INVITRO ANTI-TRYPANOSOMAL ACTIVITY OF *ASCOMATA SPP*, *GLIOCLADIUM ROSEUM* AND *EPICOCCUM PURPURASCENS* EXTRACTS.

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AUGUST, 2019.

DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Kabir Mustapha Umar and has not been presented anywhere for the award of a degree or certificate.

Signature …… Date …….

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CERTIFICATION

This Dissertation Titled (***INVITRO*** **ANTI-TRYPANOSOMAL ACTIVITY OF** ***ASCOMATA*** SPP**,** ***GLIOCLADIUM ROSEUM*** **AND** ***EPICOCCUM PURPURASCENS*** **EXTRACTS*)*** was carried out by SANI TAJO TUKUR, SPS/14/MZO/00013, Supervised by KABIR MUSTAPHA UMAR (Ph.D.)

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APPROVAL

This dissertation has been examined and approved for the award of Masters in Zoology (Parasitology).

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DEDICATION

This Dissertation is dedicated to my dear Mother. Amina Muhammad, my wife Fadimatu Aliyu Muhammad, my son, Asim and Hisham Muhammad Sani and All Relatives Including Brothers and Sisters.

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LIST OF ABBREVIATIONS

AAT	African Animal Trypanosomiasis
ANOVA	Analysis of Variance
BBB	Blood Brain Barrier
BIIT	Blood Incubation Infectivity Test
BUK	Bayero University Kano
CATT	Card Agglutination Test for Trypanosomiasis
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CTC	Capillary Tube Centrifugation
DA	Diminazine Aceturate
DFMO	Difluoromethylinithine
DNA	Deoxirybose Nucleic Acid
EDTA	Ethyline Diamine Tetra Acetic Acid
ELISA	Enzymes Linked Immunosorbent Assay
FBE	Fresh Blood Examination
FDA	Food and Drugs Administration
FIND	Foundation for Innovative New Diagnostics
HAT	Human African Trypanosomiasis
HCL	Hydrochloric Acid
HIV	Human Immuno Virus
IFAT	Indirect Immunoflourescent Antibody Test
ILRAD	International Laboratory Research on Animal Disease
KIVI	Kit For In Vitro Isolation

LAMP	Loop Mediated Isothermal Amphilification
LED	Light Emitting Diode
MAECT	Miniture Anion Exchange Centrifugation Technique
MHCT	Microhaematocrit Centrifugation Technique
MIC	Minimum Inhibitory Concentration
MSC	Master of Science
NASBA	Nucleic Acid Sequence Based Amphilification
NIITR	National Institute for Trypanosomiasi Research
NTD	Neglected Tropical Diseases
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potatoes Dextrose Agar
PNA FISH	Fluorescence *In Situ* Hybridisation
QBC	Quantitative Buffy Coat
QY	Quantum Yield
RPMI	Royal Prime Memorial Institute
SPSS	Statistical Package for Social Sciences
TBF	Thick Blood Film
USA	United State of America
UV	Ultra Violate
VAT	Variables Antigen Type

VSG	Variant Surface Glycoprotein
WBC	White Blood Cell
WHO	World Health Organisation

ABSTRACT

African human trypanosomiasis (HAT) and animal trypanosomiasis (AAT) are vector – borne parasitic diseases, which are amongst the most neglected diseases in the world. They cause major health and economic problems in rural sub- Saharan Africa. The main means of controlling the disease is limited due to parasite resistance and toxicity of the current anti-trypanosomal drugs. The development of a vaccine has been thwarted by antigenic variation of the parasite. The main aim of the current research was to evaluate the anti-trypanosomal activity of some Ascomata spp, Gliocladium Roseum and Epicoccum purpurascens extracts against Trypanosoma brucei brucei, in Vitro and Blood Incubation Infectivity Test efficacy using white albino rat as animal models. Sequential extraction of plant samples in ethyl acetate and water gave the crude extracts. In vitro assays were carried out in 96-well microtitre plates and diminazine aceturate were used as a positive control. Phytochemical screening of the extracts was conducted confirming the presence of saponin, flavonoid, alkaloid, steroid and phenol in Gliocladium roseum, saponin, steroid and phenol in Ascomata spp while Epicoccum purpurascens extract contained only alkaloid and phenol.The present study indicated that the isolated and identified fungi where Ascomate spp, Gliocaldium roseum and Epicoccum purpurascens and they all exhibited cytotoxic activities against brine shrimp larvae(table 4.1).the methanolic fungal extracts affected mortility of the parasites at 1.0 mg/ml in Ascomata spp while 1.0,0.75 and 0.50 mg/ml in Gliocladium roseum and 1.0 and 0.75 mg/ml in Epicoccum purpurascens in vitro test, and the entire tested group did not develop infection in mice inoculated with infected blood incubated with the concentrations of the extracts. There is significant difference at ($p>0.05$) as compared to control. In conclusion, the extracts showed in vitro effects. Further effort is required to isolate and purify specific compounds responsible for the antitrypanosomal activities of the studied fungi.

CHAPETR ONE

1.0 INTRODUCTION

1.1 Background of the study

Trypanosomiasis is a parasitic disease caused by haemoprotozoan belonging to the genus, *Typanosoma* of the family Trypanosomatidae, that multiplies in the blood stream, lymphatic vessels and tissues including the cardiac muscles and the central nervous system. African animal trypanosomiasis (AAT) is most common disease of domestic livestock covering 37 sub-Saharan countries located between latitude 14ºN and 29ºS and about 9 million km^2, an area which corresponds approximately to one-third of the Africa's total land area (Mattioli *et al.,* 2004). This highly fatal protozoan disease is virulent, inoculable but not contagious (except dourine, a venereal trypanosomiasis of equines). African animal trypanosomiasis the AAT is responsible for 3 million livestock and 55,000 human deaths annually in agriculture and mixed farming environments thus making it an important priority for the agricultural sector and biomedical and public agencies (Mulumba, 2003).

Human African Trypanosomiasis (HAT), commonly known as sleeping sickness, is caused by species *Trypanosoma* brucei gambiense and rhodesiense as an endemic public health threat to Sub-Saharan Africa with an estimated 55 million people at risk. The disease is important yet neglected disease which is a major cause of rural underdevelopment in Sub-Saharan Africa as it mainly affects poor and remote rural regions (Favre *et al*, 2008). It is estimated that approximately 20,000 people across Africa are infected with HAT with approximately 30 African countries affected by the disease (Sutherland *et al*, 2015). WHO describes the disease as a neurological breakdown that is

caused by the trypanosome parasite in the brain, which eventually leads to a coma or death if a patient is not treated (WHO, 2013).

Current trypanosomiasis control relies on trypanocidal drugs, use of trypanotolerant cattle breeds and control of the vector, tsetse fly of the genus Glossina. None of these methods have the full potential to work in the long-term control of the disease. Most heavily relied on are the trypanocidal drugs and this has led to an increasing problem of resistance in the target organisms (Prowse, 2005).

1.2 Statement of the Problem

Trypanosomoses are protozoan diseases, affecting both human and animals, and mainly found in tropical Africa, Latin America and Asia. In Africa, trypanosomes produce serious diseases in human beings such as West and East Sleeping Sickness caused by *T. b. gambiense* and *T. b. rodensia* respectively; while in the Americas *T. cruzi* causes the Chagas disease. Other species of Trypanosoma affect animals and produce enormous economical impact in the endemic areas. Those species could be classified as those transmitted by tsetse flies- *(T.a vivax*, *T. congolense* and *T. b. brucei*) producing a disease known as nagana and those non-transmitted by tsetse (*T.evansi* –surra, *T. equiperdum* – dourine) (Gutiérrez *et al.*, 2013).

More than 90 percent of crop production in Ethiopia is dependent on animal draught power on ploughing oxen which are mainly infected by animal trypanosomiasis, which in turn, worsens food supply and living conditions in affected areas (reviewed in Fromsa *et al.,* 2011). Due to antigenic variation shown by the trypanosome, prophylaxis of these diseases using vaccines is challenging; for that, most of the control and eradication programs against animal trypanosomes carried out in the infected areas in the world are

based on therapeutic and prophylactic measures, using trypanocidal drugs or combining both measures. However, only six compounds are available in the market (isometamidium chloride, homidium –bromide and chloride- and diminazene aceturate), pentamidine, melarsoprol and eflornithine and all of them have been on the market for over 40 years. One of the most important risks for the future use of these existing trypanocides is the development and dissemination of resistances and, for that, new drugs have been developed in the recent past and are available in the market to treat *T. evansi* (melarsomine) (Gutiérrez *et al.*, 2013). In the search for new trypanocides, a wide range of medicinal plants have been screened for antitrypanosomal activity and quite a number of them have been reported to have significant antitrypanosomal activity (Atawodi *et al.*, 2003).This is due to the fact that these plants contain phytochemicals which are responsible for this activity. A lot of plants belonging to different families have been known to have antitrypanosomal activity both *in vitro* and *in vivo* (Atawodi *et al.,* 2004; Wurochekke and Nock, 2004; Ene *et al*., 2009; Abu *et al.,* 2009; Olukunle *et al.,* 2010; Umar *et al.,* 2010, Feyera *et al.*, 2014; Kifleyohannes *et al*, 2014) due to the fact that they have been used for the treatment of trypanosomiasis traditionally.

Therefore, the search for new chemical entities that should be effective against all species of trypanosome, safe and affordable for disease-endemic countries like Nigeria is the best choice left without option to fight against the notorious impact of bovine trypanosomiasis on cattle productivity (Fromsa *et al.*, 2011) and to reduce human loss due to human trypanosomiasis. These are found in plants which are potential sources of new drugs since they contain countless number of molecules that have pharmacological effects (Newman *et al.,* 2003).

1.3 Significance of the Study

Trypanosomiasis is well known for its debilitating, anemia, edema, decreased fertility and abortions, loss of milk and meat production and work capacities and coma and death in human and therefore, exploration of the possible control mechanisms is central to addressing these losses, with the final aim of finding a novel drug which can help combat it. So this study tries to scientifically prove the claim that traditional healers make on plants used for trypanosomes control actually have the effect. If confirmed effective, the active principle will be extracted, purified and the possible mechanisms of action of active principle of the plant responsible for antitrypanosomal effects speculated. The study will also provide evidence that encourages the traditional use of the plant especially by communities which reside in areas where modern health facilities do not reach due to geographic and socio-demographic challenges.

1.4 Aim

The study was carried out to determine the *in vitro* antitrypanosomal activities of *Ascomata spp, Gliocladium roseum and Epicoccum purpurascens* extract against *T. brucei brucei.*

1.5 Specific Objectives

1. To isolate and identify endophytic fungi from the plants species of *Psidium guajava* Stem, Leaves and *Acacia nilotica* stem.
2. To evaluate the cytotoxicity of the extracts using brine shrimp lethality assay.
3. To determine the *in vitro* activity of the extracts of the fungi against *T. brucei brucei*

4. To evaluate blood incubation infectivity test of the extracts against *T. brucei brucei.*
5. To determine the metabolic profile of the ethanolic screening on extracts of endophytes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human African Trypanosomiasis

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a deadly parasitic disease endemic to sub-Saharan Africa (Stich *et al.,* 2002). HAT belongs to the so-called "neglected tropical diseases" (NTDs), a group of infections affecting the world's poor that includes, among others, leishmaniasis, Chagas' disease, dengue, leprosy, schistosomiasis and onchocerciasis. The World Health Organisation (WHO) estimates that one billion people (one sixth of the world's population) are affected by at least one of the NTDs (http://www.who.int/neglected_diseases/en/). Unfortunately, since these diseases persist almost exclusively in the most marginalised communities of undeveloped countries, very little resource is spent to lighten their tremendous social and economic burden. HAT represents a major public health threat in Africa and together with nagana, the animal form of African trypanosomiasis, is considered a main obstacle for development of rural regions of the continent (Simarro *et al.,* 2008). Since 1997, WHO has been raising awareness of this most neglected disease, favouring the establishment of national control programs and the involvement of public and private partnerships (Stich *et al.,* 2003). These efforts have significantly reduced the incidence of HAT in endemic countries by implementation of surveillance and drug availability.

2.2 The Aetiological Agent

The aetiological agent of HAT is a haemoflagellate protozoan belonging to the species *Trypanosoma brucei* (genus *Trypanosoma*, order Kinetoplastida) (Cox, 2004). Of the three subspecies of *T. brucei* only two are infectious to humans (*T. b. gambiense* and *T. b.*

rhodesiense), while *T. b. brucei* causes infection in wild and domestic animals (Barrett *et al.,* 2003). Sporadic cases of human infection with other trypanosome species have been reported (Truc *et al.,* 1998c; Joshi *et al.,* 2005), but, at least in one case, infection could be ascribed to a mutated apolipoprotein L1 found in the serum of the patient (Lun *et al.,* 2009), which is a component of the trypanolytic factor that normally protects humans from animal trypanosome infection (Pays and Vanhollebeke, 2008).

Both *T. b. gambiense* and *T.b. rhodesiense* are transmitted to the human host by the bite of an infected tsetse fly of the genus *Glossina*, which acts as vector of the disease. The two forms of HAT differ greatly (Welburn *et al.,* 2001a). *T. b. gambiense* is responsible for more than 90% of reported cases of HAT and causes a chronic form of the illness, which can last for months or years before major symptoms arise. The duration of the Gambian form has been indirectly estimated at nearly three years in absence of treatment, equally split between the two stages that is an early stage, in which the parasite are confined to the hemolymphatic system and a late, meningo-encephalitic stage of the disease (Checchi *et al.,* 2008a). On the other hand, *T. b. rhodesiense* causes an acute form that usually leads to the patient's death within weeks or few months (Brun *et al.,* 2009). However, acute Gambian and chronic Rhodesian trypanosomiasis cases, are observed (Garcia *et al.,* 2006). Moreover, despite most *T. b. gambiense* infections being fatal in absence of treatment, human trypano-tolerance, with self-resolving and asymptomatic chronic infections, has been postulated (Checchi *et al.,* 2008b). Other differences between the Rhodesian and Gambian forms lie in their clinical features and the chemotherapy protocols used, in their epidemiology and transmission and, therefore, in the control strategies applied (Fèvre *et al.,* 2006).

2.3 Disease Burden

HAT transmission is restricted to the African continent, but around 50 cases per year are diagnosed elsewhere in people who had travelled to affected regions (Sinha *et al.,* 1999; Ripamonti *et al.,* 2002; Lejon *et al.,* 2003a). Endemic foci of the disease have a discrete distribution, correlated to the presence of the tsetse flies. There are nearly 300 active foci identified, confined to an area that stretches south of the Sahara and north of the Kalahari Desert (Barrett *et al.,* 2003). *T. b. gambiense* infection is found in west and central Africa: Angola, Democratic Republic of the Congo and Sudan are the most affected countries by the Gambian form, with more than 7,000-10,000 new cases per year reported up to 2004 (WHO, 2006b). *T. b. rhodesiense* is found in the eastern and southern part of the continent. The epidemiological study identified Malawi, Uganda and United Republic of Tanzania as the countries with the highest incidence of this HAT form (50–1,500 cases per year). Uganda is the only country in Africa known to be affected by both *T. brucei* subspecies, but the distribution of these parasites is, at least for the time being, separate (Picozzi *et al.,* 2005).Compared to other parasitic diseases like malaria or worm infections, incidence of HAT is lower, but its potential to give rise to devastating epidemics as soon as active surveillance is abandoned makes this illness a major health priority in endemic countries (Cattand *et al.,* 2001). Systematic control programs established by European authorities resulted in efficacious intervention in the big epidemics that occurred between the end of the 19th and the beginning of the 20th century, bringing the disease to a nearly elimination by the 1960s (Pépin and Méda, 2001; Maudlin, 2006).

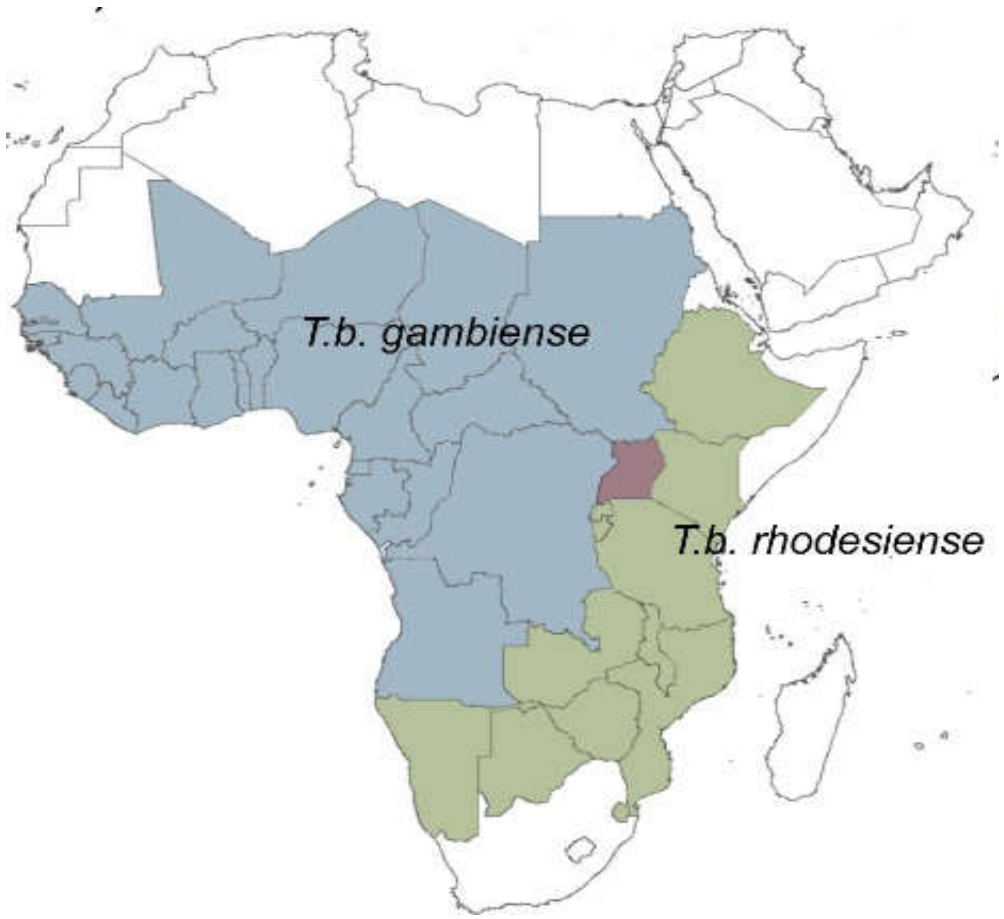


Figure 2.1. – Map of Africa showing the geographical distribution of *T. b. gambiense* and *T.b. rhodesiense*. The epidemiological status of the endemic countries is indicated with different colours. (Reproduced from Anneli *et al.*, 2017).

After the colonial era of the endemic countries, however, the number of Cases increased rapidly, due to lack of surveillance and awareness from local government, but also to poverty, political instability, wars and displacement of populations (Smith *et al.,* 1998; Cattand, 2001; Brun *et al.,* 2009). Years ago, A total of 60 million people in 36 African countries are continuously exposed to the risk of infection by one of the two forms of HAT, but only 3-4 million are under surveillance (Cattand *et al.,* 2001). For this reason, accurate epidemiological data for sleeping sickness are difficult to collect and reported

incidence of the illness is often considered an underestimate (Fèvre *et al.,* 2008; Welburn *et al.,* 2009). Despite this uncertainty, at the end of the 20thcentury, WHO estimated an annual number of cases of at least 300,000 (40,000 – 50,000 deaths), of which only 13% were identified and treated (WHO, 2001). Fortunately, improvement in control policies and new international initiatives have led, during the last decade, to a steady decline of total cases, it is estimated to be 50,000 – 70,000 (W HO, 2006b; Barrett, 2006).

2.4 Vector and Transmission

HAT is transmitted to humans through the bite of infected haematophagous arthropods belonging to the genus *Glossina*. Gambian trypanosomiasis is typically acquired from Riverine tsetse flies of the *G. papalis* group, but also *G. fuscipes*, *G. tachinoides* and *G. calliginea* can act as vectors of *T. b. gambiense* (Cattand, 2001). The reservoir of this infection is considered exclusively human. Therefore, the cyclic transmission human-fly-Human is the main cause of the persistence of the disease. Nevertheless, natural infections With *T. b. gambiense* have been reported in domesticated animals (pigs, dogs and sheep) and may occur in wild fauna as well (Njiokou *et al.,* 2006), although the epidemiological impact of this reservoir on humans remains as yet undetermined (Pépin and Méda, 2001; Brun and Balmer, 2006; Fèvre *et al.,* 2006). *T. b. rhodesiense*, on the contrary, is a zoonotic parasite found in savannah habitats. Human infection with this species is sporadic, but when it occurs the disease is highly virulent and progresses very rapidly. Rhodesiense trypanosomiasis is transmitted by tsetse flies belonging to the *G. morsitans* Group (and, at lesser extent, to *G. pallidipes*, *G. swynnertoni* and *G. fuscipes*) that have fed on infected domestic (cattle in particular) and wild (especially ungulates) animals, which, therefore, serve as the main reservoir of the parasite (Pépin and Méda,

2001; Welburn *et al.,* 2001b). Tsetse flies are usually active during the day, but some species can bite at night example *G. palpalis G. tacchinoides, G. pallidipes and G. morsitans* and both sexes can transmit the infection (Fèvre *et al.,* 2006). Interactions between vectors and parasite are complex. Only a very small percentage of natural population of tsetse flies are infected by *T. brucei* species and this can explain the absence of correlation between insect concentration and incidence of the human disease (Pépin and Méda, 2001). Other possible routes of infection with trypanosomes are through blood transfusions, infected needles or congenitally (Barrett *et al.,* 2003).

