagnetic molecule after receiving hydrogen or an electron from antioxidants. It is this binding to hydrogen that confers the radical scavenging activity. In methanol, DPPH radical is transformed into DPPH-H (diphenylhydrazine) molecules in the presence of an antioxidant, as demonstrated in the equation below.

\[ \text{DPPH}^+ + \text{A-H} \rightarrow \text{DPPH-H} + \text{A}^+ \]

Absorbance decreases as DPPH radical binds to one electron. A discoloration occurs which is stoichiometrically related to the number of electrons gained (Aksoy et al., 2013).

Preparation of Samples and Reagents

Stock concentration (1 mg/ml) of the positive control, Butylated Hydroxytoluene (BHT), was prepared, using 100% methanol. The solutions were vortexed to dissolve BHT completely. Seven (7) serial dilutions were carried out to get concentrations of 0.015625 to 1 mg/ml. Aqueous and ethanolic extracts (20 mg/ml) were prepared with distilled water and methanol, respectively. The solutions were vortexed to dissolve the extracts completely. Seven (7) serial dilutions were carried out to get concentrations that ranged from 0.3125 mg/ml to 20 mg/ml.
DPPH radical (3.94 mg) was dissolved in 20 ml of methanol to prepare DPPH solution. DDPH was weighed into a falcon tube with aluminum foil wrapped around it. The DPPH is light-sensitive and photo-bleaches on exposure to light. The DPPH solution was immediately stored in the dark for the same reason. The blanks used in the assay were absolute methanol and water without the extracts or standard, BHT.

**Assay**

Different concentration of 100 µl extracts, BHT and blanks were added into a 96-well plate in triplicate. To each well, 100 µl of 0.5 nM DPPH was added. Aluminum foil was used to cover the plates. The plates were incubated in the dark at room temperature for 20 minutes. Absorbance was read with a microplate spectrophotometer at 517nM (Tecan Infinite M200 Pro plate reader, Austria).

**Calculation**

\[
\text{% DPPH Activity} = \left( \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \right) \times 100
\]

The mean percentage antioxidant activity was plotted for positive control and extracts, and the EC50 for each was calculated. The concentration of antioxidants required to reduce initial DPPH concentration by 50% is referred to as the EC50.

### 3.3.2.2 Reducing Power Assay

**Principle**

The method used by Yang et al., 2015 was adopted.

Antioxidants donate electrons to free radicals that can lead to neutralization of the radicals. Reductants react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+),
which then reacts with ferric chloride to form a ferric ferrous complex. The complex formed has maximal absorption at 700 nm (Jayanthi and Lalitha, 2011).

\[
\text{Potassium ferricyanide} + \text{Ferric chloride} \rightarrow \text{Potassium ferrocyanide} + \text{Ferrous chloride.}
\]

**Preparation of Samples and Reagents**

Potassium ferricyanide (KFCN 1%) was prepared by dissolving 300 mg of KFCN in 30 ml of 0.2M Phosphate Buffer Saline, pH 6.6. Trichoroacetic acid (TCA 10%) was prepared by dissolving 300 mg TCA in 3 ml distilled water. Ferric Chloride (FeCl$_3$ 0.1%) was prepared by dissolving 6 mg of ferric chloride in 6 ml distilled water. Butylated Hydroxyl Toluene (BHT) was used as the standard. Stock concentration 5 mg/ml of BHT was prepared using 100% methanol. The solutions were vortexed to dissolve BHT completely. Seven (7) serial dilutions were carried out to get concentrations that ranged from 0.078125 to 5 mg/ml.

Aqueous and ethanolic extracts (20 mg/ml) were prepared by dissolving them in distilled water and methanol, respectively. Seven (7) serial dilutions were carried out to get concentrations that ranged from 0.3125 to 20 µg/ml. A control or blank was also prepared which contained all reagents except extracts or standards.

**Assay**

A solution containing 200 µl of each extract and standard in different concentrations were prepared with 200 µl of KFCN in glass tubes. The solution was incubated at 50°C for 20 minutes. Centrifuging at 300 g for 10 minutes was done, after addition of 20 µl trichoroacetic acid. New tubes were labelled and 200 µl of the upper layer of the solution was pipetted into them. Distilled water (200 µl) and FeCl$_3$ (40 µl) were added. The solutions were incubated for 30
minutes. Microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria) was used to read absorbance at 700 nM.

### 3.3.2.3 Total flavonoid Content (TFC) determination

#### Preparation of samples and reagents

The method used by Appiah-Opong et al., 2016 was adopted. Aqueous and ethanolic extracts (20 mg/ml) were prepared with distilled water and methanol, respectively. Three (3) serial dilutions were carried out to get concentrations of 20, 10 and 5 mg/ml. Quercetin was used as the standard. Stock concentration of quercetin (1 mg/ml) was prepared with methanol. A ten-fold dilution of quercetin in methanol was used as working concentration of 0.1 mg/ml. Further ten-fold serial dilutions were carried out to get concentrations that ranged from 0.000195313 to 0.1 mg/ml. Aluminum chloride solution (2% AlCl$_3$) was prepared by dissolving 140 mg aluminum chloride in 7 ml of methanol.

#### Assay

Extracts, quercetin and blanks (100 µl each) were added into wells in a 96-well plate. To each well, 100 µl of 2% AlCl$_3$ was added. The solution was incubated at room temperature for 20 minutes. Absorbance was read at 415 nM with a microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). A quercetin standard graph was plotted with absorbance against concentration. Using this graph, the flavonoid concentration in each 5 mg/ml extract and quercetin equivalence were determined.
3.3.2.4 Determination of Total Phenol Content

Preparation of samples and reagents

The method described by Acheampong, 2015 were adopted with slight modifications.

Sodium Carbonate (10 g) was dissolved in 40 ml distilled water and boiled. Few crystals of Na₂CO₃ was added to the mixture after it was allowed to cool. The solution was left to stand at room temperature for 24 hours, filtered and 10ml distilled water added to obtain 50 ml Na₂CO₃ solution.

