

**IMMUNOSTIMULATORY AND ANTIPROLIFERATIVE
EFFECTS OF KENYAN *TRAMETES VERSICOLOR*
MUSHROOM EXTRACTS ON SELECTED CANCER
AND NORMAL CELL LINES**

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**Immunostimulatory And Antiproliferative Effects Of Kenyan
Trametes Versicolor Mushroom Extracts On Selected Cancer And
Normal Cell Lines**

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**A Thesis submitted in partial fulfillment for the award of the Degree
of Master of Science in Molecular Medicine in the Jomo Kenyatta
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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my family, my wife Dorah Anzazi, whose strength, patience and encouragement enabled me to overcome the many challenges throughout my studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

%	Percentage
μL	Micro litre
°C	Degrees Celsius
ANOVA	Analysis of Variance
APC	Anaphase Promoting Complex
ATCC	American Type Culture Collection
bp	Base pare
CCK	Cell Counting Kit
CO₂	Carbon dioxide
CT	Cycle Threshold
CTMDR	Centre for Traditional Medicine and Drug Research
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immuno-sorbent Assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde- 3 phosphate dehydrogenase
H₂O	Water
HCC	Hepatocellular Carcinoma
IC₅₀	Inhibitory Concentration required to inhibit 50 % of activity.
IEC-6	Intestinal epithelial cells
<i>IL</i>	Interleukin
ILRI	International Livestock Research Institute
KEMRI	Kenya Medical Research Institute
MEM	Minimum Essential Media
mRNA	Messenger Ribonucleic Acid.
MPF	Mitosis Promoting Factor
MTT	3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium
NCBI	National Center for Biotechnology Information
ng	Nanograms

OD	Optical Density
PCR	Polymerase Chain Reaction
PS	Polysaccharopeptide
PSK	Polysaccharopeptide Krestin
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RPMI	Roswell Park Memorial Institute
SEM	Standard Error of Mean
TNF-α	Tissue Necrosis Factor- Alpha
WHO	World Health Organization
RNA	Ribonucleic Acid

ABSTRACT

Cancer is a major public health burden in both developed and developing countries. The current conventional cancer therapies like chemotherapy are expensive and inaccessible to many cancer patients. Medicinal mushrooms are becoming important as an alternative source of immune modulation and anticancer agents. This study examined the *in vitro* antiproliferative activity of an aqueous extract of a Kenyan *Trametes versicolor* (*TV*) mushroom on breast cancer (4T1), prostate cancer (DU145), hepatocellular carcinoma (HCC), rat normal intestinal epithelial cells (IEC-6) and African green monkey normal kidney (vero) cell lines using MTT assay. Polysaccharide extract of *TV* was used to evaluate the expression levels of IL-7 mRNA on IEC-6 cells using one step RT-PCR. Statistical methods (student T-test and ANOVA) were used to analyze the significance of antiproliferative activity. Double delta CT analysis was used to evaluate IL-7 mRNA expression fold change relative to GAPDH, a house keeping gene. Two-sample independent T-test was used to assess the significance in *IL-7* mRNA expression at 95% confidence interval. The results demonstrated that the *TV* aqueous extract at 1.37 µg/ml to 1000 µg/ml dose-dependently inhibited the proliferation of DU145 and 4T1 cell lines with IC₅₀ values: DU145 (71.2 µg/ml) and 4T1 (188.5 µg/ml). The aqueous extract however did not exert any significant antiproliferative effect on HCC, IEC-6 and vero cell lines (IC₅₀ >1000 µg/ml) when compared with an anticancer drug, tamoxifen ($P \leq 0.05$). There was a slight increase on the expression of IL-7 mRNA on IEC-6 cells treated with *TV* extract, with a fold change of 0.17. The increase in expression of *IL-7* mRNA was found to be significant when the mean CT value of the treated cells was compared to that of the untreated cells with a P value of 0.035. The antiproliferative activity of the Kenyan *TV* aqueous extract suggests selective inhibition of cancerous cells while its polysaccharides slightly up regulates *IL-7* mRNA expression on IEC-6 cells. Further studies with purified bioactive compounds of Kenyan *T. versicolor* to assess possible cell death mechanism is recommended. A study on the immunomodulatory potential of purified *T. versicolor* polysaccharides supplemented with other herbals is also recommended.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Cancer is a dreadful disease characterized by irregular proliferation of the cells with a manifestation of malfunctions in immunity as malignant cells manage to escape recognition and elimination by the immune system (Shurin, 2012). Cancer cells display a broad spectrum of genetic alterations that include gene rearrangements, point mutations, and gene amplifications, leading to abnormal alterations in molecular pathways involved in regulating cell growth, survival, and metastasis (Anant *et al.*, 2010). Anyone can develop cancer and the risk of being diagnosed with cancer increases with age. About 77% of all cancers are diagnosed in people 55 years of age and older (Center *et al.*, 2011).

Approximately 14 million new cases of cancer were diagnosed with about 8.2 million cancer deaths reported in 2012 with about 70% of these deaths occurring in low and middle income countries. It is projected that deaths from cancer will continue to rise with an estimate of 13.1 million deaths globally in 2030 (Wild *et al.*, 2014).

In Kenya, cancer ranks third as a cause of death after infectious and cardiovascular diseases. It causes 7% of the total national mortality each year (Odongo *et al.*, 2013). About 39,000 new cases of Cancer are diagnosed in Kenya with more than 27,000 deaths per year, with 60% of those affected by Cancer being younger than 70 years old. The leading cancers in women are Breast cancer at 34 per 100,000, Cervical cancer at 25 per 100,000), while in men are Prostate cancer at 17 per 100,000, Esophageal cancer at 9 per 100,000 (Wild *et al.*, 2014).

The National Institutes of Health estimates overall costs of cancer in 2010 at \$263.8 billion: \$102.8 billion for direct medical costs (total of all health expenditures), \$20.9 billion for indirect morbidity costs (cost of lost productivity due to illness), and

\$140.1 billion for indirect mortality costs (cost of lost productivity due to premature death) (Center *et al.*, 2011).

Chemotherapy, surgery, immunotherapy and radiotherapy remain the most effective conventional methods for cancer treatment. However, they are expensive and do cause serious side effects as these do not often show adequate differential effect between tumor and normal cells (Anant *et al.*, 2010) hence the need for affordable but effective ways of cancer management.

The efficacy of surgery, chemotherapy and radiation is limited as these treatments do not eliminate all cancerous cells, do not address the immunosuppressive nature of the disease and can further impair the patient's immune response, weakening patient's resistance to the cancer and other opportunistic infections (Shurin, 2012).

More than 50% of all modern drugs in clinical use are of natural product origins, many of which have the ability to control cancer cells (Rosangkima and Prasad, 2004). According to World Health Organization estimates, more than 80% of people in developing countries depend on traditional medicine for their primary health needs (Shah *et al.*, 2013). A survey done in the year 2008 showed that more than 60% of cancer patients use herbs as therapy (Madhuri Govind, 2008).

Several attempts have been made to explore the potential of newly discovered anticancer compounds from medicinal mushrooms as a lead for anticancer drug development (Shah *et al.*, 2013). Mushrooms have key medicinal uses including anticancer activity (Chang Wasser, 2012), immunomodulating effects, and also protects against tumor development (Finimundy *et al.*, 2013). Water extracts of *T. versicolor* mushroom from other parts of the world has been documented to have antiproliferative activity on different cancer cell lines (Chang, 2002; Ebina, 2003; Zaidman *et al.*, 2005).

Early studies have also shown that polysaccharide K (PSK), a compound extracted from *Trametes versicolor* mushroom induces gene expression leading to up

regulation of *IL-7* cytokine (Yoshikawa *et al.*, 2004). Interleukin-7 has a critical role in T-cell development and survival (Lee *et al.*, 2011), and was confirmed to increase peripheral CD4+ and CD8+ in clinical trials on patients with advanced malignancy and played a potential role in promoting T-cell recovery after chemotherapy (Mackall *et al.*, 2011). This study aimed at determining the immunomodulation and antiproliferative activity of the Kenyan *Trametes versicolor* mushroom extracts.

1.2 Statement of the problem

Cancer has a reputation of a deadly disease globally. Collectively, about half of the people receiving treatment for invasive cancer die. Survival is worse in the developing countries such as Kenya due to the high cost of diagnosis and treatment. However, the survival rates vary by cancer type, with the range running from basically all people surviving to almost none. Nearly every family has been affected by cancer, continue to suffer due to lack of affordable therapy as majority of them live below the poverty level (Odongo *et al.*, 2013).

In 2012 about 14 million new cases of cancer occurred globally (not including skin cancer other than melanoma) (Wild and Stewart, 2014). In Kenya cancer causes 7% of the total national mortality each year with 28,000 of newly diagnosed cases and over 22,000 deaths annually (Odongo *et al.*, 2013).

1.3 Study justification

Cancer is now the number three cause of death in Kenya, after infectious and cardiovascular diseases and it still remains one of the most important diseases not only in Kenya but worldwide. According to the Nairobi Cancer registry, about 41,000 new patients are registered every year with two thirds of these patients dying within 12 months.

The drugs used for cancer treatment and management are expensive or inaccessible to most of the cancer patients. Although immunotherapy is still considered an attractive therapeutic approach, its impact on clinical practice is limited. Any drugs,

compounds or supplements which are likely to reduce cancer cell proliferation and boost the immune response of a victim would go a long way in reducing the suffering due to cancer.

Commercial and wild edible mushrooms have of late been widely studied with the hope of finding a more effective drug that can prevent cancer cell growth and boost the immune system and help fight cancer. Recently, a mushroom of the *Trametes* species was identified in Kenya and it is probable that its polysaccharide extracts might have some antiproliferative activity on cancer cells and may act on the intestinal epithelial cells (IEC) resulting in up regulation of *IL-7* mRNA. Interleukin-7 then elicits favorable conditions that boost immune response to cancerous cells. A study of the immune modulation effects and antiproliferative activity of this Kenyan mushroom had not been done, hence it was important to get an effective extract that could be developed into an anticancer and/or immune booster agents.

1.4 Research questions

- What is the antiproliferative effect of the Kenyan *T. versicolor* aqueous extract on normal and cancer cell lines?
- What is the effect of the Kenyan *T. versicolor* polysaccharide extract on the expression levels of *IL-7* mRNA on IEC-6 cells?

1.5 Hypotheses

The null hypotheses states that:

1. The aqueous extract of The Kenyan *T. versicolor* does not have any antiproliferative effects on normal and cancer cell lines.
2. The Kenyan *T. versicolor* polysaccharide extracts have no effect on expression of *IL-7* mRNA on IEC-6 cells.

1.6 Study Objectives

1.6.1 General Objective

To determine the immunostimulatory and antiproliferative activity of Kenyan *T. versicolor* extracts on selected normal and cancer cell lines.

1.6.2 Specific Objectives

- To determine the antiproliferative activity of *T. versicolor* aqueous extract on selected normal and cancer cell lines.
- To determine the effects of *T. versicolor* polysaccharide extract on expression levels of *IL-7* mRNA on IEC-6 cells.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mushroom of the *Trametes* species

Mushrooms are consumed all over the world as part of the regular diet. While some are cultivated, some are just collected from the wild and consumed. In Kenya, majority of the locals depend on wild mushrooms to spice their diets as cultivated species are exorbitantly expensive and unaffordable to many. Of the 42 tribes living in the country, 38 are known to consume mushrooms (Wambua, 2004).

The mushroom *Trametes versicolor*, also called ‘Turkey tail’ grow on dead trees, logs, branches, and stumps, in woodland environment worldwide. It is occasionally also found on dead or living conifers. *Trametes versicolor* is saprophytic, which produces enzymes that decompose dead matter, providing nutrients to the mushroom. *Trametes versicolor* causes white rot in its host wood which occurs when fungi degrade the brown lignin of plant cells (Stamets, 2012).

In a recent study carried at the Kenyan Coast, mushrooms of the *Trametes versicolor* was identified in the Arabuko Sokoke forest in North coast and Kaya Teleza forest shrines at the South coast (Gateri *et al.*, 2014). This species of mushrooms has a history of use in Asia as a nonspecific immune modulator, and in breast cancer patients, they have been shown to interact with the CR3 membrane receptors for beta-glucans and its immune modulation is believed to be the primary mechanism of action of this mushroom (Zaidman *et al.*, 2005).

2.2 Active constituents of *Trametes versicolor*

Two specific polysaccharides (polysaccharide K (Krestin) and Polysaccharide peptide) have been extracted from *Trametes versicolor* and have been shown to have both anticancer and immunomodulatory activity in tissue culture, animal, and human studies (Morimoto *et al.*, 1996).

Polysaccharide K which is a protein bound polysaccharide has been studied most extensively and is in wide clinical use as an adjunctive and adjuvant cancer therapy in Japan and China (Standish *et al.*, 2008). Analyses performed by Ikuzawa and colleagues indicate that *Trametes versicolor* water extracts contain 62% polysaccharide and 38% protein (Ikuzawa *et al.*, 1988).