2.5 The Parasite Taxonomy

2.5.1 Life cycle

Trypanosomes are pleomorphic, single-celled parasites with a two-host life cycle: Mammalian and arthropod (Chappuis *et al.,* 2005; Brun *et al.,* 2009). The cycle (Figure 2.2) starts when an infected tsetse fly takes its blood meal on the mammalian host and it inoculates the metacyclic trypomastigote form of the parasite present in its saliva. Trypanosomes quickly transform into the long slender trypomastigotes and proliferate by binary fission at the site of the bite for a few days, leading to an inflammatory chancre. The parasites, then, spread to the draining lymph nodes and the bloodstream (first or early haemolymphatic stage of infection), through which they reach other organs such as the spleen, liver, heart and endocrine system. After a few weeks (*T. b. rhodesiense*) or several months (*T. b. gambiense*) trypanosomes cross the blood-brain barrier (BBB) to invade the central nervous system (CNS) through mechanisms that are still poorly understood (Enanga *et al.,* 2002): the patient is, then, said to be in the meningoencephalitic, second (or late) stage of infection. When parasitaemia in the host

increases, long slender trypomastigotes transform into non-dividing, short-stumpy trypomastigotes, which are taken up by the tsetse fly (which remains infective for its entire life), where they complete their life cycle. In the insect's midgut trypanosomes transform into the procyclic stage and after two or three weeks they migrate to the salivary glands. Here other transformations lead to their development into metacyclic forms, ready to be injected into a susceptible vertebrate host during the next blood meal.

During their whole life cycle African trypanosomes remain exclusively extracellular, thus fully exposed to the host's immune response. To survive complement-mediated lysis and specific immune attack, parasites shield invariant surface molecules with a thick coat made of variant surface glycoproteins (VSGs). Only one VSG is expressed at a time and, once neutralizing specific antibodies have been produced from the host, trypanosomes switch to a different VSG, thus allowing the population to survive (Machado *et al.,* 2006; Morrison *et al.,* 2009). This antigenic variation process explains the intermittent parasitaemia and the long asymptomatic incubation period typical of *T. b. gambiense* infection and it makes the development of a vaccine remote (Cattand, 2001).

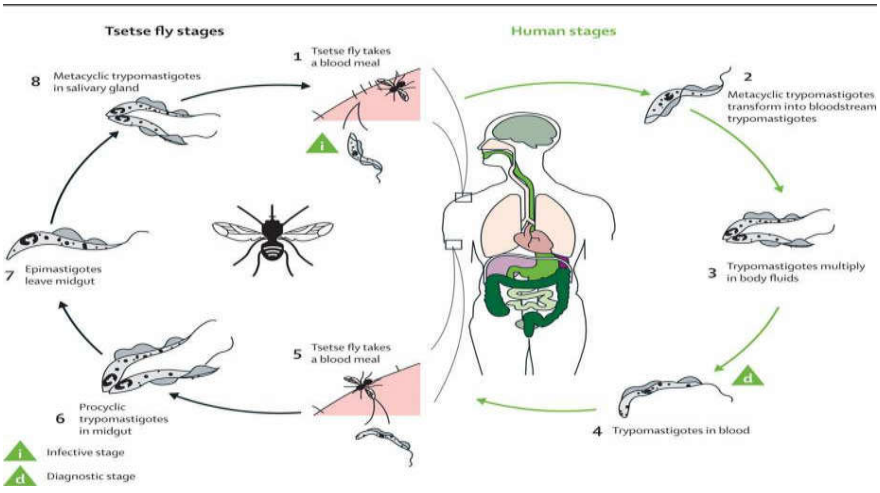


Figure 2.2 – Schematic representation of the digenetic life cycle of *Trypanosoma brucei* in the mammalian host and in the tsetse fly vector. (Blum *et al.*, 2008).

2.5.2 Cell Morphology and Cell Compartments

African trypanosomes are eukaryotic, unicellular organisms, with a spindle-shaped cell 18-20 mm long and 3 mm thick (Field *et al.,* 2004; Figure 2.3). These parasites move with a single flagellum that, in the trypomastigotes, arises from the posterior end of the cell, runs the whole length of the cell body, to which it is attached by an undulating membrane, and extends beyond it at the anterior end. Tightly connected to the basal body of the flagellum is the kinetoplast, a complex network of mitochondrial DNA composed of 30-50 DNA maxicircles (22 kb) and 5,000-10,000 minicircles (1 kb) interlocked together to form a disk-shaped structure (Chen *et al.,* 1995; Liu *et al.,* 2005). Minicircles encode for guide RNAs, which control editing specificity of maxicircle transcripts for rRNAs and subunits of respiratory complexes (Simpson and Shaw, 1989; Hong and Simpson, 2003). The kinetoplast is contained in the posterior part of the single trypanosome mitochondrion, an organelle that elongates longitudinally throughout the whole cell body. In slender trypomastigotes the mitochondrion shows only a few short cristae and little metabolic activity, due to the fact that this form of the parasite relies on glycolisis and not on oxidative phosphorylation for energy production (Schnaufer *et al.,* 2002). When the cell Enters mitosis, the kinetoplast DNA replicates and divides before the nuclear genome (Field *et al.,* 2004). The flagellum emerges from an invagination of the plasma membrane called the flagellar pocket (Field and Carrington, 2009). This structure is involved in various cell activities including immune evasion, by continuous clearance of VSG-Immunoglobulin complexes, and cell trafficking, being the only site where endocytic and exocytic processes occur (Morgan *et al.,* 2002). The organisation of the cell compartments from the flagellar pocket towards the anterior side is highly

polarised. Associated to the pocket is the Golgi complex with its endocytic and secretory vesicles confined between kinetoplast and nucleus. The single lysosome is usually close to the nucleus, which is located at the midpoint of the cell (Field *et al.,* 2004). The nuclear genome of *T. brucei* includes 11 diploid, megabase-sized chromosomes and various aneuploid, mini- and intermediate chromosomes (30-700 kb), which harbour VSGs sequences and expression sites similar to those present in the subtelomeres of the megabase chromosomes (Field *et al.,* 2004). The endoplasmic reticulum is distributed in the whole cytoplasm as well as the acidocalcisomes, acidic electron-dense granules with a diameter of around 0.2 cm, important for the homeostasis of various elements including several cations, dications (Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}) and phosphate (Docampo and Moreno, 1999; Rodrigues *et al.,* 1999). Acidocalcisomes, which have no counterpart in mammalian cells, can be visualised inside different *Trypanosoma* species by their specific staining with acridine orange (Vercesi *et al.,* 1994; Mendoza *et al.,* 2002). Glycosomes are other membrane-bound organelles, homogeneous in size (0.27 □m diameter), present throughout the whole cell body of *T. brucei*. These structures, which are closely related to peroxisomes, contain glycolytic enzymes and are particularly abundant in bloodstream trypomastigotes (240 per cell) (Opperdoes, 1987).

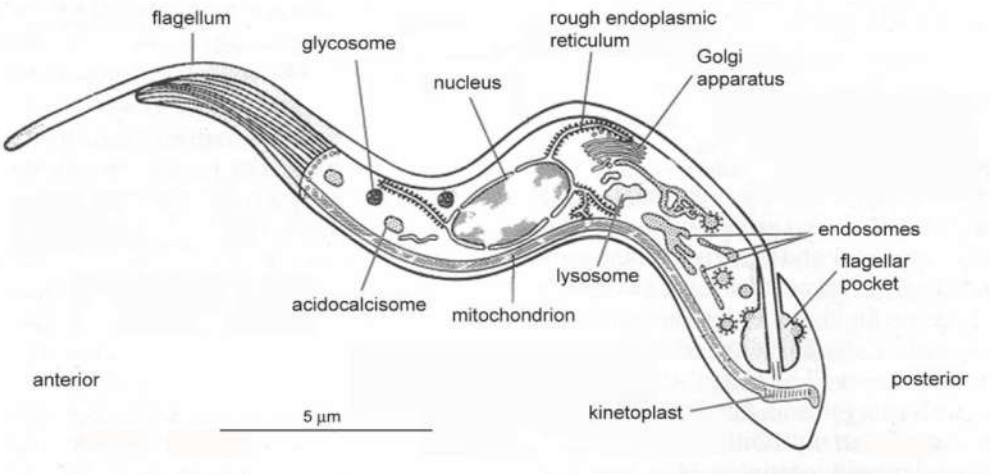


Figure 2.3 – Diagram of a bloodstream *Trypanosoma brucei* cell illustrating the major organelles. Glycosomes and acidocalcisomes are not drawn to scale. (Modified from ILRAD Reports vol. 7 (1), Jan. 1989; available online at the website http://www.ilri.org/InfoServ/Webpub/Fulldocs/Ilrad89/Trypano.htm

2.6 Control Strategies

All countries affected by HAT have national programs dedicated to surveillance of the disease (Simarro *et al.,* 2008). Control is based on two approaches: the reduction of the parasite in the reservoirs (human or animal) and the reduction of the chance of contact between vector and human host by means of vector removal (Welburn *et al.,* 2001). For *T. b. gambiense* form, where humans represent the main reservoir, active case-finding (and therefore, correct diagnosis) and treatment of the infected patients are the strategies adopted to interrupt transmission (Simarro *et al.,* 2006; Brun *et al.,* 2009). For *T. b. rhodesiense,* instead, the control of infection in livestock (and, to a minor extent, in wildlife) through the use of curative or prophylactic trypanocides or application of insecticides to the animals plays a crucial role (Hutchinson *et al.,* 2003; Fèvre *et al.,* 2006; Simarro *et al.,* 2008). For Rhodesian HAT, vector control can also be an efficient approach for containing the transmission of the disease (Welburn *et al.,* 2009). The fight against tsetse flies involves the use of insecticides and the installation of traps or screens (Torr *et al.,* 2005; Lindh *et al.,* 2009). A new technology available to decrease tsetse

population consist in the release of sterilised tsetse males (sterile insect technique, SIT), but its cost-effectiveness is disputed (Vreysen, 2001).

2.7 Diagnosis of *T. b. gambiense* Infection

Accurate diagnosis and staging of sleeping sickness infection is of the utmost importance for disease management and control (Louis *et al.,* 2001). Early detection of the disease allows not only to treat the patients with the cheaper, less toxic drugs for the first stage of the disease, but also to reduce the human reservoir of the parasite in the case of *T. b. gambiense* infection (Fèvre *et al.,* 2006). Unfortunately, diagnosis of HAT still represents a major problem, due to insensitivity and laboriousness of the techniques available especially in remote locations with limited facilities. Passive case-finding (self-reporting patients) is extremely inefficient, as infected individuals can manifest symptoms only after many months, and usually refer to local health centres when already in the second stage (Ekwanzala *et al.,* 1996; Smith *et al.,* 1998; Abel *et al.,* 2004; Odiit *et al.,* 2004). Therefore, more effective active case-finding programs have been established to screen the whole population at risk through the work of specialised mobile teams (Ruiz *et al.,* 2002; Robays *et al.,* 2004; Simarro *et al.,* 2006). HAT is diagnosed using a combination of tests. Every endemic country has adopted a specific algorithm for detection of infected subjects, but all strategies comprise a sequence of screening and confirmation steps (Figure 2.4). Suspected HAT cases are identified using indirect diagnostic tools (clinical features, serological tests and, to a lesser extent, molecular tests) and must always be confirmed by direct microscopy. On infected individuals staging of the disease is carried out to decide on the treatment protocol to use. Stage determination requires an invasive lumbar puncture and is usually performed in local treatment centres. Direct and indirect

diagnostic procedures must also be applied during follow-up periods to check for relapses, but also to monitor seropositive individuals not confirmed by parasitological techniques (Chappuis *et al.,* 2005).None of the various assays available today for HAT diagnosis is ideal: practicality, costs and low efficiency are the main drawbacks. A test's diagnostic accuracy is usually quantified by means of its sensitivity and specificity, which are respectively defined as the proportion of true positives (true positives/true positives+false negatives) and of true negatives (true negatives/true negatives+false positives) that are correctly identified by the assay (Altman and Bland, 1994; Akobeng, 2007). More useful to clinicians to decide about the probability of disease in patients are the positive and negative predictive values, which represent the proportion of people with a positive test result who actually have the disease (true positives/true positives+false positives), and the proportion of people with a negative test result who do not have the disease (true negatives/true negatives+false negatives), respectively (Altman and Bland 1994b; Banoo *et al.,* 2007). Predictive values are dependent on the population and change if the prevalence of the disease changes, while specificity and sensitivity remain unvarying. Since sensitivity and specificity are usually inversely related, the choice of an assay with a certain precision depends on the case under study: in situations where it is important to detect the highest number of affected patients (like mass screening programs), a highly sensitive test is preferable, while for case confirmation tests a high specificity is needed to avoid the risk of false positives (Empson, 2001; Nendaz and Perrier, 2004).

2.8 Chemotherapy

Since African trypanosomiasis is considered invariably fatal if untreated, chemotherapy is mandatory. Unfortunately, as for the other neglected tropical diseases, drug research and development for HAT is a minor activity due to lack of profit perspectives for pharmaceutical companies (Trouiller *et al.,* 2002). Although today new initiatives, like the Drugs for Neglected Diseases Initiative, DNDI (http://www.dndi.org/index.php), are trying to face this problem, HAT treatment still relies on only four drugs three of them introduced over 50 years ago (Ollivier and Legros, 2001). Recently, nifurtimox (an orally administered 5-nitrofuran licensed for the treatment of Chagas' disease) for use in combination with eflornithine has been added to the list (Delespaux and de Koning, 2007). The production and availability of these drugs is in constant danger and continues only to make agreements between WHO and the suppliers (Etchegorry *et al.,* 2001; Barrett *et al.,* 2003). All of these four drugs are far from ideal, with problems associated to toxicity, route of administration, low efficacy and resistance (Legros *et al.,* 2002; Fairlamb, 2003). HAT therapy is even more complicated during the second stage of the disease, since drugs have to reach the brain by crossing the blood-brain barrier, which otherwise protects parasites from compounds active in the haemolymphatic compartment (Enanga *et al.,* 2002; Lejon and Büscher, 2005).

Pentamidine and Suramin are the drugs used to treat the early stage of HAT. Pentamidine isethionate (Lomidine®) was introduced in the early 1940s and, since then, it has been used for the treatment of *T. b. gambiense* infection without being associated with any significant resistance outbreak (Delespaux and de Koning, 2007). During the colonial era of the endemic countries this compound was also widely used as mass

chemoprophylactic agent (Ollivier and Legros, 2001). Pentamidine is administered by intramuscular injection (7-10 doses of 4 mg/kg daily or every two days) and it is relatively well tolerated (Legros *et al.,* 2002). Three transmembrane transporters responsible for the selective accumulation of pentamidine inside trypanosomes have been identified (de Koning, 2001a) and two more have recently being discovered (Ortiz *et al.,* 2009), but the mechanism of action of this slow-acting diamidine is still unclear. Suramin sodium (Germanin®) is a polyanionic sulfonated naphthylamine used as first line treatment for *T. b. rhodesiense* form of HAT since 1920.

It can also be used as second drug of choice for Gambian first stage trypanosomiasis. Suramin is administered intravenously (typically, five injections every 3-7 days over a four weeks period) and can have severe adverse reactions like anaphylactic shock, neurotoxicity, kidney damage and cutaneous reactions (Legros *et al.,* 2002). As for pentamidine, the trypanocidal mechanism of suramin is poorly understood (Barrett *et al.,* 2007). This compound is known to inhibit many enzymes and a multiple mode of action is compatible with the absence of resistance cases observed in the field (Fairlamb, 2003), although resistant lines are easily selected in the laboratory (Scott *et al.,* 1996). Melarsoprol and eflornithine are the two drugs used to treat late stage HAT. Melarsoprol (Arsobal®, Mel B) was introduced in 1949 and is active on both forms of sleeping sickness.

Treatment protocols with melarsoprol traditionally consists of various intravenous injections of the drug (3.6 mg/kg body weight, dissolved in propylene glycol) separated by periods of 7-10 Days (Cattand, 2001). Only hypotheses exist on the mode in which this drug kills trypanosomes, the disruption of the parasite thiol-redox balance being one

often Proposed (Fairlamb, 2003). Melarsoprol is an organo-arsenical compound extremely toxic to humans and it is responsible for the death of up to 5% of patients to whom it is administered, due to a post-treatment reactive encephalopathy. Furthermore, over the last decade, an increasing number of clinical failures (up to 30% in some areas), particularly in the treatment of *T. b. gambiense,* have been observed (Brun *et al.,* 2001; Ollivier and Legros, 2001). Apart from poor treatment regimens, these failures have been associated, at least partially, to alterations or loss of the parasite's P2 amino-purine transporter (Carter and Fairlamb, 1993; Matovu *et al.,* 2001a), which is involved in the uptake of melarsoprol but also of diamidines like pentamidine and diminazene aceturate (a veterinary trypanocide), leading to worries about possible cross-resistance onset (Barrett and Fairlamb, 1999; de Koning 2008; Section 1.2.8). The alternative drug for treatment of melarsoprol-refractory late sage *T. b. gambiense* is eflornithine (Ornidyl®, DFMO). Introduced in 1990, this drug is not active on *T. b. rhodesiense*, is expensive (five times more than melarsoprol) and requires a high dose treatment: 400 mg/kg daily subdivided into four intravenous infusions for 7 or 14 days (Burri and Brun, 2003). Treatment failures have been observed (Balasegaram *et al.,* 2009), but it must be taken into consideration that the compound has a cytostatic effect that requires an active immune system to clear infection (Bitonti *et al.,* 1986). Nevertheless, DFMO has a much lower toxicity than melarsoprol: side effects include seizures, gastrointestinal problems and myelosuppression (Priotto *et al.,* 2006). Originally developed as a cancer treatment, the intracellular target of eflornithine is well known: the drug acts as a suicide inhibitor of the enzyme ornithine decarboxylase, disrupting the essential polyamine biosynthetic pathway of the parasite and leading to several downstream effects (Barrett *et al.,* 2007;

Delespaux and de Koning, 2007). Since DFMO registration, the first new drug candidate for HAT therapy has been an oral diamidine designed for the early stage of the disease, DB289, but, despite its efficacy, the compound's development was recently stopped due to toxicity issues (Thuita *et al.,* 2008). Diamidines remain interesting drug leads against HAT and other compounds, more potent than DB289 and including blood-brain barrier permeant derivatives, have already been identified (Wenzler *et al.,* 2009). Nitroimidazoles are another class of molecule active against trypanosomiasis (Denise and Barrett, 2001); among them, fexinidazole, developed by DNDi, is currently in Phase I trials (Torreele *at al*., 2009). Recent advances in the knowledge of parasite biology new drug targets may be validated in the future (Croft *et al.,* 2005; Barrett *et al.,* 2003 and 2007). Moreover, improved therapeutic protocols and combinations of existing drugs are under evaluation for the advantages they can offer in Particular the limitation of side effects and delay of drug resistance development, but also the reduction of costs and treatment duration). A new, shorter 10-day protocol with melarsoprol (2.2 mg/kg daily intravenously) has already been introduced with success for Gambian trypanosomiasis: it does not eliminate toxicity, but it reduces hospitalisation period and costs (Schmid *et al.,* 2005). Combination therapies of nifurtimox with either melarsoprol or DFMO have been tested for treatment of HAT second stage: the association of nifurtimox with eflornithine, in particular, has given encouraging results during early clinical trials (Bisser *et al.,* 2007; Priotto *et al.,* 2006 and 2009) and, recently, this drug combination has been included in the Essential List of Medicines for HAT treatment by WHO (http://www.who.int/neglected_diseases/en/).