Aqueous and ethanolic extracts (20 mg/ml) were prepared with distilled water and methanol, respectively. Three (3) serial dilutions were carried out to get concentrations 20, 10 and 5 mg/ml 1mg/ml stock concentration of gallic acid (i.e. standard). The gallic acid was first dissolved with 100 µl ethanol and topped up with 900 µl distilled water to get the required volume of 1ml. Ten serial dilutions were done to get concentrations that ranged from 0.001953125 to 1 mg/ml.

Absolute ethanol and water blanks without the extracts or gallic acid were prepared.

Assay

Samples (10 µl each) of extracts, gallic acid and blanks were placed in separate wells in a 24-well plate in triplicate. To each well, 0.79 µl of distilled water was added, followed by 50 µl of Folin-Ciocalteu reagent. The solution was mixed completely. The solutions were incubated in the dark for 8 minutes. To each dilution, 150 µl sodium carbonate was added, followed by two incubations at room temperature. Absorbance was read at 750 nM with a microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). Gallic acid standard graph was drawn with absorbance against concentration. Using the graph, phenolic concentration and gallic acid equivalence in each sample were determined.
3.3.3 Cell Viability Assay

Cytotoxic effects of extracts on cell lines were determined using MTT assay. MMT is a tetrazolium-based Colorimetric Assay. The method used by Appiah-Opong et al., 2016 and Ayisi et al., 2011 were adopted.

Principle

This assay is based on the ability of the cell’s mitochondrial succinate dehydrogenase to reduce yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Formazan is insoluble and purple in colour. Acidified isopropanol is used to solubilize cells and the released solubilized formazan. The absorbance of this product is measured with a spectrophotometer. Only metabolically inactive cells are unable to reduce MTT, hence this activity can only be realized with viable cells. The level of activity measured is, therefore, a measure of cell viability (Germaniuk, 2005).

Procedure

Stock solutions (100 mg/ml) of extracts were prepared. The solvent for dissolution was dimethyl sulfoxide (DMSO) with the exception of aqueous TSK that was dissolved with distilled water. After vortexing of the solutions, they were filtered and sterilized with 0.45 μm pore filters into cryotubes and stored at -20 °C until use. Sterile filtration was carried out in biosafety cabinet level II.

Working concentration of extract (1 mg/ml) was prepared. This was carried out by pipetting 10 μl of the stock solution and dissolving it in 990 μl of either DMSO or distilled water. Thereafter, a two-fold serial dilution was made of each extract to get concentrations of 0.0625 to 0.5 mg/ml. Suspension cells (Jurkat) in culture flask were then transferred into 50 ml tubes. The suspension cells were spun down and the pellets resuspended. Counting of viable cells was done with a
hemocytometer. Cell density of $1 \times 10^5$ cells/ml was done with media containing 5% Fetal Bovine Serum. In a 96-well plate, 100 μl of cells were seeded. After 24 hours, 10 μl extracts and standard were added to the cells and incubated at 37 °C, with 100% relative humidity and 5% CO₂ concentration for 48 hours. Extract concentration ranged from 62.5 to 1000 μg/ml. Positive control was curcumin at concentrations of 0 to 36.84 μg/ml. The reaction was stopped by adding 150 μl acidified isopropanol into each well. The plate were then covered with aluminum foil and incubated overnight at room temperature in the dark. Absorbance was read at 570nm with a spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria).

The procedure was repeated with monolayer cells, for example MCF-7. The difference was that since they are adherent cells that have become attached to the culture flask, trypsin was used to detach these cells followed by media to suspend the cells (Appiah-Opong et al., 2016).

Calculation of percentage cell viability:

$$% \text{ Cell Viability} = \frac{\text{Mean absorbance of Treated Cells} - \text{Mean absorbance of Blank}}{\text{Mean absorbance of Untreated} - \text{Mean absorbance of Blank}} \times 100$$

The experiment was done in triplicate. A graph of mean % viability against concentration was plotted, using Graph pad prism 5. The IC₅₀s of Curcumin and Extracts (where applicable) and the selective index were calculated. Selective index indicates whether a sample is more selective on cancer cells compared to normal cell lines. A selective index greater than 2 is desirable (Wardihan, 2013).

Calculation of Selective Index:

$$\text{Selective Index} = \frac{\text{IC50 of Sample on Chang Liver cells}}{\text{IC50 of Sample on Cancer cell lines}}$$

(Appiah-Opong et al., 2016)
3.3.4 Effect of Microsomes on Extracts

Samples that were cytotoxic on cancer cell lines, namely ETOH TS, Aqueous TS, Aqueous TSK and the standard, Curcumin, were selected for possible bioactivation of their constituents by microsomes. Microsomes used were prepared from male Sprague-Dawley rats. The most sensitive cell lines were PC-3 and Jurkat hence they were the cell lines used. MTT Assay was conducted to determine the cells viability (Lu and Waxman, 2004). The only difference between this methodology and the previous one described in Section 3.3.3 was addition of microsomes and NADPH.

In summary, the PC-3 and Jurkat cells were seeded at a density of $1 \times 10^5$ cells/well. The cells were allowed to attach for 24 hours. Extracts and curcumin at concentrations of 0 – 1000 µg/ml and 0 – 36.838 µg/ml, respectively, were used to treat the cells. A medium containing 0.3 mM NADPH and 2 µg rat liver microsome was added to each well. Each well had a final volume of 200 µl. The plates were covered with aluminum foil and incubated overnight at room temperature in the dark. Absorbance was read at 570 nM with a spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria).

**Stepwise procedure of preparing rat liver microsome**

Male Sprague-Dawley rats were sacrificed by cervical dislocation. Their livers were immediately taken out, washed with homogenizing buffer, and stored at −80°C until use. In the preparation of microsomes, buffers are needed to blend the liver and store the microsomes. These buffers are, Glycerol/phosphate buffer containing 80% of 0.1 M phosphate buffer (pH 7.5) and 20% glycerol
(v/v) and Potassium Chloride (KCl) buffer containing 0.25 M sucrose and 0.05M phosphate buffer (pH 7.5) (Warrington, 2002).

**Preparation of Buffers**

The Glycerol/phosphate buffer was prepared by adding 20 ml of glycerol and 80 ml 0.1 M phosphate buffer (pH 7.5).

