UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCES

IN VITRO INVESTIGATION OF THE RELATIONSHIP BETWEEN SCHISTOSOMIASIS AND PROSTATE CANCER

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL BIOCHEMISTRY DEGREE

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JULY, 2017

DECLARATION

I, Isaac Tuffour, do certify that this project, aside other cited works, is the result of research carried out by me under the supervision of Prof. Regina Appiah-Opong, Dr. Theresa Manful Gwira and Dr. Irene Ayi towards the award of the M.Phil. degree in Biochemistry in the Department of Biochemistry, Cell and Molecular Biology of the University of Ghana.

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ABSTRACT

Schistosomiasis is a neglected tropical disease that affects 200 million people and accounts for 100, 000 deaths annually. In endemic geographical areas, schistosomiasis has been implicated as an etiological agent in the pathogenesis of bladder, colorectal and renal carcinoma largely due to chronic infection of tissues with *Schistosoma* eggs. Several studies have also reported cases of association between *Schistosoma* infection and prostate cancer, one of the most common cancers in men. The possible causal association is however poorly understood. This study therefore aimed at experimentally investigating this association and elucidating the underlying mechanism. Urine samples from individuals living in Galilea, a Schistosomiasis endemic community in the Ga South District of Ghana were collected and screened for *Schistosoma* infection via microscopy and multiplex PCR. Soluble antigens (SEA) were prepared from *Schistosoma*-egg positive urine samples and assessed for the ability to induce cancer-like phenotypes including excessive proliferation, oxidative stress (GSH depletion) and diminished apoptosis in cultured human prostate (PNT2) cells. Cell proliferative effect of SEA was evaluated by the tetrazolium-based MTS assay. Oxidative stress-inducing effect of SEA was also determined using the fluorescent probe, O-phthalaldehyde. Apoptosis-diminishing effect of SEA was evaluated via fluorescence (Hoechst) staining and flow cytometry. 14.4% (30/209) schistosomiasis prevalence was recorded. Out of 30 s*chistosoma*-infected persons, 73% (22 persons) recorded light infections whereas 27% (8 persons) were heavily infected. Microscopic and molecular analysis revealed infecting-schistosome species to be *S. haematobium* and *S. mansoni*. 63% (19 persons) were infected with *S. haematobium* only whereas 37% (11 persons) were co-infected. Prostate cell proliferation was significantly induced by

12.5µg/ml SEA (P=0.029). Also, SEA dose-dependently depleted cellular reduced glutathione (GSH). Flow cytometric analysis and fluorescence staining revealed that SEA dose-dependently and significantly diminished apoptosis in prostate cells. Schistosomiasis still remains a major health challenge. Findings of this study suggest that *schistosoma*-infection may play a role in the pathogenesis of prostate cancer. *In vivo* studies are however needed to confirm this association.

DEDICATION

This work is dedicated to my family for their encouragement and support throughout this study.

ACKNOWLEDGEMENT

I thank the Almighty God for His enabling grace, guidance and protection.

I wish to expresss profound thanks to my supervisors, Dr. Regina Appiah-Opong, Dr. Theresa Manful Gwira and Dr. Irene Ayi for their constant dedication, guidance and motivation during the project period. I also appreciate them for their incisive reviews and evaluations that have shaped and improved the quality of this work.

A special appreciation goes to the West African Center for Cell Biology of Infectious Pathogens (WACCBIP) for funding the project.

My deepest appreciation goes to Mr. Edward Dumashie, Mrs. Yvonne Aryeetey and staff of the Parasitology Department at the Noguchi Memorial Institute for Medical Research (NMIMR) for their invaluable technical assistance. I wish to thank all staff of the Clinical Pathology Department-NMIMR for their unwavering support especially Miss Abigail Aning and Miss Eunice Dotse who immensely assisted in the maintenance of the cell lines used for this study.

Special acknowledgements go to all my friends especially, Miss Priscilla Abena Akyaw, Miss Abena Kissi-Twum, Miss Trudy Philips, Mr. Ebenezer Ofori- Attah and Mr. Philip Atchoglo for their prayers and diverse support.

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LIST OF ABBREVIATIONS

7AAD	7 Amino Actinomycin D
B-cell Lymphoma 2	B-cL2
CAD	Caspase Activated DNase
CEQ	Catechol Estrogen
CEQ-DNA	Catechol Estrogen-DNA
CHO	Chinese Hamster Ovary
COX1	Cytochrome Oxidase 1
CYP1B1	Cytochrome P450 1B
FITC	Fluorescein Isothiocynate
GSH	Reduced Glutathione
IARC	International Agency for Research in Cancer
ICAD	Inhibitor of Caspase Activated DNAse
ITS1	Internal Transcribed Spacer 1
LacdiNAc	Beta 1, 4-N-acetyl galactosamine
LacNAc	N-acetyl-D-latosamine
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl) - -2-(4Sulfophenyl)-2H-tetrazolium
NMIMR	Noguchi Memorial Institute for Medical Research
OPA	O-pthalaldehyde
PARP	Poly-ADP Ribose Polymerase
PCR	Polymerase Chain Reaction

PNT2	Human normal prostate cell
PSA	Prostate Specific Antigen
p16	Cyclin dependent kinase 4 inhibitor
p27	Cyclin dependent kinase inhibitor 1B
p53	Tumour protein 53
ROS	Reactive Oxygen Species
SEA	Soluble Egg Antigen

CHAPTER ONE

INTRODUCTION

Prostate cancer is an important global health challenge. It is among the most common cancers in men following hepatocellular carcinoma in Africa (Parkin *et al.,* 2013). Prostate cancer represents the highest form of cancer and the commonest cause of cancer-related death in men from the United States of America and Northern Europe (Siegel *et al.,* 2012). About 99% of prostate cancer cases occur in men above fifty (50) years and the disease is characterized by painful urination, blood in urine, frequent urination and sexual function disorders including difficulty in achieving erection and painful ejaculation (Miller *et al.,* 2003). It contributes enormously to global cancer related mortalities. In 2017, the American Cancer Society predicts that there will be approximately 161,360 new cases of prostate cancer in the United States of America with an estimated 26,730 deaths (Prostate cancer: statistics, 2017).

The Oncology Directorate of the two major hospitals in Ghana, namely; Komfo Anokye Teaching Hospital (KATH) and Korle-Bu Teaching Hospital (KBTH) reported that prostate cancer accounted for the highest case of solid tumours referred for radiotherapy, chemotherapy and surgery in men (Ministry of Health Report, 2011). Also, retrospective analysis of the frequency and pattern of urogenital cancer cases at KBTH from the year 1990 to 1999 indicated that prostate cancer accounted for 81.4% of all identified urogenital cancers in males. Irrespective of the overwhelming escalation of the disease and its burden globally, much is not known about its aetiology. However, factors such as old age, race, genetic and environmental influences are suspected to surge the risk of prostate cancer (Gronberg, 2003).

The role of infectious diseases in the pathogenesis of prostate cancer is largely unknown. Many studies have however, reported cases of association between the disease and schistosomiasis. Gelfand *et al.* (1970) and Vilana *et al.* (1997) reported the presence of schistosome eggs in 20% of 200 cadavers and 50% of prostate and seminal vesicles, respectively in schistosomiasis endemic regions. Similarly, several clinical cases have been reported on the presence of schistosome eggs in prostate biopsies and surgery-obtained tissues from prostate cancer patients in various geographical schistosomiasis endemic areas (Basilio-De-Oliviera *et al.,* 2002; Bacelar *et al.,* 2007; Manasseh *et al.,* 2009). Interestingly, the average age of most of these schistosomiasis-associated prostate cancer patients seem relatively lower than the age category (≥50years) normally ascribed to individuals with the disease. For instance Cohen *et al.* (1995) reported on advanced prostate cancer associated with multiple *S. haematobium* eggs in three young adults (a 27 year old and two 29 year olds) from South Africa. Interestingly, none of these patients were confirmed to have any family history of prostate cancer. This and many other related case reports suggest that infection with Schistosoma parasites and entrapment of Schistosoma eggs in prostate tissues may contribute to the pathogenesis or progression of prostate cancer.

S. haematobium infection has been classified as a Group 1 bio-carcinogen by the International Agency for Research on Cancer (IARC)-WHO. However, the cellular and molecular mechanisms linking infection with *S. haematobium* and carcinogenesis is yet to be defined. It has been known for many decades that squamous cell carcinoma of the bladder was geographically associated with urogenital schistosomiasis in regions with high risk of exposure to *S. haematobium* infection (Chen and Mott, 1989; IARC, 2012).

Schistosoma worm and egg derived estrogen-like molecules and their metabolites have been postulated to be the key carcinogenic substance implicated in Schistosomiasis linked cancers. A study conducted by Gouviea *et al*. (2013) on urine and serum samples of 40 Angolan men who were concomitantly infected with *S. hematobium* and diagnosed with bladder cancer, discovered the presence of unique estrogen-like metabolites which were not reported in the urine metabolome of healthy humans. Among the *Schistosoma* infection-associated metabolites were catechol estrogen quinones (CEQ) and their DNA adducts. These estrogen metabolites have been speculated to bind covalently to DNA bases by forming depurinated sites. Subsequently, error-prone repair of the modified DNA may generate oncogenic alterations which are evidenced in increased cell proliferation, upregulation of oncogenes, under-expression of tumor suppressor genes and diminished apoptosis (Correia da Costa *et al.,* 2014). Additionally, a study carried out by Botelho *et al.* (2009) showed an increase in cell proliferation and tumorigenesis in nude mice injected with *S. haematobium* total antigen-treated Chinese hamster ovary (CHO) cells.

Following clinical case reports, there has been no empirical data proving the relationship between schistosome infection and prostate cancer. This study therefore sought to ascertain the relationship between these two diseases. Furthermore, the study seeks to understand the biological mechanism underlying this relationship using *in vitro* molecular, cellular and biochemical approaches.

1.1 Justification

Prostate cancer cases amongst men is escalating globally, with developing countries recording the highest burden. Several studies in Schistosomiasis endemic regions have reported an association between prostatic carcinoma and schistosome infection. Most of these reported cases occur among young adults (less than 50 years) with no family history of prostate cancer; suggestive of a possible role of schistosome infection in the pathogenesis and/or early progression of prostate cancer. This possible causal association is however poorly understood and the underlying mechanism largely unknown.

Thus elucidation of the relationship between these diseases and their underlying mechanism will be necessary in diagnosing and managing prostate cancer.

1.2 Hypothesis

It is hypothesized in this study that infection of prostate cells with schistosome soluble egg antigens (SEA) promotes cancer**.**

1.3 Aim

The main objective of this study was to elucidate the possible causal relationship between schistosome infection and prostate cancer

1.4 Specific objectives

Specifically, the study sought to:

1. Identify schistosome infected persons in a schistosomiasis-endemic community by microscopy.
2. Confirm infecting-schistosome species by Polymerase Chain Reaction (PCR)
3. Determine the cancer hallmark-inducing potential of urine-derived schistosome egg soluble antigens (SEA) on cultured human prostate cells

CHAPTER TWO

LITERATURE REVIEW

2.1 Schistosomiasis

Schistosomiasis, also sometimes referred as Bilharzia, is a waterborne disease caused by blood flukes belonging to the genus *Schistosoma.* The three main schistosome species affecting man: *S. mansoni*, *S. haematobium* and *S. japonicum*, are wide spread in the tropics and sub-tropical regions (Gryseels *et al.*, 2006). *Schistosoma mansoni* is dominant in many African countries, parts of the Middle East, South-America and in the Caribbeans. *Schistosoma haematobium* is distributed in parts of Africa and in the Middle East, whereas *S. japonicum* is distributed in the Asian regions (Fig. 1). Other human-infecting species have a more limited distribution. For instance, *S. intercalatum*, *S. guineensis* and *S. mekongi* are restricted to the Democratic Republic of Congo, Central-Africa and Cambodia respectively (Webster *et al.*, 2006). Socioeconomically, the disease is reported as the second most distressing parasitic disease following Malaria (Mutapi, 2011). Schistosomiasis is more prevalent in children, who mostly contract the disease through performing domestic chores, swimming and playing in infected water bodies.