2.9 Clinical Features

HAT is difficult to diagnose at an early stage, when symptoms are largely variable and a specific and the disease can be easily confused with other common tropical infections, especially malaria (Louis *et al.,* 2001). Fever, general malaise, headache, facial oedema, anaemia, cutaneous lesions are all manifest during the haemolymphatic stage (Kennedy, 2006). The only specific features are a local inflammation at the site of the tsetse bite, called the chancre, and an enlarged posterior cervical lymphadenopathy (Winterbottom's sign). The chancre generally occurs 5-15 days after the inoculation of the parasite, but it is seldom observed in Africans (Büscher and Lejon, 2004). Palpation of enlarged neck glands and microscopic examination of the aspirate in positive cases is generally used for HAT diagnosis (Lutumba *et al.,* 2005). The diffuse inability of the local primary healthcare facilities to promptly identify the disease results in considerable delays in diagnosis and treatment, with consequent increase of risk of unfavourable prognosis and of human suffering (Bukachi *et al.,* 2009). HAT becomes more easily recognizable as the disease progresses. Later clinical features reflect the involvement of specific organs: gastro-intestinal problems, cardiovascular disturbances, endocrine dysfunction and, sometimes, eye involvement can occur (Kennedy, 2006a; Blum *et al.,* 2008). When the infection reaches the meningoencephalitic stage, signs of nervous system disruption become evident and extensive in Gambian trypanosomiasis. Sleep-pattern disturbances, with dysregulation of the circadian rhythm, represent the most typical feature at this stage and lend HAT its common name "sleeping sickness". Other neurological changes include painful peripheral sensory disturbances, tone and mobility disorders, mental

changes and psychiatric disorders. If untreated, patients stop eating, lapse into a semi-coma and finally die (Kennedy, 2006a and 2006b).

In HAT caused by members of the *T. brucei* group, i.e., *T. b. rhodesiense* and *T. b. gambiense*, the comprehension of the role played both by the parasite and the host during the host–parasite interaction is essential to understand the clinical performance of the disease. The sickness has two clear cut clinical stages, i.e., a hemolymphatic initial systemic stage and a second phase characterized by the invasion of the brain by parasites. This encephalitic stage involves sensory, motor and psychiatric disturbances, with alterations of sleep representing the most typical manifestations. The clinical presentation of HAT is thoroughly described by Vincendeau and Bouteille (2006) and I will briefly summarize its main steps. Reviews the stages that portray the clinical symptoms of HAT. The tsetse bite produces a painful reaction characterized by local erythema, heat, edema, and tenderness that leads to the appearance of the chancre. This is an ulcer where the parasites are present and it disappears after 2 or 3 weeks. Thereafter the disease evolves into the successive phases that characterize its clinical phases (Sternberg, 2004; Kennedy, 2013).

2.9.1 Clinical Presentation of HAT

The painful (infected) tsetse bite leads to the appearance of the so-called chancre. Two or three weeks later the disease evolves into two successive clinical stages. The hemolymphatic stage I is characterized by the appearance.

2.9.2 The Hemolymphatic Stage

Shortly after being infected, the patient enters the hemolymphatic stage I, a phase in which the disease is often undiagnosed and, therefore, untreated. Intermittent fever

episodes often occur as a consequence of the successive cyclical waves of trypanosome parasitaemia. However, febrile occurrences cannot be considered as useful diagnostic elements since they might be completely absent. Adenopathy, splenomegaly and even liver disturbances signal the invasion of the reticuloendothelial system by trypanosomes. Skin rashes and severe pruritus with scratching skin lesions might become unbearable for the patient and painful lymph nodes might also occur at this stage (Kennedy, 2013).

2.9.3 The Meningoencephalitic Stage II

The meningoencephalitic stage II is an insidious phase that appears slowly over a period of months or years. The parasites cross the blood–brain barrier and infect the CNS, causing disturbances to the patient's sleep pattern, as well as confusion and trouble with motor and mental coordination. Fever spikes common to the hemolymphatic stage I might still be present. Abnormal quantities of macrophages enter the CSF and non-specific perivascular inflammatory cell infiltrates occur in the leptomeninges and white matter, with a pronounced activation of microglia and astrocyte (Kennedy, 2013).

The broad spread of the meningeal inflammation to specific CNS locations leads to the appearance of the neurological symptoms common to HAT. For example, invasion of the median eminence by parasites may be the cause of sleep-wake abnormalities. At the terminal phase of the disease, disturbances in consciousness and the development of dementia with incoherence, double incontinence and seizures are consequences of CNS demyelination and atrophy (Cnops *et al.,* 2015). The patient dies by heart failure or by encephalitis in a state of cachexia and physiological collapse (Sternberg, 2004).

2.10 Serological Tests

Application of serological assays to obtain indirect indication of infection (by antibody detection) is a widely used approach to rapidly screen the population at risk in endemic **a**reas but, as for clinical features, these tests are just indicative of the presence of trypanosomes. Since immunological tests lack sensitivity and specificity, subsequent parasitological confirmation has to be performed on the positive cases identified for final diagnosis (Chappuis *et al.,* 2005). In the field, however, mobile teams often face complex situations where patients with a positive serology are not confirmed by microscopy (a phenomenon called seropositivity) and the question of whether to provide treatment to these subjects is disputed (Koffi *et al.,* 2006). The main cause of seropositivity is probably the low sensitivity of parasitological methods that gives rise to false negatives, but this phenomenon can also be explained by false serological positives generated by cross-reactivity with animal trypanosomes or other co-infecting parasites. Moreover, an efficient immune system response could allow some subjects to control the virulence of the infection and keep it under detection limits of current diagnostics (Garcia *et al.,* 2000 and 2006). The serological tests available are very useful, but they are not ideal, because of their use of not defined recombinant proteins against variable antigens. Therefore, new protein markers for HAT serodiagnosis are under study (Hutchinson *et al.,* 2004).

2.10.1 CATT/*T. b. gambiense*

Since its introduction in 1978, the card agglutination test for trypanosomiasis (CATT/*T. b. gambiense*) has been the most widely used technique for mass population screening in the field (Chappuis *et al.,* 2005). The test is a cheap, fast and simple agglutination assay able to detect specific antibodies to the *T. b. gambiense* variable antigen type (VAT)

LiTat 1.3, common in the serum of patients infected with this subspecies. Recently, a thermostable format of the test has been developed (Hasker *et al.,* 2010). To perform the assay, a drop of reagent, consisting of lyophilised, blue-stained bloodstream trypanosomes expressing the LiTat 1.3 antigen and resuspended in PBS, is mixed with a drop of fresh, heparinized, capillary blood on a white plastic card, which is then shaken for 5 minutes on a 12/220 V card rotator. The result is immediately read by the naked eye: in subjects previously exposed to *T. b. gambiense* the trypanosomes agglutinate and form a blue clot. The test sensitivity on undiluted whole blood (CATT-wb) varies from 87 to 98%, while specificity can reach 90-95% (Penchenier *et al.,* 2003; Chappuis *et al.,* 2005). Serial dilutions of blood, plasma or serum before applying the test improve its specificity. The use of end-dilution titers have been suggested as a useful tool for therapeutical decision making for CATT-wb positive patients (Simarro *et al.,* 1999; Chappuis *et al.,* 2004), although this approach can be risky because of the poor positive predictive value of the test where disease prevalence is low (Inojosa *et al.,* 2006). Moreover, a study on cost-effectiveness of different confirmation algorithms showed that the use of parasitologic confirmatory tests by concentration methods without CATT titration had a better efficiency (Lutumba *et al.,* 2007). The CATT loses its efficacy on samples from patients infected with strains of trypanosomes that lack, or do not express, the LiTat 1.3 antigen, resulting in false negatives, but the distribution of these strains seems limited (Dukes *et al.,* 1992). Similarly, false positives can occur in patients infected with other trypanosome species or parasitic diseases, which cross-react with non-variant epitopes on the surface of trypanosomes present in the reage (Jamonneau *et al.,* 2000; Inojosa *et al.,* 2006). Furthermore, the agglutination can be inhibited by

complement factors, a phenomenon called prozone, when the test is performed on undiluted, heparinized blood or serum with low dilutions (<1:4). In these cases, addition of EDTA to the dilution buffer eliminates the problem by increasing the sensitivity with only a minor loss of specificity (Pansaerts *et al.,* 1998; Magus *et al.,* 2002). Other disadvantages of CATT are that it does not work on *T. b. rhodesiense* and it does not differentiate between past and current infections: this is the reason why this test cannot be used to assess treatment efficacy during follow-up, since circulating antibodies can persist for several years after cure (Paquet *et al.,* 1992).Variations on the same theme of the CATT have been proposed as, for example, the micro-CATT (micro-card agglutination test for trypanosomiasis). This test allows the collection and storage (at 4 ºC) of dried blood samples on a filter paper (Miézan *et al.,* 1991). The micro-CATT showed the same specificity of the CATT-wb but a lower sensitivity in the field (Truc *et al.,* 2002). Furthermore, although it requires smaller volumes of reagents, it is a more expensive method than the CATT-wb because more consumables are needed to perform the assay.

2.10.2 LATEX/*T. b. gambiense*

The LATEX/*T. b. gambiense* is an agglutination card test developed as a field alternative to the CATT-wb. The reagent is based on the combination of three purified variable surface antigens (LiTat 1.3, 1.5 and 1.6) absorbed on latex particles, in order to avoid non-specific cross reactions frequent in complex antigen preparations (Büscher *et al.,* 1991). The test follows a procedure similar to the CATT, but as it is usually performed on diluted blood (≥1:4) it is more laborious to perform (Jamonneau *et al.,* 2000). The LATEX/*T. b. gambiense* showed a higher specificity (up to 99%) but a lower, or similar,

sensitivity (form 84 to 100%) when compared to the CATT assay (Truc *et al.,* 2002; Penchenier *et al.,* 2003).

2.10.3 Immunofluorescent Antibody Test (IFAT) and Enzyme-linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) and the indirect immunofluorescent antibody test (IFAT) can detect specific host antibodies (IgM, IgG), related to the presence of parasites in blood, plasma, serum or cerebrospinal fluid, after 3-4 weeks of infection. Tests use purified trypanosomal glycoproteins or lysates of whole trypanosomes of selected antigen types (Büscher and Lejon, 2004). The immunofluorescent antibody test has been used with success for HAT control in different countries (Noireau *et al.,* 1988; Simarro *et al.,* 2006) due to its high sensitivity and specificity (evaluated as superior to the CATT test). The ELISA methods can be performed with serum, filter paper eluates or cerebrospinal fluid (Lejon *et al.,* 1998), but they were also shown to be able to detect specific antibodies against *T. b. gambiense* VSGs in the saliva, with sensitivity and specificity above 90%, thus opening new perspectives for the development of a new, non-invasive serological test (Lejon *et al.,* 2006). The ELISA assay can also be used for detection of parasite antigens in patients' sera (Liu *et al.,* 1989). This approach has the advantage of demonstrating that the parasite is still, or has recently been, present in the host and does not rely on his immune response. Various antigen candidates are currently under evaluation, although their low concentration in circulation could represent a major limitation for the development of a test for African trypanosomiasis (Radwanska, 2010). Moreover, studies on animal infections raised doubts about the sensitivity and specificity of this approach (Rebeski *et*

al., 1999). Due to the need of expensive and sophisticated equipment and trained personnel, the use of IFAT and ELISA is restricted to specialised laboratories (Chappuis *et al.,* 2005).

2.11 Parasitological Methods

Direct detection of parasites in human fluids (lymph node aspirate, blood or cerebrospinal fluid) through light microscopy has always represented the "gold standard" of HAT diagnosis (Büscher and Lejon, 2004; Chappuis *et al.,* 2005). Due to risks related to available drugs, confirmation of infection by parasitological techniques for clinically or serologically diagnosed suspects should always be done before starting treatment (Louis *et al.,* 2001). Microscopy has virtually 100% specificity, but suffers from a very lowsensitivity, that significantly affects the overall efficacy of African trypanosomiasis diagnosis in the field, failing to confirm 20-30% of infections (Robays *et al.,* 2004; Lutumba *et al.,* 2005). The main cause of this limited sensitivity is represented by the low and fluctuating parasitaemia typical of *T. b. gambiense* infection: parasites seldom exceed 10,000 trypanosomes/ml (a number easily detectable) but can be present at numbers lower than 100 trypanosomes/mL (below the detection limit of the most sensitive parasitological methods in use) (Chappuis *et al.,* 2005). To decrease the number of false negatives, it is possible to examine various samples over consecutive days, or to follow-up seropositives at regular intervals, but these strategies are difficult to apply in rural settings. Processing samples with concentration techniques before viewing improves the sensitivity and increases the cost-effectiveness of confirmation algorithms (Lutumba *et al.,* 2007), but it also makes parasitological methods more laborious and expensive, limiting their routine use (Lutumba *et al.,* 2006). Despite all of

these drawbacks, light microscopy is widely used in the field because of its simplicity, its immediate results and the lack of a valid alternative.

2.11.1 Chancre Aspirate

Trypanosomes can be detected in the chancre several days earlier than in the blood (Chappuis *et al.,* 2005), especially in the Rhodesian form (Louis *et al.,* 2001), but a blood smear is more sensitive. The chancre aspirate can be viewed directly under the microscope or after fixation and Giemsa-staining. This procedure is seldom used in the field because most infections are detected when the chancre has already disappeared (Büscher and Lejon, 2004).

2.11.2 Lymph Node Aspirate

Due to its simplicity, cervical lymph node palpation is systematically done (often in association with CATT) for population screening in many endemic countries. Even if this test is considered less cost-effective than the agglutination assay (Lutumba *et al.,* 2005), in some areas it appears to be the best method for early diagnosis of human African trypanosomiasis (Vanhecke *et al.,* 2010). When enlarged lymph nodes (Winterbottom's sign) are identified, they are punctured and the fresh aspirate is spread on a slide and covered with a cover slip. The wet preparation is then quickly examined (to avoid lysis of parasites in the sample) by light microscopy at ×40 0 total magnification for the presence of motile trypanosomes. The sensitivity of this method varies between 40 and 80%, depending on the parasite strain, the stage of the disease (sensitivity is higher during the first stage) and the prevalence of other infections causing lymphadenopathy (Louis *et al.,* 2001; Büscher and Lejon, 2004).

2.11.3 Thin and Thick Blood Films

To prepare wet blood films, 5 or 10 of finger-prick blood are placed on a slide and examined microscopically under a cover slip at ×400 total magnification (Louis *et al.,* 2001). Trypanosomes are revealed by their moving between the erythrocytes. In spite of its very low sensitivity, with a detection limit as high as 10,000 trypanosomes/ml (1 parasite/200 microscope fields) this method is still used in some centres (especially for *T.b. rhodesiense*) because it is very simple, cheap and offers immediate results (Büscher and Lejon, 2004). Fixed thin blood films stained with Giemsa or Field's solutions have a similar low sensitivity (Chappuis *et al.,* 2005). The preparation of a thick blood film is, therefore, preferred since it slightly improves the sensitivity to 5,000 trypanosomes/ml (Louis *et al.,* 2001). To prepare a thick blood film, 20 ml of finger-prick blood is placed on a small area of the microscope slide and defibrinated by the use of the corner of another slide. The sample is then left to dry without being fixed, protected from direct sunlight, stained with Giemsa or Field's solution and finally observed under the microscope at x 1000 total magnification (Büscher and Lejon, 2004). This technique is the most widely used in the absence of a centrifuge, even if examination of each slide can take 10 to 20 minutes and must be carried out by an expert microscopist, since parasites are often deformed during sample preparation and are easily missed (Chappuis *et al.,* 2005).

2.11.4 Microhaematocrit Centrifugation Technique (mHCT)

The blood concentration technique of microhaematocrit centrifugation (mHCT), also called the capillary tube centrifugation technique (CTC) or Woo test (Woo, 1970), is generally used during mass surveys to test CATT-positive suspects in whom the

diagnosis could not be confirmed by classical methods (Chappuis *et al.,* 2005). The mHCT is relatively easy to perform: it consists in filling three-quarters full heparinized capillary tubes with 50-60 □l of finger-prick blood. After being sealed at their end, the tubes are centrifuged at high speed for 6-8 minutes in a haematocrit centrifuge, in order to concentrate the trypanosomes present in the blood at the level of the buffy coat. The capillary tubes are then mounted on a special holder and can then be directly viewed at low magnification (×100 or ×200 total magnification) for mobile parasites (Louis *et al.,* 2001; Chappuis *et al.,* 2005). By examining more than one capillary tube (usually 6 or 8), the sensitivity of mHCT increases, and fewer than 500 trypanosomes/ml can be detected (Büscher and Lejon, 2004). The mHCT is quite time-consuming and the presence of microfilariae in the blood can hamper detection of smaller trypanosomes (Lutumba *et al.,* 2006). The need of a Centrifuge represents the major drawback to the implementation of this technique in the field (Jannin and Cattand, 2004).

2.11.5 Miniature Anion-Exchange Centrifugation Technique (mAECT)

Since its adaptation for field diagnostic use in 1979 (Lumsden *et al.,* 1979), the miniature anion-exchange centrifugation technique (mAECT) has been continuously improved (Kimber, 1984; Zillmann *et al.,* 1996; Truc *et al.,* 1998b; Büscher *et al.,* 2009). This method purifies trypanosomes from blood using anion-exchange chromatographical columns containing diethylaminoethyl-cellulose (DEAE-52) and then it concentrates them by centrifugation before viewing (Lumsden *et al.,* 1979). The chromatographical separation depends on difference in surface charges of the blood cells and the parasites, which are less negatively charged because their glycoprotein coat keeps the negative phospholipid head groups away from the matrix. When infected blood is mixed with PSG

(phosphate saline glucose) buffer at pH 8 and is added to the column, the red and white blood cells adhere to the DEAE gel particles, while trypanosomes, being at their isoelectric point, flow through and are collected at the bottom of a sealed glass tube. After low speed centrifugation, the tip of the tube is mounted on a special holder and examined for the presence of trypanosomes using light microscopy at low magnification. Since a large blood volume (300-350 ml) is used, high sensitivity is achieved (the detection limit is less than 100 trypanosomes/ml) (Louis *et al.,* 2001). The efficiency of the technique could be further improved by processing on the column the buffy coat obtained from a larger volume of blood (5 ml) (Camara *et al.,* 2010). However, the mAECT also has some drawbacks: the manipulations are quite time-consuming, materials are expensive and an energy source is needed (Chappuis *et al.,* 2005). Initial evaluation in experimentally infected animal blood showed that this technique was more sensitive than the mHCT (Sachs, 1984). Both the mAECT and the mHCT need 30 minutes for results, but the microscopy phase for the mAECT is faster (1 minute, compared to 5 minutes for the mHTC) (Lumsden *et al.,* 1979). The mAECT is currently considered the most sensitive parasitological technique for detection of trypanosomes in blood, with sensitivity in the field in the range of 75.3-84.5% (Miézan *et al.,* 1994; Lutumba *et al.,* 2006).

2.11.6 Quantitative Buffy Coat (QBC)

The quantitative buffy coat (QBC, Becton-Dickinson) is a very sensitive technique initially developed for rapid cell count and applied to malaria diagnosis. It is a modification of the mHCT method that associates concentration of the parasites by centrifugation with their fluorescence staining using acridine orange (Bailey and Smith,

1992; Chappuis *et al.,* 2005). For sample preparation, 60 ml of finger-prick blood are loaded into capillary tubes containing EDTA, acridine orange and a small floating cylinder and are centrifuged at high speed for 5 minutes. Fluorescent DNA of motile trypanosomes can be visualised in a darkroom in the expanded buffy coat by using a UV light connected to a special objective (60×) containing the appropriate filter. The need of this fragile equipment prevents the use of this test by mobile teams, limiting its application to referral centres (Chappuis *et al.,* 2005). The QBC is a very sensitive technique, with a detection limit estimated lower than 500 trypanosomes/ml (Louis *et al.,* 2001). Compared to the mAECT, the QBC was found to have a slightly lower sensitivity both on finger-prick and venepuncture samples, but due to its simplicity and rapidity (only 15 minutes instead of the 30-45 minutes needed for mAECT result) its use was recommended for routine screening (Truc *et al.,* 1998a). Nevertheless, the costs of this technique can represent a major obstacle for its implementation in low-income countries.

2.11.7 *In Vivo* and I*n Vitro* Isolation of Trypanosome Strains

Trypanosome strains have always been isolated for research purposes from patients through inoculation of infected blood or cerebrospinal fluid into susceptible laboratory animals. Immunosuppressed African *Mastomys natalensis* rats are considered the best recipient for primary propagation of *T. b. gambiense,* with a success rate of 50% (Büscher *et al.,* 2005; Maina *et al.,* 2007b), but *Grammomys surdaster* thicket rats were shown to lead to even higher percentages of success (Büscher *et al.,* 2005). The process of isolation and propagation in rodents is problematic, especially for the low virulent Gambian strains, and requires long incubation times (Brun *et al.,* 2001). A simpler kit for *in vitro* isolation (KIVI) of trypanosome strains under field conditions proved

effective both for human and animal samples (Truc *et al.,* 1992). The procedure of the kit consists of inoculating, aseptically, several millilitres of blood in culture medium, where bloodstream parasites transform into procyclics and multiply at room temperature during the successive weeks (Büscher and Lejon, 2004). The KIVI kit can have a diagnostic value in detecting infections when other parasitological techniques fail, but it is seldom applied due to delays to obtain the results (Louis *et al.,* 2001). The long adaptation periods needed to propagate isolates can have as a consequence the selection of clones or the modification of the original isolate and, for this reasons, the number of passages must be kept at a minimum (Brun *et al.,* 2001). Recently, *T. b. gambiense* parasites were successfully amplified from Cerebrospinal fluid of HAT patients by *in vitro* culture on a fibroblast feeder layer (Giroud *et al.,* 2009), a new promising technique which was found more efficient than inoculation in BALB/c mice.