Potassium chloride-sucrose buffer (1000 ml) was prepared by dissolving 11.5 g of KCl and 85.6 g of sucrose in 500 ml of 0.1 M phosphate buffer (pH 7.5). The solution was then topped up to 1000 ml with distilled water.

Phosphate buffer (1000 ml, 0.05 M) (pH 7.5) was prepared by adding 500 ml distilled water to 500 ml 0.1 M phosphate buffer.

On the day of the experiment, centrifuge tubes were placed on ice. The liver was allowed to thaw. Potassium-sucrose buffer (140 ml) was measured into a beaker. After thawing, the liver was placed on an aluminum foil. One-fifth of the liver was removed, put back into the tube, and stored at -80°C. The liver was placed in a blender and 100 ml potassium chloride-sucrose buffer was added and blended with increasing speed. About 80% of the liquid content was put into centrifuge tubes. The unblended pieces of the liver and the remaining 20% of the liquid were added to the extra hand homogenizer. The hand homogenizer was put on ice immediately. The remaining portion of the buffer was used to wash out the remaining homogenate in the blender. About 40 ml of buffer was poured out from the blender into the beaker and kept on ice. The contents were homogenized. This liquid was added to the centrifuge tubes. The solid pieces and liquid from the beaker were added to the hand homogenizers. The pieces were completely homogenized and added to the centrifuge tubes.
The centrifuge tubes were balanced and spun for 22 minutes at 10,000 rpm at 4 °C. The centrifuge was turned on and the rotor put inside. The supernatants were poured into a clean beaker and then into ultracentrifuge tubes. The samples were balanced and the adaptor and cap put on. The ultracentrifuge tubes were added to the centrifuge, and the following parameters were set; vacuum was turned on and speed was set 33,000 rpm at 4 °C for 70 minutes. Dry cycle were initiated and the vacuum set before turning off. The supernatant was poured off. The pellets were rinsed gently with phosphate buffer. The solution was poured out. Into each tube, 2 ml glycerol phosphate buffer was put into each tube. Precut transfer pipette was used to get pellet off the sides. All tubes were transferred into the hand homogenizer. It was homogenized until it turned creamy. The liquid was poured into pre-labelled microcentrifuge tubes. An additional 1 ml was added to the hand homogenizer and homogenized. Another tube was added and distinguished from the other samples with a star on the top. All samples were stored at -80 °C (Warrington, 2002).

### 3.3.5 Statistical Analysis of Results

Results were presented as mean ± standard deviation (SD) from three independent experiments or assays. One-way and two-way ANOVA were used to test differences for single and multiple group analysis, followed by Bonferroni post-hoc test. \( P \) value less than 0.05 was considered statistically significant.
CHAPTER FOUR

4.0 RESULTS

4.1 Qualitative Screening of Secondary Metabolites

Both aqueous and ethanolic (20:80 v/v) extracts contained phenols, tannins, terpenoids, saponins and cardiac glycosides. The latter contained flavonoids in addition. Alkaloids were absent in both extracts. None of these secondary metabolites was present in Kaolin (Table 1).

Table 1. Qualitative screening of secondary metabolites in aqueous and ethanolic extracts (ETOH) of *Termitomyces schimperi* (TS), Kaolin (K) and *Termitomyces schimperi*-Kaolin (TSK).

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Phenols</th>
<th>Flavonoids</th>
<th>Terpenoids</th>
<th>Saponins</th>
<th>Alkaloids</th>
<th>Cardiac Glycosides</th>
</tr>
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<tbody>
<tr>
<td>ETOH TS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ETOH TSK</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Aqueous TS</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Aqueous TSK</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ETOH K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous K</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Means presence of the particular secondary metabolite.

- Means absence of the secondary metabolites
4.2 Antioxidant Activity of Extracts

4.2.1 Free Radical Scavenging Activity – DPPH Assay

Aqueous and ethanolic extracts of TS and TSK scavenged DPPH radical in a concentration-dependent fashion compared to the standard butylated hydroxytoluene (BHT) (Figure 2a and b). The extract with the best DPPH scavenging activity was ETOH TS, with an EC$_{50}$ of 1.285 ± 0.038 mg/ml. Kaolin had the weakest or no scavenging potential even at the highest concentration of 10 mg/ml (Table 2). EC$_{50}$ value is the concentration required to scavenge 50% DPPH radical. It was determined from the mean percentage antioxidant activity versus concentration plot.

There was an overall significant difference between EC$_{50}$ values of the various extracts, compared to the standard BHT with p-value < 0.0001 (one-way ANOVA, followed by Bonferroni post-hoc test) (Figure 3a and b).

Table 2. EC$_{50}$ values of DPPH Radical Scavenging Activities of Extracts and BHT.

| Sample / Standard | EC$_{50}$ Values (mg/ml) |  |
|-------------------|-------------------------|--|---|
|                   | TS                      | TSK | K  | p-value    |
| Aqueous           | 1.971 ± 0.231           | 4.659 ± 0.050 | > 10 | < 0.0001   |
| ETOH              | 1.285 ± 0.038           | 1.964 ± 0.124 | > 10 | < 0.0001   |
| Butylated hydroxytoluene (BHT) | 0.0407 ± 0.008 |  |   |

Values are EC$_{50s}$ ± SEM (n=3). P value ≤ 0.05 means significant difference between the extracts and BHT (one-way ANOVA and Bonferroni’s post-hoc test).
Figure 2. DPPH Radical Scavenging Activity of (a) Ethanolic and Aqueous of TS, TSK and K (b) BHT.
Figure 3. EC\textsubscript{50} values of DPPH Radical Scavenging Activity of (a) Ethanolic Samples (b) Aqueous Samples BHT.

Values represent EC\textsubscript{50}s values ± SEM (n=3). * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001 compared to standard, BHT (one-way ANOVA followed by a Bonferroni’s post-hoc test).