2.1.1 Global estimates of infection

In 2010, an estimated 238 million people were infected with Schistosomiasis, 85 percent of whom lived in Africa (Chitsulo *et al.*, 2000). Children under 14 years of age constitute the large proportion of parasite-infected persons in most Schistosomiasis endemic regions. Globally, an estimated 600 to 700 million people are at risk from the disease because they

reside in endemic regions (Thétiot-Laurent *et al.,* 2013). About 250 million people were reported to be in need of treatment for prevention of the disease in 2012 (WHO report, 2014); making it the most common parasitic infection following Malaria. Among the human schistosome species, *S. haematobium* is regarded as the world's deadliest. It is the causative agent of most of the estimated cases of Schistosomiasis that occur worldwide, most of which are reported in sub-Saharan Africa (Mutapi, 2011). As the infectious agent responsible for urogenital Schistosomiasis, *S. haematobium* is reported to infect more than 112 million people in sub-Sahara Africa alone annually (Neglected Tropical Diseases, 2011). In 2002, World Health Organization expert committee estimated that urinary Schistosomiasis in Africa is responsible for approximately 18 million cases of bladder wall pathology, 32 million incidence of dysuria, 10 million reports of hydronephrosis (distension and dilation of the renal pelvis), and 150,000 deaths from renal failure yearly (van der Werf *et al.,* 2003).

Schistosomiasis is regarded as the most fatal of the tropically neglected diseases (Schistosomiasis Facts sheet, 2014). However, global estimates concerning the number of deaths registered by the disease, vary. For instance, the 2010 Global Burden of Disease Study by Lozano *et al*. (2012) reported an estimated 12,000 direct deaths worldwide whereas the WHO in 2014 reported over 200,000 annual deaths associated with schistosomiasis (Fenwick *et al*., 2003).

Figure 1: Geographic distribution of Schistosomiasis (www.cdc.gov/parasites/schisto)

2.2 Life cycle and transmission of Schistosomiasis

Schistosomes generally have a characteristic trematode-vertebrate-invertebrate growth cycle, with human beings as the definitive host. An essential requirement for the transmission of schistosomiasis is human contact with polluted water containing the snail (intermediate host) of the genus *Bulinus*, *Biomphalaria* and *Oncomelania* (Jourdane and Cheng, 1987). All five human *schistosomes* have similar life cycles (Figure 2): parasite eggs released into the environment from infected persons, hatch on contact with fresh water to liberate the free-swimming miracidium that swims actively in the water by means of fine hairs (cilia) covering its body (Jourdane and Cheng, 1987). The miracidium survives for about 8-12 hours, during which time it infects fresh-water snails in order to develop further.

The miracidium after infection, changes into a primary (mother) sporocyst close to the site of penetration. Germ cells within the primary sporocyst eventually undergo division to produce secondary (daughter) sporocysts, which migrate to the snail's hepatopancreas and undergo further divisions to produce thousands of mammal-infectious parasites called cercariae (Wu and Halim, 2000). Cercariae emerge from the snail host in a quotidian tempo, which is highly reliant on temperature and light. The highly motile young cercariae, alternate between vigorous upward movements and sinking to maintain their position in the water (Jourdane and Cheng, 1987). The activity of cercariae, is activated by water turbulence, shadows and human skin chemicals. Permeation of the human skin takes place after the cercariae have attached to and explored the skin. Once on the skin, the parasite secretes skin protein-degrading enzymes to ensure penetration of the cercarial head. The cercarie transforms into a migrating schistosomulum as it penetrates the skin. It remains in

this stage for a period 1-2 days before locating a post-capillary venule from where it travels to the lungs to undergo additional developmental changes essential for consequent migration to the liver (Sturrock, 2001). Following eight to ten days after skin entry, the parasite migrates to the liver sinusoid; juvenile worms develop an oral sucker and beginto feed on erythrocytes (Sturrock, 2001). The almost-mature worms pair up (female worm reside in the gynaecophoric channel of the male and totally depend it for nourishment). Paired worms reposition to the mesenteric or rectal veins with *S. haematobium* ultimately migrating from the liver to the venous plexus. Upon full maturation, adult worms are unable to migrate further through the body and begin to produce eggs (Abdel-Wahab *et al.*, 1992).

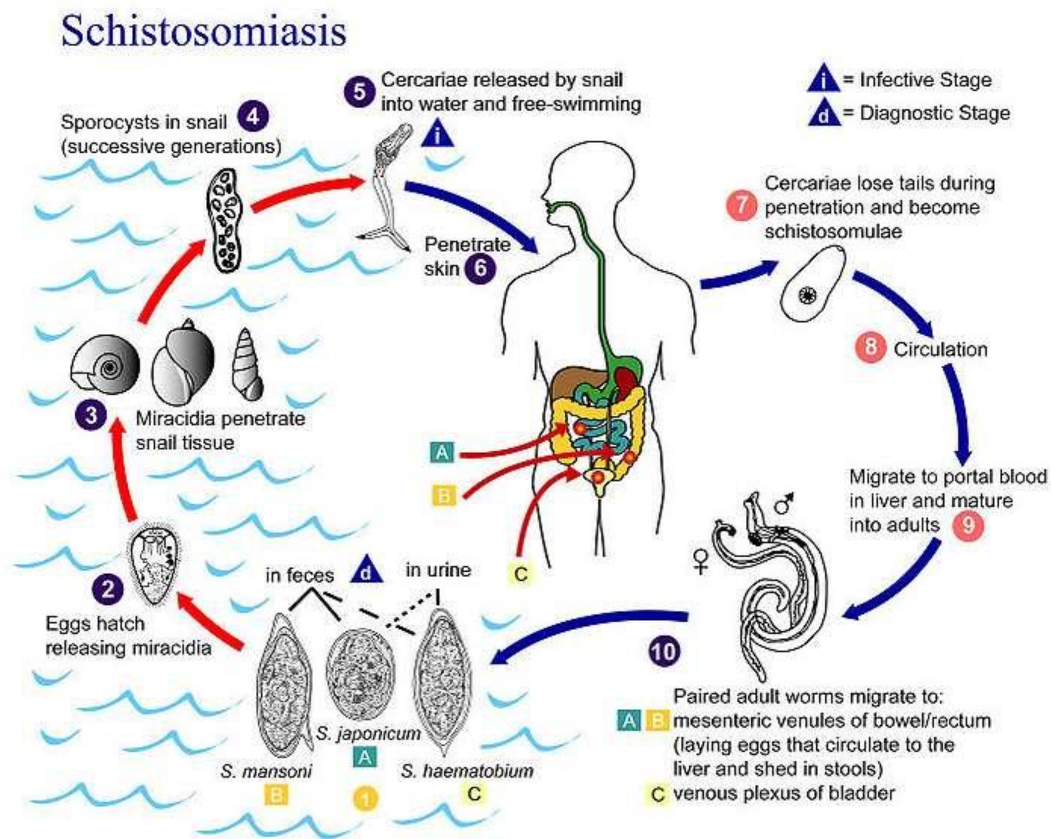


Figure 2: Life cycle of Schistosoma species (Source: http:// www.dpd.cdc.gov/dpdx)

Adult *S. mansoni* pairs residing in the mesenteric vessels may produce up to 300 eggs per day during their reproductive lives. *S. japonicum* may produce up to 3000 eggs per day (Corachan, 2002). Many of the eggs penetrate walls of the blood vessels, and through the intestinal wall, passing out of the body in faeces whereas *S. haematobium* eggs pass through the bladder wall and into the urine. About half of the eggs released by the worm pairs are either entrapped in the mesenteric veins or washed back into the liver, where they will become lodged (Shebel *et al.*, 2012). Tissue-trapped eggs undergo normal maturation, secreting antigens that provoke an acute immune response responsible for the pathology classically associated with Schistosomiasis (Corachan, 2002).

2.3 Schistosomiasis pathophysiology

Schistosomiasis is endemic in most African countries and some countries in the Middle East. *S. haematobium* which causes urinary schistosomiasis is the major causative agent. *S. mansoni* and *S. intercalatum* responsible for intestinal schistosomiasis, have also been reported in some African countries (Chitsulo *et al.*, 2000). *Schistosoma haematobium* infects an estimated 111 million people in Africa and the Middle East resulting in minimal pathology, frequently occurring in the bladder (van der Werf *et al.*, 2003). Ultrasonography is the main method for the detection of pathology in the urinary system as it is a non-invasive and an innocuous technique (Hatz *et al.*, 1998). A quarter to half of *S. haematobium*-infected patients have been reported to experience moderate to severe morbidity (Chitsulo *et al.*, 2000). Several epidemiological studies in endemic areas have reported haematuria, dysuria, anaemia and inflammatory urinary tract pathology to be the commonest forms of *S. haematobium*-linked disease (Hatz *et al.*, 1998). Due to multiple

worm infections and re-infection in highly-endemic areas, urinary schistosomiasis usually presents as a lifelong process of tissue injury (due to continued parasite-related inflammation and fibrosis). The more life-threatening, late disorders including kidney dysfunction, bladder outflow obstruction and urothelial neoplasia emerge clinically after about 20 to 30 years of infection (Hatz *et al.*, 1998). In intestinal schistosomiasis, individuals infected with *S. mansoni* can have pathologic changes in the intestines, liver and sometimes the spleen that usually manifests in abdominal pain, diarrhoea and blood in the stool (Danso-Appiah *et al.*, 2004).

2.3.1 Schistosomiasis and cancer

Helminthic infections particularly schistosomiasis remains a persistent public health problem in endemic regions. The International Agency for Research on Cancer (IARC) has classified schistosome infection as a Group 1 biological carcinogen (Correia da Costa *et al.,* 2014). This supposition is based on the myriad of studies indicating strong positive correlation (mainly in the form of case reports and case control studies) on association of the disease (schistosomiasis) and cancers particularly those related to urogenital tissues such as bladder, cervix and prostate as well as other organs such as the liver and large intestine (Madbouly *et al.,* 2007). For instance, in Africa a review on 217 cases of bladder carcinomas observed in a 5-year period showed a great number (75%) of well-defined squamous cell carcinoma. Out of these cases, 65% were infected with *S. haematobium*. Also, in a retrospective study, in which 184 biopsy reports of primary bladder tumors were assessed, schistosomal infections (*S. haematobium*) constituted 40.8% of the cases (Cooppan *et al.,* 1984). Another study conducted in Northern Tanzania by Kitinya *et al.,*

(1986) revealed that out of 172 bladder cancer cases recorded over a 9-year period, 46% had *S. haematobium* eggs in tumour tissue sections (Palumbo, 2007). In Egypt, where Schistosomiasis tops the list of endemic parasitic diseases with regards to the prevalence and intensity of infection, a consensus of available data strongly implicates a link between *S. haematobium* infection and the initiation of bladder cancer. In 1997, schistosomiasis-linked neoplasms accounted for 30.8% of the total cancer cases ranking first among all recorded cancer types among Egyptian males and second only to breast cancer in females (Ibrahim, 1986; Kahan *et al*., 1997). Helling-Giese *et al.* (1996) in a study on the gynecological relationship of schistosomiasis reported several cases describing the possible association between *S. haematobium* and squamous cell carcinoma of the cervix. An association between *S. japonicum* with colorectal and liver cancer has also been reported. A preliminary Japanese study reported 19.1% (173/907) of patients with chronic liver disease and 51% (35/68) of hepatoma patients were infected with schistosome parasites (Badawi *et al.,* 1994). Cheng and Mott. (1988) also reported a strong correlation between *S. japonicum* infection and rectal cancer in a case control study in China. Additionally, several studies have reported concomitant prostatic adenocarcinoma and schistosomiasis in some patients infected with either *S. mansoni* or *S. haematobium.* However, further investigations are required to confirm this association.

Experimentally, several animal studies have been used to clinically and pathologically evaluate the carcinogenic effects of *S. haematobium.* Kuntz *et al.* (1975) reported a noninvasive papillary and transitional bladder cell carcinoma in a talapoin monkey (*Cercopithecus talapoin*), a capuchin monkey (*Cebus appella*) and gibbons (*Hylobates lar*) when infected with *S. haematobium.* Evaluation of these types of carcinoma revealed they

were morphologically similar to those observed in humans; suggesting a link between *S. haematobium* and bladder cancer. Also, studies have shown that chistosomiasis could provide the proliferative stimulus needed to drive early cancer growth from latent tumor centers. For example, in a previous study, *S. mansoni* infection increased the risk of hepatoma associated with administration of a liver carcinogen in mice (Mostafa *et al.,* 1999). Similarly, Hashem and Boutros *et al.* (1961) reported on the evidence of hyperplasia and metaplasia in bladders of *S. heamatobium*-infected mice following treatment with sub-carcinogenic levels of acetylaminofluorene.

2.3.2 Schistosomiasis and prostate cancer

Not much is known about the aetiology of prostatic cancer. However, apart from the known implicating elements such as age, heredity, hormone levels and environmental factors, an association with infectious agents such as gonorrhea and schistosomes have been reported (Gronberg, 2003; Nelson *et al*., 2003). The relationship between schistosomiasis and cancer has been well established in bladder cancer. Geographical correlation between the two conditions, clinicopathological features of *Schistosoma* associated bladder cancer and evidence in experimental models buttress this link (El-Sheikh *et al.,* 2001). However, such comprehensive association is lacking in the case of schistosomiasis and prostate cancer, though several clinical studies have reported on a possible association.