The glucan portion of Polysaccharide K consists of β 1–4 main chain and β 1–3 side chains, with β 1–6 side chains bound to a polysaccharide moiety through O-or N-glycosidic bonds. There is wide consensus that it is the β 1–3 and β 1–6 side chains that are immunologically active. Toxicologic assessments indicate that Polysaccharide K has low toxicity with a high median lethal dose, with no reports of abnormalities in animals or humans following acute and chronic toxicity tests (Kidd, 2000).

2.3 Mushroom polysaccharides in immune modulation

Mushroom polysaccharides, including those of Polysaccharide K act as immunomodulating and antitumor materials. The fungal antitumor polysaccharide K are mainly present as glucans with different types of glycosidic linkages, but some are true heteroglycans (Moharib *et al.*, 2014).

In vitro incubation of 10-200ug/mL Polysaccharide K appeared to increase CD4+ and CD8+ T-cells and overall splenocyte proliferation (Lu *et al.*, 2010). However, in other *In vitro* studies, it was confirmed that *Trametes versicolor* Polysaccharides induces gene expression leading to up regulation of *IL-7* cytokine (Yoshikawa *et al.*, 2004). Interleukin-7 has critical role in T-cell development and survival (Lee and Margolin, 2011) and was confirmed to increase peripheral CD4+ and CD8+ in clinical trials on patients with advanced malignancy (Mackall *et al.*, 2011). An increased level of cytotoxic T-cells (CD8+) against tumors has also been noted in animals fed with *Trametes versicolor* Polysaccharides (100mg/kg) after injection of tumor cells (Lu *et al.*, 2010).

It has been reported that *Trametes versicolor* Polysaccharides in different concentrations promote the proliferation of T-lymphocytes both in human peripheral blood and mouse splenocytes, and has been shown to augment T-helper cell (CD4+) activation, and also increase the ratio of CD4+/T suppressor (CD8+) production (Cheng and Leung, 2008). In a study to investigate the effect of mushroom polysaccharides extracts *in vivo* with human ovarian xenografts tumor growth in mice, the extract was found to significantly inhibit the growth of SKOV3 cells (Yan *et al.*, 2013).

2.4 Medicinal value of *T. versicolor* and possible toxicities

The use of *Trametes versicolor* preparations have a long history of use in traditional Asian medicine and beyond (Chu *et al.*, 2002; Kidd, 2000) and its medicinal value was recorded in the *Compendium of Chinese Materia Medica* and *Shen Non-Compendium Medica* thousands of years ago in China; nowadays the therapeutic potentials of *Trametes versicolor* have been gaining acceptance among patients and scientists worldwide (Kidd, 2000; Wasser and Weis, 1999).

In Japan, preparations extracted from *T. versicolor* mushroom has been prescribed to cancer patients routinely, both during and after radiation and chemotherapy (Hiroshi *et al.*, 1995; Morimoto *et al.*, 1996; Ogoshi *et al.*, 1995; Toil *et al.*, 1992). In the United States of America, whole, freeze-dried *T. versicolor* is commonly prescribed to breast cancer patients (Deng *et al.*, 2009).

Trametes versicolor extract has been associated with minimal side effects of gastrointestinal upset (Kobayashi *et al.*, 1993) and darkening of the fingernails, but these effects have been limited and general safety has been demonstrated with daily oral doses for extended periods of time (Kidd, 2000). The extracts from *Trametes versicolor* does not seem to interact with hepatic drug metabolizing enzymes involved in the chemical processing of most chemotherapy agents (Fisher and Yang, 2002), and no genetic damage has been detected by several tests (Kobayashi *et al.*, 1993).

At doses that produced necrotic changes in tumor cells, *Trametes versicolor* extract produced no lesions in the vital organs of tumor-bearing mice after treatment for two months. It has not been associated with teratogenic effects in mice or rats (Ng, 1998). The extracts of *T. versicolor* mushroom have been reported to be non-toxic to several normal cells (Dong *et al.*, 1996; Dong *et al.*, 1997; Mwitari *et al.*, 2014); and in cancer patients on clinical trials (Kidd, 2000).

2.5 Mechanisms of action of Mushroom polysaccharides

The diversity of the polysaccharides and their derivatives is reflected in the diversity of their mechanisms of action. The indirect action (immunostimulation) is based on stimulation of host defense mechanisms, primarily on activation of T and B lymphocytes, macrophages and natural killer (NK) cells. Many mushroom β -glucans have been shown to stimulate production of several important cytokines such as *IL-7*, *IL-10*, among others (Borchers *et al.*, 1999). Besides the indirect action, several polysaccharides have shown direct effects on cancer cells. Many *in vitro* and *in vivo* studies have suggested that polysaccharides inhibit tumor cell proliferation and/or induce their death by apoptosis (Tao *et al.*, 2006; Zhang *et al.*, 2007).

2.6 Role of Interleukin 7 (IL-7) in immune modulation

The cytokine *IL-7*, a member of the small four-helix bundle family of cytokines signal through the α -receptor sub-unit to exert influence over T-cell survival, proliferation and homeostasis. The critical role of *IL-7* in T-cell development is evidenced by the finding that *IL-7* receptor mutations lead to an absence of T-cells and the development of severe combined immunodeficiency (SCID) (Lee and Margolin, 2011). Interleukin 7 is a homeostatic cytokine and functions as a limiting resource that provides continuous signal to resting naive and memory T-cells. During lymphopenia conditions, *IL-7* accumulates and this leads to an increase in both T-cell proliferation and T-cell repertoire diversity. Interleukin-7 also plays a role in B cell development and its receptor is found on immature B cell progenitors (Schluns *et al.*, 2000).

A potential therapeutic advantage of *IL-7* is its selectivity for expanding CD8⁺ T-cell populations (Rosenberg *et al.*, 2006). In murine models, recombinant *IL-7* has been found to augment antigen-specific T-cell responses after vaccination and adoptive cell therapy (Colombetti *et al.*, 2009). Another important area of investigation is the potential role of *IL-7* in promoting T-cell recovery after chemotherapy. Early phase clinical trials on patients with advanced malignancy demonstrated recombinant *IL-7* to be well-tolerated with limited toxicity at biologically active doses (in which the numbers of circulating CD4⁺ and CD8⁺ T-cells increased by 3–4 fold), suggestive of a broad therapeutic index (Mackall *et al.*, 2011).

A peculiar attribute of *IL-7*, reflecting its role in immune developmental and homeostasis, is its ability to increase the diversity of T-cells recognizing different antigens, and this may be an important phenomenon in broadening anti-tumor immune responses, especially if tumor antigens mutate under immunological pressure (Pellegrini *et al.*, 2009). Although *IL-7* itself does not contribute to the generation of *Th17* cells, the inflammatory milieu created by exogenous *IL-7*, and in some cases endogenous *IL-7*, promotes *Th17* differentiation (Kanai *et al.*, 2009).

2.7 Other Cytokines

Several early *in vitro* cancer studies have confirmed that mushroom Polysaccharides induces gene expression of several cytokines including *TNF- α* , *IL-2*, *IL-7*, *IL-6* and *IL-10* amongst others (Hirose *et al.*, 1990; Nio *et al.*, 1991; Noguchi *et al.*, 1995).

Cytokines that signal through the common γ -chain(γc) receptor, particularly *IL-2*, *IL-4* and *IL-7*, have received much attention as potential immunotherapeutics in promoting anti-tumor immune responses (Overwijk and Schluns, 2009; Rochman, Spolski *et al.*, 2009), having both innate and adaptive immunity especially in lymphocyte development, where they influence polarization, proliferation and survival (Rochman *et al.*, 2009). In preclinical studies, each of these cytokines alone or in combination has shown efficacy in enhancing tumor immunity (Capitini *et al.*, 2009). *IL-2* have been shown to significantly increase the proliferation, survival and

the expression of co-stimulatory by CD8⁺ T-cells in a study with mice (McNamara *et al.*, 2014).

Polysaccharides affect the phenotypic and functional maturation of dendritic cells from human CD14 cells, and stimulates expression of *IL-6* (Hsieh and Wu, 2001; Liu, Fung *et al.*, 1996). Interleukin-6 cytokine however induce reactions leading to the stimulation of T-cell cytotoxicity against tumor cells, intensification of antibody production by B-cells. The antitumor activity of Polysaccharide K relies on its ability to stimulate T-cells and antigen-presenting cells, which allows efficient recognition and destruction of tumor cells (Tzianabos *et al.*, 2000).

TNF- α was originally identified for its capacity to induce hemorrhagic necrosis of solid tumors with other results suggesting that prolonged continuous *TNF- α* blockage in patients may have long-term complications, including potential tumor development or progression (Kassiotis *et al.*, 2001). TNF- α or its receptors were reported to affect certain phases of the immune process, including innate immune activation or Dendritic cells maturation/recruitment (Lee *et al.*, 2005; Trevejo *et al.*, 2001), T-cell priming (McDevitt *et al.*, 2002), T-cell proliferation, T-cell recruitment, T-cell function or pathogen clearance (Boyman *et al.*, 2004). In some studies the exact mechanism is not thoroughly defined, but TNF- α is shown to be critically required for efficient T-cell immune responses (Herrera *et al.*, 2000; Hu *et al.*, 2002).

2.8 Intestinal epithelial cells (IEC) and *IL-7* in cancer management

Intestinal epithelial cells (IEC) have been implicated in *IL-7* synthesis in several cancer studies with some of the studies indicating that IEC may play an important role in mucosal immune responses by helping to regulate intestinal intraepithelial lymphocytes (IEL) (Hisataka *et al.*, 2005). The importance and usefulness of cytokine *IL-7* as a tool in immunologic activities has been demonstrated before (Gagnon *et al.*, 2010).

In a study by Mwitari *et al.*, using mushroom polysaccharides extracts of *Trametes* genus on IEC-6 cells, showed that these cells can be stimulated by the extracts to up regulate *IL-7* mRNA (Mwitari *et al.*, 2014). Another research showed that IEC are the major source of *IL-7* in the murine intestine (Shalapour *et al.*, 2010).

2.9 Cell Proliferation and its control

Cell proliferation is the process that results in an increase in the number of cells and is responsible for cell growth and replacement of dead or worn out cells. It is defined as the balance between cell division and cell loss that occur through differentiation or cell death. It is a fundamental biological process of life. It requires energy, nutrients and biosynthetic activity to duplicate all cell components during each step through the cell cycle (DeBerardinis *et al.*, 2008).

Cell proliferation is strictly controlled by many regulatory mechanisms at different points during the cell cycle. Severe genetic damage can occur if cells proceed to the next phase of the cell cycle before the previous phase is correctly completed. For instance, when S-phase cell is induced to undergo mitosis by fusion to a cell in mitosis, the Mitosis Promoting Factor (MPF) forces the chromosomes to condense. Since the replicating chromosomes are fragmented by this process, the premature entry into mitosis results in unreplicated DNA. Therefore, transition from one cell cycle phase occurs orderly and is regulated by check/restriction point (Cooper and Ganem, 1997).

There are four restriction/check points that control cell cycle; G1 checkpoint causes cell cycle arrest if there is DNA damaged by genotoxicity such as radiation or chemical modification. The S phase checkpoint ensures that cells that have not replicated their entire chromosomes do not enter mitosis. The G2 checkpoint prevents the initiation of mitosis before completion of S phase. This checkpoint ensures that incomplete replicated DNA is not distributed to daughter cells. Also the genome is replicated once in a cell cycle by preventing cells from re-entering S phase. Restriction in G2 allows repair of DNA double-strand breaks before mitosis.

Mitotic Spindle Arrest checkpoint monitors the presence of improper mitotic spindle assembling. It inhibits activation of the Anaphase Promoting Complex (APC) polyubiquitination system that leads to degradation of the anaphase inhibitor. As a result of this arrest MPF activity remains high, chromosomes remain condensed, and the nuclear envelope does not re-form (Cooper and Ganem, 1997; Lodish *et al.*, 1995)

2.10 Cell death mechanism of *Trametes versicolor* extract

Although the mechanism for direct inhibition of tumour cell proliferation induced by *T. versicolor* extracts has not yet been completely delineated, some recent studies have shown that extracts of *T. versicolor* and other mushrooms can induce apoptosis of leukemia, breast and cervical tumor cell lines (Chow *et al.*, 2003; Han *et al.*, 2001; Hsieh *et al.*, 2002). Other herbal medicines, such as sophorane from *Sophora subprostrata* and mistletoe lectin from *Viscum album* can induce apoptosis of HL-60 and U937 leukemic cell lines (Kajimoto *et al.*, 2002; Kim *et al.*, 2003). Apoptosis is a fundamental mechanism of cell death that can be engaged by a variety of cellular insults.

During apoptosis, there is a rapid reduction in the cellular volume followed by chromatin condensation, associated with characteristic internucleosomal DNA cleavage. This results in the production of nucleosomes of DNA fragments complexed with core histones, which are discrete multiples of an 180 bp subunit (Kerr *et al.*, 1972). One of the major modes of action of chemotherapeutic anti-cancer drugs on malignant cells is via the induction of apoptosis (Makin and Hickman, 2000; Sellers and Fisher, 1999). In some cases, the level of drug-induced apoptosis was shown to act as a parameter for predicting the tumor response *in vivo* (Frankfurt and Krishan, 2003; Johnson *et al.*, 2001).