The symbol * denotes comparison between BHT and TS, + denotes comparison between BHT and TSK and ¥ denotes comparison between BHT and Kaolin.
4.2.2 Reducing Power Assay

EC$_{50}$ values of all extracts were above 10 mg/ml. The extracts did not demonstrate concentration-dependent reducing power (Figure 4a and b). Absorbance recorded were below 0.5, which is lower. Only the standard BHT recorded absorbance above 0.5 (Table 3). Measuring higher absorbance values are indication of stronger reducing power potentials of samples. Only BHT had reducing power activity (Figure 4b). There was an overall significant difference between EC$_{50}$ values of the various extracts compared to standard, BHT (p-value < 0.0001, one-way ANOVA, followed by Bonferroni post-hoc test (Figure 5a and b).

Table 3. EC$_{50}$ values of Reducing Power Activities of Extracts and BHT.

<table>
<thead>
<tr>
<th>Sample / Standard</th>
<th>TS</th>
<th>TSK</th>
<th>K</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>ETOH</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT)</td>
<td>0.12 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are EC$_{50}$ values ± SEM (n=3). P value ≤ 0.05 means significant difference between the extracts and BHT (one-way ANOVA and Bonferroni’s post-hoc test).
Figure 4. Reducing Power Assay of (a) Ethanolic and Aqueous of TS, TSK and K (b) BHT.
Figure 5. EC_{50} values of Reducing Power of (a) Ethanolic Samples (b) Aqueous Samples and BHT.
Values represent EC_{50} values ± SEM (n=3). * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001 compared to standard, BHT (one-way ANOVA followed by a Bonferroni’s post-hoc test).
The symbol * denotes comparison between BHT and TS, + denotes comparison between BHT and TSK and ¥ denotes comparison between BHT and Kaolin.
4.2.3 Total Phenol Content (TPC)

At extract concentration of 5 mg/ml, aqueous TS had the highest total phenol content of 6.2531 g ± 0.1743 g / 100 g in terms of gallic acid equivalent (GAE). ETOH TSK had the lowest total phenol content of 1.6817 ± 0.4360 g/100g GAE (Table 4).

Table 4. Total Phenol Content of Extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TS</th>
<th>TSK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>6.2531 g ± 0.1743</td>
<td>3.418.24 g ± 0.0465</td>
</tr>
<tr>
<td>ETOH</td>
<td>5.7243 g ± 0.2020</td>
<td>1.6817 g ± 0.4360</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM (n=3).

4.2.4 Total Flavonoid Content (TFC)

At a concentration of 5 mg/ml, total flavonoid content in ETOH TSK and aqueous TS were 0.0077 g / 100 g and 0.0014 g / 100 g, in terms of quercetin equivalent (QUE), respectively. Flavonoids were not detected in both ethanolic *Termitomyces schimperi* and aqueous *Termitomyces schimperi* and Kaolin (Table 5).

Table 5. Total Flavonoid Content of Extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TS</th>
<th>TSK</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>0.0014 g ± 0.0002</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ETOH</td>
<td>-</td>
<td>0.0077 g ± 0.0014</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM (n=3).
4.3 **In Vitro Cytotoxicity (MTT Assay)**

Aqueous TS showed the lowest IC$_{50}$ value (i.e. concentration needed to inhibit 50% cell growth) of $377.42 \pm 32.06 \mu g/ml$ on PC-3 cell lines (Figure 6a). Ethanolic *Termitomyces schimperi* was the only extract that was cytotoxic on two cell lines; Jurkat and PC-3, with IC$_{50}$ values of $545.23 \pm 3.67 \mu g/ml$ and $862.17 \pm 23.82 \mu g/ml$, respectively (Figure 6a and 8a). In addition, the only extract combination that recorded an IC$_{50}$ value was Aqueous *Termitomyces schimperi*-Kaolin with a concentration of $748.96 \pm 319.92 \mu g/ml$ on PC-3 cell lines (Figure 6a). Only two cell lines, PC-3 and Jurkat, were sensitive to the extracts, with the former being the more sensitive cell line (Figure 6 and 8). No IC$_{50}$s were recorded on LNCaP, MCF-7, HMV II and Hep G2 (Table 6). LNCaP, MCF-7, HMV II and Hep G2 were not sensitive to any of the extracts (Figure 10 to 17).

None of the extracts showed an IC$_{50}$ value at the highest concentration of 1000 µg/ml on Chang Liver cells (Figure 18). Chang is not a cancerous cell line. A drug, which is very toxic to cancer cell lines but shows less or no toxicity of Chang, is very selective and a promising candidate for drug development.

The standard, Curcumin, demonstrated a strong concentration-dependent inhibition of cell growth on all the cancer cell lines as well as on normal cell line (Figure 6b to 18b).

Since only PC-3 and Jurkat were sensitive to the extracts, one-way ANOVA followed by Bonferroni’s post hoc-test, was conducted by comparing the relative density of all extracts to the standard. On PC-3 cell lines, there was an overall significant difference between various aqueous extracts compared to the standard, Curcumin ($p = 0.0005$), (Table 6 and Figure 7). There was no significant difference between Aqueous TS and Curcumin ($p > 0.05$). There was no significant difference between Aqueous TS and Aqueous TSK ($p > 0.05$). Difference between Aqueous TS
and Aqueous K was significant (p < 0.01). There was no significant statistical difference between Aqueous TSK and Aqueous K (p > 0.05), (Table 7).

In addition, there was significant difference between various ethanolic extracts compared to Curcumin (p < 0.0001, one-way ANOVA, followed by a Bonferroni’s post-hoc test), (Table 6 and Figure 7). Comparing activities of ethanolic samples, there was significant difference between ETOH TS and ETOH TSK, and K. There was no significant difference between ETOH TSK and ETOH K (Table 8).

On Jurkat cell lines, differences among ethanolic extracts were similar to those on PC-3 cells (Table 8).
4.3.1 PC-3 Cells

Figure 6. Cytotoxicity Effects of (a) Aqueous and Ethanolic Extracts (b) Curcumin on PC-3 Cell Line.
Figure 7. IC₅₀ values of (a) Ethanol Extracts (b) Aqueous Extracts and Curcumin on PC-3 Cell Line.

Values are IC₅₀ ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively compared to Curcumin (one-way ANOVA and Bonferroni’s post hoc test).