2.12 DNA Technologies

The applications of DNA techniques in diagnostics are quickly expanding. This is due to the many advantages these technologies offer, first of all their high sensitivity and specificity, superior to those of traditional parasitological tools. Moreover, they allow differentiating between trypanosomes of veterinary importance and, especially, between the two human pathogenic subspecies *T. b. rhodesiense* and *T. b. gambiense*, otherwise morphologically indistinguishable. This will become a crucial aspect of disease control in the case of overlap of the two human forms (Picozzi *et al.,* 2005). The continuous simplification of molecular diagnostic procedures makes their use in the field, even in relatively simply equipped laboratories, a real option (Mugasa *et al.,* 2009).

2.12.1 PCR

The polymerase chain reaction (PCR), together with other molecular biology techniques, has been widely used for research purposes to identify infecting trypanosome DNA in animal and human blood (Gibson, 2001 and 2002a; Becker *et al.,* 2004; Brun and Balmer, 2006). Different molecular probes have been tested for detection and identification of single trypanosome isolates from patients (Jamonneau *et al.,* 2001). Among them, the classical TBR 1-2 primers, which recognise a repeated satellite sequence of the subgenus *Trypanozoon*, showed high sensitivity in human blood samples (up to 100%) with a specificity higher than 92% (Penchenier *et al.,* 2000; Solano *et al.,* 2002), but these primers can not differentiate between the three *Trypanosoma brucei* subspecies. Other markers are available for detection of *T. b. gambiense* infection, such as the genes encoding for the variant surface glycoproteins LiTat 1.3 and AnTat 11.17 (Bromidge *et al.,* 1993), but these antigens are not homogeneously expressed in all isolates (Dukes *et al.,* 1992). A specific target for the Gambian form has been identified in the receptor-like flagellar pocket glycoprotein TgsGP (Berberof *et al.,* 2001), which has been shown to detect down to 10 trypanosomes/ml of human blood (Radwanska *et al.,* 2002b). For *T. b. rhodesiense*, a PCR based on the use of primers targeting the human serum resistance associated gene (*SRA*), which confers resistance to lysis by human serum and is ubiquitous in this species (Gibson *et al.,* 2002b), has been successfully applied as a molecular marker (Radwanska *et al.,* 2002a; Njiru *et al.,* 2004). Regardless of the primers utilised, PCR is generally more sensitive than other serological and parasitological diagnostic methods currently in use, showing detection limits of 0.1 trypanosome/ml of cattle blood and 1 to 40 trypanosomes/ml of human blood

(Jamonneau *et al.,* 2004). Experimental work on animals has demonstrated that its application allows detection of active infection or relapse at a very early stage (Clausen *et al.,* 1998; Bengaly *et al.,* 2001). Moreover, as PCR can be performed on samples from both blood and cerebrospinal fluid, this approach offers a possible tool not only for diagnosis but also staging of HAT (Truc *et al.,* 1999; Jamonneau *et al.,* 2001; Section 1.2.5). Unfortunately, despite the high potential as a confirmatory assay for infection, the use of this technique is restricted to research laboratories and it is not commonly used for HAT diagnosis because of the need of expensive thermocyclers and of complex DNA purification protocols. Furthermore, PCR can be relatively slow and suffers a significant risk of sample contamination (Mugasa *et al.,* 2008). Finally, this technique has not been fully validated yet: it has problems of reproducibility and can provide results at odds with those of both serological and parasitological tests (Koffi *et al.,* 2006).

2.12.2 Loop-Mediated Isothermal Amplification (LAMP)

Efforts have been made in order to simplify PCR protocols and make this technique more suitable for field requirements. One of the most promising innovations is a new isothermal PCR-like method, called loop-mediated isothermal amplification (LAMP) (Notomi *et al.,* 2000). This technique, already commercialised for various infectious diseases, allows accurate and robust amplification of the target sequence in a single heating step (60-65°C), which can be performed in a water bath, and the results can be visualised by a simple colorimetric reaction (Njiru *et al.,* 2008a and 2008b; Mori and Notomi, 2009). When applied to *Trypanozoon* isolates using primers targeting the repetitive insertion mobile element (RIME), this methodology showed a very high sensitivity (as low as 0.001 trypanosomes/ml) and reproducibility (Njiru *et al.,* 2008b).

Other probes have been designed for detection of specific trypanosome species including *T. b. gambiense* (Thekisoe *et al.,* 2007) and *T. b. rhodesiense* (Njiru *et al.,* 2008a). Moreover, the test is fast (results are available in 35 minutes) and less prone to inhibitory factors than classical PCR, a characteristic that enables the use of unprocessed template, whether buffy coat, heat-treated blood or serum (Njiru *et al.,* 2008a and 2008b), although there is some evidence of a loss of sensitivity on crude samples (Thekisoe *et al.,* 2009).

2.12.3 Molecular Dipstick Test (HAT-PCR-OC)

The molecular dipstick test is a simple detection tool for PCR products based on oligochromatography (OC). In positive samples, amplicons hybridize with a specific gold-conjugated probe and migrate on a chromatographical membrane where they result in a visible line in only 5 minutes. The test developed for *T. brucei* (HAT-PCR-OC) had 100% specificity and sensitivity on blood samples and was able to detect a single parasite in only 180 of blood (Deborggraeve *et al.,* 2006).

2.12.4 Nucleic Acid Sequence-Based Amplification (NASBA) and NASBA-OC

The nucleic acid sequence-based amplification assay (NASBA) consists in a self-sustained real-time PCR based on the amplification of RNA targets under isothermal conditions (41°C) (Deiman *et al.,* 2002). The detection of the unstable mRNA has two advantages: it is indicative of active transcription and it avoids possible DNA contamination (Büscher and Lejon, 2004). By amplification of the 18S ribosomal RNA, this assay was able to detect specifically *Trypanosoma brucei* species (without distinction between *T. b. gambiense* and *T. b. rhodesiense*) with a sensitivity of 70% on clinical samples and adetection limit of 10 trypanosomes/ml (Mugasa *et al.,* 2008). The association of NASBA with oligochromatography (OC) for amplicon detection makes

this assay even easier and cheaper. During a study conducted by Mugasa and collaborators, the NASBA-OC showed higher sensitivity than standard microscopy both on blood (73%) and cerebrospinal fluid (88.2%) (Mugasa *et al.,* 2009). Results were available in only 90 minutes (compared to the two or more hours needed for PCR or NASBA alone) and were highly reproducible.

2.12.5 Fluorescence *In Situ* Hybridisation Test (PNA FISH)

The peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) is a new technique designed to facilitate detection of trypanosomes in blood smears by the use of fluorescent molecular probes that hybridise to parasitic RNA sequences. The use of fluorescein-labelled peptide nucleic acid (PNA) probes, targeting the 18S rRNA of the subgenus *Trypanozoon*, showed a detection limit of 500 trypanosomes/ml of blood (Radwanska *et al.,* 2002c), but 90 minutes were necessary for hybridisation.

2.13 Stage Determination

When a HAT case is confirmed, the stage of the disease must be determined in order to guide treatment (Bisser *et al.,* 2002; Kennedy, 2006a; Rodgers, 2009). Since specific neurological symptoms occur late and no blood test indicating brain involvement is available, this can only be done by analysis of a cerebrospinal fluid (CSF) sample obtained from the patient by invasive lumbar puncture (Enanga *et al.,* 2002; Kennedy, 2008). Direct evidence of central nervous system (CNS) invasion is represented by the finding of trypanosomes in the CSF, but since this approach can be very insensitive, WHO also recommends the use of other indirect parameters related to the host's neuroinflammatory immune response: white blood cell count and protein concentration (WHO, 1998). Positivity of one of these two tests defines the second stage even in

absence of parasite detection or clinical symptoms (Bisser *et al.,* 2002). Nevertheless, cut-off values for these assays are disputed and the exact definition of second stage is not definitive (Kennedy, 2006 and 2006). Improved diagnostics for disease staging is urgently needed and evaluation of alternative CSF parameters for indirect stage determination is currently under investigation (Lejon and Büscher, 2005).

2.13.1 Trypanosome Detection in CSF

Detection of trypanosomes in the CSF is sufficient to classify a subject in the meningo-encephalitic stage of the illness (Lejon and Büsche r, 2005). The CSF has to be examined immediately after lumbar puncture, because trypanosomes in this medium seem to be very fragile and they start to lyse within 10 minutes (Büscher and Lejon, 2004). This technique is simple and cheap, but it suffers from insufficient sensitivity since it can detect parasites only when their concentration is >1,000 trypanosomes/ml (Lejon and Büscher, 2002). The sensitivity can be increased by single or, especially, by double centrifugation (DC) of the CSF sample. The first technique consists of viewing the sediment obtained from centrifugation of several millilitres of CSF spread on a glass slide, while in the second method this sediment is further centrifuged in a flame-sealed capillary tube which is, then, directly examined by microscopy (Cattand *et al.,* 1988). Due to the number of manipulations and the equipment required, these techniques are not widely applied (Chappuis *et al.,* 2005). A simplification of the DC test was developed by Miézan and colleagues, who showed that the direct examination of the CSF sediment at the bottom of a sealed Pasteur pipette obtained after a single centrifugation had the same sensitivity as the DC technique but it was faster (10 minutes) (Miézan*et al.,* 2000). The introduction of a new plastic collector tube, originally developed for the mAECT, could

further ameliorate this technique, increasing the sensitivity to <2 trypanosomes/ml of CSF (Büscher *et al.,* 2009).

2.13.2 White Blood Cell Count in CSF

Despite its lack of specificity, the white blood cell count is the most widely used (and often unique) technique for stage determination and follow-up (Lejon and Büscher, 2001 and 2005). The WHO upper limit for normal white cell concentration is set at 5 cells/□l, but it has been proposed to raise this value to 10 cells/l (Chappuis *et al.,* 2005) or 20 cells/□l (Lejon *et al.,* 2003b), since patients found with 6 to 20 cells/□l in their CSF were successfully cured by pentamidine (Doua *et al.,* 1996). These subjects, considered to be in the so-called "early second stage" or "intermediate stage" of the illness, often present non-concordant markers that complicate staging (Bisser *et al.,* 2002). Once the CSF is collected (at least 5 ml), the leukocyte count must be carried out quickly to prevent cell lysis (Chappuis *et al.,* 2005). In African trypanosomiasis, the CSF can present with a special type of enlarged B-cell, known as morular cells of Mott, containing cytoplasmic vacuoles filled with IgM, which can be considered pathognomic, although they are not specific for HAT (Lejon and Büscher, 2005). A recent work showed how the detection and quantification of B lymphocytes may be useful to stage the disease (Bouteille *et al.,* 2009).

2.13.3 Protein Concentration in CSF

In CSF of late stage HAT patients protein concentration is elevated (0.4-2 mg/ml) (Kennedy, 2006b), but it can also be raised during the first stage of the illness, due to the diffusion of the abundant serum immunoglobulins in the CSF (Chappuis *et al.,* 2005). Cut-off values set by WHO for sleeping sickness vary according to the quantification

method used (WHO, 1998). Recent data suggest that the protein concentration threshold of 370 mg/litre for the colorimetric method is too low and should be raised (Lejon *et al.,* 2003b). This technique is seldom applied in African sleeping sickness control centres, because the methods are sophisticated, reagents needed are unstable and the results do not offer much more information than CSF cell count (Lejon and Büs cher, 2001 and 2002).

2.13.4 Other Markers and Tests for Second Stage Determination

CSF of second stage *T. b. gambiense* patients contains high levels of immunoglobulins, especially IgM, which are considered a strong early indicator of inflammation in the CNS due to parasite invasion (Bisser *et al.,* 2002), even if other neurological infections could cause the same effect (Lejon *et al.,* 2003b). Studies on quotients CSF/serum concentration for IgM, IgG and IgA confirmed that the elevated concentration in the CSF was due to intrathecal synthesis and only partially to blood-derived immunoglobulins and that the predominant IgM presence was the most sensitive (95%) marker for brain involvement (Lejon *et al.,* 2003b). Determining the IgM content in the CSF can be very difficult to carry out under field conditions but a semiquantitative latex agglutination test for IgM in CSF (LATEX/IgM) has recently been designed for field use, thanks to its reagent stability at 45ºC for more than 2 years (Lejon and Büscher, 2 002). End titres (highest dilution causing an agglutination) ≥1:8 were shown to be 89.4% sensitive and 92.7% specific for presence of intrathecal IgM synthesis (Lejon *et al.,* 2002b). The possibility to use the LATEX/IgM agglutination test also for stage determination of *T. b. rhodesiense* infection is under evaluation (Lejon *et al.,* 2002b). Other antibodies with different affinities are being evaluated as possible second

stage markers (Lejon and Büscher, 2001). Among them, auto antibodies against brain-specific components such as neurofilaments and galactocerebrosides have been found in CSF of HAT patients (Vincendeau and Bouteille, 2006). Simple test formats to detect these antibodies not only in CSF, but also in other biological fluids like saliva, could represent a great improvement towards a less invasive assay for staging (Jannin and Cattand, 2004). Cytokines and chemokines mediate the host's neuroinflammatory immune response and high levels of these molecules in CSF have been associated to neurological involvement in *T. b. gambiense* disease (Courtioux *et al.,* 2006; Hainard *et al.,* 2009). Interleukin-10, in particular, has significantly raised concentrations in plasma (Courtin *et al.,* 2006) and CSF of infected patients and it could represent an interesting marker for treatment and follow- up, since its levels lower immediately after treatment (Lejon *et al.,* 2002a). A combination of different markers (two chemokines, CXCL8 and CXCL10 and a brain damage marker, H-FABP) was shown to correlate to HAT second stage with 97% sensitivity (Hainard *et al.,* 2009). However, the use of these indicators for staging suffers various drawbacks, such as the not direct applicability in the field, the necessity of invasive lumbar puncture and the need to exclude other brain inflammatory diseases.

Finally, the finding of trypanosome DNA in CSF by the use of PCR techniques could represent another possible tool for staging of sleeping sickness (Truc *et al.,* 1999).

2.13.5 Polysomnography

Polysomnography is a promising non-invasive diagnostic technique that could be used to discriminate between the two stages of African trypanosomiasis (Buguet *et al.,* 2005). It combines different tools (electro-encephalogram, electro-oculogram and electro-

myogram) which allow the monitoring of sleep structure and phases, both known to be seriously compromised in second stage HAT patients. The detection of abnormal sleep onset rapid eye movement periods (SOREMPs) that occur shortly after CNS involvement, has been proven to be proportional to illness severity and to recede after treatment, showing a possible application also in follow-up studies.

2.14 Other Diagnostic Approaches

Novel diagnostic approaches based on the detection of specific biomarker patterns related to the presence of the disease ("fingerprints") are under study. A proteomic signature analysis technique, for example, has been demonstrated to be much more accurate than any other diagnostic test, being able to distinguish between trypanosome-infected and non-infected serum samples with a sensitivity of 100% and a specificity of 98.6% (Papadopoulos *et al.,* 2004). The proteomic analysis is impracticable in the field but it could represent a very sensitive tool for the identification of new biomarker proteins for successive applications in diagnostics or drug discovery (Agranoff *et al.,* 2005). Following this principle, Hainard and collaborators evaluated various molecules identified by proteomic analysis as possible indicators for HAT second stage (Hainard *et al.,* 2009). Similarly, the comparison of metabolite patterns could offer another approach for discovering "fingerprints" to use for HAT diagnosis or staging. A study carried out by Wang and collaborators demonstrated the utility of the analysis of the metabolome for diagnostics, by showing a correlation between *T. b. brucei* infection in mice and systematic perturbations in the metabolites profile of the animal's urine and plasma (Wang *et al.,* 2008).

2.14.1 Follow-Up

Ideally, patients should be periodically checked (visits at 3, 6, 12, 18, 24 months) for a total period of two years after treatment to confirm complete cure (WHO, 1998), but in practice this is hardly achieved in the field, where compliance with follow-up is extremely low (Legros *et al.,* 1999). Unfortunately, unsuccesful treatment regimens are far from rare and early detection of these failures would increase the chances of the patients to be cured. Causes of drug relapses are still unclear. Many phenomena could be involved in this process, such as low drug potency, under-dosing, pharmacokinetic differences between individuals, presence of co-infections (especially HIV), and poor health of the patient at the moment of admission to the treatment centre or decreased susceptibility of trypanosomes to the drug. To assess chemotherapy success both direct and indirect diagnostic techniques are used, but standard operational criteria are still lacking. Since no blood markers for cure have been identified so far and the CATT test resulted unreliable during follow-up examinations (Lejon *et al.,* 2010), treatment failure is usually identified as a deterioration of the CSF parameters. This phenomenon normally occurs in 3-9% of late stage patients treated with melarsoprol (Maina *et al.,* 2007a). Detection of trypanosomes in blood, lymph nodes or CSF indicates treatment failure or relapse, but due to low sensitivity of parasitological techniques, increased leukocyte count and recurrence of neurological symptoms are also often evaluated (Lejon and Büsche r, 2001 and 2005; Mumba Ngoyi *et al.,* 2009). Patients hospitalised with severe brain inflammation or as previous relapsingcases are at high risk of treatment failure (Legros *et al.,* 1999; Lejon *et al.,* 2008). A recent work on *T. b. gambiense* patients identified the association between white cell count ≥ 8 cells/ml and

LATEX/IgM end titre ≥ 1:4 as being 97 % and 100% specific for relapse prediction after 12 and 18 month from treatment respectively (Lejon *et al.,* 2008). The identification of criteria for treatment outcome would have the great advantage of shortening the follow-up period of patients with low risk of failure (Mumba Ngoyi *et al.,* 2010). Since normalisation of CSF values is generally slow, the use of the PCR technique for earlier detection of relapses by mean of trypanosome DNA amplification is strongly advocated (Truc *et al.,* 1999).

2.14.2 Diagnosis of Drug Resistance

HAT treatment is complex for a number of reasons: need for hospitalisation, toxicity, stage-specificity and high costs of the drugs are all major issues in endemic countries. Moreover, the loss of efficacy of melarsoprol (the drug most used to treat the second stage of the Gambian form and the only one available for late stage *T. b. rhodesiense* infection) has become a pressing problem. In recent years the number of melarsoprol treatment failures has increased to alarming levels in some areas, reaching values of 30% in Uganda (Legros *et al.,* 1999). Drug resistance of some trypanosome strains to melarsoprol has been indicated as an important cause for these chemotherapic failures in individuals infected with *T. b. gambiense* (Brun *et al.,* 2001; Matovu *et al.,* 2001a), but other factors may contribute (Maina *et al.,* 2007a), such as differences in drug pharmacokinetics between individuals (Legros *et al.,* 1999). A major problem for drug resistance study is the difficult isolation and propagation of trypanosomes from relapsing patients, thus limiting the number of samples available for successive *in vitro* and *in vivo* drug sensitivity tests in the laboratory (Brun *et al.,* 2001).

Various mechanisms, affecting both drug levels inside the parasite and the drug target(s), can contribute to reduced sensitivity to trypanocidal compounds (Barrett and Fairlamb, 1999; Mäser *et al.,* 2003). Resistance to melaminophenyl arsenicals and diamidines has been, at least partially, ascribed to altered activity of the *T. brucei* P2 amino-purine transporter involved in their uptake (Carter and Fairlamb, 1993; Barrett and Fairlamb, 1999; Mäser *et al.,* 1999; Delespaux and de Koning, 2007). The loss of P2 function was found responsible for diminished internalisation of these classes of compounds and multidrug resistance onset (Barrett and Fairlamb, 1999). Increased drug export through over-expression of a *T. brucei* multidrug-resistance protein, called MRPA, has also been found to cause melarsoprol-resistance *in vitro*, but data were not supported *in vivo* (Alibu *et al.,* 2006). Instead, mutations in the *TbAT1* gene, which encodes for the P2 transporter, have been identified in resistant trypanosomes both in laboratory strains and field isolates (Mäser *et al.,* 1999; Matovu *et al.,* 2001a). The use of a PCR/*Sfa*NI RFLP technique (PCR amplification of the *TbAT1* gene followed by analysis of the restriction fragment length polymorphism for *Sfa*NI) allowed identification of mutated forms of the transporter, but this method was not able to identify all resistant strains, since many of them had a wild type sequence of the *TbAT1* gene (Mäser *et al.,* 1999; Matovu *et al.,* 2001b). Deletion of the whole *TbAT1* gene is also a rare, but possible, event in field isolates (Matovu *et al.,* 2001b). The treatment of patients infected with strains less sensitive to melarsoprol has both the consequences of unnecessarily exposing them to the highly toxic side effects of the arsenical compound and of contributing to the maintenance of the alleles associated with resistance in the trypanosome population (Kazibwe, 2008). In a recent paper, Kazibwe and colleagues showed how the withdrawal

of melarsoprol as first-line drug in a focus with high levels of treatment failures correlated with disappearance of mutant alleles in the trypanosome isolates, identified with the PCR/*Sfa*NI RFLP technique (Kazibwe *et al.,* 2009). This study demonstrates the potential benefits of drug resistance tests for treatment decision making and rational management of HAT in resource-poor settings.