For instance, cadaver and post-mortem studies (Gelfand *et al.,* 1970; Vilana *et al.,* 1997) reported the presence of *Schistosoma* eggs in 20% of 200 cadavers and 50% of prostate and seminal vesicles, respectively in schistosomiasis endemic areas. A histological assessment of surgical samples taken from two Iraqi patients revealed various *Schistosoma*

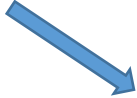
eggs associated with squamous carcinoma of prostate tissues. The author of the forementioned study questioned whether they were cases of a bladder tumour metastasizing to the prostate; however, tumours of both patients were confirmed to be primarily of prostate origin (Bacelar *et al.,* 2007). In another report, *S. mansoni* eggs were found in the prostate of a 49-year old patient with metastasized prostate cancer, treated with bilateral orchiectomy and radiotherapy (Godec *et al.,* 1992). Cohen *et al.,* (1995) also reported on concomitant presentation of *S. haematobium* infection and advanced prostate cancer in three young patients (a 27 year old and two 29 year olds) in South Africa. In a study carried out in autopsied patients with schistosome infection, 6.5% and 22% of cases had parasite-affected prostate and bladder respectively (Alexis *et al.,* 1986). An estimated 73.7% of schistosomiasis-associated cancers was reported in patients ranging between 14 and 40 years of age (*Bacelar et al*., 2007). There have also been reports of concomitant prostatic carcinoma and chistosomiasis in older individuals. For instance, Basilio-de-Oliveira *et al.* (2002) reported the case of a 68 year old Brazilian national who was diagnosed with adenocarcinoma of the prostate with his prostatic biopsy heavily infected with *S. mansoni* eggs. Manasseh *et al.,* (2009) also reported on a 70 year old Nigerian who was diagnosed with very high serum PSA, enlarged prostate and nodular hyperplasia. Histological evaluations showed many *S. mansoni* eggs surrounded by aggregates of immune inflammatory cells.In spite of the frequency of infestation of the prostate in endemic regions, the relationship between schistosomiasis and prostate cancer has been poorly reported.

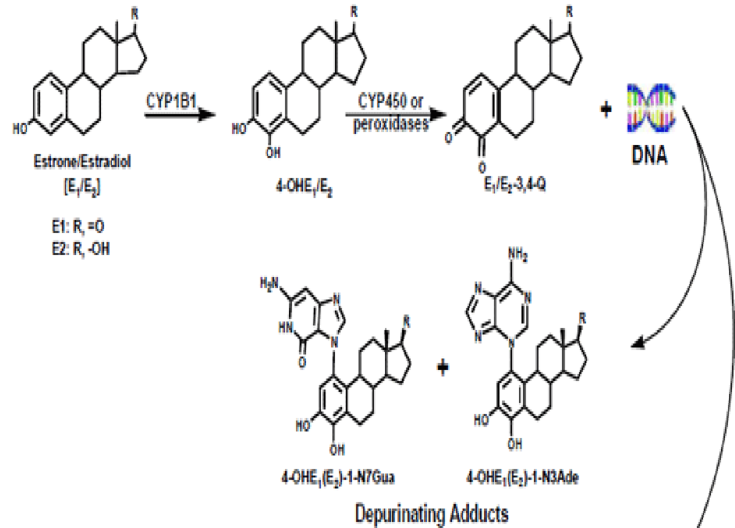
2.3.3 Mechanisms of schistosome-associated cancer

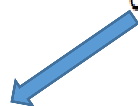
Schistosoma-infection has been implicated in the pathogenesis of bladder, colorectal and renal carcinomas in endemic (schistosomiasis) geographical areas (Ronald *et al.,* 1995). Most studies on Schistosomiasis pathology especially in relation to neoplasia have been mainly on bladder cancers. Several mechanisms have been proposed, all of which revolve around soluble antigens derived from schistosome worms or eggs. Soluble egg antigens (mainly consisting of estrogens and numerous glycoconjugates with N-acetyl-D-lactosamine (LacNAc) or Beta 1, 4-N-acetylgalactosamine (LacdiNAc)) diffuse out from the secretory glands of miracidia enclosed within the eggs and provokes acute immune responses in the infected host (Mostafa *et al.,* 1999). This results in granuloma formation around the tissue-lodged eggs and eventually leads to organ damage (Koen *et al.,* 2006). The immunogenicity of soluble egg antigens results in the mobilization of inflammatory cells to the site where eggs are lodged in the tissue and stimulate an oxidative burst. The inflammatory cells thus release reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide (Harris *et al.,* 1992; Gordon *et al.,* 1988). Activities of the generated ROS are known to be critical to tumour promotion as they bind indiscriminately to macromolecules such as DNA and may thus induce oncogenic mutations.

In another hypothesis, metabolic derivatives of egg estrogens present in soluble egg antigens, are implicated in schistosome-related cancers (Figure 3). Hydroxylation of estrogens by tissue/cellular metabolizing enzymes including Cytochrome P450 enzymes (specifically CYP1B1) catechol estrogens which are further oxidized to highly reactive semiquinones and quinones, the major carcinogenic metabolites of estrogens. Conjugation of these metabolite with glutathione and enzymatic reduction to reform catechol estrogens

are the processes by which cells protect themselves from the build-up of these reactive metabolites. However, if these defensive measures fail or are insufficient, the electrophilic nature of these metabolites (i.e. catechol estrogen semiquinones and quinones) enables them to bind covalently to macromolecules including DNA by redox recycling to form depurinating adducts; subsequent errors in repair of the modified DNA, leads to genetic aberrations that may initiate cancer (Cavalieri *et al*., 1997). Gouveia *et al*., (2013) reported on the evidence of these estrogenic metabolites and their DNA adducts derivatives in urine samples of 40 Angolan men who were concomitantly infected with *S. hematobium* and diagnosed with bladder cancer. These metabolites were however not reported in urine samples of healthy men who were recruited as controls in the study. *In vitro* investigations suggest that exposure of cells to lysates of schistosome eggs and adult parasites induce cancer hallmarks, characterized by increased cellular proliferation, migration, invasion, diminished apoptosis, upregulation of BcL2 (an anti-apoptosis protein) and downregulation of p27 (Cyclin dependent Kinase inhibitor 1B, a protein involved in cell cycle regulation) (Botelho *et al.,* 2009; 2013). A study by Tamini *et al.* (1996) also revealed a deletion of p16 gene (a Cyclin dependent Kinase 4 inhibitor) in about half of 47 tumour samples obtained from schistosomiasis-associated bladder cancer patients. Eighty six percent (6 out of 7) of Egyptian schistosomiasis-associated bladder cancer cases were reported to have mutations in certain exons within the p53 gene (Habuchi *et al.,* 1993).







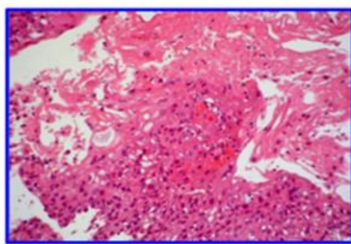


Figure 3: Carcinogenesis mediated by steroid like molecules derived from *S. haematobium* (Correia da Costa *et al.,* 2014)

2.4 Cancer Hallmarks

Generally, cancer cells in contrast to normal cells accumulate many genetic or epigenetic alteration of genes. These alteration may be caused by exposure of normal cells to radiation, infectious pathogens (e.g. Oncoviruses), oxidative stress, genotoxic chemicals and other environmental sources (Cancer Research UK, 2010). Oncogenic alterations in normal cells results in acquisition of peculiar phenotypic characteristics including; increased cell proliferation, resistance to apoptosis (insensitivity to growth-inhibiting signals), altered cell cycle, enhanced angeogenic and invasive potentials (Hanahan and Weinberg, 2000).

2.4.1 Oxidative stress

Oxidative stress refers to the state of a cell in which levels of its oxidants (mainly free radicals and reactive oxygen species, ROS) outbalances its antioxidant defenses. These oxidants (e.g. superoxide anion, hydrogen peroxide, hydroxyl radicals and organic peroxide) are mostly generated during endogenous cellular metabolic reactions and play a pivotal role in cell signaling (Jabs, 1999). However, excessive production of these oxidant leads to damage of important biomolecules (mainly lipids, proteins and DNA) resulting in consequential effect on the whole organism Oxidative damage and modification of these biomolecules have been reported to potentially induce mutations and neoplastic transformations leading to several debilitating diseases including cancer (Fang *et al.*, 2009 ; Khandrika *et al.*, 2009). The oxidative status of a cell is therefore important to its health and proper function. Reduced Glutathione (GSH), a tripeptide, is one of the key endogenous antioxidants that protects cells from oxidative damage. It quenches the

oxidative effects of free radicals and other electrophilic metabolites by serving as electron donor or reducing agent thus alternating between the reduced and oxidized (GSSG) state (Couto *et al.,* 2013). The ratio/level of GSH and GSSG content of a cell is therefore used as a measure of its oxidative status. Oxidative-stressed cells generally have a low GSH content but high GSSG content and vice versa.

2.4.2 Cell Proliferation

Normal cells grow under a tightly controlled cell division machinery. Oncogenic alterations however, results in dysregulation of the cell division controls leading to uncontrolled cell proliferation (Hanahan and Weinberg, 2011). Excessive cell proliferation has therefore been exploited over the years as one of the hallmark of carcinogenesis.

2.4.3 Apoptosis

Apoptosis is a cellular self-destruction process that is crucial for many biological events including development, tissue homeostasis and removal of unwanted or infected cells (Jeong and Seol, 2008). Oncogenic mutations of normal cells generally alters the normal equilibrium between cell proliferation and apoptosis (cell death); as cells acquire the ability to proliferate excessively with limited apoptosis. Cells undergoing apoptosis are characterized by certain morphological and biochemical changes which are exploited by several analytical techniques for characterization. Among these characteristic features include: blebbing, cell shrinkage, global mRNA decay, nuclear fragmentation nuclei/ chromatin condensation, externalization of interior membrane-bound phosphatidyl serine (PS) and mitochondrial membrane depolarization (Karam, 2009).

2.5 Review of methods

2.5.1 Detection of *Schistosoma* infection by microscopy

Microscopic detection of *Schistosoma* ova in urine and stool remains the gold standard for the detection of Schistosomiasis (Ibironke *et al.,* 2011). In this study, the conventional urine centrifugation technique described by Cheesebrough. (2005) was employed. This method mainly involves centrifugation of a given volume (mostly 10ml) of urine at a given speed and collecting pellets for microscopic observation. The urine pellets of infected persons usually contains eggs of the infecting-*Schistosoma* specie. The infecting-species are identified by morphological features of the eggs (i.e. shape of egg and characteristic position of spine) (Feldmeier and Poggensee, 1993). For instance, *S. haematobium* eggs are identified by their prominent terminal spine whiles *S. mansoni* eggs on the other hand, are recognized by their large lateral spine.

2.5.2 Confirmation of *Schistosoma* infection by Multipex PCR

The use of molecular approaches for detecting schistosome infections can be traced as far back as 1984 where the utility of rRNA probes for restricted fragment length polymorphisms (RFLPS) analysis was demonstrated by McCutchan and colleagues (McCutchan et al., 1984). In recent times, polymerase chain reaction (PCR) has shown its usefulness in the clinical diagnosis of a wide variety of pathogenic infections mainly due to its high sensitivity and specificity as opposed to other conventional approaches (Pontes *et al.*, 2002).

In this study, the multiplexed PCR technique developed by Webster *et al.* (2010) was used for identification and confirmation of the schistosome species infecting the study

participants. The system employs a universal forward primer and specific reverse primers for partial regions of mitochondrion COX1 gene of *S. haematobium, S. mansoni* and *S. bovis*; affording the advantage of detecting multiple infections in a given urine sample. Preference for this gene was due to the high abundance of mitochondrial DNA (which has highly conserved regions and structures) in the cell (Le *et al.,* 2001).

2.5.3 Determination of Oxidative stress by the O-pthalaldehyde method

O-phthaladehyde (OPA), a dialdehyde, consisting of two formyl groups attached to adjacent carbon centres on a benzene ring, is one of the vital probes that is used to measure the GSH concentration and for that matter the oxidative stress-status of a cell (Bill and Tarbell, 1954). OPA is a fluorescent probe that preferentially bind to cellular GSH to form a highly fluorescent iso-indole GSH conjugate that can be measured at wavelength of 340 nm (excitation) and 460nm (emission) (Senft *et al.,* 2000). Healthy or normal cells produce higher fluorescence as an indication of their high GSH content. Oxidative stressed cells on the other hand, produce a lower fluorescence due to their relatively low GSH concentration.