2.11 Cancer Metastasis

Cancer Metastasis is a process that cancer cells spread and migrate from the original site to other parts of the body through the bloodstream, the lymphatic system and invasion resulting to metastatic tumors. A tumor is considered metastatic when it has the capacity to spread beyond its original site and invade other tissue in the body. The steps involving metastases of cancer include; separation of cancer cells from the primary tumor, invasion to the other tissues and basement membranes, entry and survival in the circulation, lymphatic tissues, peritoneal space and colonizing distant target organs (Kerr *et al.*, 1972). The routes for spread of cancer cells include; Hematological route that involve spread of cancer cells through the blood stream to distant organs in the body. Lymphatic route that allows spread of cancer cell to lymphoid tissues and to other parts of the body. Transcoelomic routes that involve spread of malignancy into body cavities through penetrating the surfaces of pleural, peritoneal and pericardial cavities. Metastasis of cancer complicates cancer treatment and increases the rate of patient death (Berridge *et al.*, 2005).

2.12 Hepatocellular Carcinoma (HCC)

Liver cancer is the third most common cause of death in the world. Hepatocellular carcinoma (HCC) accounts for 95% of the liver cancers. It is an epithelial cancer emanating from hepatocytes (Chow *et al.*, 2003; Han *et al.*, 2001; Hsieh *et al.*, 2002). The incidence of HCC is rising in many countries with an estimated population of new cases of over 500,000 annually and an incidence of between 2.5% and 7% of patients with liver cirrhosis yearly (Kerr *et al.*, 1972). Due to a high rate of relapse, HCC has a very poor prognosis, with a 5-year survival rate of 3–5%. The available HCC drugs are unaffordable, toxic and have severe side effects. Thus it is important to develop a new agent which effectively treats, with less toxicity, minimal or no side effects and prevents recurrence of HCC. Long-term prognosis of patients with HCC is poor, and survival is mainly affected by the occurrence of metastases (Chow *et al.*, 2003; Han *et al.*, 2001; Hsieh *et al.*, 2002).

2.13 Conventional Treatment of Cancer

2.13.1 Chemotherapeutic Drugs

Chemotherapeutic agents target the process of cell division because tumor cells are more likely to be dividing more rapidly than normal cells. However their activity is not specific and they are associated with significant toxicity and severe side effects. Some therapeutic agents are discussed here below;

2.13.1.1 Alkylating Agents

Alkylating agents are highly reactive compounds that are dose-dependent and non-specific drugs that target the cell cycle. Most alkylating agents are bipolar having two groups capable of reacting with DNA. Their mode of action is through replacement of a hydrogen atom from the DNA molecule by an alkyl radical (R-CH₂-CH₂+) through a covalent linkage. Alkylation results to cleavages in DNA and in cross links of DNA double helix, interfering with DNA replication and transcription. The cell eventually dies because it cannot replicate and triggers apoptosis (Cooper and Ganem, 1997).

Clinically useful alkylating agents are categorized in five classes namely; nitrosoureas (carmustine, lomustine and semustine), ethylene derivatives (thiotepa and mitomycin) alkyl alkane sulfonates (busulfan), triazine derivatives (dacarbazine) and nitrogen mustard derivatives (ifosfamide and cyclophosphamide) (DeBerardinis *et al.*, 2008).

Experiments have demonstrated that alkylation is the cause for the main toxic effects of alkylating agents. Alkylating agents have different pharmacological effects that include mutagenesis, interference with mitosis, immunosuppression and carcinogenesis (Cooper and Ganem, 1997).

2.13.1.2 Antimetabolites

Antimetabolites directly interact with special enzymes, leading to the inhibition of the enzyme or subsequent synthesis of an aberrant molecule that cannot function normally. They are structural analogues to normal metabolites required for the synthesis of purine and pyrimidine. Upon introduction of an antimetabolite into the cell to substitute a physiological substance such as vitamins, nucleosides or amino acids, they result in inhibition of the cell processes. Some antimetabolites can be incorporated directly into DNA or RNA altering the process of replication, transcription and protein synthesis. Most antimetabolites are phase-specific and act during the S-phase of the cell when the DNA is being synthesized (DeBerardinis *et al.*, 2008).

There are three main classes of antimetabolites namely; Folic acid antagonists for example Methotrexate which competitively inhibits dihydrofolate reductase responsible for the formation of tetrahydrofolate from dihydrofolate, Pyrimidine analogues such as fluorouracil. They are analogues to pyrimidine altering nucleic acid synthesis. They inhibit enzymes involved in DNA biosynthesis leading to eventual cell death (Shalapour *et al.*, 2010).

2.13.1.3 Topoisomerase Inhibitors

Topoisomerase enzymes are excellent molecular enzymes that play a critical role in DNA topology and chromatid segregation. Topoisomerase enzymes are necessary due to the large size of eukaryotic DNA, double helix structure of DNA and association with histones. For DNA replication, transcription and recombination, double helical structure of the DNA must be relaxed. During DNA replication, the two strands of the DNA are unlinked by topoisomerase. During transcription, the translocation of RNA polymerase generates supercoiling tension in the DNA that should be relaxed. The unwinding of DNA is catalyzed by topoisomerases by creating transient breaks in the DNA (Shalapour *et al.*, 2010). There are two types topoisomerase enzyme namely; Topoisomerase I that binds to DNA molecule cuts

one strand and generates a covalent phosphoester bond between the cleaved phosphate on the DNA and a tyrosine residue in the enzyme. Topoisomerase II cuts both strands of a double-stranded DNA, pass another portion of the duplex by the generated cut, and religate the cut region. Topoisomerase inhibitors (TI) are designed to target topoisomerase enzymes because of their critical activity on replication and transcription of genetic information. Topoisomerase inhibitors act during G2 phase of cell cycle preventing cells from entering mitosis (Gagnon *et al.*, 2010).

Topoisomerase inhibitors are grouped into two broad classes; Topoisomerase I inhibitor such as camptothecin, Irinotecan and topotecan. Topoisomerase I inhibitor binds to the topoisomerase I–DNA complex making it stable thus inhibiting DNA replication. Topoisomerase II inhibitor such as etoposide and vespindolol binds to topoisomerase II–DNA complex stabilizing it thus preventing DNA replication (Hisataka *et al.*, 2005).

2.13.1.4 Antitumor Antibiotics

Antitumor antibiotics are natural products derived cytotoxic agents that act by formation of stable complexes with the nucleic acids inhibiting DNA and RNA synthesis. Clinically useful antitumor antibiotics in practice include; Bleomycin, Actinomycin-D and Antracycline antibiotics (Herrera *et al.*, 2000; Hu *et al.*, 2002).

Bleomycin is a family of glycopeptide-derived antibiotics isolated from species. Bleomycins are highly active in the G₂ and M phases of the cell cycle. They react with the DNA and cause an oxidative cleavage through production of free radicals (Boger and Cai, 1999). Bleomycin activity is limited due to altered cellular uptake, damage to normal cell DNA causing DNA lesions and inactivation by cellular enzymes (Lee *et al.*, 2005; Trevejo *et al.*, 2001).

D actinomycins are polypeptide antineoplastic antibiotics isolated from species of *Streptomyces* genus. They inhibit cell proliferation nonspecifically through formation of stable complexes with double-stranded DNA by intercalating with

deoxyguanosine and deoxycytosine residues. Actinomycin D activity also results to single-strand DNA breaks. Their activity results in ultimate reduction of DNA, RNA and protein synthesis. They are useful in the treatment of embryonic rhabdomyosarcoma, testicular tumors and pancreatic cancer (Kassiotis *et al.*, 2001).

Antracyclines are another class of anticancer antibiotics isolated from *Streptomyces peuceetius* discovered in 1960s. They play a critical role in cancer treatment since they are among the most effective anticancer treatments and have a broad spectrum of activity against many types of neoplastic disease (Tzianabos *et al.*, 2000).

Antracycline mode of action involves inhibition of DNA and RNA synthesis by intercalation between basepairs. This results to generation of free radicals that cause damage to cell membrane, proteins and DNA. They also inhibit topoisomerase enzyme preventing relaxation of supercoiled blocking DNA replication and transcription. Some of the antracyclines in clinical use include; doxorubicin and daunorubicin. A limitation in application of antracycline is severe cardiotoxicity that result to cardiomyopathy and congestive heart failure (Capitini *et al.*, 2009).

2.13.1.5 Metals

Application of metals and metal compounds in medicine has been in existence for several years throughout human history. Metals are important components of cellular systems and they play critical roles in biochemical processes in living organisms. Metals versatility in their activity is as a result of excellent characteristics such as, redox activity, reactivity to organic substrates and variable coordination modes. Alteration of some of essential metals during normal biological reactions has resulted in development of many pathological conditions such as cancer (Overwijk and Schluns, 2009; Rochman, Spolski *et al.*, 2009). Therefore, due to their critical essential role in biological systems metals and metal

compound have been researched with the objective of developing anticancer metallodrugs. Such metals in clinical application include;

2.13.1.5.1 Platinum Agents

Platinum anticancer agents include; cisplatin, oxaliplatin and carboplatin. Cisplatin is an organic metal that mechanism of action involves loss of a chloride ion after diffusing into the cell. This enables Cisplatin to cross-link with the DNA resulting to DNA intra- and interstrand eventually resulting in inhibition of DNA replication, RNA and protein synthesis. Cisplatin has been used to treat cervical, ovarian, non-small cell lung carcinoma, head and neck and testicular cancers. Its clinical use is hindered by increased toxicity such as ototoxicity, nephrotoxicity and resistance (Hirose *et al.*, 1990; Nio *et al.*, 1991; Noguchi *et al.*, 1995). Oxaliplatin is a chemotherapeutic platinum anticancer drug used in management of colon cancer (Pellegrini *et al.*, 2009). Oxaliplatin consist of a complex of a platinum atom, oxalate and diaminocyclohexane (DACH) group. It forms highly reactive complexes of platinum that inhibit DNA replication and transcription. *In vivo* and *in vitro* studies have demonstrated that oxaliplatin has broader spectrum of antiproliferative activity on several tumor model including; colon cancer and safer than cisplatin and carboplatin (Schluns *et al.*, 2000).

Carboplatin is a platinum-based antitumor agent structurally similar to cisplatin, but have organic carboxylate group instead of chlorine as in cisplatin. The carboxylate group results to increased water solubility, slower excretion and breakdown. It is less nephrotoxic and neurotoxic, but causes severe toxicity myelosuppression. It used in management of ovarian carcinoma, head and neck, lung, endometrial, bladder, esophageal, breast and cervical cancers, central nervous system or germ cell tumors; osteogenic sarcoma (Pellegrini *et al.*, 2009).

2.13.1.5.2 Arsenic

Arsenic is a naturally occurring metal found in conjunction with many minerals such as sulfur and as a pure elemental crystal. It has been used since ancient time to treat a wide variety of human diseases. In Chinese traditional medicine arsenic is used to treat cancer and other conditions. Arsenic has been previously used to treat hematological malignancies and is currently approved for treatment of refractory promyelocytic leukemia (Tao *et al.*, 2006; Zhang *et al.*, 2007).

The antitumor mechanism of action of arsenic through apoptosis through induction of p53-dependent G1 or G2/M cell cycle arrest. Despite the application of arsenic in cancer management there are reports of drug resistance, cardiotoxicity, and APL differentiation syndrome. There are other various metal such as iron, vanadium, gold, titanium, involved in cancer treatment whose discussion cannot be exhausted in this forum (Borchers *et al.*, 1999).

There has been an impressive progress in development of anticancer chemotherapy drugs but the limitation in safety profile and efficacy of chemotherapeutic anticancer agents cannot be ignored. Anticancer chemotherapeutic agents affect rapidly dividing cells without specifically targeting cancer cells. Further they only affect cell ability to divide and have minimal effect on other effects of tumour progression such as metastases and tissue invasion. Additionally, anticancer chemotherapy has notable severe side effects such as myelosuppression, alopecia, mucositis, neurotoxicity, nephrotoxicity, hair loss, skin irritation, anemia, infertility, nausea and vomiting (Borchers *et al.*, 1999). Therefore, there is a need to research and develop new anticancer drugs with minimal limitation of application such as less toxicity, highly selective, less severe side effects and affordability.

2.13.2 Surgery

Cancer surgery is a procedure of cancer management that involves removal of tumors and surrounding tissue through invasive incision in the body. Historically

surgical removal of localized tumors has been the first line of cancer treatment. Through technological improvements surgery procedures for destroying tumors have been significantly improved. Technological Progress made in ultrasound, magnetic resonance imaging, computed tomography and positron emission tomography have replaced most invasive surgical operation operations (Kidd, 2000).

Surgery objective is to either remove the tumor or the entire organ depending on the level of spread though it is limited in case of vital body organs. Surgical procedures for cancer treatment include prostatectomy for prostate cancer, mastectomy for breast cancer and lung cancer surgery for non-small cell lung cancer. Tumor oncology is limited by contradictions such as recurrence and metastasis (Kidd, 2000).

