ANTITRYPANOSOMAL ACTIVITY OF EXTRACTS FROM FOUR CYANOBACTERIA ON *TRYPANOSOMA BRUCEI BRUCEI*

BY

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DECLARATION

I declare that the work in this Dissertation entitled "**Antitrypanosomal Activity of Extracts from Four Cyanobacteria on *Trypanosoma brucei brucei***" has been carried out by me in the Department of Biochemistry, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation has been previously presented in any form for another degree or diploma at this or any other institution.

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CERTIFICATION

This Dissertation entitled "**ANTITRYPANOSOMAL ACTIVITY OF EXTRACTS FROM FOUR CYANOBACTERIA ON *TRYPANOSOMA BRUCEI BRUCEI***'' by Jerry Tersoo AGEE meets the regulations governing the award of the degree of Masters of Science in Biotechnology of the Ahmadu Bello University, Zaria, and is approved for its' contribution to scientific knowledge and literary presentation.

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DEDICATION

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ABSTRACT

African trypanosomiases (AT) are a group of hemoparasitic diseases caused by multiple flagellated organisms of the genus *Trypanosoma*. The disease affects humans and livestock animals, and it is lethal if untreated, therefore, AT is of economic and global health importance. Since there is no vaccine for prevention and available drugs produce unsatisfactory outcomes, this work was aimed at discovery of new compounds with potentials for future development of new drugs for AT. Coloured microorganisms of the phylum Cyanobacteria are known to produce therapeutic secondary metabolites, which are yet to be explored for anti-*Trypanosoma* potentials. Therefore, in search of new anti-*Trypanosoma* compounds, crude methanolic extracts of four cyanobacteria (*Microcystis aeruginosa* EAWAG198, *Microcystis flos-aquae* UTEX 2677, *Microcystis wesenbergii* and *Oscillatoria* sp) were prepared and tested for trypanosomes-killing activity. The most active crude extract (*M. flos-aquae*) was fractionated by gel filtration chromatography with Silica gel 60-120G as the stationary phase and combination of Methanol: Ethyl acetate: Hexane solvent system as mobile phase, while purity was assessed by thin layer chromatography using Hexane: Ethyl acetate (7:3 v/v). Antitrypanosomal fractions were identified by incubating each fraction with *Trypanosoma brucei brucei* cells and monitoring parasite death for 2 hrs by wet mount under 400× microscopic magnifications. Wistar rats infected with *T. b. brucei* were treated with 30, 60 and 120 mg/kg body weight dosages of *M. flos-aquae* crude extract. The most active fraction (**E**) of *M. flos-aquae* was characterized by GC/MS.. The crude extract of *M. flos-aquae* exhibited the highest *in vitro* trypanocidal activity with a percentage inhibition of 98.44 and 42.18% at 2.5 and 0.3125 mg/mL concentration, respectively, and displaying IC_{50} value of 0.4140 mg/mL. Fraction **E** exhibited the highest activity against the parasite with percentage inhibition of 74.21 % at 0.625 mg/mL with IC_{50} of

0.2991 mg/mL, and its subfraction 76 % inhibition (24 % survival) at the same concentration. Interestingly, the extract of *M. flos-aquae* suppressed parasite proliferation, and improved weight and PCV in rats at dosages above 60 mg/kg body weight. GC/MS.In conclusion, the screened cyanobacteria are high potential sources of promising bioactive compounds that could be explored for treatment of trypanosomiasis.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAT =Animal African Trypanosomiasis

DNA = Deoxyribonucleic acid

RNA =Ribonucleic acid

BG-11 = Blue-green-11

ºC =Degree Centigrade

GC-MS = Gas chromatography- Mas spectroscopy

FTIR = Fourier-Transformed Infrared spectroscopy

PBS =Phosphate Buffer Saline

PCR = Polymerase Chain Reaction

DMSO = Dimethyl Sulfoxide

HAT =Human African Trypanosomiasis

AAT= Animal African Trypanosomiasis

PCV =Packed Cell Volume

ANOVA = Analysis of Variance

TLC = Thin Layer Chromatography

R_f = Retention factor

IC_{50}= Inhibitory concentration 50

LD_{50} = Lethal dosage 50

rRNA = ribosomal Ribonucleic acid (ribosomal RNA)

rDNA = ribosomal Deoxyribonucleic acid (ribosomal DNA)

CDC = Center for Disease Control

CAS = Compound Abstract Service.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

African trypanosomiasis, commonly called sleeping sickness in humans and *nagana* in animals is a disease caused by protozoan parasites of the genus *Trypanosoma*, and transmitted to the mammalian hosts mainly by tsetse flies (*Glossina* spp) (Steverding, 2008). Sleeping sickness is caused by two subspecies of *Trypanosoma brucei* which are morphologically indistinguishable. The *Trypanosoma bruceei gambiense* causes West African sleeping sickness and *Trypanosoma brucei rhodesiense* causes East African sleeping sickness (CDC, 2015). *Trypanosoma brucei brucei* is the parental subspecies and does not infect humans, but just as *T. congolense, T. vivax,* and other animal trypanosomes, is responsible for *nagana*. According to the World Health Organization (WHO), sustained control efforts have reduced the number of new cases to less than 10,000 people per year but about 65 million people are still at risk in the 36 sub-Saharan countries that are endemic to sleeping sickness (WHO, 2017).

Chemotherapy has remained the only control measure, with only four approved drugs which have been developed more than three decades ago. Some of the chemotherapeutic drugs used for the treatment of African Animal Trypanosomiasis (AAT) are: diminazene aceturate (Berenil), suramin, melarsoprol (Arsobal) and pentamidine (Kuzeo, 1993; Fairlamb, 2003). These drugs are few and already limited by problems such as resistance by the parasites and toxic side effects (Fairlamb, 2003; Anene *et al*., 2011; Baker *et al*., 2013). In addition, there are no approved vaccines for prevention of the disease despite several efforts that have been made so far. Hence, the continuous search for new and better drug candidates.

Trypanosomes have a complicated life cycle that involves alternating between insect-adapted forms such as the procyclic forms (PCFs) in the midgut of the vector and bloodstream forms (BSFs) in the human host (Simpson *et al*., 2006). Blood Stream Forms of *T. brucei* rely exclusively on glycolysis for energy production (Verner *et al*., 2016). The glycolytic pathway of these parasites is compartmentalized within the organelles called glycosomes. Glycerol kinase is one of the glycosomal enzymes and plays a key role in the parasite energy metabolism (Minagawa *et al*., 1997; Balogun *et al*., 2010). The rudimentary mitochondrion of the parasites also houses an indispensable cytochrome-independent Trypanosome Alternative Oxidase (TAO) (Chaudhuri *et al.,* 2006). TAO is the essential terminal oxidase for re-oxidization of NADH produced during glycolysis by the trypanosomes, and in addition to glycerol-3-phosphate dehydrogenase, it is also the primary mitochondrial electron transport protein (Kido *et al*., 2010). The importance of these enzymes to the parasites and their absence in the mammalian hosts has made them a good target for anti-trypanosomal drugs search (Minagawa *et al*., 1997; Verlinde *et al*., 2001; Yabu *et al*., 2003; Balogun *et al*., 2010).

Cyanobacteria (blue-green algae) are a group of photosynthetic prokaryotic organism found in fresh and marine waters, soil, rock, wall, tree trunks and sewage. They are morphologically diverse and flourish in static and eutrophic water bodies, dominating the aquatic ecosystem through formation of blooms (Ghadouani *et al*., 2004). During cyanobacteria bloom formation in aquatic environment, there is production of secondary metabolites which are mostly targeted at inhibiting the growth of other competitors, a behavior referred to as allelopathy. Some of these metabolites inhibit the growth of other algal species (algicidal effect), while some are secreted to deter grazers which are mostly zooplankton (Pflungmacher, 2002).Bloom–forming cyanobacteria include *Microcystis* spp. *Oscillatoria* spp. *Anabaena* spp. *Nostoc* spp. and many others. These

species have been known for production of toxins such as microcystins, anatoxins and nodulalins, alongside several metabolites into the aquatic environment (Chia *et al* 2009; Singh *et al*., 2017). Cyanobacteria are acknowledged producers of diverse biologically active and structurally diverse secondary metabolites (Demay *et al*., 2019). Although, most of the isolated metabolites from cyanobacteria eventually exhibit cytotoxic effect, non-toxic metabolites from them exhibits potentials to serve as lead compounds in pharmaceutical, agricultural, or industrial applications (Tan, 2007; Burja *et al.,* 2001; Demay *et al*., 2019). The activity of these compounds against viruses, bacteria, fungi, protozoa, cancer cells and other algal species have been reported (Mundt *et al*., 2001; Portman *et al*., 2008; Mazard *et al*., 2016). A few of the secondary metabolites have been reported to possess some biological effects on trypanosomes. For instance, aerucyclamide isolated from a cyanobacterium *Microcystis aeruginosa* displayed anti-trypanosomal activity on *T.b. rhodesiense* (Portmann *et al*., 2008); Almiramides isolated from the cyanobacteria *Lyngbya majuscula* were also found to play a role in disruption of glycosome function in *T. b. brucei* (Sanchez *et al*, 2013).

Oscillatoria is a genus of filamentous cyanobacteria which is named after its oscillation movement. Studies on *Oscillatoria* spp isolated from various water bodies have reported the presence of butylated hydroxyl toluene (BHT), vitamins, minerals, viridamides, antibacterial, antifungal, anticancer and antiprotozoal compounds by the screened species (Shanab, 2007; Linington *et al*., 2007; Simmons *et al*., 2008a; Nair and Bhimba, 2013).

Microcystis is a genus of cyanobacteria characterized by colony of spherical shape cells (about 2-8µm) containing gas vesicles and constitutes the most common bloom-forming cyanobacteria in many water bodies worldwide (Mazur-Marzec *et al*., 2010). Although, *Microcystis* species have

been known to be synthesizers of hepatotoxins such as microcystin, bioactive compounds of pharmacological importance have also been isolated from them (Portmann *et al*., 2008).

Previous reports on cyanobacteria as rich sources of bioactive compounds with therapeutic potential inspired the design of this work. Research into biological activity of these compounds reported in the literature mostly focused on screening of laboratory cultures, most of which are from the marine environment; rarely species from the local environment are assessed for their pharmacological properties (Mundt *et al.,* 2001). This study was therefore designed to assess the biological activity of extracts from an indigenous freshwater cyanobacterial strain alongside three other exotics strains on *Trypanosoma brucei brucei.*

1.2 Statement of Research Problem

African human and animal Trypanosomiasis have been acknowledged as the cause of morbidity and mortality to humans and livestock throughout sub-Saharan Africa, and a major constraint to agricultural activity. An estimated 65 million people are at risk in the 36 sub-Saharan African countries. Methods of vector control through tsetse traps and spraying with insecticides such as Dichloro-Diphenyl-Trichloroethane (DDT) have a lot of limitations and have not been effective (Adamu *et al*, 2011). Chemotherapy, which is the control measure, exacerbates the situation in that it relies on a few drugs that have negative side effects, and must be administered intramuscularly or intravenously by qualified medical personnel (Fairlamb 2003; Barret *et al.,* 2007). The few available drugs are already facing the problem of emerging resistance because drugs currently in use have been employed continuously for a long time (Baker et al., 2013). Hence, the need for continuous search of better alternative drugs to tackle this majorly Africa's health problem.

1.3 Justification

Studies into natural products for discovery of novel therapeutics have been directed more towards screening of higher plants. There is less focus on marine and freshwater cyanobacteria and microalgae as promising sources of new compounds of interest in pharmacology and biotechnology. Due to structural diversity in their metabolites, the probability of rediscovery of compounds already identified in other organisms is very low. Cyanobacteria (blue-green algae) are a promising yet underexplored source for novel natural products with potent biological activities.

Although, most of the compounds isolated from cyanobacteria in the past are cytotoxic, they also produce a significant number of compounds that possess anti-infective activities (Niedermeyer, 2015). Cyclic hexapeptides isolated from *Oscillatoria* species and *Microcystis* species from marine environment have shown activity against *T. cruzi* and *T. b. rhodesiense* (Linington *et al*., 2007; Portmann *et al*., 2008). Quite a number of works have reported antimicrobial activities of marine cyanobacteria, sponges and other microalgae, but there is scarcity of information on the anti-infective potential of freshwater cyanobacteria. As a result, this study investigated the anti-*Trypanosoma* potential of methanol extracts from exotic strains and an indigenous isolate.

1.4 Aim

The aim of this work is to evaluate the extracts of *Microcystis flos-aquae, Microcystis aeruginosa, Microcystis wesenbergii* and *Oscillatoria* sp. for alternative chemotherapeutic principles against trypanosomiasis.

1.5 Objectives

The specific objectives are to:

i. Carry out molecular characterization of the isolated indigenous cyanobacterium.

ii. Determine the anti-*Trypanosoma* activity of crude extracts of the four cyanobacteria isolates on *T. b. brucei in vitro*

iii. Determine the anti-*Trypanosoma* activity of fractions from most active extract on *T. b. brucei in vitro*

iv. Detect bioactive metabolites from the most active fraction of the cyanobacterial extract

v. Carry out *in vivo* screening of the most active crude extract on *T. b. brucei* in rats.

1.6 Hypotheses

i. The isolated cyanobacterium is not *Oscillatoria* sp.

ii. Crude extracts of the four cyanobacteria has no anti-*Trypanosoma* activity.

iii. Fractions most active cyanobacterial extract has no anti-*Trypanosoma* activity.

iv. There are no bioactive metabolites in the fractions of the cyanobacteria

v. The most active cyanobacteria extract does no possess *in vivo* antitrypanosomal activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African Trypanosomiasis

African trypanosomiasis is a disease that affects both humans (HAT) and animals (AAT) across several regions in Africa. It is caused by different species of *Trypanosoma*, which are flagellated protozoan parasites transmitted by the insect vector *Glossina s*pp (tsetse flies). *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* are causative agents of HAT, while the sub-species *T.b brucei* and other species such as *T. congolense* and *T. vivax* cause AAT (Steverding, 2008). *Trypanosoma evansi* is a causative agent of *Surra* in animals such as camels, water buffaloes and horses; however, it is transmitted by blood sucking insects called tabanids (Vanhollobeke *et al*., 2006).

2.1.1 Epidemiology of the African Trypanosomiasis

The epidemiology of the African trypanosomiasis is quite complex in that transmission cycles are subject to interactions between humans/animals, tsetse flies and trypanosomes. The disease exists in two different geographical regions and shows clinically distinct symptoms and forms. The Central and West African sleeping sickness caused by *T. b. gambiense*, is a chronic infection that persists for months or even years before symptoms appear. On the other hand, the Eastern and Southern African disease caused by *T. b. rhodesiense* is very severe with symptoms of central nervous system involved and these symptoms usually occur after a few weeks (Donelson, 2003). Humans are mainly affected by *T.b. gambiense*, but the species can also be in rare instances found in animals. On the other hand the wild animals are the main reservoirs of *T.b.*

rhodesiense. The distribution of the disease throughout African continent depends primarily on the distribution of tsetse fly which serves as the transmission vector. The map of Africa showing the regions affected by trypanosomiasis has been presentes in Figure 2.1.Tsetse flies vectors are abundant throughout nearly a third of the African continent, with the flies inhabiting areas in 36 countries (Aksoy, 2003).