In our laboratory, a rapid and easy fluorescent test able to identify resistance to arsenical and diamidine drugs, by exploiting altered activity of the P2 transporter in resistant strains, was developed (Stewart *et al.,* 2005). The test utilises the diamidine DB99 [2, 5-bis(4-amidinophenyl)-3,4-dimethylfuran], which is actively transported by trypanosomes through the P2 permease and accumulates in DNA-containing organelles (nucleus and kinetoplast). DB99 is intrinsically fluorescent and its internalisation inside parasites can be monitored using a standard fluorescence microscope (330 nm, 400 nm). The test was proven able to discriminate between various sensitive (stained) and resistant (unstained) strains in mice blood, both from *in vitro* culture and field isolates, if exposure to the drug was kept inferior to 1 minute.

2.14.3 Diagnosis of *T. b. rhodesiense* Infection

T. b. rhodesiense causes an acute, febrile form of HAT in east Africa and can lead to death of the patient within months (Barrett *et al.,* 2003). The parasite is harboured in a wide animal reservoir (Smith *et al.,* 1998; Stich *et al.,* 2002), infected livestock representing a major cause of maintenance and spread of the disease (Hutchinson *et al.,* 2003; Picozzi *et al.,* 2005). Before the advent of molecular technologies, the immune trypanolysis test (Rickman and Robson, 1970; Van Meirvenne *et al.,* 1995) had been the only tool to discriminate between human infective (mainly *T. b. rhodesiense*) and non-

infective trypanosome species isolated from animals and it was widely used for epidemiological purposes.

For diagnosis of *T. b. rhodesiense* an effective serological tool, equivalent to the CATT, is still lacking and is difficult to envisage due to the higher degree of VSG variability than in *T. b. gambiense* (Louis *et al.,* 2001). Case-detection for Rhodesiense trypanosomiasis is, therefore, based on passive surveillance (Hutchinson *et al.,* 2003) and initial diagnosis relies on clinical symptoms (Garcia *et al.,* 2006). For *T. b. rhodesiense* infection biological parameters are generally more abnormal than for *T. b. gambiense* form, including severe anaemia, liver dysfunction, thrombocytopenia and cardiac failure that often causes the death of the patient (Lejon and Büscher, 2005). On the contrary, the neurological symptoms typical of the Gambian disease are not frequent in Rhodesiense trypanosomiasis and a clear distinction between first and second stage is more difficult to determine (Kennedy, 2006a). Parasitological confirmation and staging are based on the same methods used for *T. b. gambiense* disease but, since *T. b. rhodesiense* infection presents a high and persistent parasitaemia, the detection of the parasites is relatively easy (Kennedy, 2008) and direct examination of thin or thick blood smears or lymph node aspirate is usually sufficient (Smith *et al.,* 1998). More sophisticated assays like ELISA and PCR are available, but not applicable in the field (Chappuis *et al.,* 2005).

2.15 Endophytic Fungi

Fungal endophytes are microfungi that colonize living tissues of plants without producing any apparent symptoms or obvious negative effects (Hirsch and Braun 1992). Many endophytes produce unusual secondary metabolites of industrial importance

(Hawks worth *et al.,* 1995). They have protective role against insects herbivory and many are producers of novel antimicrobial secondary metabolites (Arnold *et al.,* 2003, Malinowski and Beleskey 2006 and Shiomi *et al*., 2006). Additionally, endophytic fungi are a fascinating source of new natural products which are of great potential for medicinal and agricultural applications (Aly *et al.,* 2010 and 2011). Also, endophytic fungi represent an important and quantified component of fungal biodiversity, and are known to have effect on diversity and structure of plants community (Gonthier *et al.,* .2006 and krings *et al.,* 2007). Endophytic fungi act as parasites of insects (Richard, 2014).

Endophytes describe all microbes including fungi, bacteria, cyanobacteria, and actinomycetes that exist within plant tissue. The question of how microbial endophytes enter into their host plant has been interesting to learn. Plants have been used as a remedy since ancient period of time. As per World Health Organization 80% of world population depend on traditional medicine made from plants for their health care. During the period year 2005-2006 the global market for herbal medicines counted at over US $6 billion annually. Statistical data available in USA also showed that 41 percent of pharmaceutical products originated from herbal drugs. At the present there is need for new antimicrobial agents that are extremely effective, with low toxicity and negligible environmental impact. During the period of year 1981-2002 about 60% of the new drugs registered by the FDA as anticancer, antimiraine and anti-hypersensive agents were natural products and related to them (Newman *et al*.,2003). In year 2002-2003 top 35 worldwide selling drug were natural product derived compounds (Butler, 2004).

2.15.1 Endophytes and their Potential in Drug Discovery

Endophytic microbes from medicinal plants are good source of functional metabolites (Weber *et al.,* 2004; Tejesvi *et al.,* 2007; Huang *et al.,* 2008). Endophyte infection found to alters pattern of gene expression in the host plant (Baily *et al.,* 2006). Interaction between endophyte and plant is mainly controlled by the genes of both organism and host plant modulated by the environment. Endophytes from angiosperms as well as gymnosperms have been studied for presence of novel secondary metabolites. Primary metabolites are common in all living cells and are involved in the formation of biomass and generation of energy, in contrary secondary metabolites are produced by one or few species only. These secondary metabolites are low molecular weight compounds, they are not required for growth in pure culture and are produced as an adaptation for the specific function in nature. Bioprospecting is most frequently used phrase to describe the collection and screening of the biological material for commercial purposes. The importance of natural products in the drug discovery and development has been reported briefly. The natural products produced by endophytes have vast range of bioactivities, representing a vast reservoir offering an enormous potential for exploitation in medicinal and industrial uses (Zhang *et al.,* 2006). Crude extracts from culture broth of endophytes found to show antibacterial, antifungal, antiviral, anti-inflammatory and antitumor activities (Silva *et al.,* 2007). Therefore, endophytes open up new areas for the biotechnological exploitations.

2.15.2 Host Endophyte Relationship and Effect on Metabolites Production

Microorganisms are likely to harbor metabolic pathways that lead to the production of novel secondary metabolites. Endophytes from plant origin are investigated for their

secondary metabolite production. Endophytic microbes are nonpathogenic in nature, Production of secondary metabolites enable them to survive in the plant interstitial space. Many of the important secondary metabolites have been extracted and characterized from endophytic microbes. This includes alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols and phenols. In addition natural products also often serve as lead structures whose activity can be enhanced by exploitation through synthetic chemistry (Strobel and Daisy, 2003). Endophytes are able to increases host fitness and competitive ability, by increasing nutrientional uptake, resistance to seed predators, seed germination success, tolerance to heavy metals, high salinity and good growth rate through biochemical pathways such as phytohormone indole 3 acetic acid (IAA) from fungal endophytes *Acremonium coenophialum, Aureobasidium pullulans, Epicoccum purpurascens* and *Collectotrichum* sp. along with IAA, cytokinins were also produced by an endophytic *Hypoxylon serpens*.

Plant provides spatial arrangement, shelter, nutrient and distribution to the next generation of microbes (Rudgers *et al.,* 2004). Plant may provide vital compounds for the completion of the life cycle of the endophytes (Strobel, 2002a). Current research suggested that endophyte and plant genotypic combinations together with environmental conditions are important source of variation in endophyte host interactions (Faeth and Fagan, 2002). Many factors such as season, age, environment and location these may contribute and influence the biology of endophyte (Strobel and Daisy, 2003). Endophyte derives nutrients from the plant without killing host. Endophytic microbes such as *Phomopsis*, *Phoma*, *Colletotrichum* and *Phyllosticta* have wide host range and found to colonize numerous taxonomically distinct plants (Pandey *et al.,* 2003; Jeewon *et al.,*

2004; Murali *et al.,* 2006; Sieber, 2007) developing adaptations to overcome different types of host defence. Endophyte infection found to affect concentration of abscisic acid in leaves of drought stressed grasses and this helps in the recovery of endophytes infected plant in water deficient conditions also. Endophytic microbes residing in the host tissue some time turn in to a pathogen in response to some environmental signal (Hendry *et al*. 2002). Such a change in the nature of the endophyte would also result change in its metabolite profile (Kuldav and Yates, 2000; Suryanarayanan and Murali, 2006). Endophytic microbes associated with traditionally used medicinal plants particularly of the tropics could be a rich source of functional metabolites (Tejesvi *et al.,* 2007). In this regard plant host association could also be exploited in enhancing the production of useful metabolites by the host (Wang *et al.,* 2004).

2.15.3 Physiological Role of Endophytes for Improvement of Ecological Adaptability of Host Plant

Endophytes are reported from plants found in various environment which includes tropic, temperate, aquatic, oceans, xerophytic, deserts, antarctic, geothermal soils, rainforests, mangrove swamps and also coastal forests (Hyde and Lee, 1995; Strobel *et al.,* 2002; Suryanarayanan and Murali, 2006). Endophytes found to improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens. Endophytic fungi are able to protect their host plant from drought conditions (Clay and Schardl, 2002) and salt tolerance (Waller *et al.,* 2005) and also found to increase heat tolerance so it is said that endophytes can acts as biological trigger to turn on the stress response more quickly (Redman *et al.,* 2002a). Symbiotically conferred stress tolerance involves two main mechanisms (i) activation of host stress

response rapidly after exposure to stress, (Arnold *et al.,* 2003) and (ii) biosynthesis of some of the anti-stress biochemicals by endophytes (Miller *et al.,* 2002; Schulz *et al.,* 2002). Endophytes control the activation of host stress response systems by acting as biological triggers. Enhanced drought tolerance is recognized through studies of endophyte infected species. Endophyte infected grasses respond to poor soil nutrition by an increased growth rate (Malinowski and Belesky, 2000). The most likely hypothesis related to endophytes is that the fungus survives entirely in a sterile state by establishing itself in vegetative tissues of the host including the structures associated with seeds, endocarps, stamens, styles and other structures including seed coats then the fungus grows into the newly developing plant tissues. When the seed germinates, the newly developing plant is invaded by the fungus as it becomes systemic in it, the life cycle is completed when the seed pod/seed coat is subsequently invaded by the fungus only to be perpetuated when the newly formed seed falls from the plants and is moved to new location and cycle continues again.

2.15.4 Protection from Pathogens

Present studies indicate that fungi may express different symbiotic lifestyles in respond to host genotypes and environmental factors. Both pathogenic and non-pathogenic fungi isolated from asymptomatic plant tissues, suggestive of that both pathogenic and nonpathogenic microbes express lifestyles or remain dormant in expectation of plant senescence (Schulz *et al.,* 1999). The Lifestyle expression is a post colonization phenomenon communicating between the symbiont and host. It is recognized that mutalistic fungi collectively may confer several host fitness benefits, such as growth improvement or tolerance to drought, disease, herbivory, insect and temperature to plant

(Azevedo *et al.,* 2000; Kerry, 2000; Redman *et al.,* 2001; Redman *et al.,* 2002b). The first verification that microbial endophytes associations were responsible for anti herbivory in mammals is confirmed by finding that *N. coenophialum* can produce ergot alkaloids with toxicity of cattle and some otherlive stock (Bacon *et al.,* 1977). Tanaka, in 2005 demonstrated the role of peramine in anti herbivory by *Argentine Weevil* stem, *Listronotus bonariensis* is symbiont of perennial rye grasses. It has been also documented that the janthitrems which are found in some strains of *Neotyphodium* in perennial rye grass, also found to contribute insect toxicity. Indole compounds as sesquiterpene and diacetamide along with some unidentified volatile compounds have been connected with *Epichole* species for resistance to leaf spot rust. The endophytes associated plants generally produces some metabolites that induces resistance to pathogens and it was also found that symbiotic plant activates defense system more quickly than nonsymbiotic plants after pathogen challenge.

2.15.5 Diversity and Distribution of Microorganisms Recovered as Endophytes

Endophytes lives, reproduce and forms mycelia that grow between the cells of a plant, more in the leaf sheaths and reproductive structures. When seed production starts, endophyte grows upward in the host plant. After seed formation, endophyte infects outer layers of the seed and endophytes transfer from plants in a seed production field to seed when such seeds germinate and grow, endophytic microbes starts infecting the other host. There are some major points represented as (i) Individual endophytes can switch symbiotic lifestyles and the result of symbiosis is influenced by host genotypes (ii) Mutalistic benefits conferred by endophytes are also influenced by plant genotypes (iii) The host range of endophytes is inadequately defined and which includes both monocot

and dicot species and (iv) Endophyte host plant describe adaptive symbiosis. Some endophytes have evolved with a high degree of suppleness to enter between genetically distinct plant species so provides endophytes an option to develop habitat range. Endophytic microbes can have intense effects on plant ecology, their fitness and are able to produce number of bioactive agents (Strobel and Daisy, 2003; Young *et al.,* 2012). The fossil proof shows that fungal symbionts have been associated with plants from the Ordovician period of approximately 400 million years ago, when plants first became established on land (Redecker *et al.,* 2000), migrating from aquatic to terrestrial habitats. Individual plants represent symbiotic endophytic communities associated within or on tissues below and above ground. Diverse group of this organism are able to produce bioactive agents with broad applications.

There are two major classes of fungal symbionts associated with internal plant tissues such as,

i. Fungal endophytes residing entirely within host plants and associated with roots, stems, leaves and flowers.
ii. Mycorrhizal fungi that are residing only in roots but extend out into the rhizosphere.

In count to this, fungal endophytes are also divided into two classes:

a. A comparatively minute number of fastidious species limited to a few monocot host plants (Clay and Schardl, 2002).
b. A huge number of tractable species with broad host ranges, together with monocots and eudicots (Stone *et al.,* 2000).

Considerable research have been done in class I endophytes as compare class II endophytes, corresponding largest group of fungal symbionts. This is because the class II endophytes have only been elucidated in recent times and shown to be responsible for the adaptation of some plants to high-stress environments (Arnold *et al.,* 2003; Dingle and McGee, 2003). Endophytic fungi may express different symbiotic lifestyles in response to the host genotypes and environmental factors. Lifestyle expression of endophytes is a post colonization phenomenon which involves biochemical and genetic communications between endophytic microbes and host. Basically grass species, have been entirely studied in relation to their endophytic biology (Tran *et al.,* 2010). *Clavicipitaceous* endophytes represents Class I and are small number of phylogenetically related *Clavicipitaceous* species which are fastidious in culture and also limited to some cool and warm seasonal grasses (Stone *et al.,* 2004). Transmission of these class I endophyte is mostly vertical with maternal plants passing fungi on to offspring by means of the seed infections (Saikkonen *et al.,* 2002). Class II endophyte generally comprises diverse species, all of which in general are members of the Dikarya (*Ascomycot*a or *Basidiomycota*) having ability to give habitat specific stress tolerance to host plants. The main established hypothesis says that *Clavicipitaceous* endophytes are defensive mutualists of host grasses and play role during their evolution (Lane *et al.,* 2003; Faeth and Fagan, 2002; Leuchtman, 2003; Schardl *et al.,* 2004). Class III endophytes are basically distinguished on the basis of their occurrence and horizontal transmission including vascular, nonvascular plants, some woody and herbaceous angiosperms in tropical forest and antarctic plant communities (Murali *et al.,* 2006). These endophytes are especially known for their huge diversity within individual host tissues, plants and

also populations. Class IV endophytes contains darkly melanized septa and they are restricted to plant roots. Generally these are *Ascomycetous* fungi conidial or sterile and forming melanized structures and also founds in non mycorrhizal plants from antarctic, tropical ecosystems and temperate zones.

Endophytes are very diverse and that only small minorities of all existing endophytes have been characterized. As a single leaf of a plant can harbor many different species of endophytes, may be bacterial or fungal. Tropical and temperate rainforests are the largest part of biologically diverse terrestrial ecosystem on the earth. Bills, (2002) described that metabolic distinction between tropical and temperate endophytic microbes through statistical data comparing the number of bioactive natural products from endophytes of tropical regions to that of temperate origin. And found that higher number of tropical endophytes produce enormous number of bioactive secondary metabolites than from other tropical substrata suggesting the importance of the host plants in influencing the metabolism of endophytic microbes.

Research emphasize that endophytes are usually not host specific. Same microbe isolated from different tissues or parts of the same host plant differ in their abilities for utilization of different substances (Carroll, 1986). Endophytic organisms associated with plants are varied and complex for example, fourteen distinct endophytic bacteria were isolated from *Carica papaya* L. six Gram-negative genera, which are *Pantoea ananatis*, *Enterobacter cloacae*, *Brevundimonasaurantiaca*, *Sphingomonas*, *Methylobacterium rhodesianum*, and *Agrobacterium tumefaciens,* two Gram-positive genera, *Microbacterium esteraromaticum* and *Bacillus benzoevorans* (Thomas and Clayfuqua, 2007). From last

two decades diverse endophytes have been targeted as sources of valuable bioactive compounds.

Several different bacterial endophyte species were isolated from single plant (Zinniel *et al.,* 2002). Eighteen different endophytic fungi were isolated from different tissues of bark, stem and leaf segments of five medicinal plants found within in Kudremukh range of Western Ghats of India, the dominant species isolated were *Curvulana clavata, C.lunata, C.pallescens* and *F. oxysporum*. The highest species richness as well as colonization frequency was found in the leaf segments of the host plant species (Raviraja, 2005) which confirms tissue specificity of these microbes. Subsequent identification of potential genes provides evidence of specific pathway for known alkaloids synthesis by endophytes (Panaccione *et al.,* 2001; Spiering *et al.,* 2005; Tanaka *et al.,* 2005; Wang *et al.,* 2004; Young *et al.,* 2005; Young *et al*.,2006). Consequently if endophyte can produce the same bioactive compounds as their host plants this would reduce the need to harvest slow growing rare plants and also help to preserve the worlds diminishing biodiversity.

Study represents that highest species richness and frequency of colonization found in stem. Some endophytic *A. niger, A. terreus* and *A. alternata* were organ specific (Khan *et al.,* 2010). A total of four diazotrophic endophytic bacteria were isolated from *Bambusa blumeana*. Showing big diversity with different genera as *Azospirillum*, *Escherichia*, *Pseudomonas* and *Aquaspirillum* (Wei *et al.,* 2007).Diversity of fungi in host tissues like root, stem, leaf and seed of mangrove wild legume *Canavalia cathartica* yields thirty six endophytic fungi with a maximum species in stem which followed root. The species diversity of foliar endophytes assembly changes with the leaf age (Photita *et al.,* 2004;

Suryanarayanan and Thennarason, 2004). Indicating that sampling endophytes from a plant for bioprospecting on a single time may not arrest entire variety of endophytes and their metabolites. *Calotropis gigantean* (L) R.BR showed the presence of thirteen endophytic fungal species like *Aspergillus niger*, *Aspergillus flavipes, Alternariaporri, Curvularia lunata, Fusarium oxysporum, Nigrospora sphaerica, Colletotrichum falacatum, Pestalotiopsis sydowiana, Phoma exigua, Phomopsis archeri, Leptosphaerulina chartarum,* and *Mycelia sterilia.* Endophytic diversity is generally manifested with their morphology and also with the types of benefits they offer to the host plant. Fungal diversity is much larger from endophytes of trees than endophytes of grass.