2.13.3 Radiotherapy

Cancer radiotherapy is a treatment measure that involves use of ionizing radiation to kill cancer cells. Radiation therapy is commonly applied to the cancerous tumor because has the ability to inhibit cell proliferation and kill cancer cells. Ionizing radiation mode of action is by damaging the DNA of cancerous tissue leading to cell death. The DNA damage is caused by one of two types of energies namely photon and charged particle that causes direct or indirect ionization of the atoms that make up the DNA molecule (Kobayashi *et al.*, 1993).

There are several radiation therapies in application that include; Conformal proton beam therapy used to kill tumor cells instead of X-rays, Stereotactic surgery and Stereotactic therapy that is used to treat common brain tumors, Intra-operative radiation therapy used for adjacent tissue after tumor has been removed surgically. Radiotherapy is limited by the fact that ionizing radiations have got long-term effects of causing cancer in non-cancerous cells. It is associated with severe side effects such as vomiting, oedema and infertility (Kobayashi *et al.*, 1993).

2.14 Economics of Cancer Management

The cost of treatment of cancer is high and unaffordable for patients worldwide. A case study in Canada reflects one month of a cancer treatment and management could cost approximately 10 million Kenya Shillings per patient. According to the study, chemotherapy contribute to more than 31% of the costs other costs being distributed among hospitalization (27%), Physician services (12%), diagnostic tests (2%), radiotherapy (5%), home care (5%), and prescription drugs (5%) (Yan *et al.*, 2013). The implication of prohibiting cancer drug prices is one of the calamities that have befallen cancer victims. There is need to investigate alternative affordable, less toxic, sources of treatment to cancer.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

Laboratory based experimental study design method was used.

3.2 Study site

The study was done at KEMRI (Kenya medical research institute) laboratories, Nairobi. Mushroom polysaccharide extraction, antiproliferative and Real Time-PCR assays were performed at CTMDR (Center for Traditional Medicine and Drug Research).

3.3 Ethical considerations

Ethical approval for carrying out the study was sought and obtained from the KEMRI Scientific and Ethical Review Unit (SERU), KEMRI/SERU/CTMDR/009/3045 (Appendix VIII). This study however did not involve animals or human subjects and all standard operating procedures were followed and safety measures observed.

3.4 Reagents used

The reagents used in this study included; RPMI1640, Fetal Bovine serum, Phenol red, L-glutamine, HEPES, Trypsin, sterile hand gloves, Dimethyl sulfoxide (DMSO), 5ml sterile graduated pipettes, Sequence specific primers (ILRI), RNA extraction kit, Reverse transcriptase, one step Syber green supper mix, MTT reagent, Penicillin/streptomycin, 96 well plate flat bottomed with cover, Culture flasks: (T75 and T25), Optical 96 well plates, Ethanol analar, Trypan, Micro pipettes, Eagles MEM medium, Pippete tips 20, 100, 200 μ l. These reagents were procured from Sigma Aldrich, Germany., streptomycin and penicillin were procured locally from local pharmacies while Tamoxifen was provided by CTMDR, KEMRI.

3.5 Equipment used

The following equipment were used in this study; CO₂ Incubator (Sanyo, Japan), water bath (Sakura, Japan), Liophilizer (Edward, Britain), Electric Mill (Edward, Britain), Weighing Balance (Mettler AC 1000, Switzland), Inverted Microscope (Nikon, Japan), Biosafety Cabinet ClassII (Esco, Japan), Deep Freezer (Sanyo, Japan), ELISA plate reader (Labsystems, Japan) and Hemocytometer (Esco, Japan), Real Time PCR analyzer, QuantStudio 5 (Africa Biosystems).

3.6 Cell lines

The cell lines used in this study were Breast cancer cell line 4T1; (ATCC[®] CRL-2539[™]), hepatocellular carcinoma cell line HCC; (ATCC[®] CRL-2326[™]), human prostate cancer cell line DU145, (ATCC[®] HTB-81[™]), African green monkey kidney cell line Vero; (ATCC[®] CCL81[™]) and rat normal intestinal epithelial cell line IEC-6; (ATCC[®] CRL-1592[™]) cells donated by the Center for Traditional Medicine and Drug Research (CTMDR) at Kenya Medical Research Institute (KEMRI).

3.7 Mushroom Collection and preparation

Trametes versicolor were botanically identified by a botanist and collected from Arabuko Sokoke forest at the Kenyan coast in Kilifi county and evaluated at KEMRI laboratories. Permission was sought from the Kenya Wildlife Service (KWS) and the Forestry service before collecting the mushroom from the forest. Voucher specimens were deposited at the University of Nairobi herbarium. The mushroom *T. versicolor* were cut into small pieces and dried at room temperature. The dried materials were ground in an electric Mill (Edward, Britain), to a fine powder, packed in bags, and stored at room temperature until use.

3.8 Aqueous extraction of the mushrooms

Aqueous extraction of *T. versicolor* was done as described by Mizuno (Mizuno, 1999) with modifications. A weight of 100g of the previously prepared powder of the

mushroom *T. versicolor* was measured using an electrical beam balance and put into a 500ml conical flask. Double distilled water was added until the sample was completely submerged. The mixture was then transferred into a water bath at 80°C for 3 hours and cooled to room temperature, filtered using Whatman no.1 filter paper into a clean sterile 1000ml conical flask and the solvent removed using a freeze drying machine. The resulting extract was weighed and stored in an airtight bottle at -20°C until use.

3.9 Extraction of polysaccharides

Extraction of polysaccharides was done as described by Staub (Staub, 1965) and Chihara *et al* (Chihara *et al.*, 1970). One hundred grams of the previously prepared powder of the mushroom *T. versicolor* was measured using an electrical beam balance into a 500ml conical flask. Double distilled water was added until the sample was completely submerged. The mixture was then transferred into a water bath at 80°C for 18 hours and cooled to room temperature, filtered using whatman no. 1 filter paper.

Five volumes of ethanol were added to precipitate crude polysaccharides. The precipitate was recovered by centrifugation and washed successively with ethanol, followed by drying at 50°C, to yield the crude polysaccharide. The resulting extract was weighed and stored in an airtight bottle at 4°C until use.

3.10 Qualitative phytochemical analysis of *Trametes versicolor* aqueous extract

A qualitative phytochemical analysis of the crude *T. versicolor* aqueous extract was performed using standard protocols as described by (Odebiyi and Sofowora, 1977).

3.10.1 Test for alkaloids

A weight of 0.5g of the extract was stirred with 5ml of 1% aqueous hydrochloric acid for two minutes on a steam water bath. The mixture was filtered and few drops of

Dragendorff's reagent added. The sample was then observed for color change or turbidity to draw inference.

3.10.2 Test for saponins

The persistent frothing test for saponin was used (Odebiyi & Sofowora, 1977). A volume of 30ml distilled water was added to 1g of the mushroom extract. The mixture was vigorously shaken and heated on a steam water bath. The sample was observed for the formation of stable froth to draw inference.

3.10.3 Test for phlobatannins

A weight of 0.2g of the mushroom extract was dissolved in 10ml of distilled water and filtered. The filtrate was then boiled with 2% hydrochloric acid solution and observed for deposition of red precipitate which indicates the presence of phlobatannin.

3.10.4 Test for tannins

The method of Evans (Evans, 2009) was adopted where 0.5g of the mushroom extract was dissolved in 5ml of distilled water, then, boiled gently and cooled. One ml of the solution was dispensed in a test tube and 3 drops of 0.1% ferric chloride solution were added and observed for brownish green or blue black colouration which indicates the presence of tannins.

3.10.5 Test for terpenoids

The Salkowski test was used. A weight of 5g of the extract was dissolved in 5ml distilled water. The mixture was then added in 2ml of chloroform, and 3ml concentrated Sulphuric acid was carefully added to form a layer. The solution was then observed for reddish brown colouration which confirms the presence of terpenoids.

3.10.6 Test for steroids

A volume of 2ml acetic anhydride was added to 0.5g of the mushroom extract and filtered. Two ml of Sulphuric acid was added to the filtrate and observed for colour change from violet to blue or green, which indicates the presence of steroids.

3.10.7 Test for flavonoids

A volume of 5ml diluted ammonia solution was added to 0.5g of the mushroom extract dissolved in 5ml distilled water. This was then followed by the addition of a concentrated sulphuric acid. The solution was observed for yellow colouration that disappears on standing to confirm the presence of flavonoids.

3.10.8 Test for anthraquinones

A weight of 0.5g of the mushroom extract was shaken with 10ml of benzene, filtered and 5ml of 10% ammonia solution added to the filtrate. The mixture was then shaken and observed for the presence of pink red or violet colour in the ammonia layer which indicates the presence of free anthraquinones.

3.11 Antiproliferative activity

3.11.1 Cell culture

Cells were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), L-Glutamine, 100µg/ml penicillin and 100µg/ml streptomycin and incubated at 37°C in a humidified incubator at 5% CO₂.

3.11.2 The MTT Assay

The antiproliferative activity of the aqueous mushroom extract was evaluated using MTT (3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) assay (Sigma, USA). The assay detects the reduction of MTT by mitochondrial dehydrogenase to

blue formazan product, which reflects the normal function of mitochondria and cell viability (Lau *et al.*, 2004). Formazan, which is an insoluble yellow colored product can be measured spectrophotometrically (Mosmann, 1983; Patel *et al.*, 2009) and the activity of mitochondrial dehydrogenase enzyme to produce formazan is directly and inversely proportional to the level of cell viability and inhibition respectively (Berridge *et al.*, 2005).

The cell monolayer was treated with trypsin 0.25%-EDTA 10mM after they had reached 90% confluence. A volume of 5ml of growth media was added to neutralize the trypsin enzyme. The cell suspension was transferred to centrifuge tubes and centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and cells resuspended in 5ml culture media and cell density was determined using trypan blue exclusion assay.

The five types of cell lines were seeded independently in 96-well plates with the final volume 100µl containing 24,000 cells per well, with the cell density determined using trypan blue exclusion assay. The fourth, eighth and twelfth columns of the 96 well plates contained 100µl RPMI-1640 with no cells as they were treated as blanks for each cell line. The plates were incubated at 37°C for 48 hours. The *T. versicolor* aqueous extract in phosphate buffered saline (PBS) was proportionally diluted with RPMI-1640, and 50µl of each solution was added to triplicate wells in the concentrations: 1000µg/ml, 333.33µg/ml, 111.11µg/ml, 37.04µg/ml, 12.35µg/ml, 4.12µg/ml and 1.37µg/ml with the last row of the 96 well plate left untreated to serve as a cell control. Tamoxifen was included as a positive control drug in the same concentrations as the aqueous extract. After 48 hours incubation in 5% CO₂ humidified environment at 37°C, a volume of 10µl of PBS containing 5mg/ml MTT was then added into each well including all the controls and plates further incubated for 3 hours. The medium was removed and 100µl DMSO added into each well. After the plates were shaken mildly, the absorbance of the samples was measured at 560nm with a Multiskan Spectrum Microplate Spectrophotometer.

3.11.3 Determination of Cell proliferation

Determination of cell proliferation was achieved using the formular developed by (Patel *et al.*, 2009) and (Awasare *et al.*, 2012) as follows;

$$\text{Proliferation rate} = \frac{At-Ab}{Ac-Ab}$$

$$\text{Percentage viability} = \frac{At-Ab}{Ac-Ab} \times 100$$

$$\text{Percentage inhibition} = 100 - \left(\frac{At-Ab}{Ac-Ab} \times 100 \right)$$

Where;

At = Absorbance value of test compound

Ab = Absorbance value of Blank

Ac = Absorbance value of negative control (untreated Cells)

3.12 Extraction of RNA, quantification, purity determination, RT-PCR set up and gene amplification

3.12.1 Extraction of RNA

Extraction of RNA was done according to pureLink Mini Kit (Life Technologies, USA). The IEC-6 cells (ATCC) were cultured in two T25 culture flasks in RPMI media with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic PS

(Penicillin and Streptomycin) at a cell density of 4×10^6 cells per flask. On the following day, 500 μ l of 100 μ g/ml the mushroom extract was added to one of the flasks while the other flask was left as a negative control. All the flasks were further incubated for 48 hours at 37°C. Cells were treated with trypsin to detach them and were transferred into RNase-free centrifuge tubes and centrifuged at 2,000 revolutions per minute for 5 minutes at 4°C to pellet. The media was discarded from the tube and 600 μ l of lysis buffer added. The tubes were vortexed at high speed until the cell pellet was completely dispersed and the cells appear lysed. The lysate was then passed 10 times through a 21-gauge needle attached to an RNase-free syringe. A volume of 600 μ l 70% ethanol was added to each volume of cell homogenate, vortexed to mix thoroughly. A volume of 700 μ l of the sample was transferred into a spin cartridge (inserted in a collection tube) and centrifuged at 12,000 *rpm* for 15 seconds at room temperature.