2.1.2 The Biology/Life Cycle of the African Trypanosomes

The main species of African trypanosomes that causes Human African Trypaanosomiasis and Animal African Trypanosomiasis are transmitted through the saliva of tsetse flies when an infected fly takes blood meal on an animal or human. For this reason, tsetse fly-transmitted trypanosomes are referred to as *Salivaria* (Barrett *et al.*, 2003; Stevens and Brisse, 2004). Trypanosomes transmitted by the tsetse fly, *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. congolense* and *T. vivax*, must therefore be adapted to undergo part of their life cycle in the tsetse fly host. After an infective bite by the fly, a chancre is formed at the site of the bite, after a minimum period of about five days. Formation of this chancre is usually followed by regional lymphadenopathy, from which the parasites cross to the circulatory system and other vital tissues of the body such as the brain (Stich *et al.*, 2002). The life cycle of African trypanosomes as shown on Figure 2.3, therefore, undergoes a series of transitions between the mammalian host's blood stream, tsetse mid-gut, and tsetse salivary glands. During this period, the trypanosomes pass through three developmental stages: the non-infective procyclic and epimastigote forms in the tsetse fly, and the long slender bloodstream form in the mammalian host. In each of the above-mentioned stages the parasite can undergo multiplication. However, multiplication does not occur during the infective metacyclic form, which is the final developmental stage in the

insect's salivary glands, and the short stumpy form in the mammalian hosts' bloodstream (Donelson, 2003). On reaching the tsetse fly's gut or mammalian bloodstream, the trypanosomes colonize the mid-gut (stumpy form) or bloodstream (metacyclic form) respectively. In another transition (tsetse to mid-gut to the salivary gland) the parasites differentiate into epimastigote forms which use their flagella for surface attachment. On entering the mammalian host from the tsetse salivary gland, the metacyclic trypanosomes start expressing the variant surface glycoprotein (VSG) on their cell surface and change to a slender morphology (Gull, 2002). While in the host, the parasite carries out expression of the metacylic VSGs for as long as 7 days and then change to the expression of non-metacyclic, bloodstream VSGs (Donelson, 2003). This adaptation allows the parasites to escape antibody-mediated killing and increase their population in the host, resulting to the development of a long-lasting chronic infection.

As the parasitemia progresses, long slender forms differentiate into short stumpy ones in a density dependent pattern. Short stumpy forms show several pre-adaptations for survival in the tsetse mid-gut, one of which is a partial activation of the mitochondrion. Upon uptake by the tsetse in a blood meal, the stumpy forms changes into proliferative procyclic forms. The tsetse mid-gut forms replace the bloodstream VSG coat with another protein, known as procyclin. From the procyclic population emerges the proventricular forms, which move to the salivary glands, attach, and become epimastigotes. The rapidly multiplying epimastigotes are packed closely together during the colonization of the salivary gland, where exchange of genetic material takes place in a non-obligatory fashion. Epimastigotes then finally differentiate to form the detached, non-dividing metacyclic forms, which are infective to the mammalian host when transmitted by the flies (Gull, 2002).

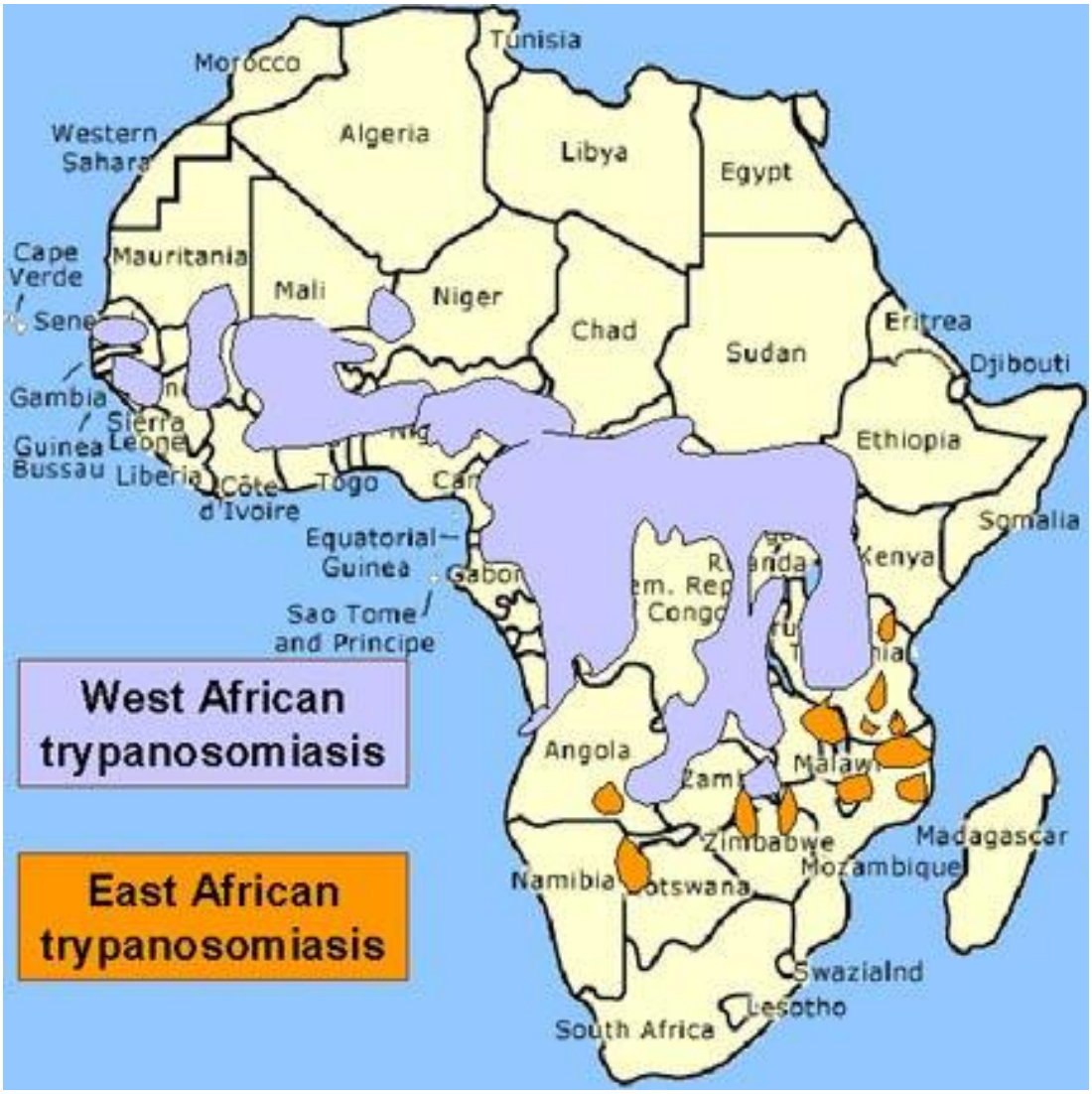


Fig. 2.1: A map of African regions affected by Human African Trypanosomiasis.

Source: https://microbewiki.kenyon.edu/index.php/African_Trypanosomiasis

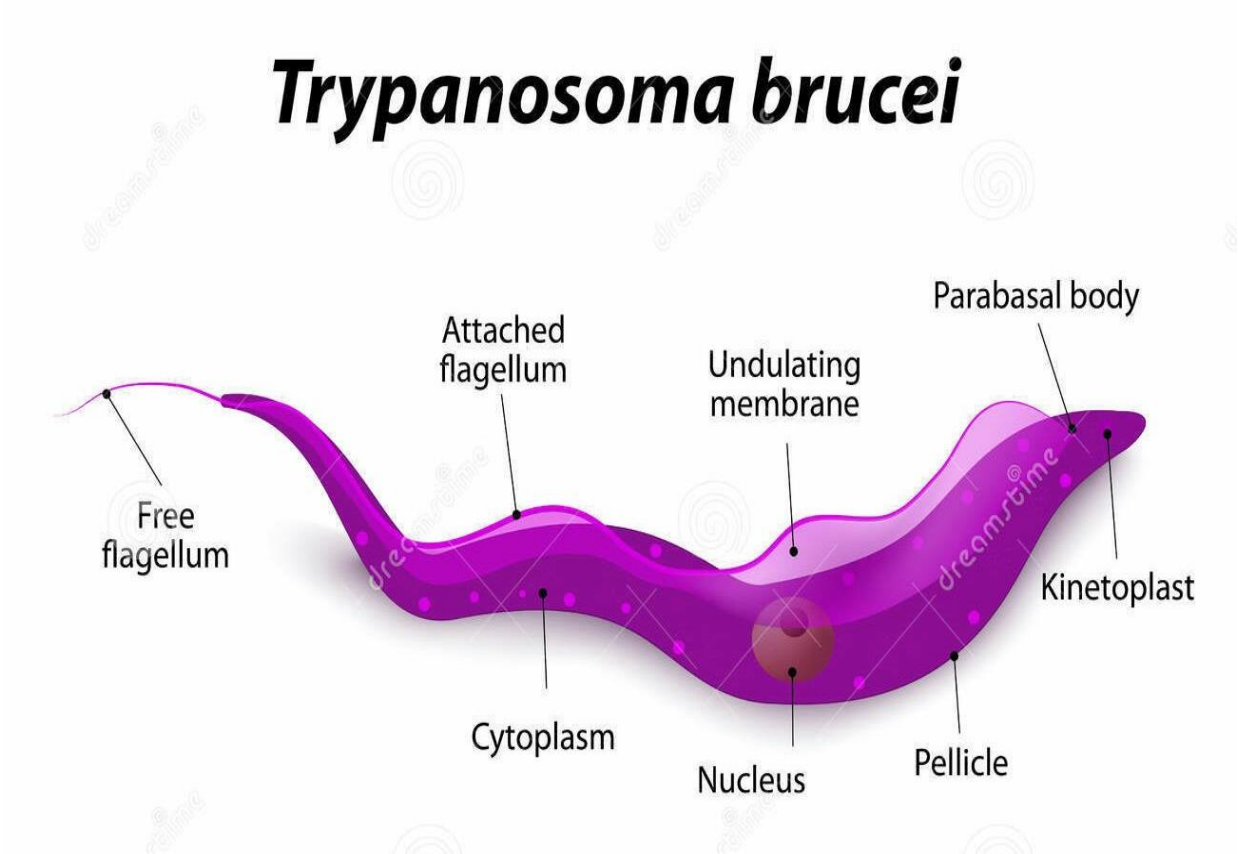


Fig. 2.2: Diagram of *Trypanosoma brucei.*

Source:https://www.istockphoto.com/vector/diagram-of-trypanosoma-cell-gm474551646-64912761

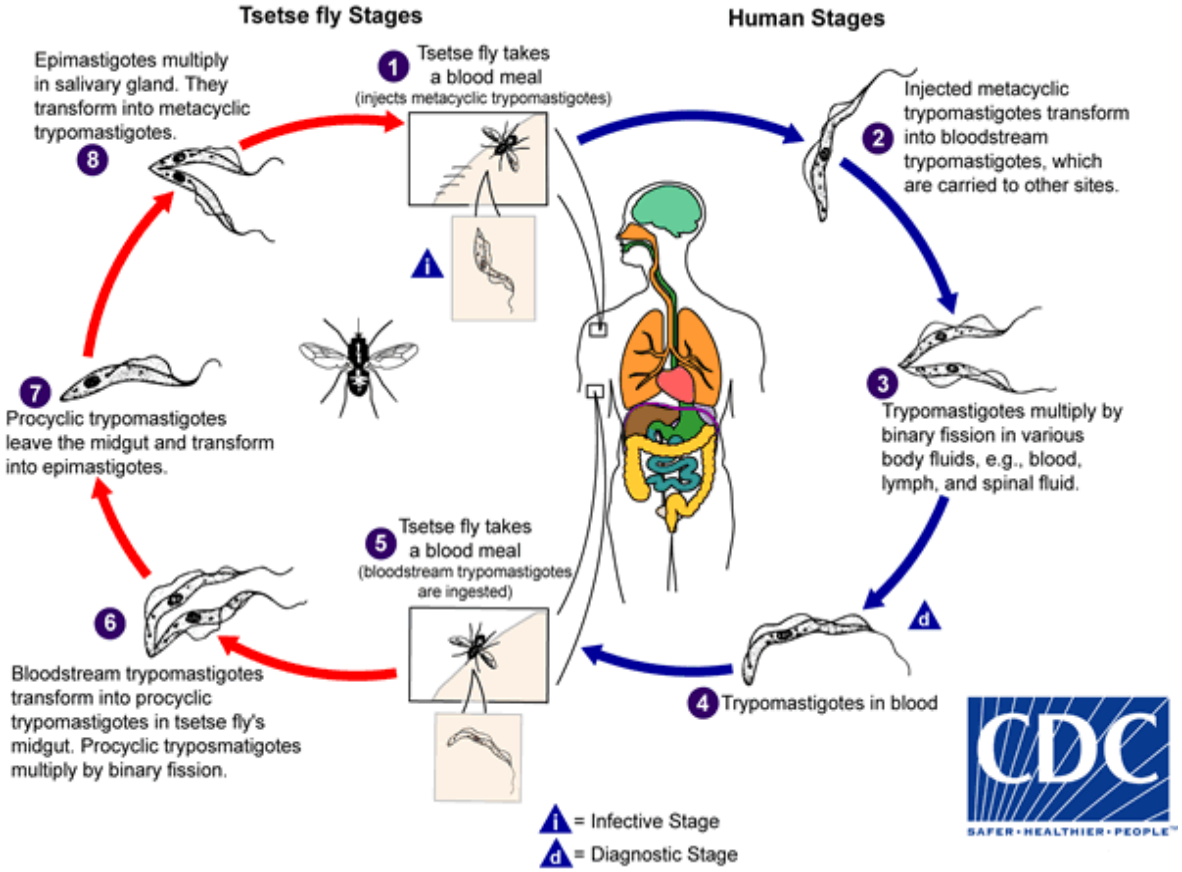


Fig.2.3: Life cycle of *Trypanosoma brucei*.

Source: https://www.msdmanuals.com/home/multimedia/image/v14456795

2.1.3 Pathogenesis of African Trypanosomiasis

When a mammalian host is bitten by a tsetse fly infected with trypanosome, the parasites undergoes multiplication in the skin, eliciting a localized host response that manifests inform of a skin lesion called chancre (Poltera, 1985). Afterwards, the parasites enter the blood circulatory system through the lymph vessels and are able to survive in the blood stream throughout the period of infection (Akol and Murray, 1982). In the blood circulatory system, the parasite is continually exposed to the immune system of the mammalian host. *T. brucei* is capable of penetrating the walls of blood capillaries, and usually invade the interstitial tissues, but always live as an extracellular parasite (Bezie *et al*., 2014). *T. congolense* is also an extracellular but intravascular blood parasite that is not capable of leaving the blood stream. However, it has a tendency to adhere to the walls of blood capillaries and small vessels (Banks, 1980).