2.5.4 Evaluation of cell proliferation by the MTS Assay

Tetrazolium-based probes such as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyphenyl)-2-(4Sulfophenyl)-2H-tetrazolium (MTS) have been widely used by researchers to assess cell proliferative effects as well as cytototoxic effects of a variety of agents including drugs and toxicants (Botelho *et al*., 2013). The assay is based on the reduction of the yellow coloured MTS tetrazolium compound by NAD(P)H-dependent oxidoreductase enzymes present in viable cells to a soluble purple coloured formazan product with an increased absorbance which could be quantified spectrophotometrically at wavelength of 490 nm

(Berridge and Tan, 1993). Thus a higher absorbance gives an indication of highly proliferating or viable cells and vice versa.

2.5.5 Evaluation of apoptosis by guava nexin based-flow cytometry

The guava nexin reagent developed by Millipore^{TM} is used to detect cells undergoing apoptosis in a given population of cells using a flow cytometric system. The reagent consists of two fluorescent labelled apoptosis-detection probes, Annexin V and 7- Amino actinomycin D (7-AAD). Annexin V selectively binds to externalized phospatidyl serine (PS) on the surface of compromised cell membranes (a feature of cells undergoing early apoptosis). 7-AAD on the hand, permeates cells with heavily compromised cell membranes (a feature of cells in late apoptosis) and selectively binds to the DNA/nucleus. By this principle, the proportion of four different category of cells (i.e. viable cells, early apoptotic cells, late apoptotic cells and necrotic cells) in a given cell population following staining with nexin reagent could be characterized and quantified in a flow cytometric setting (Appiah-Opong *et al.*, 2016).

2.5.6 Evaluation of nuclei integrity by Hoechst 33258 fluorescence staining

Hoechst 33258 dye is one of the widely used fluorescent probes for detecting nuclei/chromatin condensation. It is a DNA intercalator hence it selectively binds to the nucleus of stained fixed cells producing a characteristic blue fluorescence when observed under a microscope. Thus viable cells (characterized by intact nuclei) could be distinguished from apoptotic cells (characterized by morphologically shrunk/condensed nuclei) (Appiah-Opong *et al.,* 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design and area

The study was carried out in Galilea, a community situated along the Densu Lake in the Ga South District of the Greater Accra Region. The Galilea community is a schistosomiasis endemic area (Aryeetey *et al*., 2000). Along the banks of the lake are aquatic plants (*Ceratophyllum demersum*, *Pistia stratiotes* and *Nymphaea odorata*), which harbour the schistosome host snails (*Bulinus truncatus rohlfsi* and *Biomphalaria pfeifferi*), making the lake the main source of infection (Danso-Appiah *et al.,* 2004). Inhabitants of the community, utilize water from the lake for domestic purposes including drinking, bathing and washing clothes, whilst others, particularly children, swim and play in it. Galilea is inhabited mostly by migrant fisher folks and their families.

A total of 209 participants made up of 137 (66%) males and 72 (34%) females with ages ranging from 5 to 60 years were recruited into the study. Majority of the recruited participants were between the ages 5-14 (69%). In terms of occupation, students (174), representing 83%, constituted the largest group recruited for the study followed by those in other occupations (10%) and then fisher folks (comprising mainly of fishermen and fish sellers along the river banks) who constituted 7% of the study participants (Table 3.1).

Table 3.1: Characteristics of study participants

Characteristic		Number (%)*
Gender	Male	137 (66)
	Female	72 (34)
Age group (years)	5-14	145 (69)
	15-24	36 (17)
	25-34	11 (6)
	35-44	10 (5)
	45-54	4 (2)
	55-60	3 (1)
Occupation	Students	174 (83)
	Fisher folks	15 (7)
	Others	20 (10)

* Total number of participants = 209

3.2 Sample collection

After consents were sought from community leaders and participants, collection of urine samples was carried out. All participants (n= 209 and ≥ 5years) were registered with an identification number after they had answered questionnaires bordering on their socio-demographics, water (Densu river) contact activities, knowledge and clinical symptoms of schistosomiasis among others. Each participant was given a 50 ml container labelled with a unique identification number and was asked to provide minimal urine volume of 10 ml (collected between 10 am and 2 pm). Samples were transported to the laboratory of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana and processed to ascertain the *Schistosoma* infection status of the participants.

3.3 Ethical considerations

The study was approved by the Scientific Technical Committee and Institutional Review Board of the NMIMR (Appendix IIa). Approval for urine sample collection was also obtained from the Ga South Municipal health directorate of the Ghana Health Service (Appendix IIb).

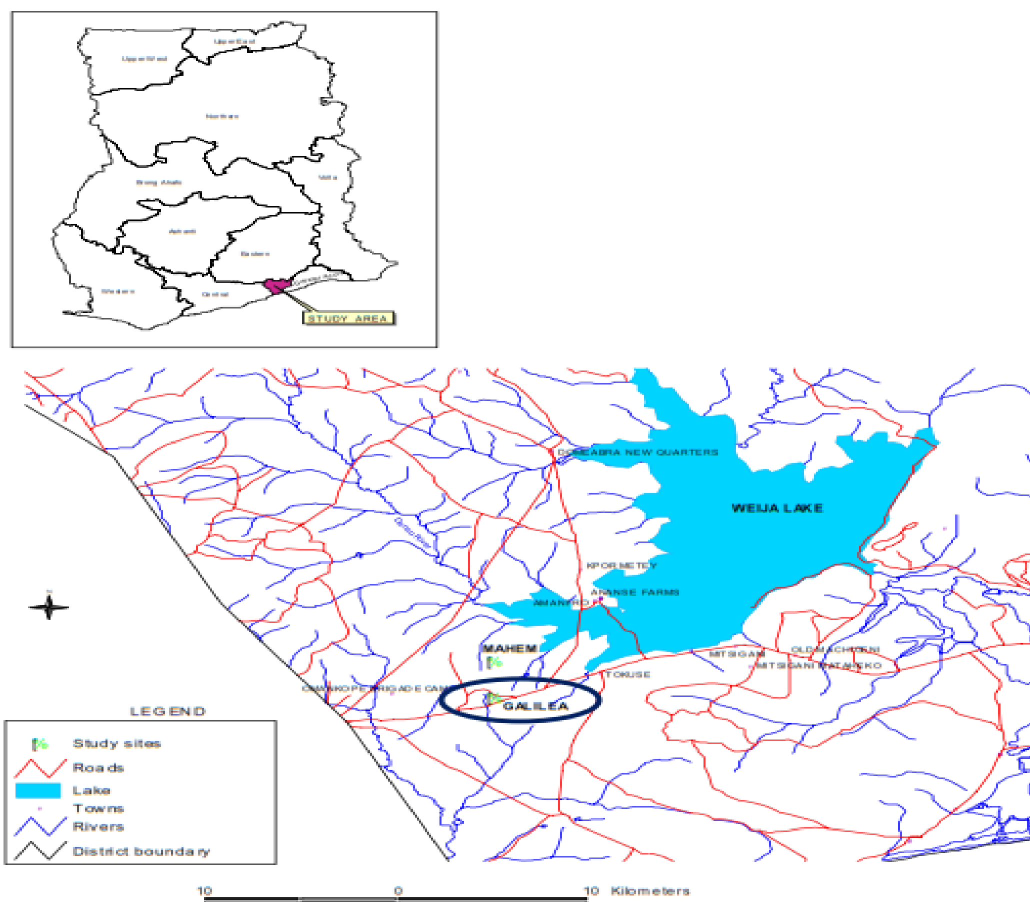


Figure 4: Geographical description of study area (Aboagye and Edoh, 2009)

3.4 Sample preparation and laboratory analysis

3.4.1 Urine analysis and microscopy

Each urine sample was tested for hematuria, proteinuria and pH using Urine Reagent Strips for Urinalysis (URS-10, USA). The presence and quantification of schistosome ova was determined by microscopy following the conventional urine centrifugation technique as described by Cheesebrough, (2005). Briefly, 10 ml of urine samples was centrifuged for 5 min at 1500 rpm (Hitachi CF7D2, Japan). Supernatant was discarded and pellets were loaded unto microscope slides. The slides were analyzed microscopically for schistosome eggs. Average egg count per participant was reported as egg per 10 ml of urine and then categorized into egg intensity classes: negative, light intensity (1 to 49 eggs per10 ml) and heavy intensity (≥50 eggs per 10 ml).

3.4.2 Molecular analysis of schistosome species

3.4.2.1 Primer selection

Schistosoma specie-specific primers amplifying the schistosome partial cox1 mitochondrial DNA region, consisting of a universal forward primer ShbmF (5′-TTTTTTGGTCATCCTGAGGTGTAT-3′) with three species specific reverse primers, ShR (5′-TGATAATCAATGACC CTGCAATAA-3′) for *S. haeamtobium,* SbR (5′-CACAGGATCAGACAAACGAGTACC-3′) for *S. bovis* and SmR (5′-TGCAGATAAAGCCACCCCTGTG-3′) for *S. mansoni*, were selected for amplification of *Schistosoma* sp DNA present in the urine samples (Sady *et al.,* 2015).

3.4.2.2 DNA Isolation

DNA was extracted from urine samples using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as instructed by the manufacturer. Briefly, 200 µl of the heat precipitated (100 ºC for 10 min) urine samples were added to 180 µl buffer ATL (containing 20 µl Proteinase K) and incubated for 2 h at 55 °C. A volume of 400 µl was then added, and incubated for 10 min after vortexing. AL Buffer was then added to sample, mixed thoroughly and incubated at 70 °C for 10 minutes. Sample was then precipitated with 400 µl of 100% ethanol and mixed thoroughly by vortexing. The mixture was carefully applied to spin columns and spun for 10 min at 10,000 rpm (Eppendorf 5417R, Germany). The spin columns were then sequentially washed with 500 µl of AW1 and AW2 buffers respectively after which the DNA was eluted with 200 µl AE buffer. The DNA obtained was stored at -20 °C until used for PCR amplification.

3.4.2.3 Multiplex PCR amplification

PCR amplification was performed in 25 µl reaction mix comprising 6.25 µl master mix (QIAGEN Multiplex PCR HotStarTaq DNA Polymerase, Hilden, Germany), 0.2 µl of 1.6 µM the universal forward primer (ShbmF), 0.1 µl each of 0.8 µM of the three reverse primers (ShR, SbR and SmR) and 2.5 µl of DNA. PCR cycling conditions employed was as follows: 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s and 72 °C for 1 min 30 s, with a final extension of 10 min at 72 °C. PCR products were visualized (375 bp for *S. mansoni*, 543 bp for *S. haematobium* and 306 bp for *S. bovis*) on a 2% agarose gel.

3.5 Preparation of schistosome egg antigens (SEA)

Urine samples of schistosome-infected persons were spun at 1500 rpm for 5 min and supernatants were discarded. Pellets (containing schistosome eggs) was suspended in Phosphate buffered saline (PBS) and washed 3 times by centrifuging at low speed. Samples were then frozen at -80°C for 10 min. The frozen samples were ultrasonified for 2 min in a repeated cycle of 5 times. When approximately 95% of the eggs were disrupted, the lysate was then centrifuged for 20 min at 1000 rpm at 4 °C (Hitachi CF7D2, Japan). The supernatant was then collected and ultra-centrifuged for 90 min at 14,000 rpm at 4 °C. The supernatant (SEA) was filter-sterilized with 0.2 µm filter and stored at -80°C until use (Botelho *et al.*, 2009). Protein concentration of SEA was determined by the Bradford method.

3.6 Cell culture

Human normal prostate cells (PNT2) was maintained in RPMI-1640 culture medium enriched with 10% Foetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin and incubated at 37°C in the presence of 5% $CO_{2.}$ The cells were sub-cultured every week. Before treatments, the cells were serum-starved overnight.

3.7 Oxidative stress assay

Oxidative stress-inducing effect of SEA was determined by evaluating the levels of cellular reduced glutathione (GSH) as described by Martin *et al.* (2008). Following exposure to SEA, cells (2×10^{5} cells/ml) were detached, homogenized by ultrasound with 5% trichloroacetic acid for 15 min. The cell homogenates were centrifuged for 30 min at 3000

rpm (Hettich zentrifugen MIKRO 200, Germany) and supernatant were treated with 10mg/ml O-phthalaldehyde (OPA). Following incubation in the dark for 15 min, GSH levels in the supernatant was obtained by measuring at wavelength of 340 nm (excitation) and 460nm (emission).

3.8 Cell proliferation assay

The proliferative effect of SEA was determined using the CellTiter 96 AQ non-radioactive cell proliferation assay as described by Soares *et al.* (2007). Briefly, cells were plated at 1×10^4 cells/well in a 96 well plate and challenged with varying concentrations of the SEA for 24 h and incubated at 37°C. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was then added to each well and cells were further incubated for 4 h at 37°C. The amount of formazan produced (gives a measure of cell viability) was measured at a wavelength of 490 nm with a microplate reader (Tecan Infinite pro).