The flow-through was discarded and the spin cartridge reinserted into the same collection tube. The remaining volume of cell homogenate was also transferred into the spin cartridge and centrifuged at 12,000 *rpm* for 15 seconds at room temperature then the flow-through and the collection tube were discarded. The spin cartridge was inserted into a new collection tube. A volume of 700 μ l of wash buffer was added to the spin cartridge and centrifuged at 12,000 *rpm* for 15 seconds at room temperature. The flow-through and the collection tube were discarded and the spin cartridge placed into a new collection tube. A volume of 500 μ l wash buffer II was added to the spin cartridge and centrifuged at 12,000 *rpm* for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge reinserted into the same collection tube.

The spin cartridge was centrifuged at 12,000 *rpm* for 2 minutes to dry the membrane where the RNA attaches to. The collection tube was discarded and the spin cartridge inserted into a recovery tube, then 30 μ l RNase-Free water added to the center of the Spin Cartridge and incubated at room temperature for 1 minute. The spin cartridge was centrifuged for 2 minutes at 12,000 *rpm* at room temperature to elute the RNA

from the membrane into the Recovery tube. The extracted RNA was labeled and immediately stored at -80°C.

3.12.2 Quantification and purity determination of RNA.

Quantification and purity determination of RNA was done using Nanodrop ND-2000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) which measures the optical difference (OD) at 260/280nm and concentration in ng/μl. RNase free water was used to blank the instrument before measuring the concentration of the RNA samples (both the treated and the untreated samples). The concentration of RNA (ng/μl) obtained was used to calculate the volume of RNA used for real time PCR at 2μg per reaction. The ratio of the absorbance (A_{260}/A_{280}) was used to determine the RNA purity where RNA with a ratio of between 1.8-2.1 was regarded as pure.

3.12.3 One step Quantitative RT-PCR set up

Sybr Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) was used to determine the levels of IL-7 mRNA relative to that of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene which was used as an internal control. All the reagents including the reconstituted primers were put on ice during the procedure. A volume of 25μl Sybr Green reaction mix was put in the optical 96 well plate (Thermo Scientific). Reverse transcription buffer 4μl, 1μl reverse transcription enzyme/platinum *Taq* Mix, forward and reverse gene specific primers (10μM) 1μl each, 18μl of RNase-free water and 5μl of RNA (2μg) were added. The volume of RNA used was calculated using the concentration of RNA (ng/μl) obtained from the Nanodrop. The plate was then covered with an adhesive cover.

The gene specific primers used were *IL-7* with primers sequence 5' to 3' forward GCAACCCTGTCATCTGCAAT and reverse TTATCCATCACCAGGAGCCC, GAPDH forward AGACAGCCGCATCTTCTTG and reverse TGACTGTGCCGTTGAACTTG (synthesized by ILRI). All primers were designed

using primer 3 and reconstituted according to manufacturer's instructions and a working solution of 10 μ M prepared by mixing 10 μ l of the reconstituted primers with 90 μ l of RNase free water. The PCR was set in triplicate for each target gene, with the control wells of the experiment left with no RNA.

3.12.4 Reverse Transcription and Gene amplification

Reverse transcription and gene amplification was performed using QuantStudio 5 real time PCR analyzer (Applied biosystems). The analyzer was pre-warmed to 50°C and reaction conditions set as: 30 minutes at 50°C (reverse transcription), 1 minute at 95°C (initial pcr activation), 15 seconds at 94°C (denaturation), 30 seconds at 60°C (annealing), 1 minute at 68°C (extension), 10 minutes at 68° C (final extension). Reverse transcription step was set to run for one cycle while gene amplification step (denaturation, annealing and extension) was set to run for 40 cycles. The plate was placed in the PCR analyzer immediately after adding the amount of RNA and the machine started.

3.13 Data management and analysis

The data collected in this study was both qualitative and quantitative. Data generated from *in vitro* antiproliferative activity and gene expression levels of *T. versicolor* extract was quantitative data while the data from phytochemical analysis of the *T. versicolor* extract was qualitative.

Results were expressed as the mean \pm standard error of the mean (SEM) for all the different concentrations of the mushroom extracts. Statistical differences between the concentration means were assessed by One-way ANOVA and the means subjected to Tukey's post-hoc test for pairwise comparison with values of $P \leq 0.05$ considered as statistically significant. Generated dose response curves were used to determine IC₅₀ values. Student's unpaired t-test was then used to compare the IC₅₀ values of *T. versicolor* to that of Tamoxifen for statistical significance.

Expression levels of IL-7 mRNA was calculated in fold change using the mean CT values of IL-7 relative to that of GAPDH mRNA using double delta CT method ($2^{-\Delta\Delta C_T}$) (Schmittgen *et al.*, 2000). The results were presented in a standard error bar graph. The mean CT values for mRNA *IL-7* from the treated and untreated IEC-6 cells were subjected to a two-sample independent t-test for assessment of statistical significance at 95% confidence interval. Data analysis was done using Excel spread sheets and Minitab statistical software version 17.0.

CHAPTER FOUR

RESULTS

4.1 Qualitative phytochemical analysis of *T. versicolor* aqueous extract

Results from the phytochemical analysis of the aqueous extracts of the Kenyan *T. versicolor* mushroom revealed the presence of saponins, tannins, steroids, terpenoids and flavonoids while anthraquinones, alkaloids and phlobatannins were absent (Table 4.1)

Table 4.1: Qualitative phytochemical screening of *T. versicolor* aqueous extract

Phytochemical	<i>T. versicolor</i> aqueous extract
Saponin	+
Tannin	+
Steroid	+
Alkaloid	-
Terpenoid	+
Flavonoids	+
Anthraquinone	-
Phlobatannin	-

The (+) sign denotes presence of phytochemicals while (-) sign denotes absence of phytochemicals.

4.2 Antiproliferative potential of *T. versicolor* aqueous extract

This study evaluated the antiproliferative potential of *T. versicolor* aqueous extracts on five cell lines namely 4T1, DU145, HCC, IEC-6 and Vero cell lines. The *T. versicolor* aqueous extract at 1.37 to 1000 μ g/ml exhibited dose-dependent inhibitory effects on the proliferation of DU145 and 4T1 cells with more than 90% and 70% suppression respectively at the highest concentration (Table 4.2). However, the extract induced a low level of suppression on the proliferation of HCC, normal IEC-6 and normal Vero cells showing values of 41.19%, 43.85% and 42.64% respectively at the highest concentration (Table 4.2).

Table 4.2: Percentage viability and percentage inhibition for effect of aqueous extract of *T. versicolor* mushroom on 4T1, DU145, HCC, IEC-6 and Vero cell lines.

Concentration (μ g/ml)	% Viability					% Inhibition				
	4T1	DU145	HCC	IEC-6	Vero	4T1	DU145	HCC	IEC-6	Vero
Untreated cells	100	100	100	100	100	0	0	0	0	0
1.37	96	84	95	92	97	4	16	5	8	3
4.12	94	74	90	89	94	6	26	10	11	6
12.35	83	68	89	86	92	17	32	11	14	8
37.04	70	52	85	70	88	30	48	15	30	12
111.11	60	41	81	65	86	40	59	19	35	14
333.33	31	35	65	60	74	69	65	35	40	26
1000.00	22	9	59	56	57	78	91	41	44	43

Results are expressed as the Mean of three independent experiments of three wells each which were calculated from the OD values obtained from a Spectrophotometer.

Table 4.3 shows the concentrations producing 50% growth inhibition (IC₅₀) of the *T. versicolor* extract on the five cell lines of which DU145 proliferation was most potently suppressed with the lowest IC₅₀ value (71.16µg/ml) followed by 4T1 cells (IC₅₀ = 188.53µg/ml) after incubation with the *T. versicolor* extract. The *T. versicolor* extract did not record any IC₅₀ values against HCC, Vero and IEC-6 cell lines even at the highest concentration (1000µg/ml). The IC₅₀ values of *T. versicolor* against all cell lines used were statistically compared to those of the reference drug (Tamoxifen) where results revealed that there was no statistical difference between the IC₅₀ values of *T. versicolor* extract and Tamoxifen against DU145 and 4T1 cells (Table 4.3).

Table 4.3: Statistical comparison on the IC₅₀ values between *T. versicolor* extract and Tamoxifen (reference drug) on the 5 cell lines

Cell line	Drug	IC ₅₀ (µg/ml) ± SEM
4T1	1	188.53±4.81 ^a
4T1	2	163.33±8.82 ^a
DU145	1	71.16±3.48 ^b
DU145	2	63.33±3.48 ^{bc}
IEC-6	2	42.20±5.64 ^{cd}
HCC	2	41.37±5.49 ^{cd}
Vero	2	36.46±2.69 ^d
IEC-6	1	-
HCC	1	-
Vero	1	-

1= *T. versicolor*, 2= Tamoxifen (Reference drug). Results are expressed as the Mean ± SEM of three independent experiments of three wells each. Values that do not share a superscript are significantly different (P>0.05). i.e.: IC₅₀ values on 4T1 cells treated with drug 1 and drug 2 are statistically not different. Drug 1 (*T. versicolor*) did not produce IC₅₀ values on IEC-6, HCC and Vero cell lines (IC₅₀ > 1000µg/ml).

All cell lines treated with the aqueous extract of *T. versicolor* had a general reduction in their viability with the extract showing a general increase in the percentage inhibition on all cell lines used (Table 4.2). The mushroom extract demonstrated a dose dependent inhibition of cell proliferation in all cell lines used. As the concentration of the mushroom extract increased from 1.37 to 1000 μ g/ml, the percentage viability decreased, showing the lowest viability at 1000 μ g/ml and the highest in untreated cells with HCC and DU145 cell lines showing the highest and lowest viability respectively (Appendix V).

A general reduction on the proliferation rate against all cell lines used was recorded. As the concentration of the mushroom extract increased, the proliferation rate decreased with DU145 and 4T1 cells showing the lowest proliferation rates at the highest concentration (1000 μ g/ml). In all cell lines, the proliferation rate was lowest at 1000 μ g/ml and the highest in untreated cells (Figure 4.1). There was a significant difference between proliferation rate at 1000 μ g/ml and untreated cells in all cell lines. ($P < 0.05$; Table 4.4).

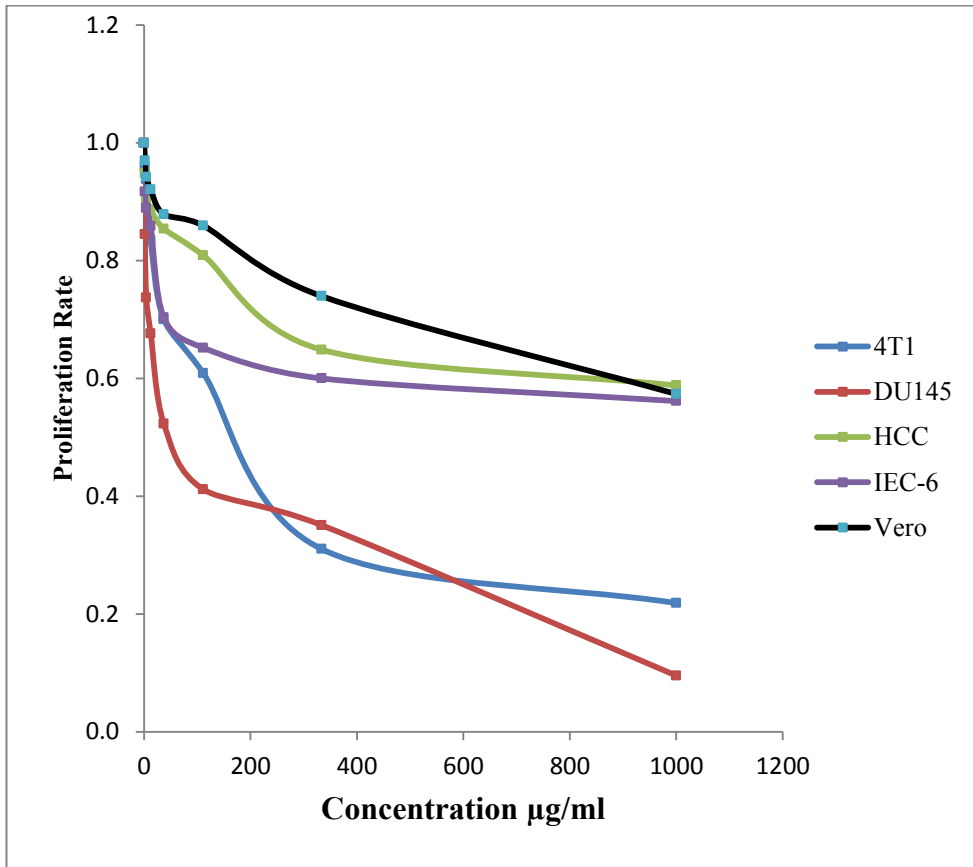


Figure 4.1: Dose response curve showing the proliferation rate of aqueous extract of *T. versicolor* on the cell lines.