African trypanosomes have developed very complex evasion mechanisms to enable their survival in chronically infected hosts. These evasion mechanisms include: antigenic variations, downregulation of nitric oxide production, activation of the complement system resulting to persistent hypocomplementeamia, marked immunosuppression, and polyclonal β-lymphocyte activation (Hudson *et al*., 1976). Anaemia is the most common symptom in clinical and experimental disease conditions, while splenomegaly, meningoencephalitis and cachexia occur during the extravascular localization of the trypanosome (Rifkin and Lsberger, 1990).

2.1.4 Prevention and Control of African Trypanosomiasis

The two main approaches for prevention and control of African Trypanosomiasis are chemotherapy and vector control. Chemotherapy has relied on few available drugs while vector control has been achieved with the use of tsetse traps and insecticidal sprays such as DDT

(Adamu *et al.*, 2011). Research over the past many years has led to development of about only four clinically approved drugs namely; pentamidine, suramin, efflornithine and melarsoprol for treatment of Sleeping sickness (Steverding, 2010), three out of the four mentioned were developed more than five decades ago (Fairlamb, 2003). For animal trypanosomiasis, homidium (ethidium), diminazine aceturate (berenil) or isomethamidium (samorin) are currently in use.

Diminazene is an aromatic diamidine from surfen C, which is marketed as the diaceturate salt. This consists of two amidinophenyl moieties linked by a triazene bridge, p,p-diamidinodiazo-aminobenzene diaceturate and N-1,3-diamidinophenyltriazene diaceturate tetrahydrate. Diminazene aceturate has recently become the most used drug for trypanosomiasis in domestic animals. It has proven to be highly effective against *T. congolense* and *T. vivax*, but in contrast less effective against and *T. evansi* and *T. b. brucei* infections. Studies on its mechanism of action shows that diminazene interferes with nuclei acid synthesis and specifically binds to DNA by non-intercalative mechanism, thereby inhibiting DNA and RNA synthesis (Mehlhorn, 2008). For *T. b. brucei*, diminazene tends to enter the parasite through the P2 nucleoside transporter that is also able to transport other diamidines and melamine-based arsenicals. Mild negative side effects are often reported including nausea, vomiting and albuminuria, but in severe cases reversible paralysis and coma are seldom seen.The adverse effect seen in animals is usually severe cerebral haemorrhages (Holmes *et al.*, 2004).

2.2 Ethnobotanical Treatment of Trypanosomiasis

Several plants have been are used traditionally for treatment of African trypanosomiasis and laboratory investigations of most of the plants have also demonstrated antitrypanosomal activity. A survey on the ethnomedicinal use of plants in Kaduna state for treatment of trypanosomiasis

showed that *Khaya senegalensis* accounted for 23.3% usage while *Terminalia avicennioides, Ximenia americana, Anona senegalensis and Azadirachta indica* accounted for 12.7%, 16.3, 10.7% and 9.3%, respectively (Maikai *et al*., 2010). In Nupe land (North Central Nigeria), plants such as *Acacia nilotica, Bombax buonopozense, Terminalia avicennioides* and *Zanthoxylum zanthoxyloides* are traditionally used for treatment of sleeping sickness (Mann *et al*., 2011). Investigation of methanol extracts of *Acacia nilotica, Heterotis rotundifolia, Bombax buonopozense, Zanthoxylum zanthoxyloides, Pterocarpus erinaceus* and *Terminalia avicennioides* showed promising *in vivo* antitrypanosomal activity against *T. b. brucei* (Mann and Ogbadoyi, 2012). In another investigation, Atawodi *et al*. (2003) reported the *in vivo* antitrypanosomal activity of extracts from *Khaya senegalensis, Securidaca longepedunculata, Piliostigma reticulatum, Adansonia digitata, Terminalia avicennoides, Parkia clappertioniana, Prosopis africana, Anchomanes difformis, Lannea Kerstingii* and *Cassytha* spp against *T. brucei brucei* and *T. congolense*. Longdet *et al*. (2014) investigated the antitrypanosomal activity of *Nauclea latifolia* methanolic extract against *T. congolense* in rats. In another study, the ethyl acetate leaf extract of *Cymbopogon citratus* showed strong antitrypanosomal activity on *T.b brucei*-infected rats (Longdet *et al*., 2018).

2.3 Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes. Although often referred to as blue-green algae, cyanobacteria are not directly related to higher algae (Mazard *et al*., 2016). Cyanobacteria are believed to be among the oldest organisms that exist on earth with records of fossils dating back 3.5 billion years (Schopf, 1987; 1993). They are key players in the Earth's transition from a carbon dioxide-rich atmosphere to the present relatively oxygen-rich atmosphere because of their

oxygenic photosynthesis (Bekker *et al*., 2004). Cyanobacteria present a diverse range of morphology, including unicellular, surface-attached, filamentous colony- and mat-forming species (Mazard *et al*., 2016). Base on the broad taxonomic diversity that occur across the phylum, cyanobacteria are found in a diverse range of both aquatic and terrestrial habitats, ranging from arid deserts to freshwater and marine ecosystems across a range of oligotrophic and eutrophic conditions. They can also inhabit extreme habitats such as Antarctic dry valleys, thermophilic lakes Arctic, Lava caves and subsurface of calcareous rocks (Steunou *et al*., 2006; Comte *et al*., 2007; Saw *et al*., 2013).

2.3.1 Taxonomy of Cyanobacteria

Several techniques have been developed to identify cyanobacterial species. These include light microscopy (compound, inverted and epifluorescence microscopes) and methods comparing DNA-sequences. Hoffmann *et al.* (2005) proposed classification system of cyanobacteria based on genetic relationships, mainly 16S rDNA sequences, thylakoid arrangements and morphology. The distribution of 16S rDNA in prokaryotes is universal; and the phylogenetic analysis of the 16S rDNA has revealed close relationships among cyanobacteria and has a key role in inferring phylogenetic relationships and in identification of cyanobacteria. Traditionally, microscopy is used to identify organisms based on morphological characteristics. However, a major drawback of this approach is that morphology may change depending on the environmental conditions (Evans *et al.,* 1976; Garcia-Pichel *et al.,* 1996). Another limitation is that microscopy-based identification equires time and certain level of expertise to determine key morphological features (Scholin *et al.,* 2003). Nowadays DNA sequence analysis is a most reliable method to identify cyanobacteria (Anjos *et al.,* 2006; Roeselers *et al.,* 2007) up to species level and helpful in

preparation of phylogenetic trees. The phylogenetic analyses are reliably used to estimate the evolutionary relationships amongst organisms. Nubel *et al*. (1997) developed and tested a set of primers for specific amplification of the 16 S rRNA from various cyanobacteria and plastids. Sequencing of the PCR amplified DNA at the small sub-unit ribosomal DNA (Robertson *et al.*, 2001; Anjos *et al.,* 2006) and the phycocyanin DNA-region (Neilan *et al.*, 1997; Janson and Granéli, 2002; Hameed, 2008) has been used to determine phylogenetic relationships in cyanobacteria. Sequence analyses include alignment of sequences, construction of a phylogenetic tree and testing the reliability of the constructed phylogenetic tree with bootstrapping (Ludwig and Klenk, 2005; Hameed, 2008).

2.3.2 Growth Pattern of Cyanobacteria

Generally, batch cultures of microorganisms including cyanobacteria, undergo four growth phases: the lag phase, exponential phase, stationary phase, and declining or death phase (Figure 2.4). The initial cell density, nutrient concentrations, temperature, and many other factors determines the duration of each growth phase. In the lag phase, cyanobacteria cells acclimatize to the medium conditions and the cells synthesize new biomolecules needed for cell multiplication. During exponential phase, the cells adapt and divide rapidly and the cell components all components increase at the same rate. Following the exponential growth phase the rate of growth begins to decline but the biomass continues to rise. During the stationary phase, the nutritional content of the medium is exhausted and the cells cease to multiply but the cells continue to increase in size. Senescence or the death phase eventually sets in, where cells begin to die and the number of living cells reduces. Monod equation is the most commonly used model (Monod, 1949): $\mu = \frac{\mu \max S}{Ks+S}$

Where μ_{max} represent the maximum specific growth rate, S stands for the substrate concentration, K_s is the saturation constant of the rate-limiting substrate when the specific gowth rate is half of the maximum.

In general, the growth of cyanobacteria is influenced by changes in nutrient availability as well as environmental factors such as light intensity, dissolved oxygen (DO), temperature, salinity and pH. Just like other photosynthetic phytoplankton, at optimal environmental temperatures and light intensity the biomass accumulation in cyanobacteria is directly proportional to the amount of nutrients available (particularly Nitrogen and Phosphorus) in the water column. Nitrogen types can also affect the rate of growth in cyanobacteria. Changes in the growth rate and morphology of *Cylindrospermopsis raciborskii* under $NO3^-$ versus on $NH4^+$ conditions has been investigated (Saker and Neilan, 2001).Differences in cyanobacterial growth rates when cultivated on $NO3^-$ versus on $NH4^+$ are frequently observed for individual strains. In some cases even within the same species there is usually a variation, as some strains may grow faster on $NH4^+$ while the growth of some may be favoured on $NO3^-$ (Berg and Sutula, 2015).

Increase in media pH and DO concentrations depress the growth of *Microcystis* species. Decrease in the fresh and dry weight biomasses of *Oscillatoria* sp. cultured in BG-11 medium was observed in a 4-weeks growth experiment (Yadav *et al*., 2016). It was suggested that increase in the medium pH over time could be the reason for the decrease in biomass yield. In the natural aquatic environment there is constant water column disturbance, and this has been shown to also affect cyanobacteria growth. Water flow rate influences the growth of cyanobacteria by making the adaptive phase shorter, with longer logarithmic phase resulting to

higher specific growth rate (Lin *et al*., 2012). To understand the influence of water flow on the growth of *Microcystis* and its relationship with amount of nutrient available, Lin *et al*.(2012) investigated the effect of water flow on *M. aeruginosa* under high nutrient condition (using BG-11 medium) with controlled irradiance and temperature. It was found that high nutrient enhanced the resilience of *Microcystis* towards water flow.

Environmental temperature also influences the growth of cyanobacteria, but unlike other phytoplankton, cyanobacteria typically show higher growth rates at higher temperatures and lower growth rates at colder temperatures. Studies have shown that cyanobacterial isolates from temperate regions typically have optimum growth temperature between 25 and 35 °C (Reynolds, 2006; Lurling *et al*. 2013). An investigation of eight cyanobacterial isolates under laboratory culture conditions showed that the optimum growth *Microcystis aeruginosa* strains were 30-32.5 °C, and 32.5 °C for *Aphanizomenon gracile*. Lower optimum growth temperature (27.5 °C) were observed in *Planktothrix agardhii* and *Cynlindrospermopsis raciborskii*, while that of *Anabaena* sp. was at 25 °C (Lurling *et al*., 2013). Gao *et al*. (2020) assessed the growth of axenic *Microcystis auruginosa* in BG-11 medium under static and flask-shaking conditions for 21 days. Growth curve obtained from this investigation showed that the cyanobacteria attained its exponential phase between day 14 and day 16. Highest cyanobacterial cell number for the various treatments group was recorded at day 21.

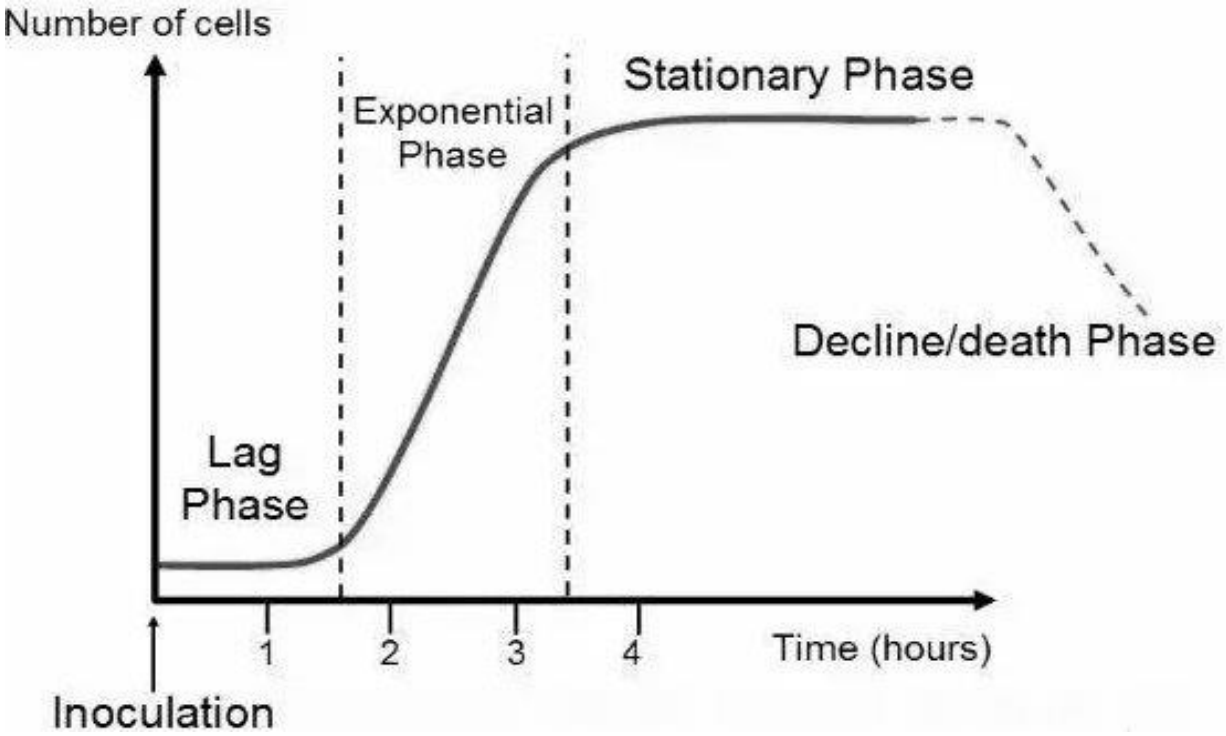


Fig. 2.4: A typical bacterial growth curve. Source: https://orbitbiotech.com/bacterial-growth-curve-generation-time-lag-phase-log-phase-exponential-phase-decline-phase/

2.4 The Genus *Microcystis*

Microcystis is a genus of cyanobacteria characterized by colony of spherical shape cells (about 2-8µm) containing gas vesicles and constitutes the most common bloom-forming cyanobacteria in many water bodies worldwide (Mazur-Marzec *et al*., 2010). The most common members of this genus include *Microcystis wesenbergii, M. aeruginosa* and *M. flos-aquae*. Although, *Microcystis* species have been known to be synthesizers of hepatotoxins such as microcystin, bioactive compounds of pharmacological importance have also been isolated from them (Portmann *et al*., 2008).