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK and ^ denotes comparison between Curcumin and Kaolin.
4.3.2 JURKAT Cells

Figure 8. Cytotoxicity Effects of (a) Aqueous, Ethanolic Extracts and (b) Curcumin on JURKAT Cell Line.
Figure 9. IC\textsubscript{50} values of (a) Ethanolic Extracts and (b) Aqueous Extracts and Curcumin on JURKAT Cell Line.

Values are IC\textsubscript{50} ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post-hoc test).

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK, and ^ denotes comparison between Curcumin and Kaolin).
4.3.3 LNCAP Cells

Figure 10. Cytotoxicity Effects of (a) Aqueous, Ethanolic Extracts and (b) Curcumin on LNCaP Cell Line.
Figure 11. IC$_{50}$ values of (a) Ethanolic (b) Aqueous Extracts and Curcumin on LNCaP Cell Line.

Values are IC$_{50}$ ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post-hoc test).

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK, and ^ denotes comparison between Curcumin and Kaolin).
4.3.4 MCF-7 Cells

Figure 12. Cytotoxicity Effects of (a) Aqueous, Ethanolic Extracts and (b) Curcumin on MCF-7 Cell Line.
Figure 13. IC\textsubscript{50} values of (a) Ethanol (b) Aqueous Extracts and Curcumin on MCF-7 Cell Line.

Values are IC\textsubscript{50} ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post-hoc test).

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK and ^ denotes comparison between Curcumin and Kaolin.
4.3.5 HMV II Cells

Figure 14. Cytotoxic Effects of (a) Aqueous, Ethanol Extracts and (b) Curcumin on HMV II Cell Line.
Figure 15. IC₅₀ value of (a) Ethanolic, (b) Aqueous Extracts and Curcumin on HMV II Cell Line. Values are IC₅₀ ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post-hoc test.

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK, and ^ denotes comparison between Curcumin and Kaolin.
Figure 16. Cytotoxicity Effects of (a) Aqueous, Ethanolic Extracts and (b) Curcumin on HEP G2 Cell Line.
Figure 17. IC\textsubscript{50} values of (a) Ethanolic (b) AqueousExtracts and Curcumin on HEP G2 Cell Line.

Values are IC\textsubscript{50} ± SEM (n=3). P values \(\leq 0.05\), \(\leq 0.01\) and \(\leq 0.001\) and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post-hoc test).

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK, and ^ denotes comparison between Curcumin and Kaolin.
Figure 18. Cytotoxicity Effects of (a) Aqueous, Ethanolic Extracts and (b) Curcumin on CHANG Cell Line.
Figure 19. IC₅₀ values of (a) Ethanolic (b) Aqueous Extracts and Curcumin on CHANG Cell Line.

Values are IC₅₀ ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post-hoc test).

The symbol * denotes comparison between Curcumin and TS, + denotes comparison between Curcumin and TSK and ^ denotes comparison between Curcumin and Kaolin.
### 4.4 IC<sub>50</sub> Values of Extracts without Microsomes

Table 6. Summary of IC<sub>50</sub> values of Extracts and Curcumin on selected cancer cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Sample</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; values (µg/ml)</th>
<th>p value</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample</td>
<td>TS</td>
<td>TSK</td>
<td>CURCUMIN</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>545.23 ± 3.67</td>
<td>&gt;1000</td>
<td>3.89 ± 0.76</td>
</tr>
<tr>
<td>PC-3</td>
<td>Aqueous</td>
<td>377.42 ± 32.06</td>
<td>748.96 ± 319.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>862.17 ± 23.82</td>
<td>&gt;1000</td>
<td>12.02 ± 1.35</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>12.23 ± 2.61</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>16.86 ± 0.95</td>
</tr>
<tr>
<td>HMV II</td>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>7.60 ± 1.41</td>
</tr>
<tr>
<td>HEP G2</td>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>12.92 ± 1.89</td>
</tr>
<tr>
<td>CHANG</td>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>12.92 ± 0.34</td>
</tr>
</tbody>
</table>

The values are IC50s ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 and are denoted by the symbol *, ** and ***, respectively, compared to Curcumin (one-way ANOVA and Bonferroni’s post hoc test).
Table 7. Comparison of cytotoxicity of aqueous samples towards PC-3 cells.

<table>
<thead>
<tr>
<th>Bonferroni's Multiple Comparison Test</th>
<th>p value</th>
<th>Significant P &lt; 0.05</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin vs Aqueous TS</td>
<td>&gt; 0.05</td>
<td>No</td>
<td>Ns</td>
</tr>
<tr>
<td>Curcumin vs Aqueous TSK</td>
<td>&lt; 0.01</td>
<td>Yes</td>
<td>**</td>
</tr>
<tr>
<td>Curcumin vs Aqueous K</td>
<td>&lt; 0.001</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>Aqueous TS vs Aqueous TSK</td>
<td>&gt; 0.05</td>
<td>No</td>
<td>Ns</td>
</tr>
<tr>
<td>Aqueous TS vs Aqueous K</td>
<td>&lt; 0.05</td>
<td>Yes</td>
<td>*</td>
</tr>
<tr>
<td>Aqueous TSK vs Aqueous K</td>
<td>&gt; 0.05</td>
<td>No</td>
<td>Ns</td>
</tr>
</tbody>
</table>

One-way ANOVA followed by Bonferroni's multiple comparison.

Table 8. Comparison of cytotoxicity of ethanolic samples towards both PC-3 and Jurkat cells.

<table>
<thead>
<tr>
<th>Bonferroni's Multiple Comparison Test</th>
<th>p value</th>
<th>Significant P &lt; 0.05</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>CURCUMIN vs ETOH TS</td>
<td>&lt;0.001</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>CURCUMIN vs ETOH TSK</td>
<td>&lt; 0.001</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>CURCUMIN vs ETOH K</td>
<td>&lt; 0.001</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>ETOH TS vs ETOH TSK</td>
<td>&lt; 0.001</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>ETOH TS vs ETOH K</td>
<td>&lt; 0.001</td>
<td>Yes</td>
<td>***</td>
</tr>
<tr>
<td>ETOH TSK vs ETOH K</td>
<td>&gt; 0.05</td>
<td>No</td>
<td>Ns</td>
</tr>
</tbody>
</table>

One-way ANOVA followed by Bonferroni's multiple comparison.
4.5 Selectivity Index

Aqueous TS extract showed the highest selectivity, SI value of 2.65 on PC-3 cell line. All other extracts recorded SI value less than 2.0. Curcumin had the highest selectivity of 3.32 on Jurkat cell line (Table 9).