Table 4.4: Effect of aqueous extract of *T. versicolor* mushroom on the proliferation rate on 4T1, DU145, HCC, IEC-6 and Vero cell lines

Concentration ($\mu\text{g/ml}$)	<u>Proliferation rate</u>				
	Cell line				
<i>T. versicolor</i>	4T1	DU145	HCC	IEC-6	Vero
Untreated cells	1.00 \pm 0.02 ^a	1.00 \pm 0.01 ^a	1.00 \pm 0.01 ^a	1.00 \pm 0.01 ^a	1.00 \pm 0.01 ^a
1.37	0.96 \pm 0.01 ^a	0.84 \pm 0.00 ^{ab}	0.95 \pm 0.00 ^{ab}	0.92 \pm 0.01 ^b	0.97 \pm 0.01 ^{ab}
4.12	0.94 \pm 0.02 ^a	0.74 \pm 0.04 ^{bc}	0.90 \pm 0.01 ^{abc}	0.89 \pm 0.00 ^b	0.94 \pm 0.01 ^{abc}
12.35	0.83 \pm 0.02 ^{ab}	0.68 \pm 0.01 ^{cd}	0.89 \pm 0.02 ^{bc}	0.86 \pm 0.00 ^b	0.92 \pm 0.01 ^{bcd}
37.04	0.70 \pm 0.01 ^{bc}	0.52 \pm 0.02 ^{dc}	0.85 \pm 0.02 ^{bc}	0.70 \pm 0.01 ^c	0.88 \pm 0.03 ^{cd}
111.11	0.61 \pm 0.04 ^c	0.41 \pm 0.05 ^{cf}	0.81 \pm 0.01 ^c	0.65 \pm 0.03 ^{cd}	0.86 \pm 0.01 ^d
333.33	0.31 \pm 0.01 ^d	0.35 \pm 0.01 ^f	0.65 \pm 0.01 ^d	0.60 \pm 0.06 ^{dc}	0.74 \pm 0.001 ^c
1000.00	0.22 \pm 0.01 ^e	0.10 \pm 0.01 ^g	0.59 \pm 0.02 ^d	0.56 \pm 0.02 ^e	0.57 \pm 0.02 ^f

Proliferation rates in the same cell line were compared to each other among the different concentrations of the extract. Results are expressed as the Mean \pm SEM of three independent experiments of three wells each. Values followed by the same superscript in the same cell line are not significantly different ($P>0.05$). In each cell line, the proliferation rate on the untreated cells is statistically different to that on the highest concentration (1000 $\mu\text{g/ml}$). i.e: Values followed by superscript 'a' and 'e' on 4TI cell line are statistically different.

4.3 RNA quantification and purity

In order to get the concentration and purity of the extracted RNA, the total RNA products from the treated and untreated IEC-6 cells were used. The results from a NanoDrop ND-2000 showed a concentration of 448.9ng/ μl with a purity level of 1.8 for the RNA from treated cells whereas the RNA from the untreated cells had a concentration of 521.7ng/ μl and a purity level of 1.9 (Table 4.5). The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants such as protein. Pure RNA has an A_{260}/A_{280} ratio of 1.8–2.1.

Table 4.5: Concentration and purity levels of RNA products from IEC-6 cells

RNA product	Concentration (ng/μl)	A₂₆₀/A₂₈₀ Ratio (purity)
Test (Treated)	448.9	1.8
Control (Untreated)	521.7	1.9

RNA concentration and purity was measured on a NanoDrop ND-2000, blanked with RNase free water which was also used to elute the RNA during extraction. RNA with a A₂₆₀/A₂₈₀ ratio of between 1.8-2.1 is considered to be pure.

4.4 Expression Levels of *IL-7* mRNA

The *T. versicolor* polysaccharide extract used in this study (100μg/ml) exerted a minimal increase in the expression of *IL-7* mRNA when relatively compared to that of GAPDH mRNA with a fold change of 0.17 (Table 4.6). The CT values obtained from GAPDH together with those of *IL-7* were used to calculate the fold change using the fomular; $\text{Fold change} = 2^{-\Delta\Delta\text{CT}}$. A summary of the fold change analysis is illustrated in Table 4.6.

Table 4.6: Expression fold change of *IL-7* mRNA using a house keeping gene (GAPDH) with double delta CT ($\Delta\Delta$ CT) method.

Average CT <i>IL-7</i> with Drug (TE)	Average CT, GAPDH with Drug (HE)	Average CT <i>IL-7</i> , No Drug (TC)	Average CT GAPDH, no Drug (HC)	Change in CT value (Δ CT) with Drug (TE-HE)	Change in CT value (Δ CT) with No Drug (TC-HC)
25.122± 0.627	23.638± 0.708	22.136± 0.514	23.205± 0.921	1.484	-1.069
Expression fold change ($2^{-\Delta\Delta$ CT) was calculated by first obtaining Double Delta ($\Delta\Delta$ CT) value = ((TE-HE) - (TC-HC)). A $\Delta\Delta$ CT value of 2.553 was obtained, which was then applied on the formula ($2^{-\Delta\Delta$ CT) to give an expression fold change for <i>IL-7</i> mRNA as 0.17.					

The mean CT value for *IL-7* mRNA from the treated IEC-6 cells was 25.122 where as that of the untreated cells was 22.136 ($P > 0.05$), (Figure 4.2). However, this increase in mRNA *IL-7* expression was found to be statistically significant when compared to the mean CT value of mRNA *IL-7* from the untreated cells ($P = 0.035$) at 95% confidence interval (Table 4.7).

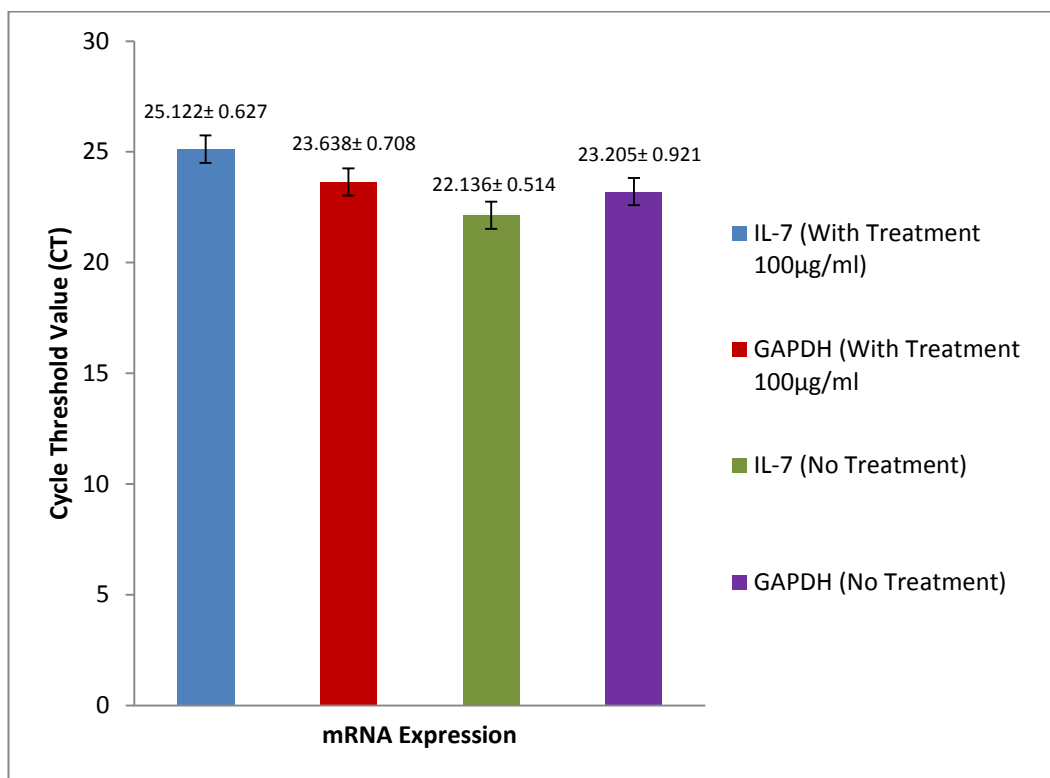


Figure 4.2: Cycle Threshold (CT) levels for *IL-7* and *GAPDH* genes.

Table 4.7: Comparison of CT means for mRNA *IL-7* Expression

mRNA <i>IL-7</i>	Mean	SEM	P value
Test	25.122	0.514	0.035
Control	22.136	0.627	

Comparison of the CT means for mRNA *IL-7* was performed using CT values of a qRT-PCR experiment run in triplicate. The means for the test (Treated) and the control (untreated) samples were subjected to a Two-sample independent T-test at 95% confidence interval resulting to a P value of 0.035.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

This study evaluated the antiproliferative activity of aqueous extracts of Kenyan *T. versicolor* mushroom on normal and cancer cell lines namely, 4T1, DU145, HCC, IEC-6 and Vero, and also the expression levels of IL-7 mRNA using *T. versicolor* polysaccharide extract . The aqueous extract of the Kenyan *T. versicolor* mushroom used in this study exhibited antiproliferative activity on two cancer cells with high safety levels on normal cells. Previous studies have shown that aqueous extracts of *T. versicolor* mushroom contain natural polysaccharides and some phytochemicals which have been proven to have some anticancer activity (Ooi and Liu, 2000).

The findings of this study revealed that aqueous extracts of the Kenyan *T. versicolor* mushroom significantly inhibited the proliferation of breast cancer cells (4T1) and human prostate cancer cells (DU145) in a dose dependent manner. The antiproliferative activity of the *T. versicolor* extract was reflected by its high percentage inhibition and the relatively low IC₅₀ values which were significantly not different to those of Tamoxifen, the reference drug used. These findings are consistent with previous studies where water extract of *T. versicolor* was reported to have significant inhibitory effects on human breast cancer cells, prostate cancer, human leukemia, and lymphoma cells (Lau *et al.*, 2004). In another study done by Ho *et al.*, ethanol-water extract of *T. versicolor* exhibited significant antiproliferative activity on three human breast cancer cell lines (MDA-MB-231, MCF- 7 and T-47D) in a dose-dependent manner *in vitro* (Ho *et al.*, 2005). Torkelson *et al.* achieved similar findings in a clinical trial on women with breast cancer where oral preparations of *T. versicolor* administered to the subjects showed significant antiproliferative activity (Torkelson *et al.*, 2012).

However, the aqueous extracts of *T. versicolor* in the present study showed a very low inhibition on the proliferation of human liver cancer cells (HCC), rat normal intestinal epithelial cells (IEC-6) and African green monkey normal kidney cells (Vero). This is shown by their high IC₅₀ values where even the highest concentration of the mushroom extract used could not inhibit at least 50% of the cells. In a study on water extracts isolated from *T. versicolor* mushroom, the results recorded no antiproliferative activity on three human liver cancer cells (HepG2, 7721, PLC) and human normal liver cells (WRL) (Zhou et al., 2007), which corresponds with the results of this study as *T. versicolor* extract failed to inhibit the proliferation of human cancer liver cells (HCC). In the same study, Zhou *et al* recorded that the water extract of *T. versicolor* showed antiproliferative activity on four breast cancer cell lines (T-47D, Bcap37, ZR7-30, MCF- 7) while it inhibited the proliferation of only one liver cancer cell line (7703) out of four liver cancer cell lines used (Zhou et al., 2007). This therefore might be suggestive that *T. versicolor* extract acts on liver cancer cells selectively.

The results of this study suggests that the aqueous extract of the Kenyan *T. versicolor* mushroom is more active in the inhibition of breast and prostate cancer cells than human liver cancer cells and normal cell lines. This was reflected by the low IC₅₀ values of 71.16µg/ml and 188.53µg/ml on prostate cancer (DU145) and breast cancer cells (4T1) respectively compared to liver cancer cells (HCC), rat normal intestinal epithelial cells (IEC-6) and African green monkey normal kidney cells (Vero) both of which had an IC₅₀ value >1000µg/ml. These results tend to agree with previous reports that *T. versicolor* extracts can selectively suppress the proliferation of various cancer cells with no antiproliferative activity in normal cell lines suggesting its high level of safety (Ho *et al.*, 2005; Zhou *et al.*, 2007). In all cell lines used in this study, there was a significant difference in the proliferation rate between the untreated cells and the highest concentration (1000µg/ml) of the *T. versicolor* extract used. This tendency however suggests that the extract used in this study has a dose-dependent activity on the cell lines used.

The antiproliferative activity of the *T. versicolor* aqueous extract used in this study can be associated with the phytochemicals found to be present in the extract. Saponins, flavonoids and Taninns, which were present in the extract have been documented from previous research to possess some antiproliferative activity on different cancer cell lines (Mithraja *et al.*, 2011; Rivière *et al.*, 2009; Yildirim and Kutlu, 2015). Flavonoids have demonstrated antiproliferative activity in prostate, renal, colon, non-Hodgkin's lymphoma, gastric carcinomas and neuroblastoma cancer cells (Chandrappa *et al.*, 2014; Priya *et al.*, 2014; Senderowicz, 2001) Tannin isolated from *Cuphea hyssopifolia*, *Phyllanthus niruri* and *Cistus ladanifer* among other medicinal plants has been documented to have antiproliferative effects against breast, human promyelocytic leukemia HL-60, ovarian and pancreatic cancer cell lines (Barrajón-Catalán *et al.*, 2010; Jia *et al.*, 2013; Wang *et al.*, 2000).