2.5 The Genus *Oscillatoria*

Oscillatoria is a genus of filamentous cyanobacteria that constantly undergoes oscillation movement; hence, the name oscillatoria. Morphologically *Oscillatoria* species show resemblance with other genera such as *Lyngbya* and *Phormidium*. Studies on species of *Oscillatoria* isolated from various water bodies have reported the presence of vitamins, minerals, butylated hydroxyl toluene (BHT), viridamides, and several other antimicrobial compounds (Mundt *et al*., 2003; Simmons *et al*., 2008a; Linington *et al*., 2007).

2.6 Antiprotozoal Activity of Some Algal and Cyanobacterial Extracts

Although, very little research has focused on the potentials of algae and cyanobacteria (blue-green algae) as a source of antiprotozoal compounds, studies have shown promising antimalarial activity as well as trypanocidal and antileishmanial activity in *Pelvetia babingtonii, Fucus evanescens* (Nara *et al*., 2004) as well as *Ulva lactuca* and *Sargassum natans* (Orhan *et al*.,

2006). Leon-Deniz *et al*. (2009) reported the anti-*Trypanosoma* activity of 29 tropical marine algae against *Trypanosoma cruzi*. The strongest and most rapid inhibition capacity was shown by *Turbinaria turbinate* (IC_{50} 7.55 ±1.52 µg mL^{-1}), *Dictyota caribaea* (IC_{50} 7.73 ± 1.41 µg mL^{-1}), and *L. variegata* (IC_{50} 9.72 µg mL^{-1}) within 48 hours. For the members of Chlorophyta (green algae) screened, *Halimeda incrassata* and *Rhipocephalus phoenix*, a 100% reduction of parasite was also observed at the high concentrations. *Halimeda incrassata* (IC_{50} 10.26±1.53 µg mL^{-1}) and *R. phoenix* (IC_{50} 16.32 ± 2.17 µg mL^{-1}) at 7 days showed high *in vitro* activity (Leon-Deniz *et al.*, 2009). *In vitro* trypanocidal activity of different crude extracts and fractions of two cyanobacteria *Nostoc commune* and *Rivularia biasolettiana* isolated from Ireland exhibited mild to strong anti-*Trypanosoma* activity against *T. b. rhodesiense* and *T. cruzi* with no cytotoxic effect when tested on L6 cell lines (Broniatowska *et al*., 2011).

2.7 Bioactive Metabolites from Cyanobacteria

Cyanobacteria such as *Microcystis, Nostoc, Anabaena, Oscillatoria* and *Lyngbya* are well known to be synthesizers of variety of bioactive compounds (Sivonen and Borner, 2008). Growth stage significantly influences secondary metabolite synthesis and their interaction with other organisms. Some species reach their maximal metabolite yield during the stationary phase, others during the exponential phase (Skulberg, 2000; LeFlaive and Ten-Hage, 2007).

Generally, secondary metabolites in microorganisms are usually produced during the stationary phase, but it has been proposed that cyanobacteria contain secondary metabolites at all growth stages (Repka *et al*., 2004). Some of these compounds produced by cyanobacterial species are also known to be toxic (Welker, 2008; Tango and Butler, 2008). However, the cyanobacterial

secondary metabolites cyanobacteria are rich sources to obtain novel bioactive compounds used for the development of medicines and chemicals of agricultural importance. Some of the metabolites secreted extracellulary by cyanobacteria act as allelo-chemicals in the natural aquatic environment (Pflungmacher, 2002). Cyanobacterial bioactive metabolitess that have been characterized as algicides target the photosystem II of photosynthesis in plants and therefore are termed natural herbicides (Priyadarshani and Rath, 2012).

Approximately 58% of cyanobacterial metabolites reported in literature is from Oscillatoriale, and 35% of natural products published is from the genus *Lyngbya*, a member of the family (Shah *et al*., 2017). The antimicrobial compounds isolated from microalgae are chemically characterized as peptides, amides, alkaloids, acetogenins, polyphenols, terpenes, shikimates, polyketides and xanthones (Ghasemi *et al*, 2004; Mayer *et al*., 2017). From the literature, about fourteen major activities have been reported, including neurotoxicity, lethality, anti-inflammatory, antiprotozoal, hepatotoxicity, dermatoxicity and cytotoxicity, antioxidant, anti-microalgal, antiviral, antibacterial and antifungal activities as well as enzyme inhibition activities (Demay *et al*., 2019). In addition, microalgae are also a good source of other important compounds such as vitamins, amino acids, fatty acids, simple hydrates, siderophores, and other compounds that are essential in supporting the growth of other microorganisms (Gademani and Portmann, 2008). Lipopeptides produced by cyanobacteria have been identified as interesting biochemically active compounds. Approximately 85% of them are bioactive, including cytotoxic (41%), antitumour (13%), antibiotic (12%), enzyme inhibitor (8%), antifungal (4%) antiviral activities (4%) and the remaining activity (18%) include tumour promoters, herbicides, antimycotic, antimitotic, antimalarial, algicidal, cell-differentiation promoting activity, multi

drug resistant reversers, antifeedant and UV absorbing activity which are used as sunscreens (Burja *et al.,* 2001). The number of cyanobacterial metabolites family with their corresponding activities reported in the literature is shown on Figure 2.4.

Although, compounds isolated from cyanobacteria are from many chemical classes, polyketide and peptide and structural elements are the dominant among cyanobacterial metabolites identified so far (Burja *et al*., 2001; Walker, 2006; Simmons *et al*., 2009). The peptides comprise branched, cyclic, and linear structures as well as lipopeptides, depsipeptides, and peptides with unique modifications like N- and O-methylation, sulfation, glycosidation, halogenation, oxidation, heterocyclization, dehydration, prenylation and ketide extensions (Tidgewell *et al*., 2010 ; Jones *et al*., 2010; Vining *et al*.,2015). Studies have shown that these compounds are often synthesized through combined polyketide synthases and non-ribosomal peptide synthetases (PKS/NRPS) (Rinehart *et al*., 1994; Dittman et al., 2001). This results in a high prevalence of non-proteinogenic amino acids as building blocks of these compounds (Niedermeyer, 2015). Several cyanobacterial and algal bioactive metabolites were discovered through screening studies that make use of target organisms which are not related to those for which the metabolites were poduced (Priyadarshani and Rath, 2012).

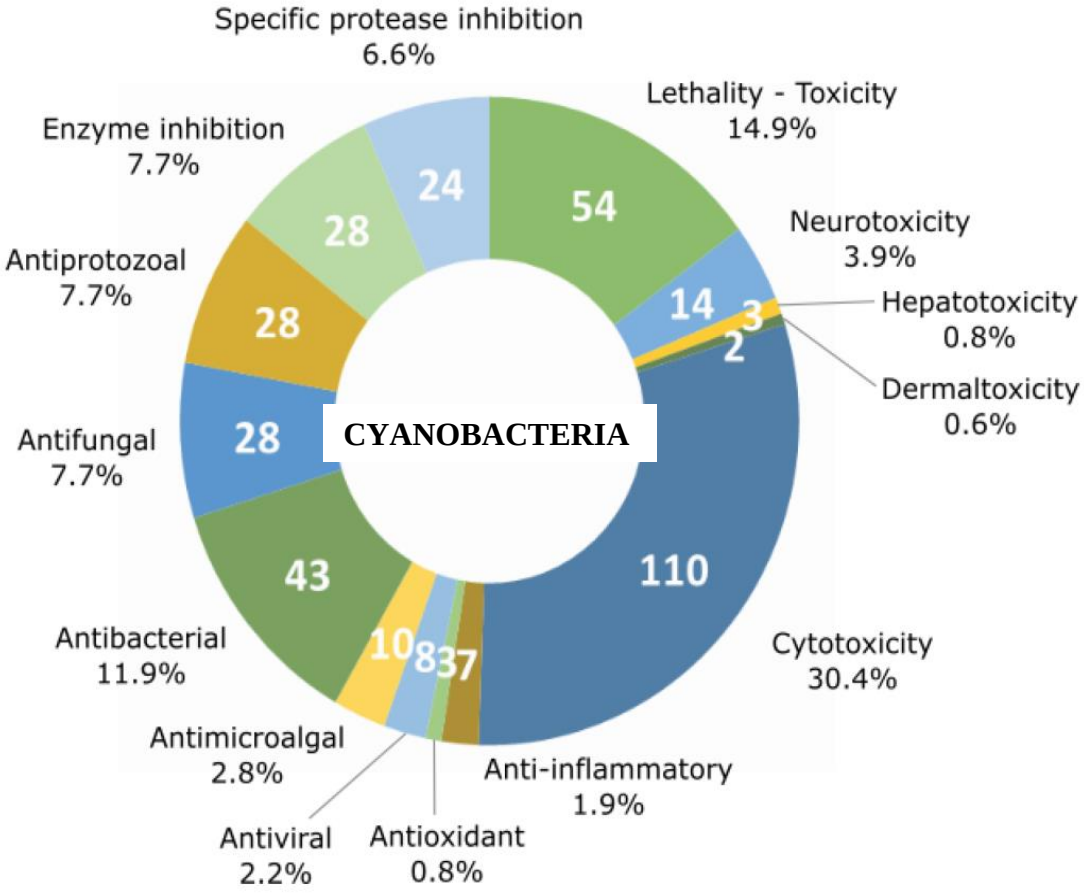


Fig.2.5: Number of isolated compounds from cyanobacteria and percentage of various activities reported (Adopted from Demay *et al*., 2019).

2.8 Metabolites from Cyanobacteria with Antiprotozoal Activity

Several studies have reported the antiprotozoal activity of secondary metabolites isolated from microalgae including cyanobacteria. The bioactive compounds widely acknowledged include terpenes, alkaloids, peptides, lipopeptides, polyphenols, acetogenins, long chain fatty acids, polyketides and xanthones (Simmons *et al*., 2009; Demay *et al*., 2019). The names and structures of these metabolites are presented on Figures 2.6, 2.7 and 2.8.

2.8.1 Metabolites with Antitrypanosomal Activity

Two cyclic hexapeptides, identified as venturamides **A** and **B** were isolated from the Panamanian collection of the marine cyanobacterium *Oscillatoria* sp. and tested against *Trypanosoma cruzi* (Linington *et al*., 2007). The A and B forms of the compounds exhibited moderate activity against *T. cruzi* with IC_{50} values of 14.6 and 15.8 μM, respectively. The cytotoxicity test of the two compounds showed a mild cytotoxicity to mammalian Vero cells with IC_{50} values of 86 and 56 μM, respectively, with selectivity index (SI) of less than 6. In a different study, related cyclic peptides identified as aerucyclamides **B** and **C** isolated from the cyanobacterium *Microrcystis aeruginosa* also showed anti-trypanosomal activity against *T. b. rhodesiense* with IC_{50} values of 15.9 and 9.2 μM, respectively (Portmann *et al*, 2008). Aerucyclamide **C** and **B** had an SI of 12 and 8 respectively against L6 cells. In a study, Sanchez *et al*. (2013), reported the inhibition of *T. b. brucei* by two linear peptides, almiramides B and C extracted from a marine cyanobacterium *Lyngbya majuscula* (Sanchez *et al*., 2010), at low micromolar concentrations. The Almiramide B and C showed inhibition with IC_{50} values of 6 and 3 μM respectively, with SI of 11 and 9 respectively compared to Vero cells. The Compound also did not show inhibition of growth when tested on Zebra fish embryos. Further investigation to understand their mechanism of

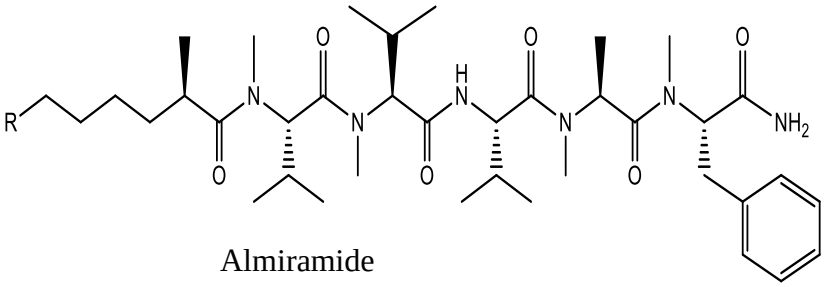
action revealed that the compounds were involved in interference with the trypanosome's glycosomal function by disruption of membrane assembly machinery (Sanchez *et al*., 2013).

An anti-trypanosomal lactam, named Hoshinolactam isolated from a marine cyanobacterium species collected at the coast near Hoshino, Okinawa in Japan exhibited potent anti-trypanosomal activity against *Trypanosoma brucei* with an IC_{50} value of 3.9 nM (Ogawa *et al*., 2017). Hoshinolactam did not show any toxicity against human fetal lung fibroblast cells (IC_{50} >25 µM). A cyclic polyketide-peptide hybrid possessing a *tert*-butyl group named Janadolide, has been isolated from the cyanobacteria *Okeania* sp. collected near coast of Janado, Okinawa (Ogawa *et al*., 2016). Janadolide showed potent antitrypanosomal activity against *T. b. brucei* with an IC_{50} value of 47 nM, which has shown to be more potent than the commonly used therapeutic drug, suramin (IC_{50} value of 1.2 µM). Janadolide exhibited no cytotoxicity against human cells even at 10µM concentration. Abdelmohsen *et al*. (2012) reported that the dibenzodiazepine alkaloid (diazepinomicin) isolated from a strain of *Micromonospora* sp. RV115 associated with the Croatian marine sponge *Aplysina aerophoba* showed activity against *T. brucei* trypmastigote forms and inhibited the parasite protease rhodesain.

2.8.2 Metabolites with Antiplasmodial Activity

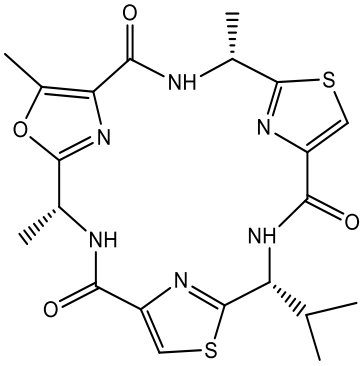
A number of screening studies have reported on the bioactivity of cyanobacteria metabolites against *Plasmodium* spp. Cyclic depsipeptides named Dudawalamides A–D belonging to the kulolide superfamily, were isolated from the cyanobacteria *Moorea producens*, collected from Dudawali Bay, in Papua New Guinea (Almaliti *et al*.,2017). Dudawalamide A and D showed potent activities against *Plasmodium falciparum* with IC_{50} values of 3.6 and 3.5 µM respectively. A new polyhydroxy macrolide, bastimolide **A** with a 40-membered ring was isolated from *Okeania hirsuta*. Bastimolide **A** exhibited a portent activity against four resistant strains of

Plasmodium falciparium with IC_{50} values between 80 to 270 nM (Shao *et al*., 2015). Two new cyclic depsipeptides, companeramides A and B were isolated from marine cyanobacterial collection in Coiba National Park, Panama (Vining *et al.,* 2015). Companeramides A and B exhibited moderate anti-plasmodial activity. Antimalarial activity of Gallinamide A isolated from cyanobacteria has also been reported (Linington *et al*., 2009). Two cyclic hexapeptides, venturamides A and B have been isolated from the Panamanian marine *Oscillatoria* sp. through antimalarial bioassay-guided isolation (Linington *et al*., 2007). Dragonamide and its analogues isolated from *Lyngbya majuscula* and *Lyngbya polychroa* have been reported to exhibit antimalarial activity (Gunasekera *et al*., 2008; Balunas *et al*., 2010). Carmabin A, dragomabin, and dragonamide A showed good antimalarial activity with IC_{50} values of 4.3, 6.0, and 7.7 μM, respectively.