Table 9. Selectivity Indices of Extracts and Curcumin on selected cancer cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Aqueous TS</th>
<th>ETOH TS</th>
<th>Aqueous TSK</th>
<th>ETOH TSK</th>
<th>Aqueous K</th>
<th>ETOH K</th>
<th>Curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>1.00</td>
<td>1.83</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>3.32</td>
</tr>
<tr>
<td>PC-3</td>
<td>2.65</td>
<td>1.16</td>
<td>1.34</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.07</td>
</tr>
<tr>
<td>LNCaP</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.06</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.77</td>
</tr>
<tr>
<td>HMV II</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.7</td>
</tr>
<tr>
<td>HEP G2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
4.6 *In Vitro* Cytotoxicity of Selected Extracts and Standard in Presence of Microsomes.

Both ETOH TS and Aqueous TS demonstrated a concentration-dependent inhibition of cell growth. IC$_{50}$ of ethanolic *Termitomyces schimperi* was reduced from 545.23 ± 3.67 µg/ml to 31.56 ± 2.98 µg/ml on Jurkat cell line (Figure 20). On PC-3, the IC$_{50}$ was reduced from 862.17 ± 23.82 µg/ml to 28.86 ± 0.26 µg/ml (Figure 21). In effect, cytotoxicity of ethanolic *Termitomyces schimperi* extract was potentiated by 17 and 30-fold on Jurkat and PC-3 cell line, respectively (Table 10).

Also, the IC$_{50}$ of aqueous *Termitomyces schimperi* was reduced from >1000 µg/ml to 28.24 ± 9.53 µg/ml on Jurkat cell line (Figure 20). On PC-3, the IC$_{50}$ reduced from 377.42 ± 32.06 µg/ml to 206.08 ± 23.85 µg/ml (Figure 21). The cytotoxicity of aqueous *Termitomyces schimperi* extract potentiated by 35- and 2-fold on Jurkat and PC-3 cell line, respectively. The IC$_{50}$s for aqueous *Termitomyces schimperi*-Kaolin on both cell lines were greater than 1000 µg/ml (Table 10).

Table 10. Potentiation ratios, IC$_{50}$ values of Extracts and Curcumin with and without microsomes on PC-3 and Jurkat cell lines.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>JURKAT</th>
<th></th>
<th>PC-3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Microsome</td>
<td>With Microsome</td>
<td>Potentiation</td>
<td>No Microsome</td>
</tr>
<tr>
<td>Aqueous TS</td>
<td>&gt; 1000</td>
<td>28.24 ± 9.53</td>
<td><strong>35.41</strong></td>
<td>377.42 ± 32.06</td>
</tr>
<tr>
<td>ETOH TS</td>
<td>545.23 ± 3.67</td>
<td>31.56 ± 2.98</td>
<td><strong>17.28</strong></td>
<td>862.17 ± 23.82</td>
</tr>
<tr>
<td>Aqueous TSK</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>-</td>
<td>748.96 ± 319.92</td>
</tr>
<tr>
<td>Curcumin</td>
<td>3.89 ± 0.76</td>
<td>1.07 ± 0.17</td>
<td><strong>3.64</strong></td>
<td>12.02 ± 1.35</td>
</tr>
</tbody>
</table>
4.6.1 Effect of Bioactivated Extracts on Jurkat Cells

Figure 20. Cytotoxic Effects of (a) Extracts alone and Extracts incubated with microsomes (b) Curcumin alone and Curcumin incubated with microsomes on Jurkat Cell Line.
4.6.2 Effect of Bioactivated Extracts on PC-3 Cells

Figure 21. Cytotoxic Effects of (a) Extracts alone and Extracts incubated with microsomes (b) Curcumin alone and Curcumin incubated with microsomes on PC-3 Cell Line.

There was significant statistical difference between extracts without microsomes and extracts incubated with microsomes (p < 0.0001, F value = 274.5) two-way ANOVA followed by a Bonferroni’s post-hoc test. The difference between the two cell lines PC-3 and Jurkat was statistically significant (p = 0.0160, F value = 6.551). There were significant statistical
difference between aqueous TS with and without microsomes on both PC-3 and Jurkat with p-values <0.01 and <0.001 respectively (Figure 22).

Difference between ETOH TS with and without microsomes on PC-3 and Jurkat were also statistically significant p values of <0.001 on both cell lines (Figure 22). Difference between aqueous TSK with and without microsomes on Jurkat was statistically insignificant (p value > 0.05). However, on PC-3, there was statistically significance difference (p value <0.001) (Figure 22).

4.6.3 Effect of Microsomes on Extracts

![Graph showing IC50 values for different conditions](image)

**CELL LINES**

Figure 22. Effects of Microsomes on Extracts and Curcumin.

Values are IC₅₀ ± SEM (n=3). P values ≤ 0.05, ≤ 0.01 and ≤ 0.001 denoted by the symbol *, ** and ***, respectively, compared to the sample (one-way ANOVA and Bonferroni’s post hoc test. The symbol * denotes comparison between TS and TS+Microsomes, + denotes comparison between TSK and TSK+Microsomes and - denotes comparison between Kaolin and Kaolin+Microsomes.
CHAPTER FIVE

5.0 DISCUSSION

The results obtained suggest that the 80% ethanolic extract of *Termitomyces schimperi* (TS) contained phenols, tannins, saponins and cardiac glycosides, which is consistent with the findings of Anane-Adjei *et al.*, 2016. The difference between the aqueous and ethanolic extracts of TS was the absence of flavonoids in the latter. This difference could be due to the type of solvent used in the extraction. Different solvents extract different bioactive compounds or metabolites (Iloki-Assanga *et al.*, 2015). Furthermore, the water and ethanol systems had major effects on phenolic composition and antioxidant activities of extracts (Sun *et al.*, 2015). Therefore, to maximize the yield of flavonoids and other phenolic compounds that have good antioxidant properties, the use of a water and ethanol system that extracts greater amount of phenolic compounds should be appropriate.