The percent frequency of occurrence of endophytic fungi found to increase between seed, rootstem, leaf segments in host *C.cathartica*. The mean percent frequency of endophytic fungi was highest in pod (11.6%), followed by leaf (9.8%) and root (8.9%). In *C. cathartica*, *Chaetomium globosum* was the dominant endophytic fungus and root showed its dominance as single species (*C. globosum*) (Seena and Sridhar, 2004). In mangrove *C. cathartica*, multispecies dominance was seen. *A. flavus, A. niger, Fusarium oxysporum* and *Nonsporulating* Sp. *Acremonium, Colletotrichum* and *Fusarium* were common endophytes in halophytes, mangrovesand sea grass (Beena *et al.,* 2000; Kumaresan and Suryanarayanan, 2001; Ananda and Sridhar, 2002; Maria and Sridhar, 2003; Devarajan *et al.,* 2002). Tissue specificity of endophytic fungi in whole-stem and xylem has been reported in tree species *Pinus* and *Fagus* (Petrini and Fisher 1988). Some fungi were restricted to specific organ or tissue e.g. leaf: *Cladosporium oxysporum*, *Aspergillus* sp., pod: *Aspergillus fumigatus*, seed: *Eurotium chevalieri*. *Ascomycetes*

usually are the dominant decomposers of plant detritus in mangrove and marine habitats (Kohlmeyer and Volkmann-Kohlmeyer, 1991). However, endophytic fungi were dominated by mitosporic fungi in mangroves and halophytes (Beena *et al.,* 2000; Suryanarayanan and Kumaresan, 2000; Ananda and Sridhar, 2002; Seena and Sridhar, 2004). Importance of endophytic fungi in grasses has been understood better than non-grass endophytic fungi (Hyde and Soytong, 2009). The endophytic fungi of non-grass are important because of deterring or decreasing insect herbivory, enhancing drought/disease resistance in plants.

2.15.6 Habitat and Physical Environment

Endophytes have been isolated from the roots and aerial parts of a diverse range of hosts such as bryophytes, pteridophytes, gymnosperms, angiosperms and algae (Wang *et al.,* 2006; Kharwar *et al.,* 2008). Some physical factors, such as temperature, rainfall, edaphic factors and UV radiation also found to affect endophytic communities directly or indirectly. Along with some indirect effect of soil physical and chemical factors including pH, salinity and soil texture. Both biotic and abiotic effects mainly influence the dynamic patterns of endophytic microbe in relation to plant host (Rosenblueth and Martinez, 2004).

2.15.7 Endophyte Host Interaction

Endophytes interact with host mutualistically. Endophytic fungi found to produce alkaloids in the plants. Some of the agronomic species infected with endophytes showed resistance to toxic effects on vertebrate and invertebrate herbivores. Endophytic bacteria found within plant tissue as biotrophic symbionts and either obligate or facultative. Endophytic bacteria can colonize thousands of different plant species, while some of

them are restricted to selected plant families. Bacterial endophyte produces large number of phytohormones, such as auxins, cytokinins and the gibberellins. Examination indicates that interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environmental conditions (Battistoni *et al.,* 2005; Rosenblueth *et al.,* 2006). There is inadequate data on the endophyte host molecular interactions.

2.15.8 Plant Colonization with Endophytes and Effect of Plant Age on Endophytes

The growth stimulation by the endophytes may also be by nitrogen fixation, phosphate solubilization, production of siderophore and also supply of essential vitamins to plants (Sevilla *et al.,* 2001; Verma *et al.,* 2001; Hurek and Reinhold-Hurek, 2003; Iniguez *et al.,* 2004; Wakelin *et al.,* 2004; Pirttila *et al.,* 2004) phytohormones, production of volatile substances like 2-3 butanediol and aceotin for plant growth (Ryu *et al.,* 2003). Endophyte produces adenine ribosides that found affect nematodes propagation (Sturz *et al.,* 2004) for eg. Endophytic *Herbaspirillum seropedicae* and *Clavibacter xylii* are genetically modified for excretion of deltaendotoxin of *Bacillus thurengensis* to control insect pests, as the future application for agriculture related areas. Bacterial endophytes isolated from leaves of *Ocimumsanctum* shows growth promoting benefits (Tiwari *et al.,* 2010). Bacterial endophytes were isolated from the Balloon flower (*Platycodon grandiflorum*) showed that the population of low G+C Gram positive bacteria (LGCGPB) gradually increased 60-80% from 1 to 6 years with maximum hydrolytic enzyme activity and it is presumed that elder balloon flower plants invite more potential antifungal endophytes, therefore plant age is presumed to influence diversity of balloon flower endophytic

bacteria (Shah *et al.,* 2010). Some bacterial endophytes find their mode of entry through cracks generally formed at the emergence of lateral roots and also at the zone of elongation seed sections carries diversity of endophytes (Coombs *et al.,* 2003b). Presence of endophytic organisms were noted in almost all parts of host plant such as roots, stems, leaves, seeds, fruits, tuber, ovules and also inside legume nodules (Benhizia *et al.,* 2004). Plants have considerable role in controlling endophytes colonization and such type of finding are useful to study plant endophyte relationship (Rubini *et al.,* 2005).

2.15.9 Plant Growth Promoting Endophytes

Management of advantageous microbial communities to favor plant growth could better understand by physiological and molecular interactions between microbe and plant. Endophytes show numerous direct and indirect mechanisms to promote plant growth and health. Consideration of these mechanisms can progress the value of poplar and some other plants as feed stocks for biofuel production and plant growth (Dell Amico *et al.,* 2005).Non reducing disaccharide trehalose is main storage carbohydrate of bacteria, it can be produced in plants to a much lesser extent than sucrose, this sugar thought to play a vital role in plants controlling their partitioning of carbon into cell wall biomass (Ramon and Rolland, 2007). Activity levels of trehalase enzyme responsible for degrading trehalose sugar strongly induced by infection with the trehalose producing microbes for example *Plasmodiophorabrassicase*. Alteration in biosynthesis and metabolism of trehalose also increasetolerance to drought, salt, and cold. Therefore several endophytic bacteria from poplar were able to metabolize trehalose (Taghavi *et al.,* 2005; Porteous *et al.,* 2006). Plant-associated bacteria can also indirectly benefit plant growth by preventing the growth of plant pathogens through, antibiosis (Ramos-Gonzalez

et al.,2005), production of some hydrolytic enzymes (Krechel *et al.,* 2002) along with induction of plant defense mechanisms (Spencer *et al.,* 2003; Jeun *et al.,* 2004; Kloepper *et al.,* 2004; Ryu *et al.,* 2004; Zhang *et al.,* 2004). Plant growth promoting endophytic bacteria were isolated from *Brachiaria* hybrid CIAT 36062 and introduced into *Brachiaria* hybrid cv. *Mulato,* positive for nif H gene sequence, and inoculated *Mulato* plant showed higher chlorophyll and total nitrogen contents in leaves. DNA sequence analysis demonstrated that the nif H gene found were highly similar to *Klebsiella pneumoniae* and some other N_2-fixing organisms. For this reason plant research area are now diverted to use endophytes in development of agriculture crops and forest regeneration.

2.15.10 Natural Products of Endophytes

The requirement for new antimicrobial agents generally, comes from the increasing resistance of pathogenic microbes towards antibiotics. Many microorganisms of agricultural concern are also known to acquired resistance to commonly used antimicrobial chemical compounds. So the interest in natural methods of pathogen control through new, eco-friendly agents is increased. The biologically active natural products from endophytes are excellent resources for medicine, agriculture and industry (Guo *et al.,* 2008). Amines and amides are very common metabolite products from endophytes and have shown to be toxic to insect but not to mammals. Bioactive metabolites, such as steroids, terpenoids and diterpenes also are generated by endophytes. Endophyte also produces extracellular hydrolyases to establish a resistance mechanism against plant invasion which includes some of the extracellular enzymes like cellulases, proteinase, lipases and esterases. The actions of these enzymes found to support the hypothesis of co-evolution between endophytes and their hosts. Number of secondary metabolites produced by fungal endophytes is larger than that of any other

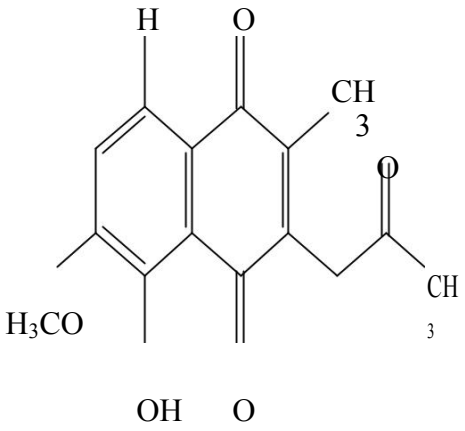
endophytic microorganisms (Zhang *et al.,* 2006). Endophytic fungi are a promising source of novel compounds. About 51% of biologically active substances from fungal endophytes were previously unknown (Strobel, 2002a).

2.15.11 Secondary Metabolites from Endophytes as Antibiotics

Plants with ethno botanical history are generally expected to be powerful source of endophytes producing active natural products (Strobel *et al.,* 2004). As more than 3000 diseases are clinically described today less than one third of these can be treated symptomatically, and even less than that needs new therapeutic agent with infectious disease control (Strobel and Daisy, 2003). In recent time it is found that isolated secondary metabolites of endophyte are synthesize via variety of metabolic pathways, for example polyketide, isoprenoid or amino acid derivation. And belongs to different structural groups such as phenols, isocoumarins steroids, xanthones, perylene derivatives, depsipetides quinines, furandiones, terpenoids, and cytochalasines. Fungus *Podospora* Sp. endophytic from the plant *Laggera alata* (Asteraceae) shows presence of xanthones sterigmatocystin. Also chaetoglobosin A and rhizotonic acid were reported from endophytic *Chaetomium globosum* in *Maytenus hookeri* and *Rhizoctonia* Sp. in *cynodon dactylon* correspondingly to be active against the gastric ulcer.

Novel fungal genus *Cinnamomum zeylanicum* found to be producing extremely bioactive volatile organic compounds (VOCs). Endophytic *Muscodoralbus* produces a mixture of VOCs consists primarily of various alcohols, acids, esters, ketones and lipids (Strobel *et al.,* 2001). Cryptocandin A an antifungal lipopeptide was isolated from endophytic *Cryptosporiopsis quercina* containing a number of unusual hydroxylated amino acids and a amino acid, 3 hydroxy-4-hydroxymethylproline active against human fungal pathogens

which including *Candida albicans* and *Trichophyton* Sp. and also against some of plant pathogenic fungi such as *Sclerotinia Sclerotiorum* and *Botrytis cinerea.* The endophytic *Chloridium* Sp. from *A.indica* produces Javanicin (2.7) which is highly active against *Pseudomonas* Spp. (Kharwar *et al.,* 2008). A tetramic acid cryptocin (Figure 2.8) was obtained from *C.quercina* possessing strong activity against *Pyriculariaoryzae* plant pathogenic fungi. Ambillic acid is a highly functionalized cyclohexenone possess strong antifungal activity found to produce by endophytic fungus. *Pestalotiopsis jester* is an endophytic fungi produces the extremely functionalized cyclohexenone epoxide jesterone and hydroxy jesterone, exhibiting excellent antifungal activities against a variety pathogenic fungi of plants. Fungal endophytes isolated from *A.indica* and *Acalypha* species shows potent antibacterial activity against human pathogenic bacteria such as viz, *B. subtilis, K. pneumoniae* and *S. aureus* (Gangadevi *et al.,* 2008). Dual biological control reported for the fungal entomopathogens, *Beauveria bassiana* (Bals.-Criv.) Vuill. (*Ascomycota Hypocreales*) and *Lecanicillium* Spp (*Ascomycota hypocreales*). The mechanism of antibiosis includes production of antibiotic compounds, bioactive volatile organic compounds and some enzymes (Ownley *et al.,* 2010). Some endophytic fungi were found to produce bioactive secondary metabolites not related to the products produced by their host. Fungal endophyte *Phomopsis* Sp. YM 311483 produces four new ten membered lactones active against *Aspergillus niger*, *Botrytis cinere* and *Fusarium* Sp. (Huang *et al.,* 2008). *Fusarium Spendophytic* from *Selaginella pollescens* active against *Candida albicans* (Strobel and Daisy, 2003). Endophytic fungi isolated from *Rhizophora mucronata, Avicenna officialis* and *Avicenna* marina showed maximum antibacterial activity against bacterial pathogens.



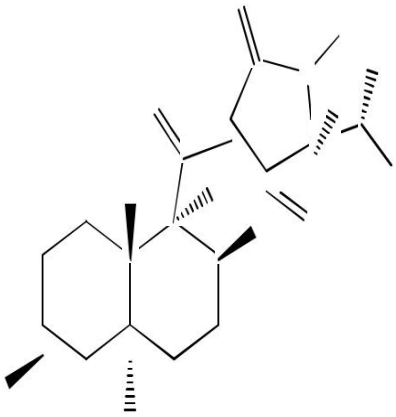


Figure 2.4: Javanicin **Figure 2.5: Cryptocin**

An endophytic bacterial strain *Bacillus subtilis* ZZ120 was isolated from stem of *Prunus mume* showed inhibition against *Fusarium graminearum*, *Alternaria alternata, Rhizoctonia solani, Cryphonectria parasitica* and *Glomerella glycines*. Gram negative endophytic bacteria *Pseudomonas* Sp. have been investigated as biological control agent with respect to production of antimicrobial metabolites (Nielson *et al.,* 2002).

Endophytic Gram positive bacteria like *Bacillus* Sp. have also been isolated from cotton, cucumber root and citrus plants (Mahaffee and Kloepper, 1997; Reva *et al.,* 2002). Plant pathogenic fungi were also control by bacterial endophytes (Berg and Hallmann, 2006). Coronamycin characterize a complex peptide antibiotic with activities against pythiaceaus fungi, human fungal pathogen *Cryptococcus neoformans* and also against the malarial parasite *Plasmodium falciparum* was produced by a Verticillate *Streptomyces spendophyte* from an epiphytic vine *Monstera* Sp. (Ezra *et al.,* 2004,). During the isolation of endophytes, actinomycetes generally appeared much later than endophytic bacteria and fungi and are also able to produce various metabolites. Igarashi isolated 398

actinomycetes showing excellent antagonistic activity against phytopathogenic fungi and bacteria. Extracts from *Streptomyces* Sp. collected from *Allium fistulosum* suppresses infection of *Alternaria brassicicola* on Chinese cabbage seedlings (Igarashi *et al.,* 2002).They also recognized several new bioactive compounds from endophytic actinomycetes. Two new novobiocin analogs were found to produce by *Streptomyces* Sp. from *Aucubajaponica*, and also cedarmycins by *Streptomyces* Sp. from *Cryptomeria japonica.* Anew naphthoquinone antibiotic, alnumycin, was also isolated from endophytic *Streptomyces* Sp. of *Alnus glutinosa. Streptomyces* Sp.NRRL30562 from snake vine plant was found to produce new peptide antibiotic, designated as munumbicins A-D40 with a broad spectrum of activity against several human, phytopathogenic fungi and bacteria. *Streptomyces* Sp. NRRL30566, from a fern-leaved grevillea (*Grevillea pteridifolia*) tree, reported to produce original kakadumycin chemically related to echinomycin (Castillo *et al.,* 2007). Endophytic actinomycetes from *Azadirachta indica (*A.I*)* were also evaluated in response to their antimicrobial action against pathogenic fungi and bacteria. A total of 54.5% actinomycetes were commonly recovered from roots followed by stems (23.6%) and leaves (21.8%) dominant genus found as *Streptomyces*, *Streptosporangium*, *Microbispora* and *Streptoverticillium.*

Isolates having sharp activities against *Pseudomonas fluorescens*, *E. coli* and antagonistic activity against root pathogens *Pythium* and *Phytophthora* sp. (Verma *et al.,* 2008). Biologically dynamic species of *Streptomyces* were isolated from species of *Nothofagus* and some other plants from the southern Patagonia. Having activity against plant pathogens, like *Pythium ultimum, Sclerotinia sclerotiorum*, *Mycosphaere llafijiensis* and *Rhizoctonia solani* (Castillo *et al.,* 2007). Endophytic actinomycetes were isolated from the *Trewia nudiflora* Linn with antifungal activity against *Penicillium avellaneum* UC

4376. Large numbers of endophytic actinomycetes were isolated from total of 26 medicinal plants species from Panxi plateau showing huge spectrum of antimicrobial activities with valuable reservoirs of novel bioactive compounds (Zha, 2010). Endophytic fungi *Microdiplodia hawaiiensis* CZ315 isolated from *Garcinia mangostana* showing antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli*, *Pseudomona aeruginosa*, *Salmonella typhi* and *Micrococcus luteus*, with the minimum inhibitory concentration(MIC) in range of as *S. aureus* (MIC 25 µg/ml), *B. subtilis* (MIC 5µg/ml), *M. luteus* (MIC 25 µg/ml), *E. coli* (MIC 20 µg/ml), *S. typhi* (MIC 20 µg/ml) and *P. aeruginosa* (MIC 10 µg/ml), respectively. *Streptomyces aureofaciens* found to produce 4-Arylcoumarins with antifungal potential (Thongchai *et al.,* 2005). Fungal endophytes from coffee plants exposed the presence of various genera of fungi including *Acremonium, Beauveria, Cladosporium, Clonostachys* and *Paecilomyces* and found pathogenic against coffee berry borer. Metabolites produced include bassiani, beauvericin, bassianolide, beauveriolide, bassiacridin, oosporein and tenellin (Quesada Morage and Vey, 2004).

2.15.12 Endophytes and Protozoal Activity

Protozoan parasites of genera *Leishmania* and *Trypanosoma* are the etiological agents of severe neglected tropical diseases (NTDs). Extract of the endophytic fungi *Cochliobolus* Sp. from host *Piptadenia adiantoides* (UFMGCB-555) able to kill 90%of the amastigote-like forms of *Leishmania amazonensis.* The resulted compound isolated were of cochlioquinone A and isocochlioquinone A (Campos *et al.,* 2008). Endophytic symbiont offers new strategies for mitigating the impacts of global change on native plant communities and agricultural crop production.

2.15.13 Endophytes in Phytoremediation

For phytoremediation enrichment requires for both rhizosphere and endophytic microbes. Endophytes are able to degrade toxic compounds (Arnold *et al.,* 2007). The outcome of contaminants in the rhizosphere-root system mostly depends on its physicochemical properties. Plants take up organic xenobiotics as well as weak electrolytes and these compounds enter the xylem tissues faster and so that the microflora can degrade it (Trapp *et al.,* 2000). Once taken up, plants and endophytes metabolize the contaminants, like toluene and trichloroacetic acid (Newman *et al.,* 1997). Alternatively, plants also release volatile compounds into the environment by evaporation through leaves or trunk (Schroder *et al.,* 2002). Phytoremediation of volatile compounds along with water soluble organic xenobiotics can be improved using recombinant endophytes with degradation pathway (Barac *et al.,* 2004; Lelie, 2004). Endophytic bacteria with a toluene degradation pathway were found able to decrease toluene phytotoxicity and evapo transpiration from yellow lupine plant. Purpose of modified and engineered endophytic bacteria to improve phytoremediation has several advantages over the application of engineered rhizospheric bacteria (Lodewyckx *et al.,* 2001; Ryan *et al.,* 2007a; Ryan *et al.,* 2007b; Doty 2008).

There will be an advantage for endophytic microbes possessing the degradation characteristics (Germaine *et al.,* 2006; Germaine *et al.,* 2009; Mastretta *et al.,* 2009). Several pathways for degradation of organic contaminants are generally located on plasmids and transposable elements. One hypothesis suggests that horizontal gene transfer of mobile elements can plays an essential role in adapting the endogenous plant endophytic community. Heavy metal resistant endophytic bacteria were characterized from roots tissues of *Brassica napus* for their potential to promote the growth and lead

accumulation in plant (Sheng *et al.,* 2008). Two lead resistant endophytic bacteria were isolated from *Brassica napus* growing in heavy metal-contaminated soils, the two isolates were identified as *Pseudomonas flurescens* G1 and *Microbacte rium* G16 exhibiting different heavy metal resistance characteristics along with root elongation, production of indole acetic acid and siderophores. It can establish stable endophyte association with host plant.

2.15.14 Endophytic Microbes in Agriculture

Plant growth pramoting rhizobia and nitrogen fixing bacteria enhance the plant growth (Lewandowski *et al.,* 2003; Gray, 2005). However, little is known about the nitrogen-fixing bacteria associated with endophyte. Nitrogen fixing bacteria have great potential in agricultural system. *Azoarcus* Sp. is nitrogen fixing microbe associated with sugarcane and kallar grass and showed endophyte plant interaction. The whole genome sequencing of *Azoarcus* sp. strain has been done (Krause *et al.,* 2006). Giving complete sequence of 4,376,040 base pair long and containing 3,992 predicted protein-coding sequences which help to study *life* cycle of nitrogen fixing endophytes and also their role in biotransformation of agriculture products (Simanjuntak *et al.,* 2010).