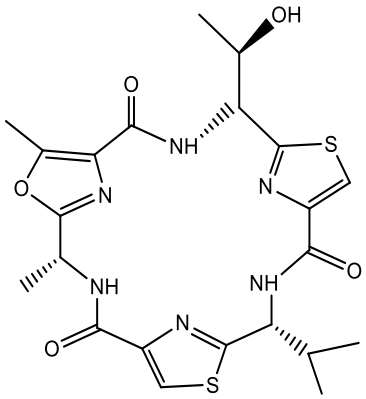


Almiramide

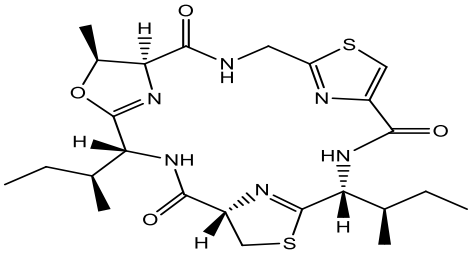
Name	R
Almiramide A	CH_3CO-
Almiramide B	CH≡C-
Almiramide C	CH_2=CH-



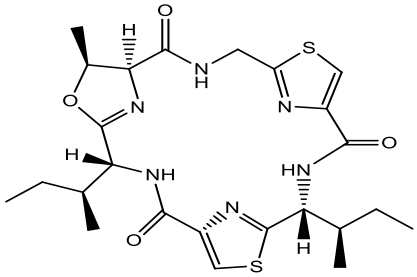
Venturamide A



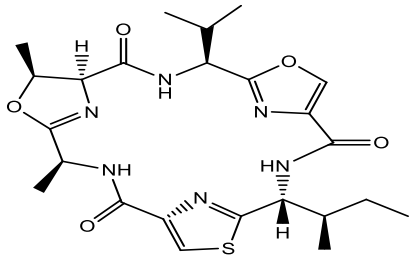
Venturamide B



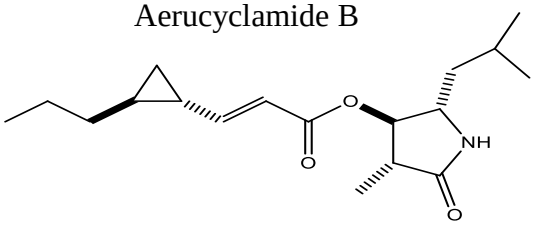
Aerucyclamide A



Aerucyclamide B



Aerucyclamide C



Hoshinolactam

Fig. 2.6: Structures of metabolites from cyanobacteria with antiprotozoal activity

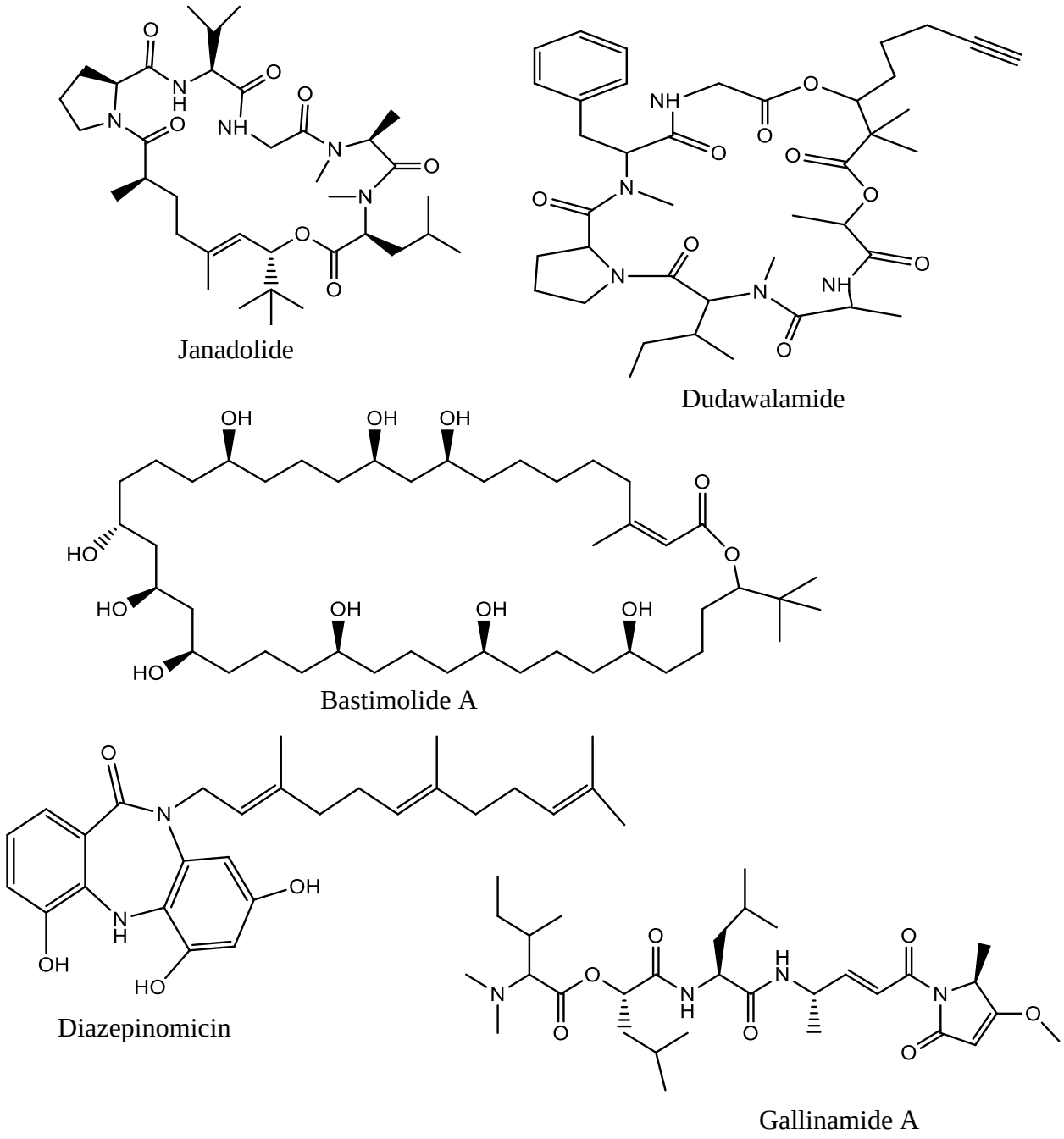
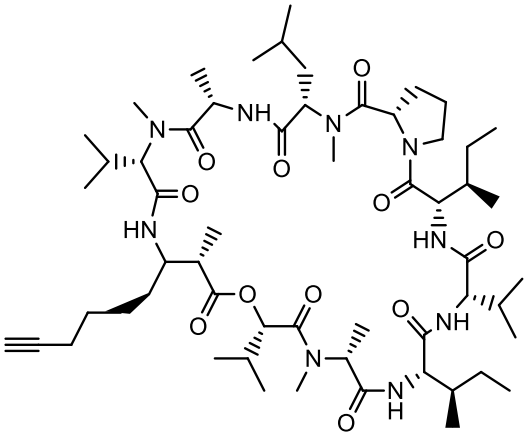
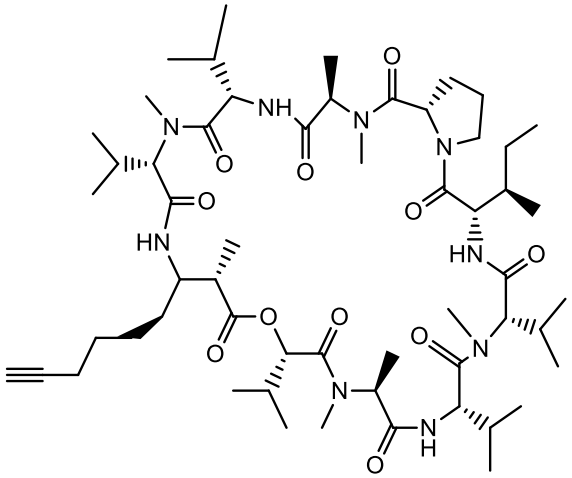


Fig. 2.7: Structures of metabolites from cyanobacteria with antitrypanosomal and antiplasmodial activity



Companeramide A



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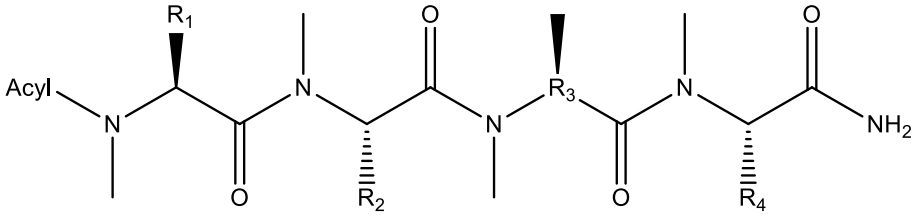
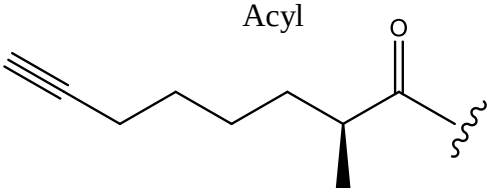
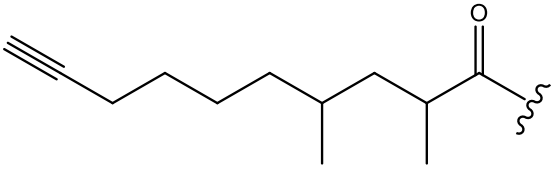


Dragonamide

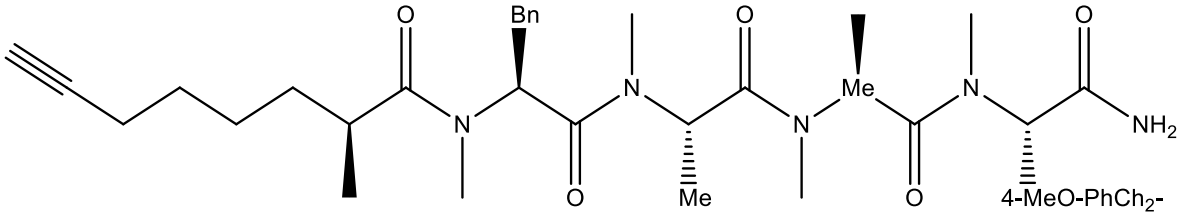
Acyl



Dragonamide A: R_1, R_2 &R_3 = *i*-Pr



Carmabin: R_1= Bn, R_2 &R_3 =Me



Dragomabin

Fig. 2.8: Structures of metabolites other marine organisms with antiprotozoal activity

2.8.3 Metabolites with Anti-leishmanial Activity

In a study to screen marine cyanobacteria from the Caribbean coast of Panama, two antileishmanial lipopeptides, named almiramides B–C and their non-active analog, almiramide A were identified from *Lyngbya majuscula* (Sanchez *et al*., 2010). When compared with the most closely related secondary metabolite dragonamide A, almiramides B–C with an extra Alanine residue, no methyl group on Val1 and the opposite configuration of the α-carbon of the lipophilic side chain, exhibited better antileishmanial activity with IC_{50} of 2.4, and 1.9 μM, respectively. Cyclic depsipeptides, Dudawalamides A–D isolated from *Moorea producens*, showed effective activity against *Leishmania donovani* with an IC_{50} value of 2.6μM (Almaliti *et al*.,2017).

Two lipodepsipeptides, Viridamines A and B isolated from *Oscillatoria nigro-viridis* have been reported for antileishmanial and antimalarial activities (Simmons *et al*., 2008a).

2.10 Cyanobacteria as Source of Nutritional Supplements and Pigments

As phototrophs, cyanobacteria capture light as their energy source through a variety of photosynthetic apparatus that are rich in pigments and chromophores. Several chromophores from the light-harvesting complexes such as phycobilins and chlorophylls have been reported to possess beneficial health effects, example, providing micronutrients and macronutrients, aiding in digestion, *et cetera* (Mazard *et al*., 2016). There is a high market demand for the use of cyanobacteria as beneficial human food/health supplements, and they are now being widely utilized in the nutraceutical industry. Globally, the estimated market value of microalgae is US$ 6.5 billion, and US$ 2.5 billion out of the money is generated by the health food sector (Hemantkumar *et al*., 2019).

One of the most widely used species is the salt tolerant *Spirulina* (*Arthrospira platensis* and *Arthrospira maxima*). *Spirulina* cells have a high nutritional value and high digestibility, due to their richness in various nutrients and high protein content (Kwei, 2012). They also exhibit additional health benefits as a source of antioxidants, coenzymes and vitamins (Krishnaraj *et al*., 2012). Marine phytoplankton including cyanobacteria, are a rich source of pigments and carotenoids (Takaichi, 2011). These have been historically used as colouring agents and colour enhancers and are now gaining prominence due to health concerns over the use of synthetic chemical colouring agents.

2.11 Other Metabolites of Marine Origin with Anti-*Trypanosoma* Activity

Bioactive compound of marine origin, previously reported for anti-*Trypanosoma* activity are predominantly from marine sponges and ascidians. Extracts from two marine sponges (*Spongia* sp. and *Ircinia* sp.) yielded a series of linear furanoterpenes and meroterpenes, as well as di- and triterpenes with varying anti-*Trypanosoma* activity when a tested on *T. b. rhodesiense* (Orhan *et al.*, 2010). According to their report, 4-hydroxy-3-tetraprenylphenylacetic acid showed the highest activity with an IC_{50} value of 1.4 μM against *T. b. rhodesiense.* The related structure heptaprenyl-*p*-quinol (having a longer isoprene chain and a hydroquinone terminal unit), 11-β-acetoxyspongi-12-en-16-one (the diterpene) and Demethylfurospongin-4 also demonstrated a promising activity against the parasite (Orhan *et al*., 2010).

Several bioactive molecules with varying degrees of trypanocidal activity have been identified from marine sponges of the genus *Agelas*. The sterol, 24-ethyl-cholest-5α-7-en-3-β-ol obtained from the *n*-hexane extract of *Agelas oroides* exhibited a limited activity against *T.b. rhodesiense*

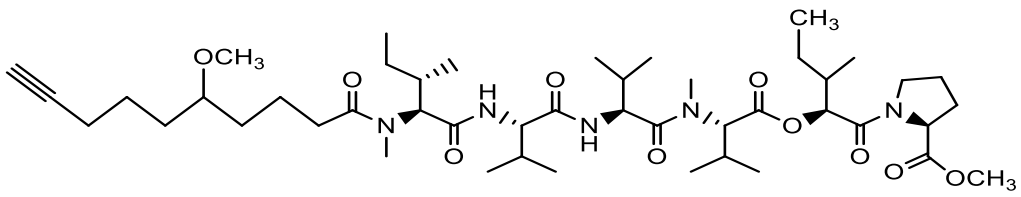
with an IC_{50} value of 34.2 μM (Tesdemir *et al.*, 2007). A series of steroidal saponins isolated from the Caribbean sponge *Pandaros acanthifolium* have shown trypanocidal activity against T. b. *brucei, T. b. rhodesiense* and *T. cruzi* (Regalado *et al*., 2010; Regalado *et al*., 2011). Several other studies have also reported bioactive compounds from marine sponges with trypanocidal activity (Kossuga *et al*., 2008; Feng *et al*., 2010; Pimentel-Elardo *et al*., 2011; Chianese *et al*., 2012). Figure 2.9 shows the structures of these compounds.