3.9 Apoptosis assay

The effect of SEA on apoptosis was elucidated by flow cytometry**.** Briefly, cells (2×10^5 cells/ml) were cultured overnight in 24 well plates and challenged with varying concentration of SEA for 24 h. The cells were then harvested and treated with equal volumes of guava nexin reagent (contains Annexin V- FITC and7-AAD), incubated for 20 min in the dark at room temperature and analyzed immediately after incubation with a flow cytometer (Guava easyCyte HT).

3.10 Nuclei integrity analysis

Effect of SEA on cell nuclei integrity was determined using the cell permeable DNA dye Hoechst 33258 according to Uto *et al.* (2013). Briefly, cells were cultured overnight in 24-well plates at a density of 2×10^5 cells/ml and treated with varying concentration (0 - 12.5μg/ml) of SEA for 24 h. The cells were collected, washed once with PBS and fixed with 1% glutaraldehyde. After 30 min incubation at room temperature, cells were centrifuged and supernatants were discarded. Cell pellets were then stained with 1 mM Hoechst 33258. Stained cells were observed under a fluorescence microscope equipped with a digital camera to determine the extent of nuclei condensation.

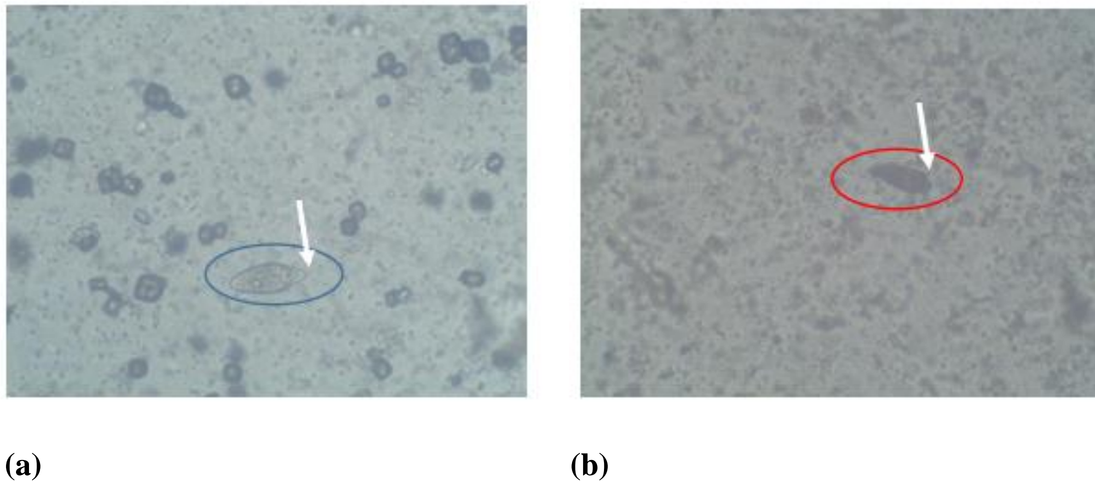
CHAPTER FOUR

RESULTS

4.1 Identification of schistosome-infected persons

4.1.1 Microscopic Analysis

Urine samples obtained from all study participants were analyzed for the presence of *Schistosoma* eggs by the conventional urine centrifugation method. Out of the 209 participants recruited and sampled, 30 (14.4%) had *Schistosoma* eggs present in their urine samples (Fig 4.1).



(a) **(b)**

Figure 4.1 *Schistosoma* eggs in urine of infected participants (a) *S. haematobium* egg (terminal spine indicated with arrow) (b) *S. mansoni* egg (lateral spine indicated with arrow). Eggs were visualized under an Olympus CX12 compound microscope (magnification x20).

4.1.2 Prevalence and intensity of schistosome infection

Schistosome eggs were found in 30 (14.4%) of the urine samples. Out of the 30 infected persons, 21 (70%) were males whereas 9 (30%) were females. Regarding intensity of infection, 8 (27%) of the infected participants were heavily infected with schistosome eggs

whereas 22 (73%) recorded light infections (Table 4.1). Both *S. haematobium* and *S. mansoni* eggs were found in 11 urine samples; 10 of which were from male participants and 1 from a female participant. The remaining 19 urine samples (11 males and 8 females) were found to be infected with S. *haematobium* eggs only. None of the urine samples screened were found to be infected with S. *mansoni* eggs only (Fig 4.2)

Table 4.1: Prevalence and intensity of schistosome infection among participants

	Male	Female	Total
Number sampled	137	72	209
Number Infected	21	9	30
Prevalence (%)	15.3	12.5	**14.4**
Intensity of Infection			
Light infection: 1-49 eggs/10 ml	15	7	22
Heavy infection: ≥ 50 eggs/10 ml	6	2	8

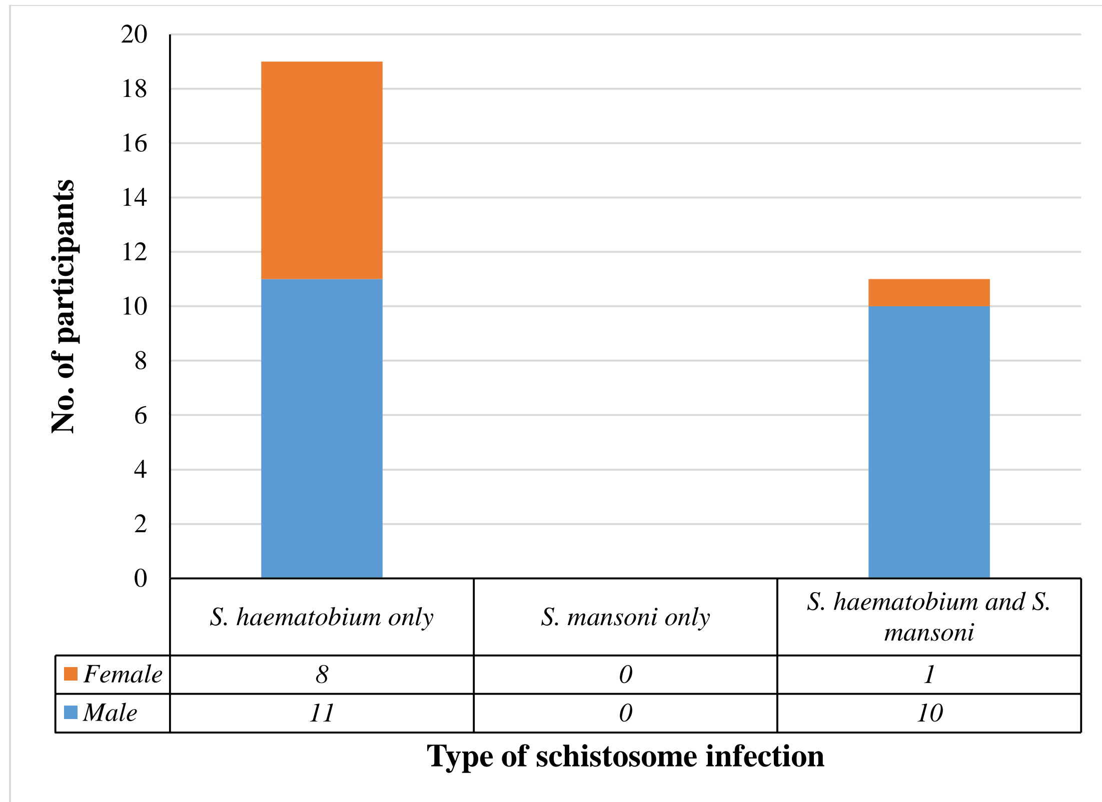


Figure 4.2: Type of schistosome infection identified among infected participants

4.2 Molecular Analysis

4.2.1 Molecular detection of *S. haematobium* and *S. mansoni* species in infected participants

Confirmation of infection and identification of infecting species was done by detecting the presence of schistosome DNA in all the 209 urine samples. A universal forward primer and reverse primers specific for partial regions of mitochondrion COX1 gene of *S. haematobium*, *S. mansoni* and *S. bovis* were used. In all, 21 (10%) participants were found to be positive for schistosome infection. Out of the 21 infected participants, 7 were found with *S. haematobium* infection only, 9 were found to have *S. mansoni* infection only and 5 were found to have both *S. haematobium* and *S. mansoni* infection (Fig 4.4).

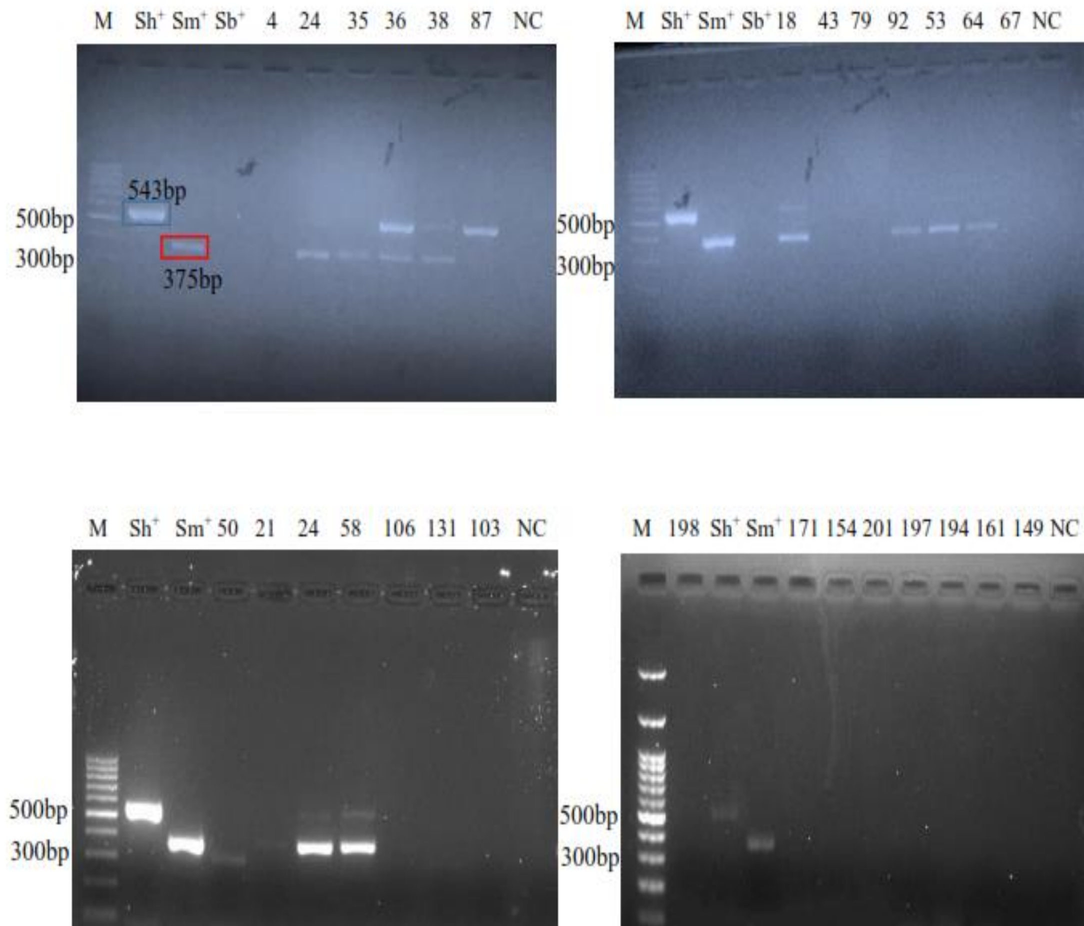


Figure 4.3: Representative gels showing PCR amplification of the partial COX1 gene of *Schistosoma* species. **M**- 100 bp Molecular weight marker, **Sh⁺**- Positive control (*S. haematobium* DNA), **Sm⁺**- Positive control (*S. mansoni* DNA), **Sb⁺**- Positive control (S. *bovis* DNA), **NC**- Negative control (nuclease free-water), **Numbers** (4, 24, 35 etc.)-.DNA of participants. All amplicons were run on a 2% agarose gel.