No secondary metabolites were found in Kaolin (Table 1). Kaolin is composed of kaolinite (10-95%) and other clay minerals (Adamis and Williams, 2005). This signifies that there was no cross contamination between kaolin and the TS extracts during the experiment.

In determining the antioxidant potential of the extracts, two major assays were carried out, namely, reducing power and DPPH radical scavenging assays. In the reducing power assay, the EC$_{50}$ values of all the extracts were greater than 10 mg/ml (Table 3) (Figure 4 and 5). None of the extracts was able to significantly donate electron(s) to reduce ferricyanide (Fe$^{3+}$(CN)$_6$) to ferrocyanide (Fe$^{2+}$(CN)$_6$) to form an intense Prussian blue colour complex. Comparing the reducing power of *T. schimperi* to other species such as *T. robustus*, the latter’s EC$_{50}$ was lower with a value of 1.24 ± 0.01 mg/ml (Obodai *et al.*, 2014).
In the DPPH radical scavenging assay, TS alone or in combination with kaolin stabilized the DPPH radical into a stable diamagnetic molecule by donating electrons or hydrogens to the radical (Aksoy et al., 2013). However, this effect was not remarkable compared to the radical scavenging effect of the standard compound, Butylated hydroxytoluene (BHT). There was an overall significant difference between EC\textsubscript{50} values of the various extracts compared to BHT (p-value < 0.0001, one-way ANOVA and Bonferroni post-hoc test). The general trend of DPPH radical scavenging activity of all samples (highest to lowest) was BHT > ETOH TS > ETOH TSK > Aqueous TS > Aqueous TSK > ETOH K = Aqueous K. In comparison with other Termitomyces species such as T. robustus, T. albiminosus and T. reticulatus, TS showed higher antioxidant activity (Obodai et al., 2014; Loganathan et al., 2010). The antioxidant activity may be due to constituents of Termitomyces schimperi such as phenols. Natural phenols have been shown to exert antioxidant activity by promoting endogenous antioxidant capacity and/or quenching free reactive species (Chen et al., 2011). Comparatively, among Termitomyces species such as T. clypeatus, T. globulus, T. heinii, T. indicus and T. mammiformis, TS has very high phenolic content (Carmen, 2016). In this study, kaolin reduced the antioxidant activity of the TS extracts which may be as a result of reaction or chelation with some of the bioactive compounds or metabolites thereby reducing their ability to donate electron or hydrogen to the DPPH radical (Table 2). On the contrary, other studies have shown that kaolin boosts the antioxidant activity of grapevines and a tea sample, Camelia sinesis (Bernado, 2015; Yazici and Goksu, 2017).

The cytotoxic activities of the extracts on the growth of prostate (PC-3 and LNCaP), T-lymphocyte (Jurkat), liver (Hep G2) and skin (HMV II) cancer cells and normal Chang Liver cells were determined using MTT assay. This assay is based on the ability of cell’s by
mitochondrial succinate dehydrogenase to reduce yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple formazan. Noteworthy, only metabolically inactive cells are unable to reduce MTT, hence this activity can only be realized with viable cells. The level of activity measured is, therefore, a measure of cell viability (Germaniuk, 2005). Aqueous extract of *Termitomyces schimperi* showed the lowest IC<sub>50</sub> value of 377.42 ± 32.06 µg/ml on PC-3 cell line. Ethanolic extract of *Termitomyces schimperi* was the only extract that was cytotoxic on both Jurkat and PC-3 cell lines with IC<sub>50</sub> values of 545.23 ± 3.67 µg/ml and 862.17 ± 23.82 µg/ml, respectively. In addition, the only extract combination that recorded an IC<sub>50</sub> was Aqueous *Termitomyces schimperi*-Kaolin with an IC<sub>50</sub> of 748.96 ± 319.92 µg/ml on PC-3 cell line (Table 6). Only two cell lines PC-3 and Jurkat were sensitive to the extracts, with the former being the more sensitive cell line (Figure 6 and 8). LNCaP, MCF-7, HMV II and Hep G2 were not sensitive to any of the extracts (Figure 10 to 17). Curcumin had the highest cytotoxic effect compared to all the extracts. To the best of our knowledge, this is the first time cytotoxic activity of *Termitomyces schimperi* has been demonstrated on this panel of normal and cancer cell lines.

In order to determine whether the extracts were more selective to cancer cell lines than to normal cell lines, Chang liver cells were used in this study. Chang liver cells are normal cells, so extracts that are not cytotoxic to them, could be promising candidates for drug development. None of the extracts was cytotoxic on Chang liver cells. The IC<sub>50</sub> values of all the extracts were greater than 1000 µg/ml (Table 6) (Figure 18 and 19). Curcumin was very cytotoxic on the Chang liver cells which is consistent with literature (Appiah-Opong *et al.*, 2016; Anane-Adjei *et al.*, 2016; Acheampong, 2015). Out of the three (3) extracts that demonstrated some degree of cytotoxicity on cancer cells, only the aqueous extract of *Termitomyces schimperi* demonstrated selectivity on
cancer cells with selective index of 2.65 on PC-3 cell line. In the case of curcumin, it exhibited selectivity on Jurkat cell line. The selectivity of the aqueous extract of TS is commendable because most of the side effects associated with anticancer drugs are due to their lack of selectivity.

In order to determine whether kaolin may have potentiated the cytotoxic activity of *Termitomyces schimperi*, all the extracts (TS alone, kaolin alone, and the combination of TS and kaolin) were compared among themselves on the two most sensitive cell lines, PC-3 and Jurkat. Among the ethanolic extracts, there were statistical differences between TS alone and kaolin, TS alone and the combination of TS and kaolin. However, there was no statistical difference between kaolin and the combination of TS and kaolin. These imply that, kaolin could have interacted with the constituents of TS and resulted in a product with a lower cytotoxic effect. The cytotoxic effect of this end-product was not statistically different from that of kaolin alone. Likewise, among the aqueous extracts, kaolin was shown to reduce the cytotoxic potential of TS. However, the reduction in effect was not to a greater extent as observed with the ethanolic extract of TS. This could be due to the higher cytotoxic potential of the aqueous extract of TS (IC$_{50}$ value of 377.42 µg/ml) compared to the ethanolic extract of TS (IC$_{50}$ value of 862.17 µg/ml) on PC-3 cell line (Table 6). Based on these results, it can be concluded that kaolin had no significant potentiating effect on TS.