Pyridoacridines, ascididemnin and 12-deoxyascididemnin, isolated from an Australian ascidian *Polysyncraton echinatum* also exhibted selective sub-micromolar activity against *T.b. brucei* with IC_{50} values of 0.032 and 0.077 μM (Feng *et al*., 2010). A series of dimethylthio, spiro-pentacyclic and fused penta- and hexacyclic diketopiperazines isolated from the marine-isolated fungus *Aspergillus fumigatus* and *Nectria inventa* also showed varying activity against *T. b. brucei* (Watts *et al*., 2010). Two brominated β-phenyl ethylamine-based alkaloids, identified as convolutamines I and J, were reported from a Tasmanian bryozoan *Amathia tortusa* with IC_{50} values against *T.b. brucei* of 1.1 and 13.7 μM, respectively (Davis *et al*., 2011).

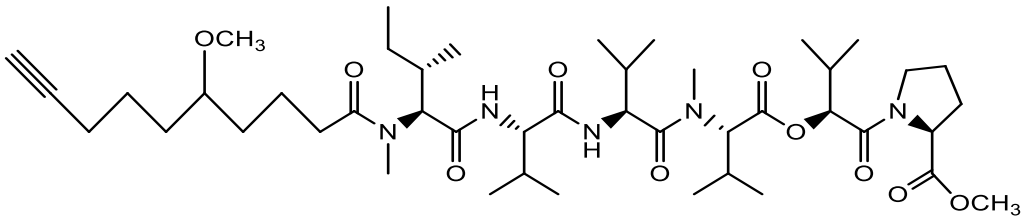
2.12 Extraction of Metabolites from Cyanobacteria

Natural products obtained from microorganisms are limited in their variability and availability, inconsistent product quality, low yield; and their yield/quality depends on the extraction process (Zhang and Furusaki, 1999; Mendiola *et al*., 2007). To optimize product yield from microalgae, an appropriate extraction method is essential; especially to achieve high recovery with low losses. There are several reported methods of extraction, each with its advantages and disadvantages. Generally, an extraction method will be advantageous if it involves a shorter

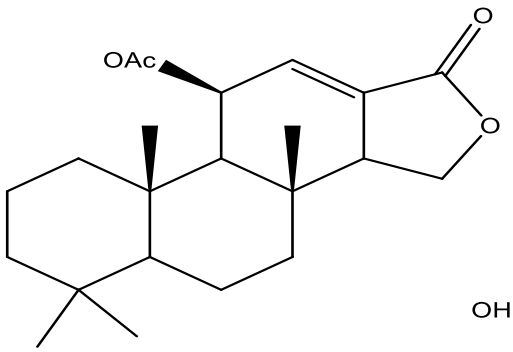
extraction time, less toxic solvent, environmentally friendly and cheap cost of capital as well as operating costs (Kwei, 2012). Reported extraction methods include: Traditional solvent extraction, pressurized liquid extraction, microwave extraction, supercritical fluid extraction, detergent treatment extraction, lysozyme extraction, solid/liquid extraction and freeze-thaw (Jaime *et al*., 2005; Santoyo *et al*., 2009, Kwei, 2012).



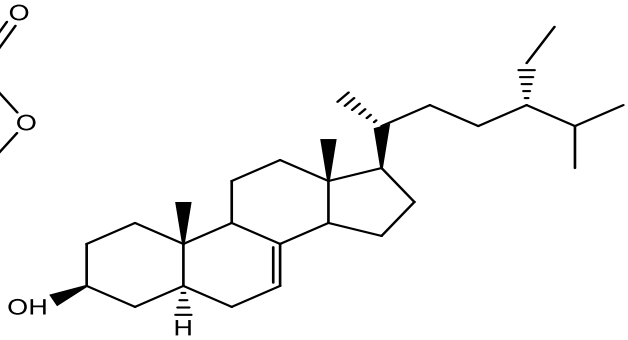
Viridamine A



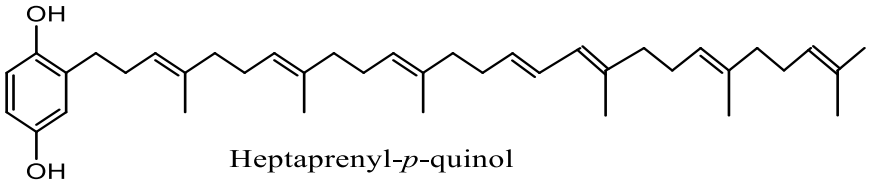
Viridamine B



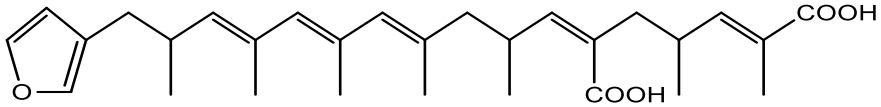
11-β-acetoxyspongi-12-en-16-one



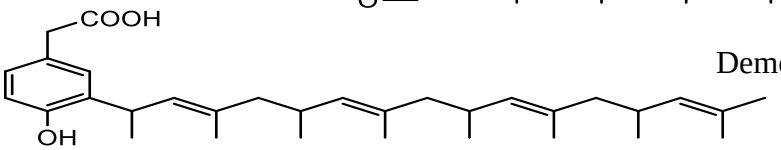
24-ethyl-cholest-5α-7-en-3-β-ol



Heptaprenyl-*p*-quinol



Demethylfurospongin-4



4-hydroxy-3-tetraprenylacetic acid

Fig. 2.8: Structures of metabolites from other marine organisms with antitrypanosomal activity

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

All reagents used were of high analytical grades and were obtained from Sigma (USA). The reagents used for extraction and chromatography included: Methanool, Hexane, Ethyl acetate, silica gel 60-120G, DMSO, capillary tube, glass slide, cover slip, Aluminium-coated TLC plates.The reagents used for molecular aspect of the work were procured from Inqaba Biotech West Africa Ltd. These included: DNA kit, DNA primers, Agarose gel, Loading dye and gel dye (SafeView^{TM}), DNA ladder, proteinase K, TAE buffer, dNTPs, pipette tips, PCR tubes and $MgCl_2$ and Hand gloves.

3.1.2 Cyanobacteria

The cyanobacteria strains; *Microcystis wesenbergii*, *Microcystis flos-aquae* UTEX 2677, *Microcystis aeruginosa* EAWAG 198 and *Oscillatoria* sp. were obtained from the Phycology laboratory, Department of Botany, Ahmadu Bello University, Zaria. The *Oscillatoria* sp was isolated from Samaru stream, Zaria and it is being maintained in the laboratory.

3.1.3 *Trypanosoma brucei brcei*

Trypanosoma brucei brucei was obtained from the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria. The parasite was maintained in the laboratory by continuous passage in rats until when needed.

3.1.4 Experimental Animals

Fifty rats were obtained from the Department of Biological Sciences, Bayero University, and Kano and transported to the Department of Biochemistry animal house, Ahmadu Bello University, Zaria. The animals were provided with food and water *ad libitum*. Ethical clearance was obtained from Ahmadu Bello University Committee for Animal Health and Care (ABUCAUC) with approval number: ABUCAUC/2021/135.

3.2 Methods

3.2.1 Propagation of Trypanosomes in Rats

Parasitized blood from a mouse that was previously infected with *T. b. brucei* was inoculated into rats via intraperitoneal route. Development of parasitemia was confirmed by examination of the rat's tail blood and estimated by the Marching Method (Herbert and Lumsden, 1976). Briefly, this method is carried out by microscopic counting of parasites per field in blood diluted with phosphate buffered saline (PBS, pH 7.2). Logarithm values of these counts are then obtained by matching with the table of Herbert and Lumsden which are converted to antilogs to provide absolute number of trypanosomes per ml of blood. Blood was collected from rats with high parasitemia of about 10^7cells/mL of blood, and the whole blood was used for DNA isolation.

3.2.4 Culturing of Cyanobacteria

The four cyanobacterial cultures (*Microcystis wesenbergii, M. flos-aquae, M. aeruginosa and Oscillatoria* sp.) were obtained from the Phycology laboratory, Department of Botany, ABU, Zaria. The strains were cultured using BG-11 nutrient media as described by Nair and Bhimba

(2013), with slight modifications. Appendix I shows detail composition of the media. For each of the strains, about 20 mL of the cyanobacteria cultures at exponential phase was inoculated into sterile 2000 mL of BG-11 medium in 5000 mL conical flasks and covered with aluminum foil paper. All the samples were incubated in a growth chamber at 25±2 ºC under illumination (1500 lux) using white 40W fluorescent bulbs for 12 hr day/night cycles. The cultures were subjected to constant aeration using an aerator (Shining Beach, SB9905). The flasks were monitored on a regular basis to determine the growth rate; this was done by counting cells using a haemocytometer, and absorbance of the cultures were taken after two days intervals using a spectrophotometer at 750 nm.

The cyanobacteria were harvested at day eighteen post inoculation, corresponding to the late exponential growth phase of the cyanobacteria. The cyanobacteria biomass was separated from the culture medium by centrifugation at 4000 rpm for 10 minutes and their pellets were washed with distilled water to remove salts contained in the media. The pellets were stored in a freezer at -4°C until when needed for further use.

3.2.5 Extraction of *T. b. brucei* and *Oscillatoria* sp. DNA

A whole rat blood parasitized with *T. b. brucei* was used for DNA extraction using a DNA mini kit (Zymogen) according to the manufacturer's instructions. Whole blood was used because the primers used are specific for *Trypanposma* spp so they coud pick the parasites DNA. For the trypanosome, 400 µL of the genomic lysis buffer was added to 100 µl of parasitized rat blood, and mixed completely by vortexing for 4-6 seconds, then allowed to stand for 5-10 minutes at room temperature. The sample was the transferred to the Zymo-Spin™ IICR Column in a collection tube, then centrifuged at 10,000 *x g* for one minute. The flow through was discarded

the Collection. The Zymo-Spin™ IICR column was transferred to a new collection tube and 200 µL of DNA pre-wash buffer was added to the spin column. I was then centrifuged at 10,000 *x g* for one minute. 500 µL of genomic DNA wash buffer was added to the spin column and centrifuge at 10,000 x *g* for one minute. The spin column was transferred to a clean microcentrifuge tube, and 50 µL DNA elution buffer was added. This was incubated for 5 minutes at room temperature and then centrifuged at 10,000 *x g* for 30 seconds to elute the DNA. The eluted DNA was quantified using a Nanodrop.

For the cyanobacteria, the weighed cyanobacteria fresh biomass (20 mg) was put in the ZR BashingBead^{TM} lysis tube, and 750 µL of BashingBead^{TM} buffer was added and the tube was capped tighly. The tube was subjected to shaking using a votexing machine for 10 minutes in order to disrupt the cells. The BashingBead^{TM} lysis tube was centrifuged at 10,000 × g for 1 minute, and then 400 µL of the supernatant was tranfered into a Zymo-Spin filter attached to a collection tube and centrifuged again at 8,000 × g for 1 minute. 1,200 µL of genomic lysis buffer was aadded to the filtrate in the collection tube, and 800 µL of the mixture was tranfered Zymo-Spin column attached to a collection tube and centrifuged at 10,000 × g for 1 minute; this step was repeated once again. About 200 µL of DNA pre-wash buffer was added to the Zymo-Spin column attached to a new collection tube and centrifuged at 10,000 × g for 1 minute. The Zymo-Spin column was then attached to a clean 1.5 mL microcentifuge tube and 100 µL of DNA elution buffer was added to the column matrix and centrifuged at 10,000 × g for 30 seconds. The eluted genomic DNA was quantified using a Nanodrop.

3.2.6 Molecular Identification of *Trypanosoma brucei brucei*

Molecular identification of the *T. b. brucei* was carried out by polymerase chain reaction (PCR), using Internally Transcribed Spacer (ITS-1) primers that targeted the ITS-1 region of rRNA gene in *Trypanosoma* species. The following primers were used for PCR as previously described by Adams *et al.* (2006) for identification of *Trypanonosoma* spp. in cattle in Tanzania : TRYP1:5'-AAGCCAAGTCATCATCCATCG-3'; TRYP2:5'-TAGAGGAAGCAAAAG-3'; TRYP3:5'-TGCAATTATTGGTCGCGC-3'; TRYP4:5'-CTTTGCTGCGTTCCTT-3'. PCR was carried out in a 25 µL final reaction volume containing: 2.5 µL Taq buffer, 0.5 µL of each primer (10 µM concentration) , 1.5 µL $MgCl_2$, 5 µL, 0.5 µL dNTPs and 14 µL nuclease free water to a final volume of 25 µL. Cycling conditions were set as follows: 1 cycle at 95 ºC for 5 minute (initial denaturation), followed by 35 cycles at 94 ºC for 1 minute, 54 ºC for 1 min, 72 ºC for 30 s, and final extension 72 ºC for 10 min. For nested PCR, two consecutive PCR reactions were carried out; a set of outer primers TRYP 3 and TRYP 4 were used in the first-round reaction, followed by the inner primers TRYP 1 and TRYP 2 in the second round. Exactly 0.5 µL of the PCR product from the first reaction was used as template DNA in 25 µL volume of the second-round reaction in a new PCR tube. The cycling conditions applied for nested PCR were the same as the conditions used for the standard PCR reaction. Two PCR samples were prepared; one with a master mix prepared withindividual components and the other one with a ready-made master mix.

The PCR products for both *T. b. brucei* and *Oscillatoria* sp were separated by gel electrophoresis on 1.5 % Agarose gel stained with SafeView^{TM} Classic. To prepare 1.5 % agarose in 50 mL volume, 0.75 g of the agarose was weighed and put in 50 mL TAE buffer. The mixture was

shaken to vigorously to dissolve the agarose and then heated in a microwave for 2 minutes until a clear solution was obtained. Exactly 10 µL of SafeView^{TM} Classic dye was added to the solution and then allowed to cool to 40 ºC. The prepared agarose was cast in the gel tank and allowed to set. To load the PCR amplicons (DNA) samples on the agaros gel, 2 µL of gel loading dye blue was mixed with 10 µL of the PCR amplicons and was transferred into the wells using a pipette. In the first well, 10 µL of the DNA maker (purple100 bp ladder) was loaded. The parasite was not isolated from the infected blood before genomic DNA isolation was done. Therefore, two negative controls were included; the well in lane 3 was loaded with PCR mixture without the template DNA, while lane 4 was loaded with DNA extracted from blood of uninfected rat. The tank was couple to the power pack and the gel electopheresis was rant at 80 V, 200 A for 45 minutes. The gel was removed from the tank and viewed/captured with a gel documentation system.