4.3 Cancer hallmark-inducing potential of schistosome soluble egg antigens (SEA)

4.3.1 Oxidative-stress inducing effect of SEA on PNT2 prostate cells

In comparison to serum-fed cells, SEA dose-dependently and significantly depleted cellular GSH levels in treated cells (P=0.001 and P=0.001 for 6.25 and 12.5 µg/ml SEA, respectively). Additionally, 12.5 µg/ml SEA significantly depleted GSH levels in comparison to the untreated serum starved cells (negative control) (P=0.023) (Fig 4.4)

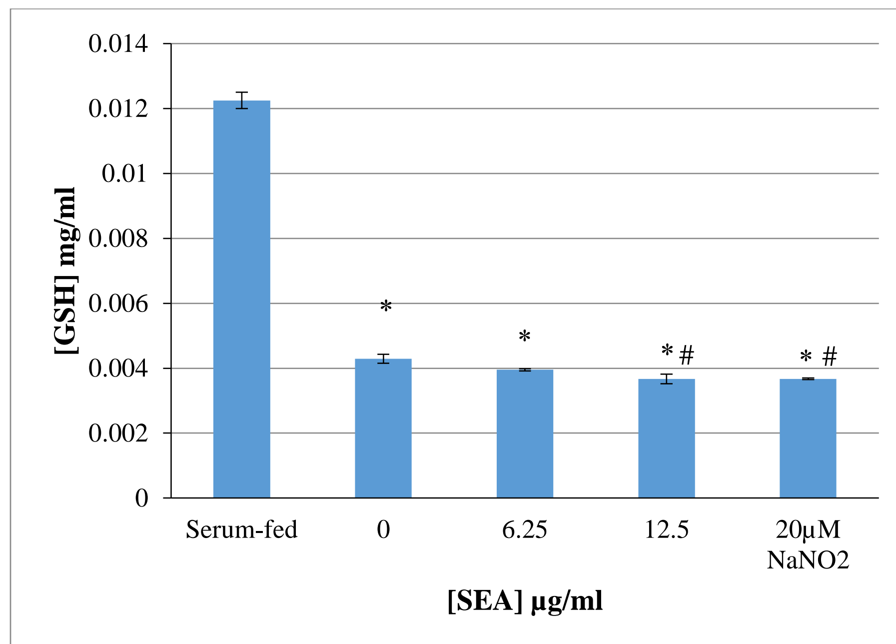


Figure 4.4: SEA depletes GSH levels in prostate (PNT2) cells. Statistical significance by student's t test; *p≤ 0.05 (Serum fed vs treated cells) #p≤ 0.05 (untreated vs treated cells). Results are representative of three independent experiments. Shown are mean ± SD.

4.3.2 Cell proliferative effect of SEA on PNT2 prostate cells

The SEA dose dependently increased cell viability and proliferation up to the concentration of 12.5 µg/ml (P=0.03). Subsequently, there was a drastic decline at the concentrations, 25 µg/ml and 50 µg/ml (Table 4.2).

Table 4.2 SEA induces proliferation of PNT2 cells

[SEA] µg/ml	Cell proliferation values[1,2]	p-value[3]
0	0	
3.125	6.16 + 6.16	0.201
6.25	25.66 + 3.68	0.091
12.5	32.53 + 1.48	0.029*
25	-21.84 + 10.84	0.293
50	-30.53+ 12.33	0.244

[1] Cell proliferation values are means + SD. [2] Results are representative of three independent experiments. [3] Statistical significance by student's t test; $^{*}p \leq 0.05$.

4.3.3 Effect of SEA on PNT2 prostate cell apoptosis

SEA treated cells recorded a concentration-dependent decrease in percentage of apoptotic cells relative to the untreated cells/negative control (i.e. 11.8%, 9.3% and 4.9% for the negative control, 6.25µg/ml SEA and 12.5µg/ml SEA treated cells, respectively). Serum-fed cells (Positive control) recorded the lowest percentage (3.3%) of apoptotic cells (Fig 4.5). Conversely, SEA treated cells recorded a concentration-dependent increase in the percentage of viable cells. The increase in percentage of viable cells followed the order: Serum-fed cells (94.8%)>12.5µg/ml SEA-treated cell (93.1%) >6.25µg/ml SEA-treated (89.5%) > untreated cells (87%). There was significant difference in the percentage of viable cells between the serum-fed cells and untreated cells (P=0.03) and 6.25µg/ml SEA-treated cells (P=0.02). There was also a significant difference in the percentage of apoptotic

cells between the serum-fed cells and untreated cells (P= 0.0317) and SEA-treated cells (P= 0.02) (Fig 4.6).

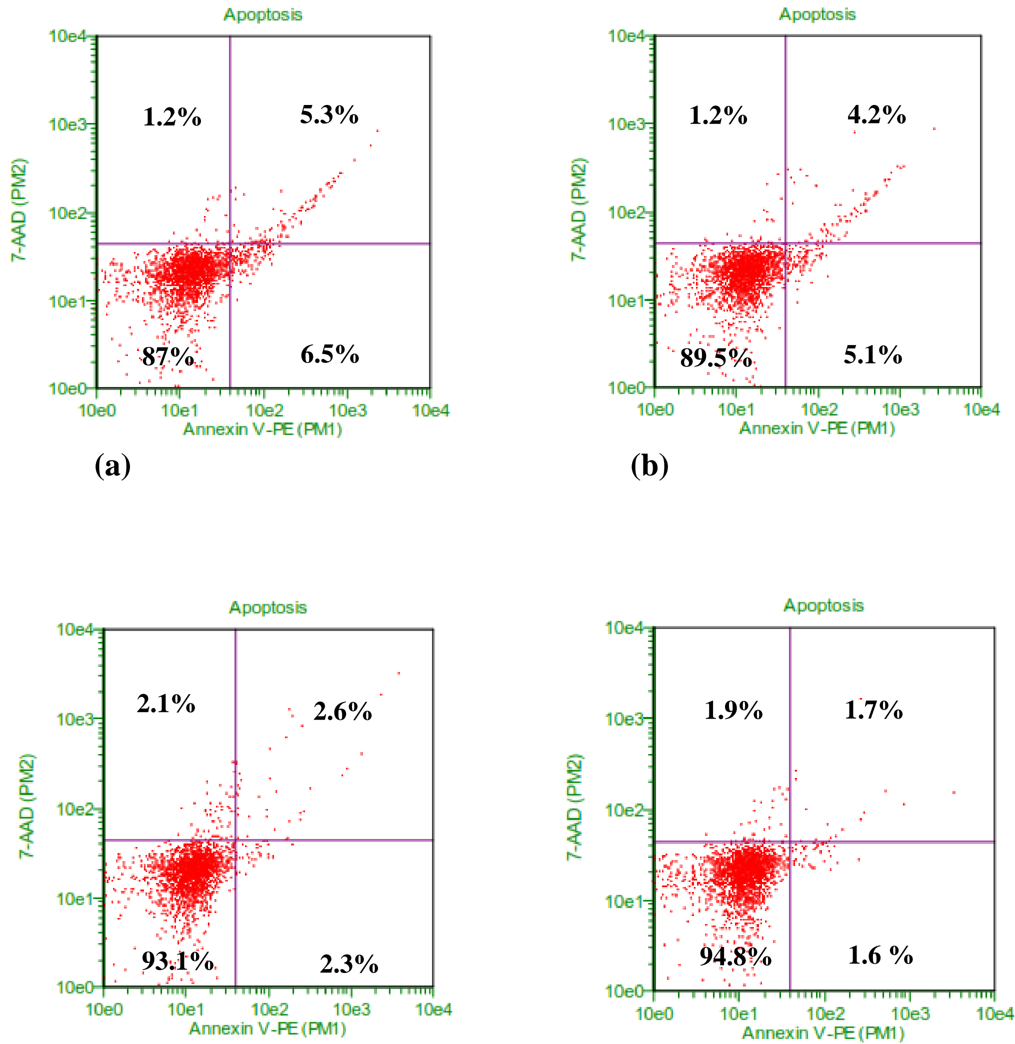


Figure 4.5: SEA induces apoptosis in PNT2 cells. (a) Negative control (b) 6.25µg/ml SEA (c) 12.5 µg/ml SEA (d) Serum-fed (Positive control). **Lower left quadrant**- Viable cells, **Lower right quadrant** -Early Apoptotic cells, **Upper left quadrant**- Nuclear debris, **Upper right quadrant**-Late apoptotic cells. Data was acquired by Guava EasyCtye^{TM} flow cytometer.

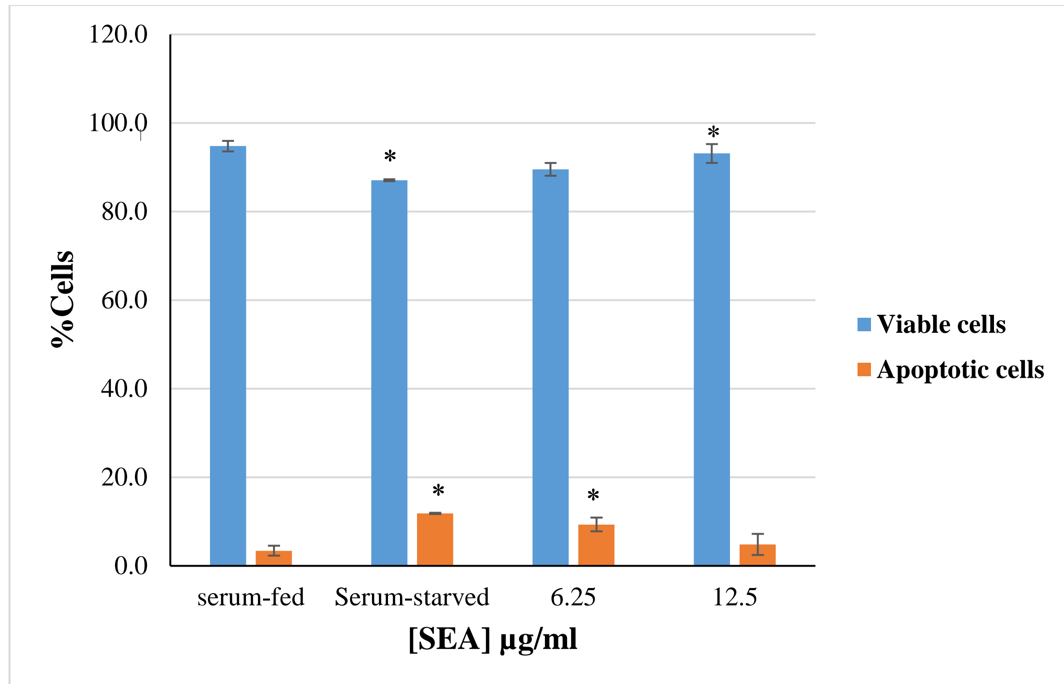


Figure 4.6: Comparison of apoptosis and cell viability among various experimental groups. Statistical significance by student's t test; $^{*}p \leq 0.05$ (serum-fed vs treated groups) Results are means $\pm$ SD. Results are representative of three independent experiments.

4.3.4 Effect of SEA on nuclei integrity of PNT2 prostate cells

As shown in Figure 4.7, the untreated cells displayed the most nuclei morphological changes characterized by chromatin condensation/nuclei shrinkage. The SEA-treated cells exhibited a dose-dependent reduction in the number of morphologically condensed nuclei. The serum-fed cells on the other hand presented intact nuclear morphology.

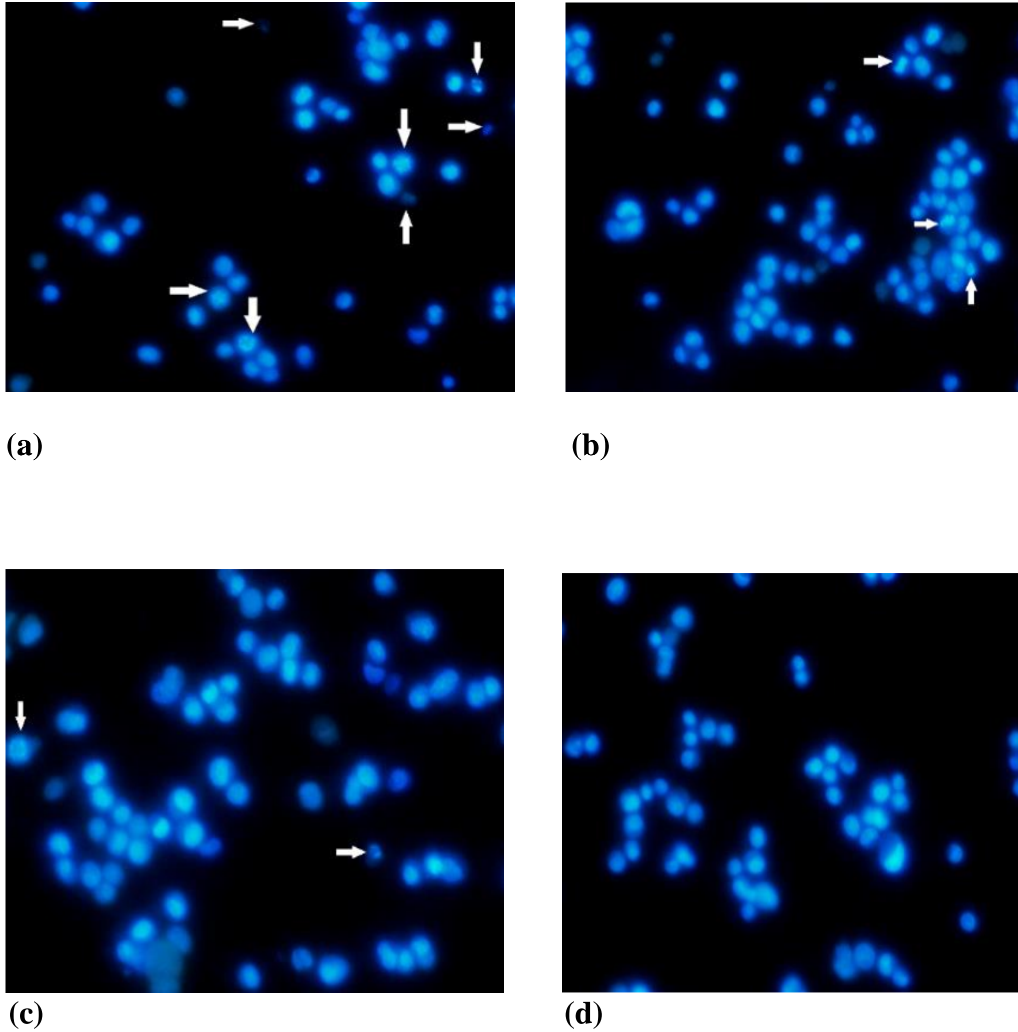


Figure 4.7: SEA diminishes nuclei condensation in PNT2 cells. (a) Negative control (b) 6.25µg/ml SEA (c) 12.5 µg/ml SEA (d) Serum-fed (Positive control). Condensed and morphologically altered nuclei indicated by arrows. Cells were stained with Hoechst 33258 dye and nuclei visualized with an Olympus DP 72 fluorescence microscope (magnification 40x)