Actinobacteria are gram-positive, filamentous bacteria capable of secondary metabolite production like antibiotics. Biologically active endophytic *Actinobacteria phylum* were isolated capable to suppress wheat fungal pathogens *Rhizoctonia solani*, *Pythium* Sp. and *Gaeumannomyces graminis*. Plant pathogenic microorganisms are main threat to food production. Increasing use of chemical causes several harmful effects, as well as pathogens develops resistance to them. So biological control has been now considered an alternative to reduce chemicals in agriculture. Literature describes about potential use of

plant associated microbes to stimulate plant growth and protection. *Micromonospora* Sp. isolated from tomato plant showed a strong inhibitory activity against *Fusarium oxysporum* Sp. Taechowisan and Lumyong (2003) isolated endophytic actinomycetes from the roots of *Zingiber officinale* and *Alpinia galangal* with antifungal activity against *Candida albicans. Streptomyces aureofaciens* showed the most powerful antagonism against pathogenic organisms.

Endophytic fungi *Beaveria alternate*, *Phoma* Sp., *Acremonium strictum* showed their effect on the causal agents of maize seedling like blight stalk and root rot (Orale *et al.,* 2009). Endophytic actinomycetes from a field-grown plant can successfully colonize tissue cultured seedlings of a plant, and than the seedlings could becomes resistant to plant pathogens. Endophytic actinomycetes are promising for positive interactions affecting plant growth. Seedlings of *Mountain laurel* inoculated with an endophytic *Streptomyces padanus* shows high salt-tolerance similar observation obtained by plant barley by endophytic *Piriformospora indica* (Waller, 2005). Igarashi, (2002) isolated pteridic acids A and B as plant growth promoters with auxin like activity from endophytic *Streptomyces hygroscopicus* TP-A045 from host *Pteridium aquilinum,* accelerating development of adventitious roots in hypocotyls of kidney beans. It seems that the endophytes mostly adapted for living inside plants are naturally selected. Research on endophytic actinomycetes will contribute to the development of novel technologies and methodologies in the agricultural field.

2.15.15 Secondary Metabolites from Endophytes with Further Interesting Activities

Compounds with immunosuppressive activities were obtained from endophytic fungi such as subglutinols A and B which are noncytotoxic diterpene pyrones from *Fusarium subglutinans,* endophyte from *Triptergium wilfordii*. Aurasperone A from endophytic *Aspergillus niger* isolated from *Cynodondactylon* is xanthine oxidase inhibitor. Endophytes do produces secondary metaboliteswhen placed in culture, however, the temperature, the composition of the medium ,and the degree of aeration will affect the amount and kind of compound that are produced by an endophytic fungus (Strobel *et al.,* 2004; Gunatilaka, 2006; Ke *et al.,* 2010; Hanhong *et al.,* 2011). The host endophyte interaction provides nutrient and shelter for endophyte, which in substitute improve plant growth and health. Many endophytic bacteria are closely related to environmental and clinical isolates whose genomes have been or are in the process of being sequenced.

2.15.16 Plant Endophyte Interaction Affect Metabolite Production

Secondary metabolites are seemingly not essential for growth and reproduction and are usually suppressed by high specific growth rates of producing culture metabolites. Some endophytes can be cultured from small piece of their host plant in appropriate growth medium. Not all endophytes present can be cultured in this way (Rosenblueth and Martinez, 2006). Historically, many compounds have been isolated from the natural environment, particularly plants and many of the drugs available commercially are derived from plant-based chemicals.

Plants have been viewed as a major source of new lead compounds for drug discovery, attention has more recently turned to endophytes as these microorganisms are have great

potential as sources for new bioactive compounds. This may be the case because endophytes have developed close biological associations with their host, leading to the production of high number of biologically active metabolites. Endophytic fungi are the unseen members of the microbial world and because they generally exist asymptomatically, they have received less attention than pathogenic microbes. Thus, they represent an under-utilized resource in the search for new compounds.

The search for secondary metabolite is important. As described earlier are some examples of bioactive products from endophytic microbes and their potential in the environmental, pharmaceutical and agrochemical arenas.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Plant Materials

Plants samples of *Psidium guajava* and *Acacia nilotica* stems and leaves respectively were collected from the Botanical garden in the Department of Biological Sciences Bayero University Kano. The plants samples were collected in black polythene bags with appropriate labelling and taken directly to the laboratory for the analysis.

3.2 Isolation and Identification of Endophytic Fungi

Fourty grams of potatoes dextrose ager (PDA) was measured and mixed with 0.04 gm of streptomycin. The mixture was later transferred into 1000 ml conical flask and made up to one liter of distilled water (Julieta *et al.,* 2012). The medium was then autoclaved at 121^{o}C for 15 minutes and poured into petri dishes after cooling. The plant materials were rinsed gently in running water to remove dust and debris.

Samples were then cut into small pieces for further processing under aseptic conditions. Highly sterile conditions were maintained for the isolation of endophytes. Sterile glassware and mechanical materials such as scissors, forceps etc., were used in the experiments. Leaves were cut in to 3-4 mm in diameter and 0.5-1 cm in length with and without midribs. Stem samples were cut in 0.5-1cm length.

The isolation of endophytic fungi was done according to the method described by Petrini *et al.* (1986). The surface sterilization was done by treating each plant material with 70% ethanol for 1minute followed by immersion in 0.3% commercial bleach and again in 70% ethanol for 30 second. Finally the plant materials were rinsed 3 times with sterile

distilled water. The plant pieces were bloated on a sterile bloating paper. One segment was then placed on medium supplemented with antibiotic streptomycin 100/µg/ml concentration. The dishes were sealed with paraffin and kept under dark condition at room temperature until fungal growths were initiated within 2 weeks of inoculation. The first day of visual growth was observed for each organism and results were recorded. Isolation from the master plate was done by transferring the hyphal tips to fresh potato dextrose agar plate (PDA) without addition of any antibiotic to obtain the pure culture. The pure culture was then taken to BUK central laboratory for identification.

3.3 Fungal Identification

The fungi were identified on the basis of their morphological and cultural characteristics using a microscope as described by Watanabe, (2010). Fungi were grown on specified media at specified culture condition for identification.

A strand of hyphae was placed on a microscopic grease-free clean slide and a drop of distilled water was added. The mixture was covered with cover slip and observed using x40 objective lens. The micrographs were compared using standard fungal atlas, of Watanabe, (2010).

3.4 Fermentation of fungi for the production of metabolites

3.4.1 Preparation of inoculum

The inoculum was prepared according to the method described by Ghisalberti (2002). The Czapek's dox solution agar was also used for the evaluation of the growth rate where the growth of the organism was observed.

3.4.2 Fermentation in Liquids Medium

The Czapex medium was constituted with 30 gram sucrose, 3 gram $NaNO_3$, 1 gram Di potassium phosphate, 0.5 gram Magnesium sulphate, 0.5 gram potassium chloride and 0.01 gram of ferrous sulphate in 1000 microlitre of distilled water. A Sample of 10 days fresh mycelia from the fungus grown on PDA in a petri dish at 27^0C, were inoculated aseptically into a 1 litre flask containing 300 millilitre of liquid medium. The flasks were kept stationary for 30 days for growth (Lin *et al.,* 2002). The flasks were examined periodically for any contamination.

3.4.3 Extraction of the Metabolites from the Liquid Medium

Extraction of the metabolite from the liquid medium was performed as described by (Chaudhary *et al.,* 2004). The culture medium and mycelia were separated by filtration. The mycelia were extracted in methanol for 7 days and later with ethyl acetate. The liquid broth was extracted with ethyl acetate 4 times. The organic extract were evaporated using a rotary evaporator at 40 ^{0}C and solid residues were obtained

3.5 Brine Shrimp Lethality Bioassay

The Artemia Salina eggs were incubated in natural sea water (33 g/L) at room temperature under constant aeration for 48 hours, the phototropic nauplii were then ready for use in assays. Different concentrations of the ethanolic extracts from the 2 different fungi were prepared in dimethyl sulphoxide (DMSO) to obtain each of the three concentrations at 1000, 100 and 10 µg/ml. Ten of the napuliis were collected by pipette from the lighter side of the hatching chamber and were put in vials containing 4.5 ml of sea water each with different concentration of extracts for the test. Ten of the naupliis were put in 4.5 ml sea water with 0.2 % DMSO for the control set up. After 24 hours,

surviving shrimps in each vial were viewed with magnifying glass, counted and the survival data recorded.

3.6 Determination of LC_{50} of fungal extracts

The lethal concentration of fungal extracts resulting in 50% mortality of brine shrimp (LC_{50}) was determined using probit analysis.

3.7 Parasite Culture medium

The medium used for the *in vitro* cultivation of *Trypanosoma brucei brucei* was RPMI (royal pack memorial institute) 1640. It was prepared by adding 10% Goat serum, 1% Glucose, and 40 μg/ml of ampicillin according to the manufacturer's instruction.

3.8 Test Organism for In Vitro Assay

Trypanosoma brucei brucei strain was used as the test organism. It was obtained from the Department of Vector and Parasitology of National Institute for Trypanosomiasis Research (NIITR). Kaduna State, Nigeria. The parasite was maintained in the laboratory by continuous passage in rats until required. Each cycle of passage was done when parasitaemia was in the range of 35 – 40 parasites per field, which corresponded to an interval of 6 days-post infection in rats. For several passages, about 3 ml of blood was obtained from an infected rat by cardiac puncture after light chloroform anesthesia into 5 ml syringe and emptied into a vial containing 9 ml of normal saline (NS). About 10^6 parasites in 0.2 ml blood/PBS solution was injected intraperitoneally into a rat previously unexposed to trypanosomal infection.

3.9 Passaging of Trypanosomes

Passaging is simply referred to as the act of inoculating the parasite from an infected animal to the uninfected animal in order to maintain the parasite until required. Two hundred microliters of blood was collected and mixed with 0.9 ml of normal saline. The animal was then restrained in an upside down position and inoculated with the parasites intraperitoneally. Parasitaemia was checked daily by microscopic examination of the blood from the infected animal for the presence of *trypanosoma brucei brucei.*

3.10 Harvesting Blood of Rising Parasitaemia

The harvesting of blood from rising parasitaemia was done by cardiac puncture as described by Bulus *et al.,* (2012), the albino rat with high parasitaemia was sacrificed using chloroform for few seconds. The animal was later placed on the dissecting board and the fore limbs and hind limbs were pinned. The dissecting scissors was used to cut the animal open. Ten millilitres of PBS was poured on the heart after cardiac puncture to maintain the pH of the solution. The blood was collected into the syringe and transferred into the effondorf tube for the analysis. Parasitaemia was monitored daily in blood obtained from tail vein by nipping the tail. A drop of blood was placed on a clean microscope slide, covered with cover slip and observed under a light microscope (Olympus microscope B×3 M, Japan) at ×400 magnification. The number of parasites was determined microscopically at ×400 magnification using the "Rapid Matching" method of Herbert and Lumsden (1976). The method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with Normal Saline.

3.11 Preparation of Carbon Dioxide

Preparation of carbon dioxide was conducted by transferring HCL into a wash bottle. Sodium bicarbonate was placed in another bottle connected with rubber tube linking the two bottles together. Another rubber tube was also linked to 2000 ml polythene. The wash bottle was then pressed to pass the Hydochloric acid through the pipe to the sodium bicarbonate container where carbon dioxide and atmospheric air was collected to half-filled the 2000 ml polythene, i.e. 1000 ml polythene (Bulus *et al.,* 2012). The carbon dioxide was introduced into the desiccator with the micro titer plate containing the test organism and extracts of different concentrations in the wells, was used as part of the requirement for the cultivation of *Trypanosoma brucei brucei in vitro,* (Bulus *et al.,* 2016).

3.12 In Vitro Activities of Ethyl Acetate Crude Extract of *Ascomata, Gliocladium Roseum and Epicoccum Purpurascens*

Ten milligram per millilitre of each extract was weighed and dissolved in 1 ml of culture medium as a stock solution and followed by serial dilution using the culture medium to obtain different concentrations of 1.0 mg/ml, 0.75 mg/ml, 0.50 mg/ml, 0.25 mg/ml and 0.1 mg/ml. Measurement of the *in vitro* antitrypanosomal activities was conducted in duplicate in 96 wells microtitre plate. One millilitre of each extract was incubated in a 96 wells microtitre plate with 10 ul of the parasite suspension. The control wells contained 100 µl of the culture medium with parasites and without extract solution being added to it. The level of parasite motility was monitored on the free clean glass slides with cover slip under microscope by ×40 objective lens. The number of the motile parasites were counted after 1^{st}, 2^{nd}, 4^{th}, 6^{th} and $8^{th}$for *Ascomata* extract, *Gliocladium roseum* extract,

Epicoccum purpurascens extract. A decline in the number of parasite was taken as an indication of extract activity against *Trypanosoma brucei brucei* for the incubation periods conducted.

3.13 Blood Incubation Infectivity Test of *Epicoccum Purpurascens* Extract

After 8 hours of incubation, each remaining incubation mixture from each well of the plate was then inoculated into a healthy albino rat to determine if the parasites have lost infectivity or not. The parasitaemia was checked daily for 10days according to the method of Herbert and Lumsden (1976).

3.14 Phytochemical Screening

The methanol extracts of the fungi were tested for the presence of bioactive compounds by using standard tests (Prashant *et al.,* 2011).

3.14.1 Test for Saponins (Frothing Test)

One gram of the extract was dissolved in 10 ml of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. If a honey comb was formed which persisted for up to 30 minutes indicated presence of saponins.

3.14.2 Test for Flavonoids (Alkine reagent test)

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour which becomes colourless on addition of dilute acid, indicated the presence of flavonoids.

3.14.3 Test for Alkaloids (Dragendroffs test)

Extracts were treated with dragendroff reagent (solution of potassium bismuth iodide). Formation of red precipitate indicated the presence of alkaloids.

3.14.4 Test for Steroids (Salkowski test)

Two millilitre of the extract, 1.0 ml of conc H_2SO_4 was added carefully alongside of the test tube. A Red colour produced indicated the presence of steroids.

3.14.5 Test for Phenol (ferric chloride test)

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

3.15 Statistical Analysis

The data obtained from this study were summarized as mean ± standard deviation, Analysis of variance (ANOVA) were applied using statistical software Statistical Package for Social Sciences (SPSS) version 23.0. The statistical method was performed at 95% confident interval (C.I) to determine the significant differences between the concentrations and controls for endophytic fungal extracts at p values less than 0.05 ($P< 0.05$) were considered significant while the Probit analysis was applied to analyse the impact of toxicity effect of the extracts.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Isolation and Identification of Endophytic Fungi

The isolated and identified fungi were ***Ascomata spp, Gliocladium roseum and Epicoccum purpurascens.***