The Polysaccharide extract of the Kenyan *T. versicolor* mushroom used in this study showed a slight increase in the expression of IL-7 mRNA on IEC-6 cells. A study done by Mwitari *et al.*, with Polysaccharide extracts obtained from mushroom *Trametes robiniophila murr* grown on media supplemented with Chinese herb showed a slight up regulation of *IL-7* mRNA to almost 2 folds on IEC-6 cells (Mwitari *et al.*, 2014). These results are consistence with the results of the present study though it is probable that the low fold change on the expression of IL-7 mRNA in this study was due to the use of crude polysaccharides which were used independently compared to the polysaccharides used in the above mentioned study which were obtained from Mushrooms supplemented with other herbs (Mwitari *et al.*, 2014). In other several *in vitro* studies, purified PSK compound extracted from medicinal mushrooms was found to slightly induce gene expression of several cytokines including *IL-7* (Hirose *et al.*, 1990; Nio *et al.*, 1991; Noguchi *et al.*, 1995).

In another study using unsupplemented polysaccharides extracted from a medicinal mushroom *Agaricus blazei* Murill, the levels of *IL-7* gene was slightly up regulated while a significant rise in other immune modulating cytokines was also documented (Johnson *et al.*, 2009). The immunomodulatory activity of a mushroom extract can

be affected by the content and type of glycans present which might differ from samples collected on different regions of the world (Ooi and Liu, 2000).

The findings of this study on the antiproliferative nature of the aqueous extract of the Kenyan *T. versicolor* mushroom are consistent with previous results on *T. versicolor* mushrooms collected from other parts of the world. However, it is probable that the phytochemicals found to be occurring in the aqueous extract of the Kenyan *T. versicolor* used in this study are responsible for the antiproliferative nature of the extract. It is also evident from the results of this study that the polysaccharide extract of the Kenyan *T. versicolor* mushroom has a potential to up-regulate the expression of *IL-7* mRNA.

5.2 Conclusions

The current study with a Kenyan *T. versicolor* mushroom has demonstrated significant antiproliferative activity of aqueous extract *T. versicolor* *in vitro* against DU145 and 4T1 cancer cell lines. This significant antiproliferative activity reflects that the mushroom is endowed with potent antiproliferative properties.

The aqueous *T. versicolor* extract used in this study had high levels of safety on the normal cell lines used with the highest IC₅₀ values (>1000µg/ml). This indicates low or minimal toxicity levels.

The polysaccharide extract of *T. versicolor* used in this study could slightly up regulate the expression of *IL-7* gene. This is suggestive that the Kenyan *T. versicolor* mushroom is a potential candidate for immunomodulation activity against cancer.

Based on the findings of this study therefore, the null hypotheses is rejected.

5.3 Recommendations

The results of this study suggests the need for a quantitative analysis and study of the phytochemicals of the Kenyan *T. versicolor* mushroom. *In vitro* and *in vivo* anti-

proliferative activity and evaluation of cell death mechanisms using purified active compounds extracted from the Kenyan *T. versicolor* mushroom is recommended. A broad study on the immunomodulatory potential of purified *T. versicolor* polysaccharides supplemented with other herbals is also recommended.

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APPENDICES

Appendix I: Interleukin-7 and GAPDH Gene Sequences in Fasta Format From NCBI

Appendix I. a: Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapdh), mRNA

>NM_017008.4 Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapdh), mRNA

GGGGCTCTCTGCTCCTCCCTGTTCTAGAGACAGCCGCATCTTCTTGTGCAG
TGCCAGCCTCGTTCATAG

ACAAGATGGTGAAGGTCGGTGTGAACGGATTTGGCCGTATCGGACGCCT
GGTTACCAGGGCTGCCTTCTC

TTGTGACAAAGTGGACATTGTTGCCATCAACGACCCCTTCATTGACCTCA
ACTACATGGTCTACATGTTC

CAGTATGACTCTACCCACGGCAAGTTCAACGGCACAGTCAAGGCTGAGA
ATGGGAAGCTGGTCATCAACG

GGAAACCCATCACCATCTTCCAGGAGCGAGATCCCGCTAACATCAAATG
GGGTGATGCTGGTGCTGAGTA

TGTCGTGGAGTCTACTGGCGTCTTACCACCATGGAGAAGGCTGGGGCTC
ACCTGAAGGGTGGGGCCAAA

AGGGTCATCATCTCCGCCCTTCCGCTGATGCCCCATGTTTGTGATGGGT
GTGAACCACGAGAAATATG

ACA ACTCCCTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTGCTTA
GCCCCCTGGCCAAGGTCAT

CCATGACAACTTTGGCATCGTGGAAGGGCTCATGACCACAGTCCATGCCA
TCACTGCCACTCAGAAGACT

GTGGATGGCCCCTCTGGAAAGCTGTGGCGTGATGGCCGTGGGGCAGCCC
AGAACATCATCCCTGCATCCA

CTGGTGCTGCCAAGGCTGTGGGCAAGGTCATCCCAGAGCTGAACGGGAA
GCTCACTGGCATGGCCTTCCG

TGTTCCCTACCCCCAATGTATCCGTTGTGGATCTGACATGCCGCCTGGAGA
AACCTGCCAAGTATGATGAC

ATCAAGAAGGTGGTGAAGCAGGCGGCCGAGGGCCCACTAAAGGGCATCC
TGGGCTACACTGAGGACCAGG

TTGTCTCCTGTGACTTCAACAGCAACTCCCATTCTTCCACCTTTGATGCTG
GGGCTGGCATTGCTCTCAA

TGACAACCTTTGTGAAGCTCATTTCCTGGTATGACAATGAATATGGCTACA
GCAACAGGGTGGTGGACCTC

ATGGCCTACATGGCCTCCAAGGAGTAAGAAACCCTGGACCACCCAGCCC
AGCAAGGATACTGAGAGCAAG

AGAGAGGCCCTCAGTTGCTGAGGAGTCCCCATCCCAACTCAGCCCCAAC
ACTGAGCATCTCCCTCACAA

TTCCATCCCAGACCCCATACAACAGGAGGGGCCTGGGGAGCCCTCCCTT
CTCTCGAATACCATCAATAA

AGTTCGCTGCACCCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Appendix I. b: Rattus norvegicus interleukin-7 (IL-7) mRNA

GenBank: AF367210.1

>AF367210.1 Rattus norvegicus interleukin-7 (IL-7) mRNA, complete cds

TCCCGCAGACCATGTTCCATGTTTCTTTTAGATATATCTTTGGAATTCCTC
CCCTGATCCTTGTGCTGCT

GCCGGTCACATCGTCTGACTGTCACATTAAAGACAAGGACGGTAAAGCC
TTTGGGAGTGTGCTGATGATC

AGCATCAATCAACTGGACAAAATGACAGGAACTGATAGTGATTGCCCAA
ATAATGAACCAAACCTTTTTTA

AAAAACATCTATGTGATGATACAAAGGAAGCTGCTTTTCTAAATCGTGCT
GCTCGCAAGTTGAGGCAATT

TCTTAAAATGAATATCAGTGAGGAATTCAATGACCATTTACTAAGAGTTT
CAGACGGCACACAAACACTG

GTGAACTGCACAAGCAAGGAAGAAAAACCATAAAGGAACAGAAAAAG
AATGATCCATGTTTCCTAAAGA

GACTACTGAGAGAGATAAAAACCTTGTTGGAATAAAATCTTGAACAGCAG
TATATAAACAGAAAATGTAGT

AGCAACCTCCAAGAACTACTGCCTTGCATACTTGGAGAGTTTGGAAACC
TCCCAGAAGTTTCTGGATGC

CTCCTGGTCAAATAAAGCAAGCAACTGGGAAATCTACAGTGATAGACAT
AAGAAATGCAACTGACTCCTG

CGGTCAGCATATGCCTATGGAAATTTATCAACAGTACTGATTTTGTAAAG
CAACCCTGTCATCTGCAATG

CTTTTAAAGCCTTTCCAAGTGTTTCTACCATTATCCAGTCTACAAACAAGG
CTCTGAAGATTCAGAGTCG

CCACTGTTTTGTTAGCAACCATGCATCGGTGAACCACTGGGGGAGTGGAA
CTGTCCTGTGGAAGACTGCA

GATACAGGAGGGCTCCTGGTGATGGATAATGCTCTTGAAAACGAGAGTC
ACATCTTAAAGCAGCAGCAGC

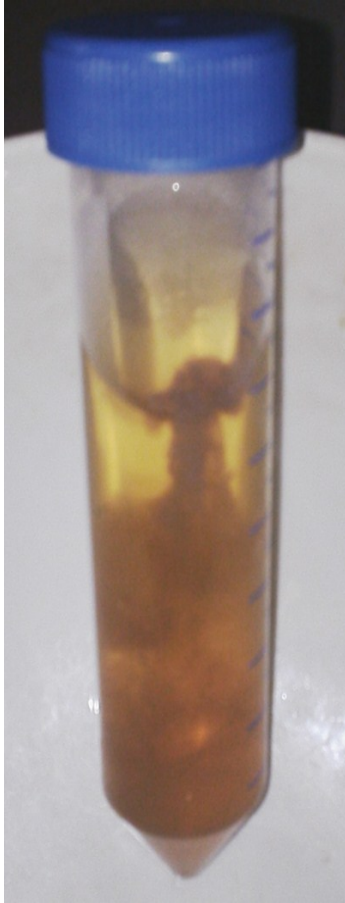
AGCAGCAAAAAGAAGGTTAAGGCATCAACAAATGTAGTTAAATATGAAT
GTATAACACATAGCTTCAGTA

CAGAGCATAGCAGATATTTTTAAATAAAAAGTATTCTTAAAAATAGAAATG
CACTTAGTTCAAAGATACTG

AACCTTAGTATTCAGTCACTTTTGTCATATATGTATAATAAAGCTTATATA
ACTGAAAAAAAAAAAAAAAAA

AAAAAAAA

Appendix II: *Trametes versicolor* polysaccharides precipitated in ethanol



Appendix III: A plate displaying *T. versicolor* growing at Arabuko Sokoke forest

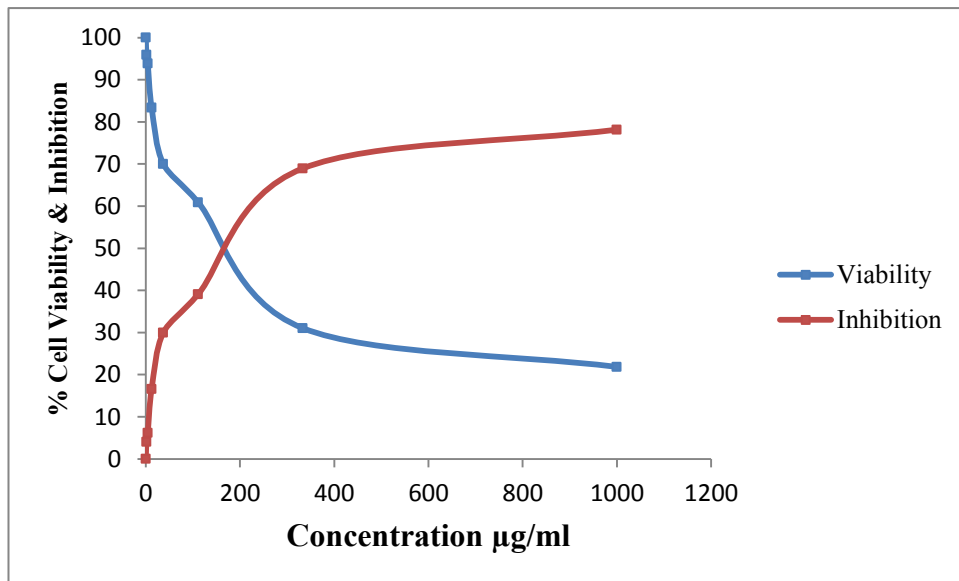


Appendix IV: *T. versicolor* Aqueous extract stored in an air tight bottle

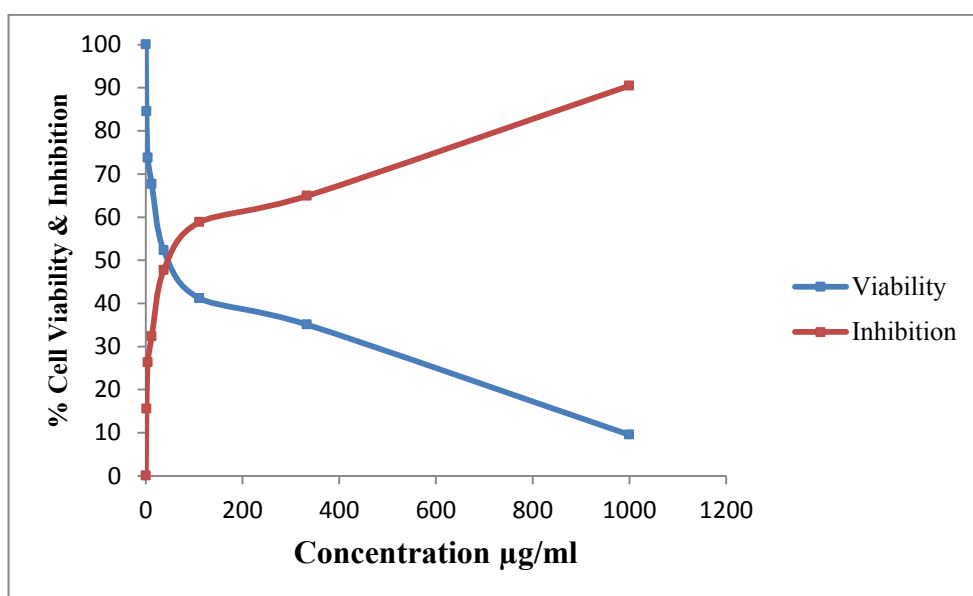


Appendix V: Dose response curves showing the effect of aqueous extract of *T.versicolor* on the percentage inhibition and viability on cell lines.