CHAPTER FIVE

DISCUSSION

Schistosomiasis (one of the major neglected tropical diseases) has been implicated in several case-report studies as a possible aetiological agent in prostate cancer pathogenesis. In this study, we elucidated the oncogenic effect of schistosome soluble egg antigens (SEA) derived from urine of experimentally confirmed schistosome-infected persons, on normal prostate cells by exploring certain cellular events characteristic of carcinogenesis including oxidative stress, cell proliferation and apoptosis.

Despite intense efforts being put in place to eradicate helminthic diseases, some communities in Ghana still remains a hotspot for schistosomiasis. Studies conducted by Aboagye and Edoh (2009) and Aryeetey *et al.* (2000) , revealed that some communities near the Densu river, including Manheim and Galilea were endemic for schistosomiasis, with urinary schistosomiasis (caused by *S. haematobium*) being the most prevalent. In this study, Galilea was chosen as the site for urine sample collection. Male participants constituted 66% (137/209) and female participants constituted 34% (72/209). The disparity (bias towards male) in gender with regards to the study is attributed to the purpose of the investigation which was geared towards probing the association between schistosomiasis and prostate cancer, a disease associated with males only. Additionally, because most of the recruitment took place close to the Densu river, most of the participants encountered were males; who engage relatively in more water contact activity as compared to the female counterparts. Most of the recruited participant (68%) were children and teenagers between 5 to 14 years and adolescents within the age range of 15 to 24 years (17%), majority of whom were students (83%). This may be explained by the fact that children, teenagers and

young adults constitute the group of persons that engage in most of the water contact activities (especially fishing, swimming, washing, fetching of water and playing) that are associated with the transmission of schistosomiasis (Wagatsuma *et al.,* 2003; Useh and Ejezie, 1999).

Microscopic detection of parasite eggs present in urine or stool still remains the gold standard for the detection of schistosomiasis (Ibironke *et al.,* 2011). The conventional urine centrifugation technique described by Cheesbrough (2005) was employed prior to microscopic evaluation and a 14.4% (30 out of 209 participant) infection prevalence was recorded in this study. This figure is lower than an earlier reported prevalence of 49% for the community (Galilea) by Aboagye and Edoh in 2009. This wide disparity in prevalence may be attributed to the increasing knowledge of the disease among indigenes as revealed by responses to administered questionnaires as well as the periodic mass administration of Praziquantel (the main chemotherapy for schistosomiasis) in the schools and the community over the years. With regard tothe infected participants, more males (n=21) were found to be infected than females (n=9) possibly due to the relatively fewer females (n=72) recruited in the study as opposed to male participants (n=137). Comparing prevalence within groups, a disease prevalence of 15.3% (21 out of 137) was recorded for males and a 12.5% (9 out of 72) prevalence for females. This observation is expected considering the fact that socio-cultural practices such as fishing, farming and other recreational activities expose males to infected water bodies than their female counterparts (Nsowah-Nuamah *et al.,* 2001). Of the 30 infected persons, *S. haematobium* infection was identified in 19 participants. Interestingly, 11 participants had mixed infections with *S. hamematobium* and *S. mansoni*. Conventionally, *S. haematobium* is responsible for urinary schistosomiasis

hence it is normal to detect *S. haematobium* eggs in the urine of infected persons. *S. mansoni* on the other hand is responsible for intestinal schistosomiasis hence their presence in an infected person is detected mainly through microscopic analysis of eggs in stool and not the urine. Detection of *S. mansoni* eggs in urine sample of some infected persons in this study gives an indication of the community's endemicity to both species. The presence of both schistosome species may be blamed to insanitary behavourial activities such as urination and defecation by *S. mansoni* infected persons into the waterbodies. Also, schistosome worms are unable to discriminate between species, so in a situation where an individual has both *S. haematobium* and *S. mansoni* infection, there could be a possible interspecies pairing/hybridization (Tchuenté *et al.,* 1997; Steinauer *et al.,* 2008; Huyse *et al.,* 20013; Webster *et al.,* 2013). A pairing between a female *mansoni* worm and a male *haematobium* worm will result in the relocation of the paired worms from the liver to the venous plexus of the bladder where they mature into adult parasites and may produce parthenogenetic *S. mansoni* eggs (Khalil and Mansour, 1995; Southgate *et al.,* 1998). A similar observation was reported in an epidemiological study in two schistosomiasis-endemic communities in the Northern part Senegal. In that study, Meurs *et al.* (2012) reported that 15% of subjects that had mixed *S. haematobium* and *S. mansoni* infections, manifested ectopic egg excretion (i.e. *S. masoni* eggs in urine and *S. haematobium* eggs in faeces) as results of heterospecific pairing.

Diagnosis of *Schistosoma* infection in asymptomatic and chronically infected persons is a clinically important challenge. Asymptomatic and chronically infected persons pass few eggs in urine and stool as most (eggs) get trapped in tissues hence their infection status is often missed by microscopy; which is the gold standard (Ibironke *et al.,* 2011).

Additionally, conventional microscopy is very subjective and bedeviled with the challenge of accurate deciphering of closely related schistosome species due to similar egg morphologies. As infection levels decline over the years, low-level parasitemia may remain undetected in infected persons who may subsequently develop severe pathology and also serve as reservoir of infection (Koukounari *et al.,* 2009). Alternative techniques that overcome these aforementioned challenges are therefore being developed. In recent times, polymerase chain reaction (PCR) assay has shown potential as an effective method of diagnosis due to its relatively high specificity and sensitivity (Mharakurwa *et al.,* 2006). In this study, the mitochondrion COX1 gene-based multiplexed PCR technique developed by Webster *et al.* (2010) was used for identification and confirmation of the schistosome species infecting the study paticipants. Whereas no participant was detected to have *S. mansoni* infection only via microscopic evaluation of urine samples, PCR amplification in our study, revealed that 9 participants carried single infections with *S. mansoni*. This may be attributed to the fact that the infected participants were lightly infected or chronically infected hence passed few or no *S. mansoni* eggs in the urine thus resulting in microscopy-negatives. This also alludes to the high sensitivity of molecular techniques such as PCR than conventional microscopy. Interestingly, fewer (21) infection-positive participants were detected by PCR as compared to microscopy which recorded 30 infected participants. This observation may be due to the likely circulation of genetically-hybrid schistosome eggs with similar ("*haematobium*-like or *mansoni*-like") morphologies as a result of the suspected heterospecific pairing. Contrary to the beliefs that heterospecific (between phylogenetically distant) pairing always resulted in parthenogenetic eggs and offsprings, recent studies have experimentally demonstrated that interspecie pairing could generate

hybrids (eggs and for that matter offsprings) that might remain undetected by conventional PCR (Huyse *et al.,* 2013). In their novel discovery, they amplified the nuclear internal transcribed spacer (ITS1) rDNA and partial mitochondrial COX1 fragment of both eggs and miracidia (derived from a heterologous pairing of *S. haematobium* and *S. mansoni* worms) and identified two ITS1 rDNA genotypes within a single egg and miracidia (one identical to *S. mansoni* and the other identical to *S. haematobium*) suggestive of a hybrid descent. However, analysis of the COX1 clones showed that they always belonged to either one of the parental species, pointing to the limitation of using the mitochondrion COX1 gene as a biomarker for identifying hybrid infections. This may explain why the suspected hybrids were undetected in our molecular investigation culminating in the recorded low number of infected participants as compared to microscopy.

Several *in vitro* studies have identified increased reactive oxygen species (ROS) as initiation agents in cancer (Botelho *et al.,* 2013). The production of ROS and associated oxidative stress have been implicated in cellular abnormalities such as imbalance of intracellular calcium homeostasis, disruption of membrane lipids and DNA aberrations (Shukla *et al.,* 2011). Most importantly, ROS generation in cells elicits a corresponding protecting effect from endogenous cellular antioxidants including GSH and other essential enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione transferase. Extreme accumulation of oxidants (ROS) may result in depletion and reduction of cellular antioxidant defenses resulting in stress condition that leads to the fore-mentioned deleterious consequences. Earlier studies (Gouveia *et al.,* 2013: Bothelho *et al.,* 2013) have revealed the presence of estradiols in *Schistosoma* eggs and implicated them in pathogenesis of cancer via an oxidative stress route. Cellular/tissue enzymes including

Cytochrome P450 (CYP 1B1) and peroxidases metabolically oxidize these estradiols into highly reactive quinone metabolites (Correia da Costa *et al.,* 2014). Conjugation of these metabolites with GSH and enzymatic reduction to reform catechol estrogens are the processes by which cells protect themselves from the accumulation of these reactive metabolites. Thus accumulation of these metabolites results in depletion of GSH and accumulation of GSSG resulting in stress condition. In this study, the observed significant increase in the depletion of prostate cell GSH levels may be attributed to the accumulation of reactive quinone metabolites with increasing concentration of SEA. The probe, OPA binds to free cellular GSH to form a highly fluorescent iso-indole GSH conjugate (Senft *et al.,* 2000). The concentration of GSH in unstressed (serum-fed) cells, are relatively high hence resulting in the observed increase in fluorescence intensity upon treatment with OPA. Conversely, the dose-dependent decrease in fluorescent intensity recorded for the SEA treated cells may be attributed to the dose-dependent decrease in GSH levels possibly arising from the metabolic accumulation of reactive quinone metabolites at high concentrations of SEA which preferentially conjugate to GSH thus depriving OPA of enough substrate (GSH) for conjugation. This observation is consistent with an earlier study by Botelho *et al*. (2013) who challenged HCV 29 (normal urothelial) cells with *S. haematobium* soluble egg antigens to investigate the mechanism underlying of schistosome-associated bladder cancer.

The oncogenic effects of schistosome infection have been reported in various geographical settings; providing evidence that alterations of certain cell proliferation-associated genes (downregulation of p27, deletion of p16 and mutation in exons 5, 6, 8 and 10 of p53) are responsible for schistosomiasis-associated bladder cancers (Botelho *et al.,* 2009; Tamini *et*

al., 1996; Habuchi *et al.,* 1993). Probing further, Correia da Costa *et al.* (2014) have implicated estradiol-quinones (the major carcinogen of estrogen) as the key genotoxic agent responsible for its ability to form depurinating adducts that have oncogenic consequences. In this study, the cell proliferative effect of SEA was elucidated by the MTS cell proliferation assay. A dose-dependent increase in absorbance due to increase in cell proliferation was recorded for SEA-treated cells (up to 12.5µg/ml concentration) as compared to the serum-starved control. This is attributed to the concentration-dependent increase in the levels of metabolically generated estradiol-quinones leading oncogenic genotoxicities characterized by increased cell proliferation. On the other hand, extreme oxidative stress on cells have been implicated in programmed cell death (apoptosis) via the intrinsic (mitochondrion-dependent) pathway (Bohm and Schild, 2003). Extreme cellular oxidative stress induces the loss of the mitochondrial membrane potential ($\Delta\psi$) and cytochrome c release from the mitochondria to the cytosol, leading to caspase-9-dependent activation of caspase-3 and finally cell death (Armstrong, 2006). Thus the observed decrease in absorbance/cell proliferation at the very high SEA concentrations (25 µg/ml and 50 µg/ml) may be attributed to the cytotoxic effect of the excessive accumulation of highly reactive estradiol-quinone intermediates.