According to literature, researches on cytotoxicity of kaolin started several decades ago. Experimentally, effects of kaolin in both *in vitro* and *in vivo* test systems are contradictory. Some studies have reported that kaolin is cytotoxic while others have reported the opposite. In a study, kaolin was reported to have low cytotoxicity (Daniel and Le Bouffant, 1980). In another study, two kaolin samples which were well-crystallized and disordered kaolinites were relatively non-
cytotoxic at 80 µg/ml (Gormley et al., 1983). Kaolin in its pure form showed marked cytotoxicity (Robertson et al., 1982). Kaolin was cytotoxic to alveolar macrophage in an experiment (Low et al., 1980). The major reason attributed to these contradictory results is that kaolin samples vary markedly. Kaolin has variable amounts of ancillary minerals other than kaolinite such as quartz which is a major determinant of toxicity and also, the crystallinity of kaolinite could be either be high or low (Adamis and Williams, 2005). Also, kaolin that had cytotoxic effects in vitro could not be reproduced in vivo. In a study, kaolin caused cell membrane damage of epithelial cells in a concentration-dependent fashion in vitro (Michel et al., 2014). In another study, kaolin had very little cytotoxic effect on papillary thyroid cancer cells (Zhang et al., 2016). Doxorubicin fortified with kaolin, showed significant increase of its cytotoxic effect in a dose-dependent manner (Zhang et al., 2017).

It is worth emphasizing that the extract with the lowest IC$_{50}$ value (377.42 ± 32.06 µg/ml) in this study was the aqueous extract of Termitomyces schimperi. However, according to the American National Cancer Institute, the IC$_{50}$ end-point to adjudge a promising extract for additional purification is a value less than 30 µg/ml (Ferreira et al., 2011). Based on the National Cancer Institute (NCI) criterion, aqueous extract of TS cannot be described as potent or promising. However, it is well-known that some extracts may contain prodrug-like compounds and as such their cytotoxic properties can only be evidenced or enhanced after they are metabolized into their active form. In vitro drug metabolism or bioactivation was achieved by incubating the drugs rat liver microsomes. Liver microsomes are rich in cytochrome P-450, flavin monoxygenase (FMO) and some phase II enzymes, such as uridine glucuronide transferase (UGT). In a study, methoxymorpholinyl doxorubicin (MMDX), a prodrug, was incubated with liver microsomes and NADPH. Cytochrome-P450 enzymes present in the microsomes bioactivated MMDX and
increased its cytotoxicity on cultured tumour cells (breast MCF-7, brain U251, lung A549, and colon LS180 cell lines). Nanomolar IC$_{50}$ were recorded and cytotoxicity was potentiated up to 100-fold (Lu and Waxman, 2004). In another experiment, morpholino derivative of doxorubicin (MRA), when also bioactivated by liver cytochrome P450 enzymes, increased its cytotoxicity on cultured tumour cells by 50-100-fold (Lewis et al. in 1992).

In this study, the extracts that demonstrated the anti-proliferative activity on the cancerous cell lines were selected for the liver microsomes-dependent bioactivation studies. The rat liver microsomes potentiated cytotoxicity of the ethanolic extract of *Termitomyces schimperi* by 17 and 30-fold on Jurkat and PC-3 cell lines, respectively (Table 10 and Figure 20 to 22). Likewise, cytotoxicity of aqueous extract of *Termitomyces schimperi* on Jurkat and PC-3 cell lines were potentiated by 35 and 2-fold respectively. However, the IC$_{50}$ value of aqueous *Termitomyces schimperi*-Kaolin combination on both cell lines were greater than 1000 µg/ml (Table 10 and Figure 20 to 22). As explained above, the presence of kaolin in the mixture may have formed a complex with the TS which were not metabolized by the microsomes.

There was a statistical difference between aqueous and ethanolic extracts of *Termitomyces schimperi* with and without liver microsomes (Figure 22). There was no statistical difference between aqueous extract of *Termitomyces schimperi*-Kaolin combination with and without liver microsomes (Figure 22). Based on these results, it has been proven that both aqueous and ethanolic extract of *Termitomyces schimperi* contain bioactivatable compounds and also the metabolites have appreciable cytotoxic activity. Moreover, these findings on bioactivation with microsomes give some credence to the rather low cytotoxicity of the eleven compounds isolated from *Termitomyces schimperi* in the other study of this project (Anane-Adjei et al., 2016). Some
of the isolated compounds if not all may be prodrugs. A further bioactivation study using the isolated compounds is highly recommended.

In addition, the higher efficacy of the finished product claimed by the herbalists as mentioned in the anecdotal report, could be explained by the fact that the herbal preparation is administered orally and, as such, some biotransformation of the constituents into active forms may have taken place.
CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

Conclusion

Termitomyces schimperi (TS) without Kaolin has good cytotoxic activity on PC-3 and Jurkat cell lines. However, the antioxidant effect of TS was enhanced by kaolin. The ethanolic extract (20:80 v/v) of the mushroom (ETOH TS) has the best DPPH free radical scavenging activity. Its phenol content may be largely responsible for quenching the radical. However, ETOH TS is thirty-two times less potent than BHT in scavenging DPPH radical. The mushroom had no reducing power potential at the highest concentration of 10 mg/ml. The most cytotoxic extract was aqueous TS on PC-3 cell lines with a selectivity index of 2.65.

Termitomyces schimperi has components that act like prodrugs and were bioactivated with microsomes.

Recommendation

These studies are recommended: in vitro studies on the effects of microsomes on isolated compounds of Termitomyces schimperi, studies on animal models, using both extracts and isolated compounds and mechanistic studies, such as apoptosis.
7.0 REFERENCES


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