FUNGAL MICROGRAPHS ISOLATED

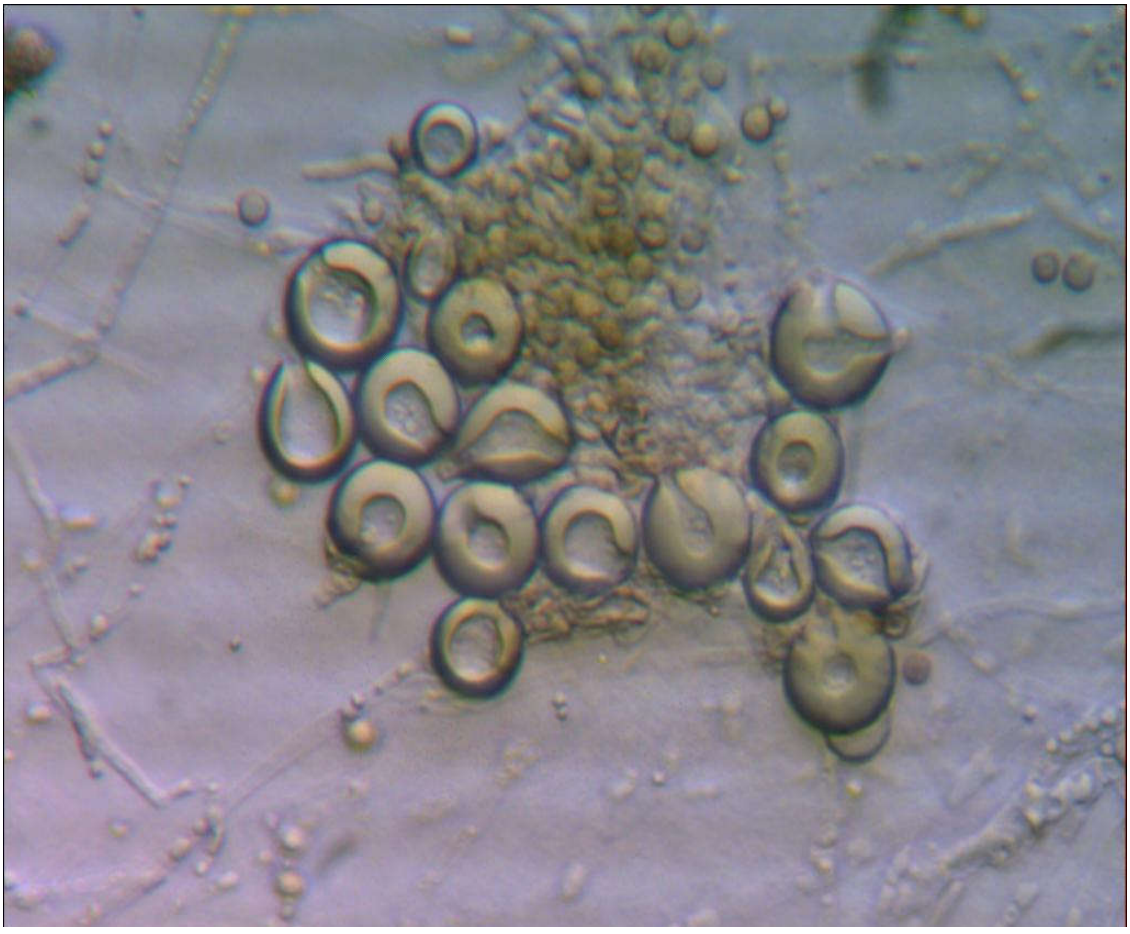


***Plate I: ASCOMATA* SPP (× 400)**

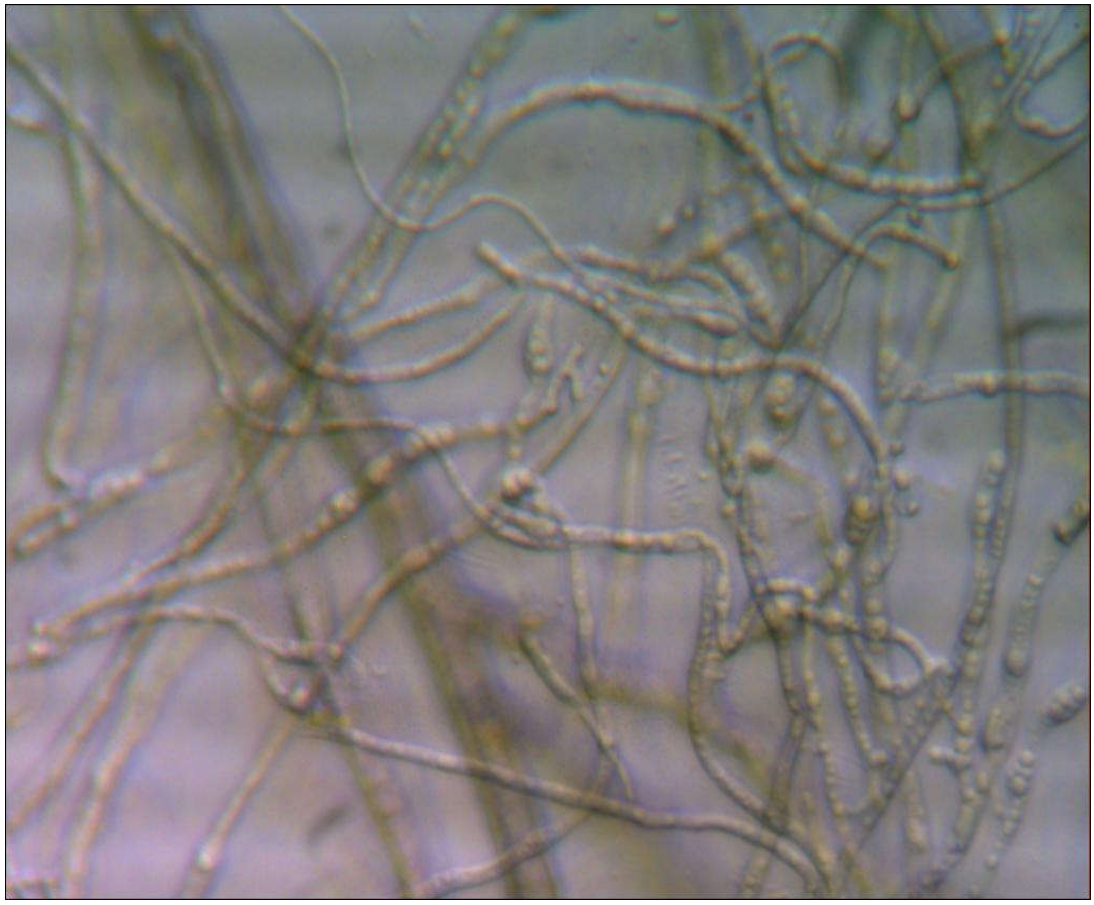


Plate II: ***GLIOCLADIUM ROSEUM*** **(× 400)**

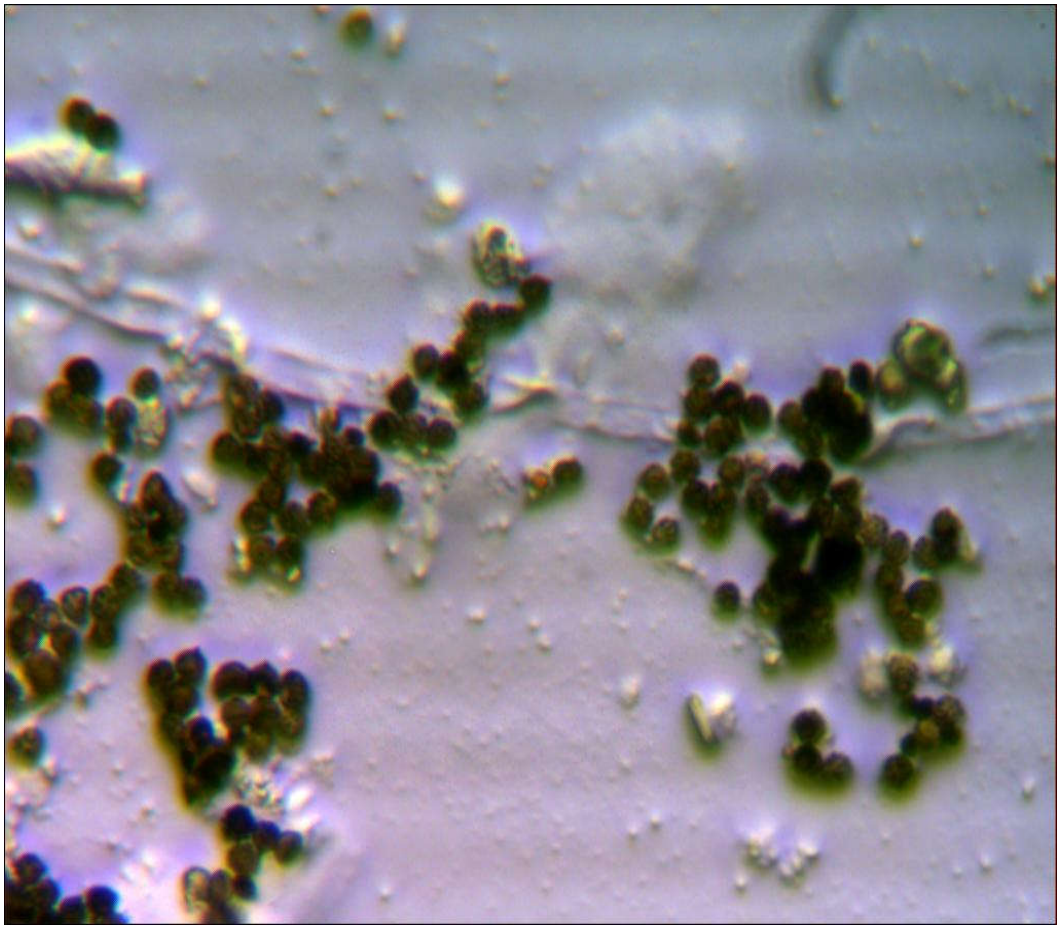


***Plate III: EPICOCCUM PURPURASCENS* (× 400)**

4.1.2 Phytochemical Screening

Phytochemical screening revealed the presence of various phytochemicals in the extracts. Methanolic extract of *Gliocladium roseum* was found to contain all the phytochemicals tested such as saponins, flavonoid, alkaloids, steroid and phenol, *Ascomata* spp was found to contain only saponin, steroid and phenol while alkaloid and phenol in *Epicoccum purpurascens* (Table 4.1).

Table 4.1: Phytochemical Constituents of Methanolic Fungal Extracts

Secondary metabolites.	AS	GR	EP
Saponin	+	+	-
Flavonoid	-	+	-
Alkaloid	-	+	+
Steroid	+	+	-
Phenol	+	+	+

Key: + = presence, - = absent.

4.1.3 Toxicity Testing Criterion Used

Clarkson's toxicity criterion for the toxicity assessment of fungal extracts classifies extracts in the following order: Extracts with LC_{50}above 1000 µg/ml are non-toxic, $LC_{50}$of 500 - 1000 µg/ml are low toxic, extracts with $LC_{50}$of 100 - 500 µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic (Clarkson *et al*., 2004).

Table 4.2: Determination of LC_{50} of fungal extracts

FUNGAL EXTRACTS	LC_{50} VALUES (µg/ml)	TOXICITY PROFILE
Ascomata spp	0.80	Highly toxic
Gliocladium roseum	0.38	Highly toxic
Epicoccum purpurascens	1.15	Highly toxic

The survival rate in both the concentrations (1.00, 0.75, 0.50, 0.25 and 0.10 mg/ml) and Diminazine aceturate but the higher the time factor the decrease in the survival of the parasite in the inoculum mixture at both the treatment and positive control. However, the lowest number of the survival rate in the table above shown in the treatment at 1.0 mg/ml and positive control. This shows that the treatment has effect on Trypanosomes as recommended in standard drugs, because there is no significant difference between the Positive control and treatment. This shows that the methanolic extract of *Ascomata* spp had the intensity or the effect on trypanosome when compared with negative (untreated) control which, at 8 hours post inoculation time of observation, there are highest survival count of the parasite (Table 4.3).

Table 4.3: Anti-Trypanosomal Activity of the Methanolic Extract of *Ascomata* spp against *Trypanosoma brucei brucei*

CONCENTRATION mg/ml	Count of motile trypanosomes at different time intervals				
	TIME (HOUR)				
	1st	2nd	4th	6th	8th
1.00	12.50 ± 1.70[b]	9.25± 1.70[b]	7.25± 2.21[a]	5.75 ± 0.95[a]	1.75 ± 0.96[a]
0.75	9.50 ± 4.50[a]	9.25 ± 1.29[b]	7.75 ± 2.50[a]	7.25 ± 1.25[ab]	4.50 ± 1.29[b]
0.50	13.25 ± 1.25[b]	10.75 ± 2.21[b]	8.25 ± 1.25[ab]	6.25 ± 0.95[b]	5.50 ± 2.08[ab]
0.25	12.25 ± 0.95[b]	10.25 ± 0.95[b]	10.75 ± 1.50[b]	8.75 ± 1.70[ab]	5.25 ± 0.50[ab]
0.10	11.25 ± 2.06[b]	11.00 ± 1.63[bc]	9.75 ± 2.50[b]	9.50 ± 1.73[ab]	4.50 ± 1.29[b]
DA 3.33	7.75 ± 0.50[a]	6.75 ± 0.50[a]	6.00 ± 0.81[a]	4.75 ± 0.50[a]	3.75 ± 0.95[b]
CONTROL	15.75 ± 0.95[c]	13.50 ± 1.73[c]	13.00 ± 2.21[c]	12.25 ± 2.75[c]	11.25 ± 0.95[c]

Values are expressed as mean ±SD; the mean difference is significant at the 0.05 level, DA= Diminazine Aceturate. abc = superscript. Note that any value with the same superscript are not significantly different

The survival rate in both the concentrations (1.00, 0.75, 0.50, 0.25 and 0.10 mg/ml) *Gliocladium roseum* extract and Diminazine Aceturate but the survival rate of the parasite decreased with time of exposure in the inoculum mixture at both the treatment and positive control. The *Gliocladium roseum* extract had the highest effect on *T. brucei brucei*, due to the fact that, the lowest number of the survival rate in Table 4.4 right from the 4^{th} hour of observation when compared with positive control to the highest time of observation. There is significant difference between the positive control and highest level of concentrations at 1.0, 0.75 and 0.5 mg/ml as they are more effective than the positive control. This shows that the methanolic extract of *Gliocladium roseum* had the intensity or the effect on Trypanosoma brucei brucei when compared with negative (untreated) control which, at 8 hours post inoculation time of observation, there are highest survival count of the parasite (Table 4.4).

Table 4.4: Anti-Trypanosomal Activity of the Methanolic Extract of *Gliocladium roseum* against *Trypanosoma brucei brucei*

CONCENTRATION mg/ml	Count of motile trypanosomes at different time intervals				
	TIME (HOUR)				
	1st	2nd	4th	6th	8th
1.00	11.75 ± 1.25[a]	4.00 ± 1.82[a]	0.00± 0.00[a]	0.00± 0.00[a]	0.00± 0.00[a]
0.75	11.50 ± 2.64[a]	4.00 ± 3.74[a]	0.00± 0.00[a]	0.00± 0.00[a]	0.00± 0.00[a]
0.50	10.75 ± 1.50[a]	6.00 ± 2.70[b]	0.25 ± 0.50[a]	0.00± 0.00[a]	0.00± 0.00[a]
0.25	13.50 ± 2.64[a]	9.00 ± 0.81[ab]	8.25 ± 2.62[ab]	7.25 ± 0.95[ab]	4.75 ± 0.50[b]
0.10	10.75 ± 1.50[a]	8.50 ± 1.91[ab]	7.03 ± 2.16[ab]	5.50 ± 2.38[b]	3.75 ± 1.50[b]
DA 3.33	9.25 ± 0.95[a]	5.75 ± 1.50[b]	4.75 ± 0.50[b]	4.00 ± 0.81[b]	3.00 ± 0.81[b]
CONTROL	15.00 ± 1.63[b]	13.00 ± 1.41[c]	12.50 ± 1.29[c]	11.50 ± 1.29[c]	11.00 ± 0.81[c]

Values are expressed as mean ±SD; the mean difference is significant at the P> 0.05 level, DA= Diminazine Aceturate. abc = superscript. Note that any values with the same superscript are not significantly different.

The survival rate in both the concentrations (1.00, 0.75, 0.50, 0.25, and 0.10 mg/ml) and Diminazine Aceturate but the higher the time factor the decrease in the survival of the parasite in the inoculum mixture at both the treatment and positive control. However, the lowest number of the survival rate in the above table shown in the treatment especially at 1.0 and 0.75 mg/ml as they are more effective than the positive control. This shows that the methanolic extract of *Epicoccum purpurascens* had the intensity or effect on *Trypanosoma brucei brucei* when compared with negative (untreated) control which, at 8 hours post inoculation time of observation, there are highest survival count of the parasite (Table 4.5).

Table 4.5: Anti-Trypanosomal Activity of Methanolic Extract of *Epicoccum purpurascens* against *Trypanosoma brucei brucei*

CONCENTRATION mg/ml	Count of motile trypanosomes at different time intervals				
	TIME (HOUR)				
	1st	2nd	4th	6th	8th
1.00	10.50 ± 1.73^{ab}	10.25 ± 0.95^{b}	6.25 ± 1.50^{a}	1.75 ± 0.95^{a}	0.50 ± 0.50^{a}
0.75	12.00 ± 1.41^{b}	10.50 ± 1.29^{b}	8.50 ± 1.73^{ab}	2.75 ± 0.50^{ab}	0.50 ± 0.06^{a}
0.50	12.50 ± 1.29^{b}	10.25 ± 0.95^{b}	8.50 ± 1.91^{ab}	4.75 ± 1.71^{b}	1.00 ± 1.41^{a}
0.25	12.00 ± 2.82^{b}	9.50 ± 1.73^{ab}	7.50 ± 2.38^{ab}	4.50 ± 2.64^{b}	3.75 ± 2.36^{b}
0.10	13.75 ± 2.21^{b}	10.75 ± 2.21^{b}	9.75 ± 2.06^{ab}	6.00 ± 1.63^{c}	4.75 ± 2.62^{ab}
DA 3.33	7.25 ± 0.95^{a}	7.00 ± 0.81^{a}	5.75 ± 0.50^{a}	3.75 ± 0.95^{ab}	2.75 ± 0.50^{a}
CONTROL	15.75 ± 0.95^{c}	13.50 ± 1.73^{c}	11.75 ± 2.21^{c}	11.50 ± 2.08^{d}	10.25 ± 0.95^{d}

Values are expressed as mean ±SD; the mean difference is significant at the P> 0.05 level, DA= Diminazine Aceturate. abc = superscript. Note that any values with the same superscript are not significantly different.

The invitro antitrypanosomal activities of *Epicoccum purpurascens* extract was confirmed by Blood incubation infectivity test. All the untreated control mice dead within mean of 5 days post observation. While all mice inoculated with inoculum mixtures and standard drugs did not develop infection during the observation period (table 4.6). This result was in agreement with the finding of Yusuf *et al*. (2012) who reported that test substances inhibited healthy mice from developing infection for more than 10 days. The complete immobility of the parasites invitro may not necessarily indicate that the parasites were dead, but rather the parasites may have lost their infectivity (Yusuf *et al*., 2012).

Table 4.6: Effects of *Epicoccum purpurascens* extract on infectivity against *T. brucei brucei*

	Observation period in days									
Concentration(mg/ml)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	4	22	12	5	6	11	8	10	12	21
0.75	2	23	13	10	8	5	3	1	2	28
0.5	5	31	25	13	4	4	5	7	4	12
0.25	3	36	17	11	3	4	4	3	3	1
0.1	0	32	12	15	6	5	2	4	14	10
Control DA	5	26	12	18	9	6	4	0	0	0
Control	1	46	56	128						

4.2 Discussion

For several decades trypanosomiasis continued to contribute adversely to economic and social wellbeing of sub Saharan Africans (WHO, 2006). Despite the enormity of helath and economic implication of African trypanomiasis, current chemotherapeutic options are very limited and far from ideal for both human and livestock (Legroos *et al*., 2002). So the need for safer, cheapter, available sources of medications cannot be overemphasized.

The present study was designed to determine the invitro antitrypanosomal activities of three fungal endophytes obtained from *Psidium guajava* stem, leaves and *Acacia nilotica* stem. The isolates (*Ascomata* spp, *Gliocladium roseum* and *Epicoccum purpuraseens*), both the three fungal extracts exhibited antitrypanasomal activity.

Invitro evaluation of fungal extract for antitrypanosomal effect is a rapid and cheap test for evaluating the efficacy of plants against trypanosomes. Some plants have been reported to exhibit *in vitro* trypanocidal effect measured by cessation of motility of the parasites (Atawodi and Shehu, 2010; Habila *et al.,* 2011), while some are reported to exhibit trypanostatic effect measured by reduction in motility of the parasites(Atawodi *et al.,* 2003; Atawodi, 2005). Anti-trypanosomal activity of *Gliocladium roseum* extract reported in the present study is comparable to a report by Adeyemi *et al*. (2009) which showed that possesses impressive *in vitro* trypanocidal effect against *T. b. brucei*. The anti-trypanosomal effect exhibited by *Gliocladium roseum* extracts may be attributed to the presence of some secondary metabolites like flavonoids, tannins, alkaloids, saponins, steroid, and phenol, *Ascomata spp* were found to contain only saponin, steroid and phenol while alkaloid and phenol in *Epicoccum purpurascens* (Table 4.1).

This study on anti-trypanosomal activity of *Gliocladium roseum* showed that methanolic extracts exhibited good anti-trypanosomal *in vitro* activities against *T. b. brucei*. In the present study however, the focus was on methanolic extracts of *Ascomata spp*, *Gliocladium roseum* and *Epicoccum purpurascens* which showed *in vitro* activity against *T. b. brucei* blood stream forms. However, the major constituents of methanolic extract of *Gliocladium roseum* were flavonoids, saponins, alkaloids, steroid and phenol. Thus, the observed *in vitro* activities of the methanolic extract of *Gliocladium roseum* may be attributed to the presence of these secondary metabolites (Kamath *et al.,* 2010). The results obtained from treatment with methanolic extract of *Ascomata* spp*, Gliocladium roseum and Epicoccum purpurascens* could be effective in the management of African trypanosomiasis, since there was considerable extension of life span of infected animals compared with the negative (untreated) control (Table 4.3, 4.4 and 4.5).

There was also appreciable decline in parasitaemia for all animals treated with the methanolic extracts. This result confirmed many pharmacological uses of *Gliocladium roseum* extracts (*Kamath et al*., 2008). The metabolic profile of the methanolic screening of *Gliocladium roseum* extract (Table 4.1) contains saponins, flavonoid, alkaloid, steroids and phenol. There are reports of anticancer activity of various extracts of Endophytic fungi (Guo *et al.*, 2008) including that of methanol extract of *Epicoccum purpurascens* by a group in India (Strobel *et al*., 2004). The use of extracts of *Epicoccum purpurascens* in the treatment of diabetes has also been documented (Nielson *et al.*, 2002).

The invitro antitrypanosomal activities of *Epicoccum purpurascens* extract was confirmed by Blood incubation infectivity test. All the untreated control mice dead within mean of 5 days post observation. While all mice inoculated with inoculum mixtures and

standard drugs did not develop infection during the observation period (table 4.6). This result was in agreement with the finding of Yusuf *et al*. (2012) who reported that test substances inhibited healthy mice from developing infection for more than 10 days. The complete immobility of the parasites invitro may not necessarily indicate that the parasites were dead, but rather the parasites may have lost their infectivity (Yusuf *et al*., 2012).

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

African human trypanosomiasis (HAT) and animal trypanosomiasis (AAT) are vector – borne parasitic diseases, which are amongst the most neglected diseases in the world. They cause major health and economic problems in rural sub- Saharan Africa. The main means of controlling the disease is limited due to parasite resistance and toxicity of the current anti-trypanosomal drugs. The development of a vaccine has been thwarted by antigenic variation of the parasite. The main aim of the current research was to evaluate the anti-trypanosomal activity of some *Ascomata* spp*, Gliocladium Roseum* and *Epicoccum purpurascens* extracts against Trypanosoma brucei brucei, invitro and Blood Incubation Infectivity Test efficacy using mice as animal models. Sequential extraction of plant samples in ethyl acetate and water gave the crude extracts. Invitro assay were carried out in 96-well microtitre plates and diminazine aceturate were used as a positive control. Phytochemical screening of the extracts was conducted confirming the presence of saponin, flavonoid, alkaloid, steroid and phenol in *Gliocladium roseum*, saponin, steroid and phenol in *Ascomata* spp while *Epicoccum purpurascens* extract contained only alkaloid and phenol. The present study indicated that the isolated and identified fungi where *Ascomate* spp, *Gliocaldium roseum* and *Epicoccum purpurascens* and they all exhibited cytotoxic activities against brine shrimp larvae (table 4.1). The methanolic fungal extracts affected mortility of the parasites at 1.0 mg/ml in *Ascomata* spp while 1.0, 0.75 and 0.50 mg/ml in *Gliocladium roseum* and 1.0 and 0.75 mg/ml in Epicoccum purpurascens in vitro test, and the entire tested group did not develop infection in mice

inoculated with infected blood incubated with the concentrations of the extracts. There is significant difference at ($p>0.05$) as compared to control. In conclusion, the extracts showed in vitro effects. Further effort is required to isolate and purify specific compounds responsible for the antitrypanosomal activities of the studied fungi.

5.2 Conclusions

From the foregoing discussion, it can be concluded that:

1. The isolated and identified fungi were *Ascomata, Gliocladium roseum and Epicoccum purpurascens.*
2. The metabolic profile screening of the extracts for saponins, flavonoid, phenols, steroid and alkaloid were confirmed positive in *Gliocladium roseum,* saponin, steroid and phenol in *Ascomata* while *Epicoccum purpurascens* contained alkaloid and phenol.
3. The fungal extracts exhibited cytotoxic activity against brine shrimp larvae (Table 4.1).
4. The methanolic extracts of *Ascomata spp*, *Gliocladium roseum* and *Epicoccum purpurascens* could be said to possess anti- trypanocidal effects against *T. brucei brucei* thereby leading to the usage of the plants in African traditional medicine.
5. Blood incubation infectivity test (BIIT) has been shown to be more sensitive in evaluating *in vitro* antitrypanosomal effects of fungal extracts when compared with *in vivo*.

The methanolic extracts of *Ascomata spp*, *Gliocladium roseum* and *Epicoccum purpurascens* cures experimental African trypanosomiasis in rats and it is potentially useful as an anti-sleeping sickness agent.

5.3 Recommendations

- ✓ Further work is needed to be done in order to isolate and purify specific compounds responsible for the antitrypanosomal activity of the studied fungi.
- ✓ The methanolic extract of *Ascomata spp*, *Gliocladium roseum* and *Epicoccum purpurascens* should be tested on other trypanosomes species.
- ✓ The effects of the extracts on blood parameters need to be investigated.
- ✓ It is therefore important to investigate *in vivo* anti-trypanosomal efficacy of these extracts against *T. b. brucei.*
- ✓ Although the fungal extracts are highly toxic under preliminary studies further investigation on toxicological issues have to be confirmed in repeated administration of the extracts beyond this.

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