3.2.7 Molecular Identification of the *Oscillatoria* spp.

Total genomic DNA was isolated from the *Oscillatoria* sp. using a Zymogen mini kit according to manufacturer's instructions. PCR was carried out to amplify the 16S rRNA genes of the cynobacteria as described previously by Nubel *et al*. (1997) using specific primers for cyanobacteria. Forward primer (CYA106F): 5'-CGGACGGGTGAGTAACGCGTGA-3' and reverse primer (CYA781R): 5'-GACTACTGGGGTATCTAATCCCATT-3'. PCR was carried out using a 25µL reaction volume containing: 2.5 µL Taq buffer, 0.5µL of each primer (10µM concentration), 1.5 µL $Mgcl_2$, 5µL, 0.5µL dNTPs and 14µL nuclease free water to a final volume of 25 µL. Cycling conditions were set as follows:1 cycle at 95 ºC for 5 minute (pre-denaturation), followed by 35 cycles at 94 ºC for 1 minute, 60 ºC for 5s, 72 ºC for 1min, and

final extension 72 ºC for 10 min. The PCR products for *T. b. brucei* were also separated by gel electrophoresis on 1.5 % Agarose gel stained with SafeView^{TM} Classic dye as earlier described.

3.2.8 Preparation of Crude Extract

This was done according to Nair and Bhimba (2013), with modifications. A weighed biomass of the pellet was ground in a ceramic mortar with pestle, then transferred into a beaker and 100 mL of methanol per 10 g was added. This was then subjected to shaking on an orbit shaker (Lab-Line instrument, inc.3827-1/34) at 200 rpm for 4 hours and left to stand for 48 hours. The supernatant was sieved through a Whatman No.1 filter paper and the extracts were concentrated (by removing the solvent) at 40 ºC in a water bath. The yield of the extracts were determined and the crude extract were stored a -20 ºC until when needed for further analysis.

3.5 *In vitro* Assay for anti-*Trypanosoma* Activity with Crude Extracts

The extracts obtained was not readily soluble in water, therefore a stock concentration of 20 mg/mL of the different crude extracts was made by dissolving in 2% Dimethyl sulfoxide (DMSO) prepared with phosphate buffered saline (PBS pH 7.2). Serial dilution of this stock solution was done using PBS to obtain extract concentrations of 5, 2.5, 1.25 and 0.625 mg/mL. Assessment of the *in vitro* antitrypanosomal activity was performed in triplicates in 96 wells microtitre plate. In wells of the microtitre plates, 20 µL of each extract concentration was incubated with 40 µL of the infected blood (obtained from a donor rat with about 10^9 *T. b. brucei* cell per mL of blood) at 37 °C. This was done to achieve effective test concentrations of 2.5, 1.25, 0.625 and 0.3125 mg/mL. For control, the 20µL of extract was replaced with the diluent

(2% DMSO in PBS). Parasite count was done on a glass slide (covered with a cover slip) and observed under a microscope at ×400 magnification. The number of motile parasites was counted at 2 hrs after incubation with the extracts. Cessation of motility of parasites in the extracts treated blood compared to that of parasite-loaded control blood without extracts was taken as a measure of trypanocidal activity.

Results of the assay were expressed as percentage survival (% survival), representing the amount of motile parasites observed per microscope field after 2 hrs incubation with the test agents. This was calculated according to the formula:

$$\% \text{ Survival} = \frac{No.\ of\ \ motile\ parasites\ in\ treatment\ wells}{No.\ of\ motile\ parasites\ in\ the\ control\ wells} \times 100$$

3.6 Fractionation of the Crude Extract

Fractionation was carried out as described previously (Ode *et al*., 2011) with modifications as described below. The crude extract of *Microcystis flos-aquae* (1.38 g) was subjected to column chromatography to separate the extract into its component fractions in a column of 16 x 3 cm dimension. Silica gel 60-120G was used as the stationary phase while varying solvent combinations of increasing polarity (Hexane, Ethyl acetate and Methanol) were used as the mobile phase. In the setting up of column chromatography, the lower part of the glass column was stuffed with cotton wool with the aid of glass rod. The slurry prepared by mixing 50 g of silica gel and 100 mL of hexane was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a beaker below. The set-up was considered to be in order when the solvent drained freely without conveying either the silica gel or glass wool into the beaker. At the end of the packing process, the tap was locked, and the

column allowed for 24 h to stabilize, after which, the clear solvent on top of the silica gel was let to drain down to the silica gel meniscus.

The dry packing method was used. Briefly, 5g of the silica gel was mixed with 1.38 g of the crude extract. The dry mixture was then gently layered ontop of the silica gel, and the column tap was opened to allow the eluent to flow at the rate of 40 drops per minute. Elution of the extract was done with solvent systems of gradually increasing polarity using hexane, ethyl acetate and methanol. The following percentage ratios of solvent combinations were sequentially used in the elution process; Hexane: Ethyl acetate, 100:0, 80:20, 60:40, 40:60, and 20:80; Ethyl acetate: methanol 60:40, 50:50; Ethyl acetate: methanol 0:100. The eluted fractions were collected in aliquots of 20 mL in 25 mL glass vials. A total of 57 fractions were collected and analyzed by thin layer chromatography (TLC).

3.6.1 Thin Layer Chromatography (TLC)

The content of each glass vial was spotted on pre-coated (silica gel GF254) Aluminum plates and placed in chromatographic tank containing solvent to separate the different compounds based on their relative mobility in solvent systems and reactions with ultra-violet light. The mass of the different fractions was determined on a weighing scale. For the TLC, pre-coated silica gel (GF254) on aluminum plate was used. With a non-heparin coated (plane) capillary tube, a spot of the sample (2 µL) was applied on the plate about 1.0 cm from the edge. It was dried at room temperature, and the strip was lowered into a small chromatographic jar containing the solvent system; Hexane: Ethyl acetate, 7:3. The jar was covered with a glass lid, and the solvent allowed ascending until the solvent front was about three-quarter of the length of the strip. The strip was removed and dried by a hot air dryer and viewed under U.V lamp at 365nm. The 57 fractions

were pooled based on the TLC results to obtain 6 fractions. To pool the fractions, 2 mL of methanol was used to reconstitute each of the concentrated fractions. The resultant solutions were then merged accordingly, and the solvent (methanol) evaporated at room temperature. The dried fractions were weight using a sensitive digital scale and store at –4 °C until when need for further analysis.

Retardation factor (R_f) was calculated using the equation below:

$$R_f = \frac{Distance\ travelled\ by\ the\ compound}{Distance\ travelled\ by\ the\ solvent}$$

3.7 *In Vitro* Assay for anti-*Trypanosoma* Activity with *M. flos-aquae* fractions

The *in vivo* antitrypanosomal activity of the various fractions obtained was tested on *T. b. brucei* in 96 wells titer plates as ealier described for the crude extract, but with three concentrations (1.25, 0.625 & 0.3125 mg/L). The fractions obtained were also not readily soluble in water, therefore stock solutions were prepared by dissolving them dissolved in 2% DMSO in phosphate buffered saline (PBS pH 7.2). Parasite count was alsodone at 2 hrsincubation with the fractions.

3.7.1 Sub-fractionation of Active Fractions

The active fractions were further subjected to fractionation using column chromatography and thin layer chromatography as earlier described. However, wet packing method was used in this case due to the limited amount of the starting material (fractions). Here, a 10 cm × 2 cm column was packed. Briefly, 2 mL of methanol was used to dissolve each fraction: fraction B (60 mg), fraction E and F (40 mg). Each of the solutions was added to 2 g silica gel and mixed thoroughly before allowing them to dry. The mixture was then loaded on a stacked column containing 20 g

silica gel. The eluent were collected at 50 drops/min speed in 10 mL for each fraction using methanol, hexane and ethyl acetate by varying the solvent combination. For fraction B, Hexane: Ethyl acetate (2:3) was used to elute 9 fractions, and 100% methanol was then used to elute the last fraction. While for fraction E, ethyl acetate: methanol (1:1) was used to elute 9 fractions and the last fraction eluted with 100% methanol. This solvent combination was used based on the results from the first fractionation. A total of thirty fractions were obtained (ten from each fraction), sub-fractions of B named B_1-B_{10}, E named E_1-E_{10} and F named F_1-F_{10}. All the subfractions were then tested for *in vitro* antitrypanosomal activity in a 96 wells titer plate as ealier described for the crude extract, but at 0.625 mg/mL.

3.8 Characterization of the Most Active Fractions

The most potent fraction (**E**) and sub-fraction (**E2**) were further analyzed for detection of the bioactive compounds using Gas chromatography/ Mass spectrometry (GC/MS). GC was carried out using a GC/MS equipped with a HP-5ms Ultra Inert MA capillary column of 30 m x 250 μm x 0.25 μm in dimension (Agilent Technologies 19091S-433UI, USA). The GC operating conditions were as follows: the temperature was held at 50 ºC for 1 min, and increased from 50 ºC to 200 ºC at a rate of 3 ºC/min and then with final isothermal hold at 300 ºC at a rate of 3 ºC/min for 15 min, with final equilibration held at 350 ºC for 1 min. Helium was the carrier gas used, and 3 μL of the sample was injected in the split mode with the injector temperature at 250 ºC and pressure at 4.4867 psi. Interpretation of the mass spectroscopy was carried out using database of the National Institute Standard and Technology (NIST) which has more than 62000 patterns. The spectrum of the unknown component was compared with the spectrum of the known compounds stored in the NIST library.

3.9 *In vivo* Antitrypanosomal Activity of Most Active Crude Extract

3.9.1 Determination of Acute Toxicity

The acute toxicity test (LD_{50}) of *M. flos-aquae* crude extract was carried out according to the method of Lorke (1983). This method involved two treatment phases. Phase 1 required nine animals. The nine rats with weight in the range of 70-80 g were divided into three groups of three animals each. Each group of the animals was administered 10, 100 and 1000 mg/kg body weight doses of the crude extract orally. The animals were placed under observation for 24 hours to monitor their behavior as well as mortality. Phase 2 involved the use of three animals, which were distributed into three groups of one rat each. The rats were administered higher doses (1600, 2900 and 5000 mg/kg) of the crude extract and then observed for 24 hours for their behavior as well as mortality.

The LD_{50} value was calculated using the equation:

$$LD_{50} = \sqrt{(D0 \times D100)}$$

Where D_0 = highest dose that gave no mortality

D_{100} = lowest dose that produced no mortality

3.9.2 *In vivo* Antitrypanosomal Assay

A total of twenty four (24) rats were used for the *in vivo* assay, and were divided into six groups of 4 rats each. Rats in the infected groups were infected intraperitoneally with blood parasitized with *T. b. brucei* (10^4 cells/mL of blood), and the level of parasitemia was monitored daily by the method of Herbert and Lumsden (1976). At the fourth day post-infection, the animals were treated accordingly for six (6) consecutive days as follows:

Group I: treated with 30 mg/kg body weight of the extract

Group II: treated with 60 mg/kg b.w of the extract

Group III: treated with 120 mg/kg b.w of the extract

Group 1V: infected and treated Diminazene aceturate (3.5 mg/kg b.w)

Group V: infected but not treated (administered 2 % DMSO used as extract diluent)

Group VI: not infected and untreated (normal control).

The extract was administered orally once in a day, while 3.5 mg/kg b.w Diminazene aceturate (Sequzene®) was administered intraperitoneally.

3.9.2 Determination of Parasitemia and Weight of rats

Blood films were collected from the caudal vein of the rats after sterilization of the tail with methylated spirit. Trypanosome count was determined by examination of the wet mount on the microscope at ×400 magnification using the rapid matching method (Herbert and Lumsden ,1976). The weight of the animals was taken and recorded after two days intervals using a weighing scale

3.9.3 Determination of Packed Cell Volume (PCV)

Packed Cell Volume (PCV) was determined at the first and last days of the experiment using haematocrit method as described by Bain *et al*. (2016). For the initial PCV, two third of a heparin coated capillary tube was filled with blood obtained from the tail of the animals. One end of the tube was sealed with crista seal, and the capillary tube was then subjected to spinning at 10,000 rpm for 5 minutes in a microhaematocrit centrifuge. The percentage of packed red blood cells was estimated using a microhaematocrit reader. For determination of terminal PCV, the animals

were sacrificed at day eleven of the experiment and blood collected in heparin coated tubes, and then loaded in the capillary tubes for centrifugation.

3.10 STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 8.0.2. One way analysis of variance (ANOVA) was used to compare the mean values at 95% confidence interval. T-test was used to compare the means of initial and terminal PCV.

CHAPTER FOUR

4.0 RESULTS

4.1. Molecular Identification of the *T. b. brucei*

The PCR analysis yielded an amplicon of approximately 430 bp in size on lane 1 of the 1.5 % agarose gel when estimated with the 50 bp DNA ladder (Plate I). Lane 2 which was the sample prepared with a ready-made PCR master mix did not amplify the targeted DNA. The negative controls (Lanes 3 & 4) did not show any band on the agarose gel. The PCR amplicon size observed in lane 1 corresponds to the expected DNA band size for *T.b. brucei*. ITS-1 region of trypanosomes rRNA is of variable length in the known trypanosome species. Hence, it is a good target for molecular identification of species in the genus *Trypanosoma*.

4.2 Microscopic and Molecular Characterization of the Isolated Cyanobacteria

Microscopically, the indigenous cyanobacterial isolate was identified as *Oscillatoria* sp. Microscopic examination of *M. flos aquae*, *M. wesenbergii* and *M. aeruginosa*, which have already been characterized also confirmed that they were the right species been cultured (Plate II).The 16S rRNA of the *Oscillatoria* sp was targeted using generic primers. The PCR of each run yielded a single DNA band on the agarose gel. The size of the PCR amplicon of approximately 700 bp was estimated when the cyanobacterial DNA was compared with a 100 bp DNA ladder (Plate III).