Cancer cells have rewired genetic architecture (upregulated oncogenes and down regulated tumour suppressor genes) that enables them to grow uncontrollably with diminished or inhibited apoptosis (Fearon and Vogelstein, 1990). To understand schistosomiasis-associated carcinogenesis, Botelho *et al.* (2010) demonstrated that inactivation of p27 and upregulated expression of Bc-L2 contributed to cancer hallmarks (mainly stimulated cell proliferation and inhibited apoptosis) in Chinese Hamster Ovary (CHO) cells challenged

with lysates of *S. haematobium* eggs and adult parasites. p27 (Cyclin dependent kinase inhibitor 1B) is a tumour suppressor protein that regulates cell cycle progression at G1 phase by binding to and inhibiting activation of cyclin D and cyclin dependent Kinase 4 (Chu *et al*., 2007). Its inactivation/downregulation therefore, results in unregulated and excessive cell proliferation. On the other hand, Bc-L2 (B cell lymphoma 2) is a protein localized to the outer membrane of mitochondria, and plays a crucial role in promoting cell survival and antagonizing the actions of pro-apoptotic proteins (proteins that promote apoptosis).

This phenomenon may explain the observed dose-dependent increase in percentage of viable cells with corresponding concentration dependent decrease in percentage of apoptotic cells in SEA-treated cells relative to the negative control (SEA-untreated serum-starved cells).

Apoptosis is a highly regulated and controlled process that is characterized/preceded by distinct cell morphological changes including: blebbing, cell shrinkage, global mRNA decay, nuclear fragmentation and chromatin condensation (Karam, 2009). These cell morphological changes are triggered by multiple cellular pathways of which two have been well characterized namely: the intrinsic pathway and the extrinsic pathway. Both pathways are mediated by a group of cysteine proteases known as caspases. Caspases initially exist as zymogens but are activated by proteolytic cleavage of specific fragments (Gonzalez *et al*., 2010). They are classified into initiator Caspases (Caspase 8, 9, 10) and effector Caspases (3, 6, 7).

As an effector Caspase, upon activation, Caspase 3 mediates apoptosis by cleaving (inactivating) PARP (Poly-ADP Ribose Polymerase); a protein which plays a key role in

cellular assembly and is involved in repair of single strand DNA breaks. Additionally, activated Caspase 3 activates the cytoplasmic endonuclease CAD (Caspase Activated DNase) by cleaving off its inhibitory subunit ICAD (Inhibitor of CAD) (Liu *et al*., 1996). Activated CAD translocates into the nucleus and cleaves DNA into internucleosomal fragments and induces nuclei condensation/shrinkage; morphological phenotypes characteristic of apoptosis. In this study, previously established apoptosis-diminishing effect of SEA was further investigated by elucidating its (SEA) effect on nuclei morphology of prostate cells using the DNA/nuclei-intercalating fluorescence dye, Hoechst 33258. Fluorescence microscopic evaluation revealed a dose-dependent decrease in the number of morphologically condensed nuclei in SEA-treated cells. However, control (untreated) cells displayed numerous morphologically condensed nuclei. This observation suggests that SEA may induce oncogenic alterations in cells that highly favour cell proliferation and diminish/inhibit activities of key mediators of the apoptosis cascade.

CHAPTER SIX

6.1 Conclusion

Schistosoma soluble egg antigens induced oncogenic phenotypes including oxidative stress, increased proliferation and diminished apoptosis in cultured normal human prostate cells. Findings of this study have revealed that schistosomiasis still remains a major public health challenge and there may be hybrid *Schistosoma sp*ecies in circulation. This study, for the first time has provided empirical evidence on the possible role of schistosome-infection as an aetiological agent in the pathogenesis of prostate cancer.

6.2 Recommendations

Molecular techniques such as ITS1 rDNA genotyping should be used to complement currently used diagnostic tools so as to identify hybrid infections. Subsequently, the role of hybrid genotypes should be investigated for their implication on disease outcome.

Other oncogenic hallmarks such as cell cycle alteration, expression patterns of oncogenes and tumour suppressor genes should be investigated to strengthen research findings. *In vivo* studies should also be conducted to confirm this association. Lastly, a more comprehensive study that looks at the effect of the individual *Schistosoma* species on prostate cells is highly recommended.

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APPENDICES

APPENDIX I

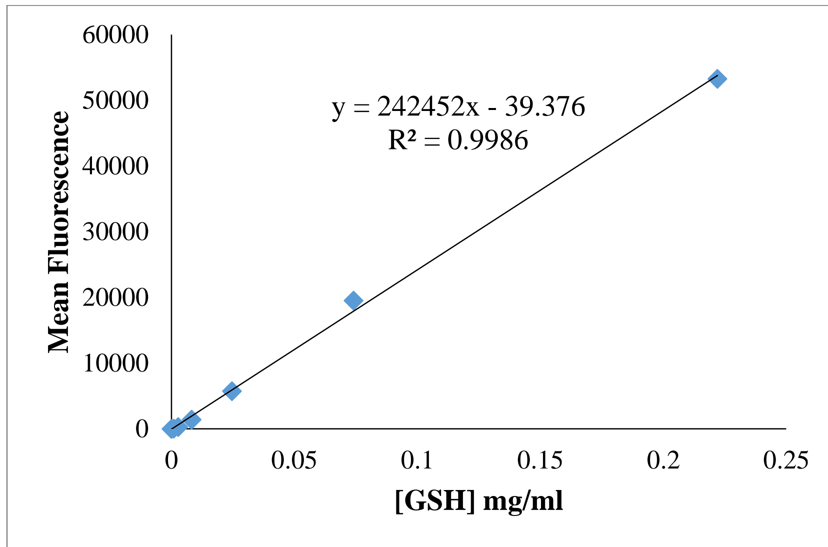


Figure A1: Reduced Glutathione (GSH) standard curve

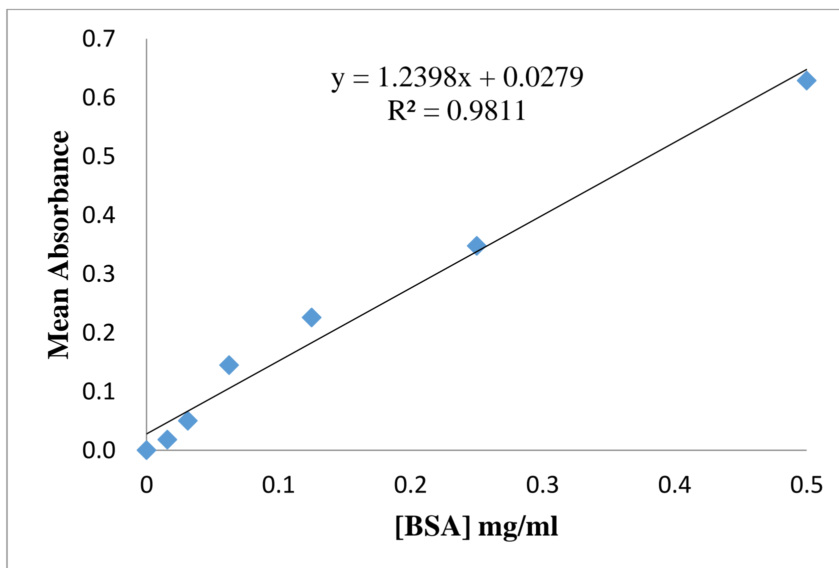
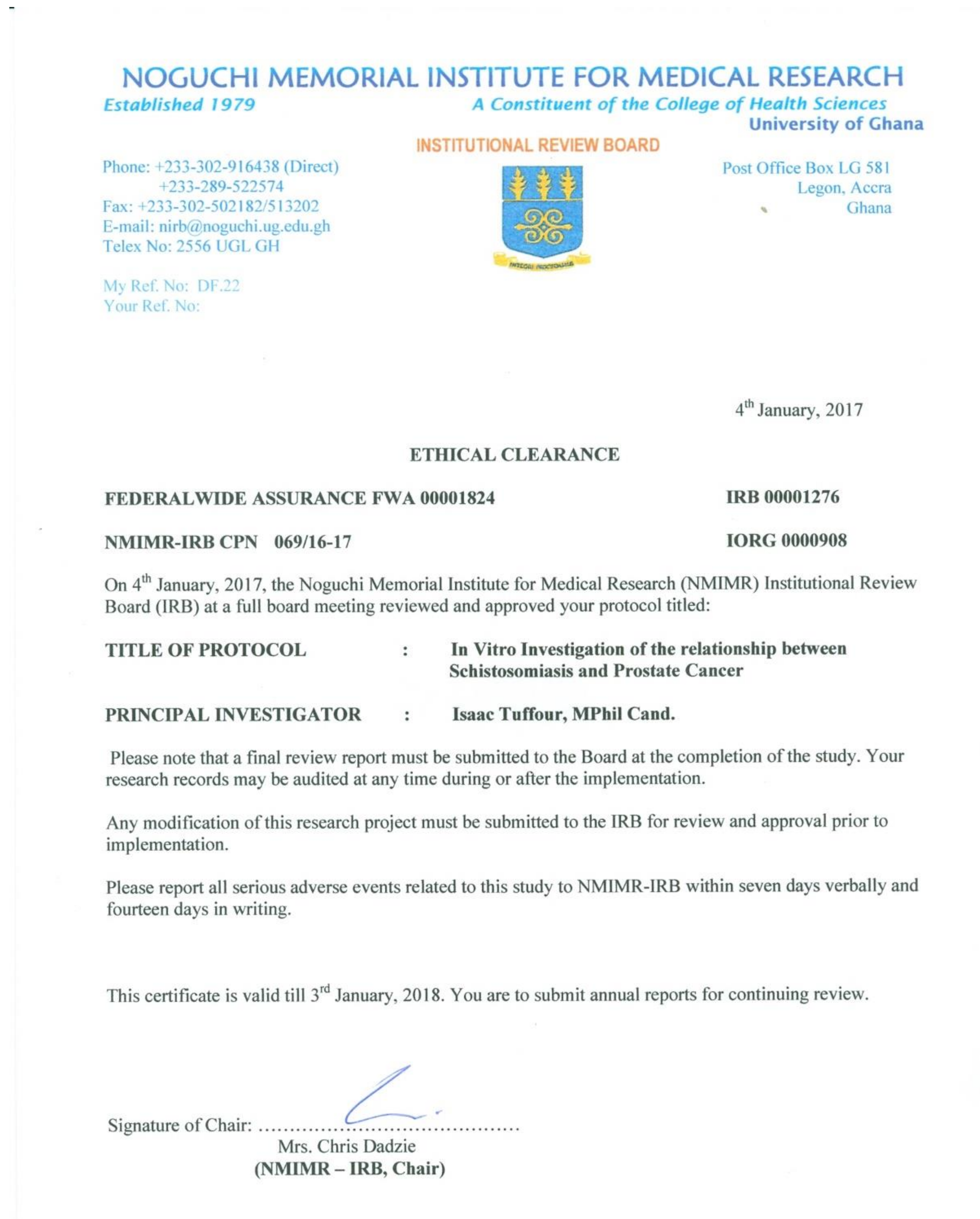


Figure A2: Bovine Serum Albumin (BSA) standard curve

APPENDIX II



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4th January, 2017

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 069/16-17 **IORG 0000908**

On 4th January, 2017, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **In Vitro Investigation of the relationship between Schistosomiasis and Prostate Cancer**

PRINCIPAL INVESTIGATOR : **Isaac Tuffour, MPhil Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 3rd January, 2018. You are to submit annual reports for continuing review.

Signature of Chair: ....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

Ethical approval certificate for the study

In case of reply the number and date of this letter should be quoted.

My Ref. No.
Your Ref. No.

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6th October 2016

THE MUNICIPAL DIRECTOR OF HEALTH SERVICES
GA SOUTH MUNICIPAL HEALTH DIRECTORATE
WEIJA

RE: REQUEST FOR PERMISSION TO OBTAIN URINE SAMPLES FROM RESIDENTS IN COMMUNITIES ALONG THE WEIJA LAKE

Approval is hereby given by the Regional Health Directorate to enable Mr. Isaac Tuffour, Principal Investigator, Department of Clinical Pathology and Dr. Irene Ayi, Co-Supervisor, Department of Parasitology from the Noguchi Memorial Institute for Medical Research to collect urine samples from residents in the communities along the Weija lake under the Ga South Municipality as per attached.

Kindly provide the needed assistace to enable a successful exercise.

Thank you.

DR. LINDA A. VANOTOO
REGIONAL DIRECTOR OF HEALTH SERVICES
GREATER ACCRA

cc: Dr. Irene Ayi ✓
Co-Supervisor
Department of Parasitology
Noguchi Memorial Institute for Medical Research

Letter of approval for sample (urine) collection