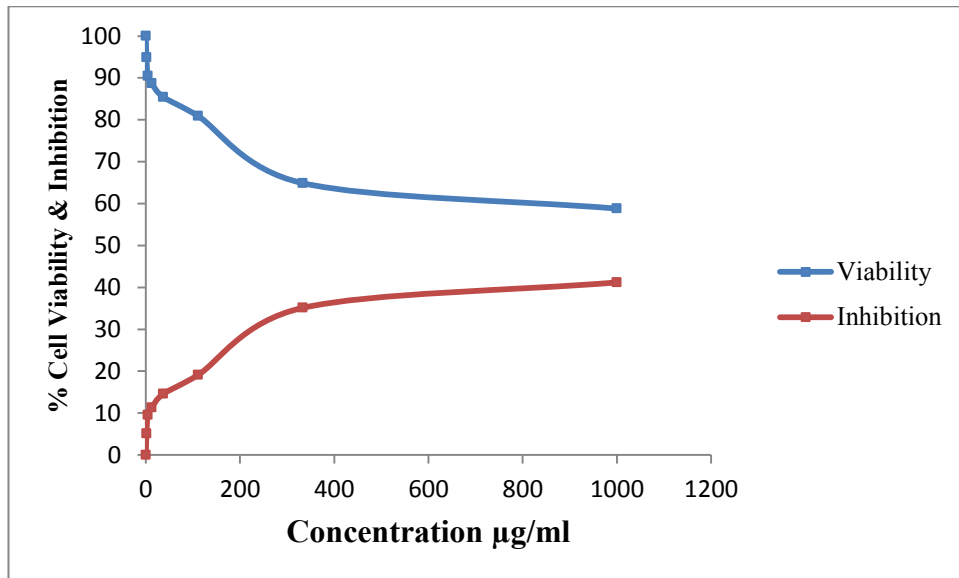
Appendix V. a: Dose response curve showing the effect of aqueous extract of *T.versicolor* on the percentage inhibition and viability on 4T1 cell line.



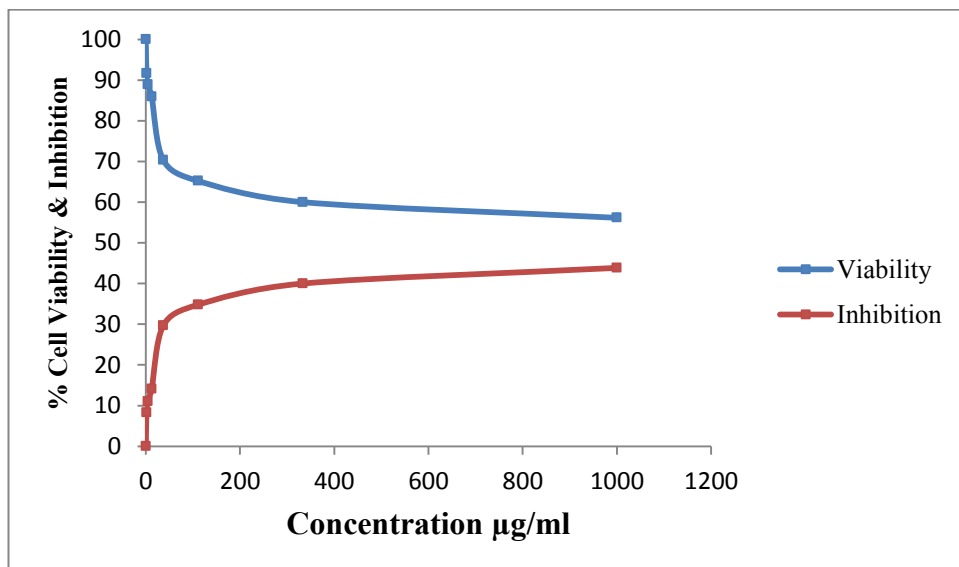
Appendix V. b: Dose response curve showing the effect of aqueous extract of *T.versicolor* on the percentage inhibition and viability on DU145 cell line.



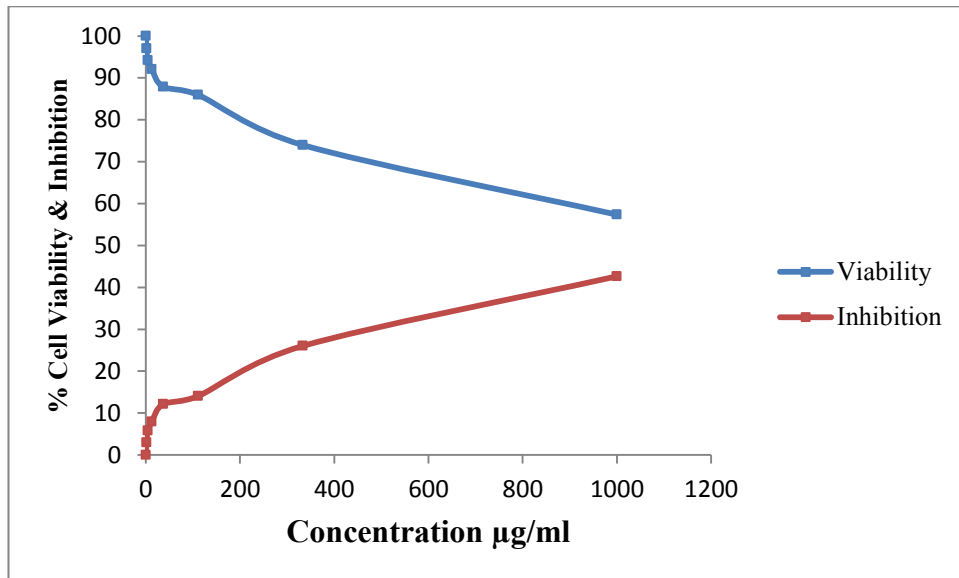
Appendix V. c: Dose response curve showing the effect of aqueous extract of *T.versicolor* on the percentage inhibition and viability on HCCcell line



Appendix V. d: Dose response curve showing the effect of aqueous extract of *T.versicolor* on the percentage inhibition and viability on IEC-6 cell line.



Appendix V. e: Dose response curve showing the effect of aqueous extract of *T.versicolor* on the percentage inhibition and viability on Vero cell line.



Appendix VI: Expression levels of IL-7 and GAPDH genes expressed as means of CT values

IL-7 with <i>T. versicolor</i> extract (100µg/ml)	GAPDH with <i>T. versicolor</i> extract (100µg/ml)	IL-7 with no Drug	GADH with no Drug
25.122 ± 0.627	23.638 ± 0.708	22.136 ± 0.514	23.205 ± 0.921

Appendix VII: Two-Sample T-Test Results from Minitab

Two-sample T-Test for IL-7

IL-7	N	Mean	StDev	SE Mean
Test	3	25.122	0.891	0.514
Control	3	22.136	1.09	0.627


Difference = μ (1) - μ (2)

Estimate for difference: 2.986

95% CI for difference: (0.405, 5.567)

T-Test of difference = 0 (vs \neq): T-Value = 3.68 P-Value = 0.035 DF = 3

Appendix VIII: Copy of Ethical approval letter from SERU (KEMRI)


KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **June 04, 2015**

**TO: JOHN KAHINDI CHENGO,
PRINCIPAL INVESTIGATOR**

**THROUGH: DR. PETER MWITARI,
THE DIRECTOR, CTMDR,
NAIROBI**

Dear Sir,

*Forwarded
Peter 9.6.2015*

**RE: KEMRI/SERU/CTMDR/009/3045 (RESUBMITTED INITIAL SUBMISSION):
IMMUNOSTIMULATORY AND CYTOTOXICITY EFFECTS OF KENYAN TRAMETES
VERSICOLOR MUSHROOM POLYSACCHARIDE EXTRACTS ON IEC-6 CELLS IN
CANCER MANAGEMENT-(VERSION 1.1 DATED 26TH MAY, 2015)**

Reference is made to your letter dated 26th May, 2015. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on May 29, 2015.

This is to inform you that the Committee notes that the issues raised during the 236th C meeting of the SERU held on 30th April, 2015 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **4th June, 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **June 03, 2016**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **April 22, 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

**PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT**

In Search of Better Health

Appendix IX: Front page of Published Manuscript



Antiproliferative Activity of Kenyan *Trametes versicolor* Aqueous Extract on Selected Cancer and Normal Cell Lines

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Abstract

Cancer is a major public health burden in both developed and developing countries. The current conventional cancer therapies like chemotherapy are expensive and inaccessible to many cancer patients. Commercial and wild edible mushrooms are becoming more important for their nutritional value and are becoming an alternative source of immune modulation and anticancer agents. Although previous studies with *Trametes versicolor* mushroom from various parts of the world have demonstrated antiproliferative activity on various cancer cell lines, the antiproliferative activity of the recently identified Kenyan *T. versicolor* mushroom have not been studied. This study examined the *in vitro* antiproliferative activity of an aqueous extract of the Kenyan *T. versicolor* mushroom on breast cancer (4T1), prostate cancer (DU145), hepatocellular carcinoma (HCC), rat normal intestinal epithelial cells (IEC-6) and African green monkey normal kidney (vero) cell lines using MTT assay. The results demonstrated that the *T. versicolor* extract at 1.37 µg/ml to 1000 µg/ml dose-dependently inhibited the proliferation of DU145 and 4T1 cell lines with IC₅₀ values: DU145 (71.2 µg/ml) and 4T1 (188.5 µg/ml). The extract however did not exert any significant antiproliferative effect on HCC, IEC-6 and Vero cell lines (IC₅₀ > 1000 µg/ml) when compared with a chemotherapeutic anticancer drug, gemcitabine (p<0.05), confirming the tumor-selective cytotoxicity on cancer cell lines and its safety on normal cell lines. In all cell lines, the extract showed a significant difference in inhibition of cell proliferation between the untreated cells and the highest concentration (1000 µg/ml) (p<0.05). Presence of phytochemicals such as saponins, terpenes, steroids, terpenoids and flavonoids in the *T. versicolor* extract used might be the probable reason for its antiproliferative activity.

Keywords: *Trametes versicolor*; Antiproliferation; Cancer; Cell line; Inhibition; Viability

Introduction

Cancer is a disease characterized by irregular proliferation of cells with a manifestation of malfunctions in immunity as malignant cells manage to escape recognition and elimination by the immune system [1]. Cancer cells display a broad spectrum of genetic alterations that may include gene rearrangements, point mutations, and gene amplifications, leading to disturbances in molecular pathways regulating cell growth, survival and metastasis [2]. Anyone can develop cancer and the risk of being diagnosed with cancer increases with age. About 77% of all cancers are diagnosed in people 55 years of age and older [3].

Chemotherapy, surgery, immunotherapy and radiotherapy remain to be the most effective conventional methods in cancer treatment. However, they are expensive and can cause serious side effects as these do not often show adequate differential effect between tumor and normal cells [2], hence the need for affordable but effective ways of cancer management.

More than 50% of all modern drugs in clinical use are of natural product origins, many of which have antiproliferative ability [4]. According to World Health Organization estimates, more than 80% of people in developing countries depend on traditional medicine for their primary health needs including cancer management [5]. A survey done in the year 2008 showed that more than 60% of cancer patients use herbs as therapy [6]. An attempt has been made to explore the potential of newly discovered anticancer compounds from medicinal mushrooms as a lead for anticancer drug development [5]. Mushrooms have key medicinal uses including anticancer activity [7], immunomodulating effects and antiproliferative activity [8]. *Trametes versicolor* mushroom, also called "Turkey tail" has been widely studied

and its antiproliferative activity on different cancer cell lines has been well documented [9-11].

In Japan, polysaccharide-Krestin (PSK) extracted from *T. versicolor* mushroom is prescribed to cancer patients routinely, both during and after radiation and chemotherapy [12-15]. In the United States of America, whole, freeze-dried *T. versicolor* is commonly prescribed to breast cancer patients [16].

In some studies done in China to test for the antiproliferative activity of *T. versicolor* extract, results showed that the extract could inhibit the proliferation of four breast cancer cells (T-47D, Hsp37, ZR75-30, MCF-7), B-cell lymphoma (Raji), human promyelocytic leukemia (HL-60, NB-4) and liver cancer cell line (7703) [17,18]. However, the extracts of *T. versicolor* mushroom have been reported to be non-toxic to several normal cells [19-21] and in cancer patients on clinical trials [22-24]. Differences in the ecological zones where mushroom exist and the influence of some environmental factors may account for the overall composition and activity of compounds present in mushrooms [25,26]. This study therefore investigated the antiproliferative potential

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