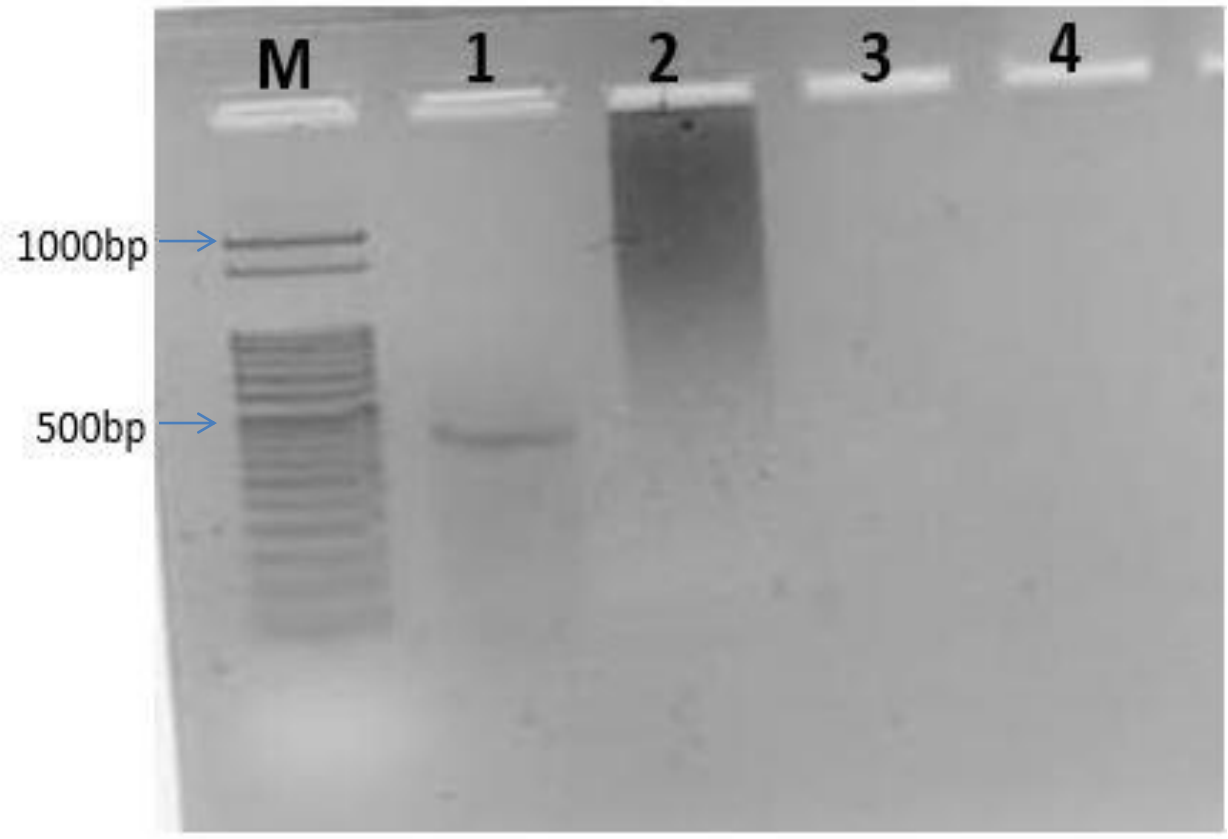


Plate I: A 1.5 % Agarose gel electrophoregram of the amplified ITS-1 region of *T. b. brucei* Marker (**M**) 50 bp, Lane 1: a DNA band corresponding to *Trypanosoma brucei brucei*. Lane 2: smear obtained from positive sample with a ready-made master mix. Lane 3: loaded with PCR mixture without trpanosome's template DNA. Lane 4: loaded with DNA from unparasitized rat.

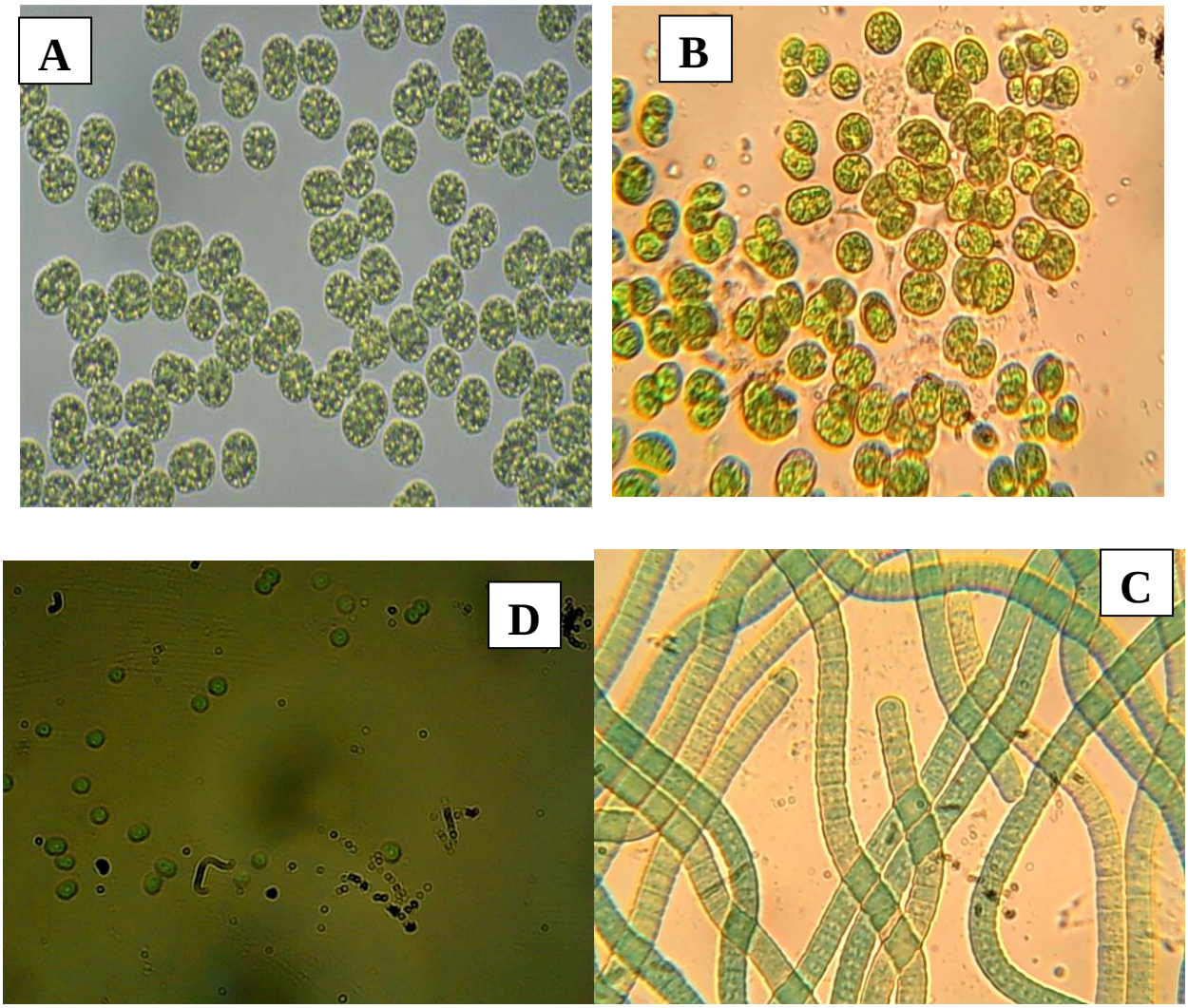


Plate II: Photomicrographs of the screened cyanobacteria; *Microcystis aeruginosa* 1000 ×(**A**), *Microcystis wesenbergii* 400× (**B**), *Oscilllatoria* sp 400 ×(**C**) and *Microcystis flos-aquae* 400 ×(**D**)

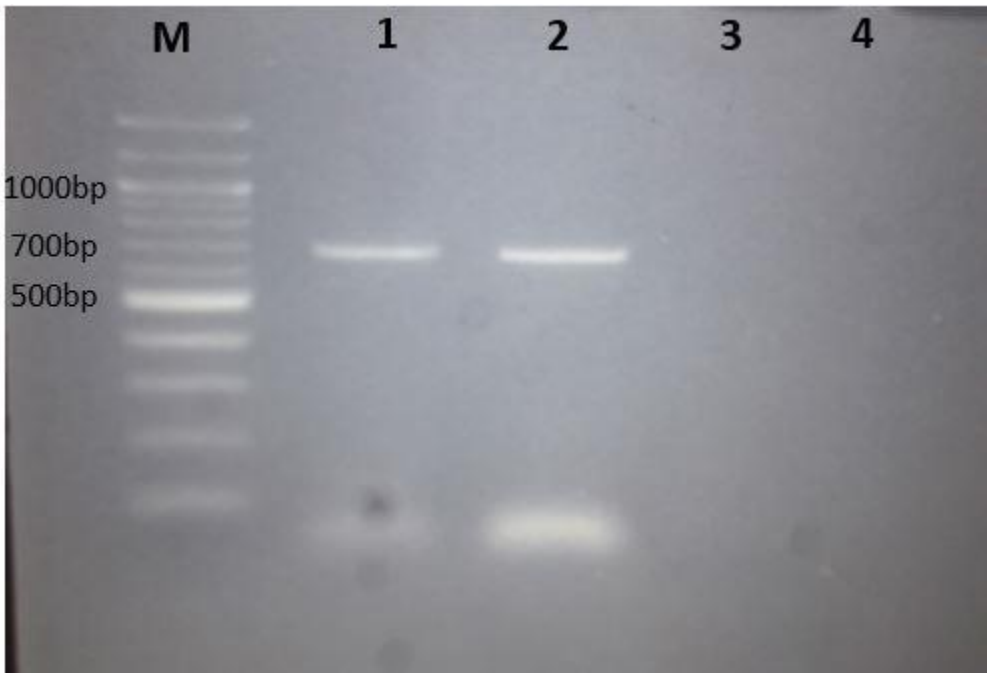


Plate III: A 1.5 % Agarose gel electrophoregram of the amplified 16S rDNA region of the *Oscillatoria* sp. Marker (M) of 100 bp was used, Lanes 1and 2: an approximately 700 bp amplicon size. Lanes 3 and 4: Negative control (PCR reaction mixture without template DNA).

4.3. Growth Pattern of the Colony Forming Cyanobacteria

Growth stage determines the type and concentration of metabolites present in microorganisms, including cyanobacteria. The growth pattern of the colony-forming cyanobacteria was therefore monitored for the 18 days of culturing. Result obtained show that there was no significant difference ($P > 0.05$) in the growth rates of the species (Figure 4.1). However, *M. flos-aquae* and *M. wesenbergii* were at late exponential phase, while *M. aeruginosa* was at the early stationary phase at the time harvested.

4.4. Percentage Yields of the Crude Methanol Extract

The cyanobacterial biomass was harvested by centrifugation and extracted using absolute methanol. Results of the biomass harvested and the crude methanol extract yield is shown on Table 4.1. From the results obtained, *M. wesenbergii* yielded dry 14.5g biomass, *M. flos-aquae* yielded 54.9 g, *M. aeruginosa* yielded 16.2g and *Oscillatoria s*p. yielded 10.0 g biomass. When solvent extraction was carried out methanol, 0.30 g (2.07 %) crude extract of *M. wesenbergii* was ontained, *M. flos-aquae* yielded 3.68g (6.70 %), *M. aeruginosa* yielded 0.55 g (3.39 %) and *Oscillatoria* sp. yielded 0.15 g (1.5 %) crude extract. These cryde extract obtained were used for the *in vitro* studies. About 4 g of *M. flos-aquae* extract was further obtained and used for *in vivo* studies.

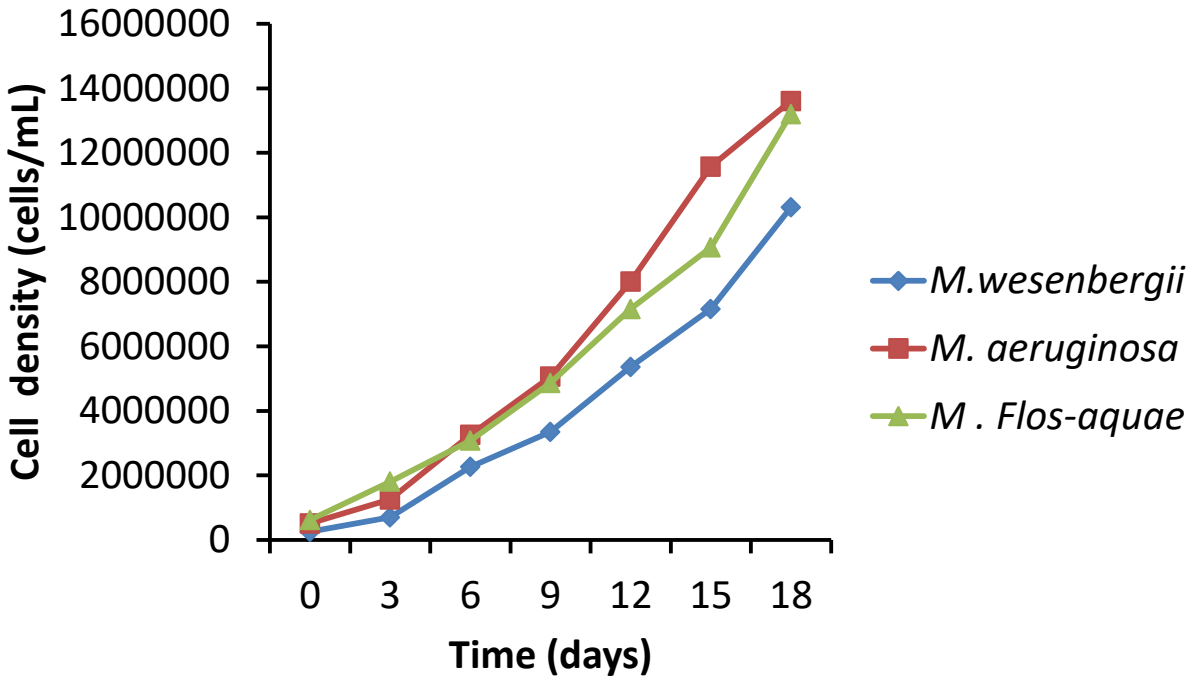


Fig 4.1: Growth pattern of the unicellular cyanobacteria cultured for a period of 18 days in BG-11 media. Results are mean ± SD of triplicate determinations.

Table 4.1: Biomass and crude cyanobacterial extracts yield of the cyanobacteria used for *in vitro* assays

S/N	Cyanobacteria species	Weight of biomass (g)	Weight of extract (g)	Extract yield (%)
1.	*M. wesenbergii*	14.5	0.30	2.07
2.	*M. flos-aquae*	54.9	3.68	6.70
3.	*M.aeruginosa*	16.2	0.55	3.39
4.	*Oscillatoria* sp.	10.0	0.15	1.50

4.5. *In vitro* Antitrypanosomal Activity of the Cyanobacterial Crude Extracts on *T.b brucei*

Results of the *in vitro* antitrypanosomal assay carried out on 96 wells titer plate in triplicate is shown on Figure (4.2). From the result, mean parasite suvival of 1.56, 12.5, 36.72 and 57.82 % when *T. b brucei* were incubated with 2.5, 1.25, 0.625 and 0.3125 mg/mL respectively of *M. flos-quae* crude extract for 2 hours was observed. Results also showed 10.9, 26.57, 55.47 and 89.06 % survival when incubated with 2.5, 1.25, 0.625 and 0.3125 mg/mL respectively of *M. wesenbergii* for 2hrs. Figure 4.2 also show 8.59, 26.56, 45.32 and 84.38 % survival when parasites were incubated with 2.5, 1.25, 0.625 and 0.3125 mg/mL respectively of *Oscillatoria* sp., while incubation with the same concentrations of *M. aeruginosa* for 2 hrs gave 39.06, 76.57, 87.50 and 99.22 % parasite survival respectively. Diminazene aceturate was used as a standard drug to compare its activity with the extracts. An average of 7.82 % survival was observed when incubated with 0.3125 mg/mL of the diminazene aceturate, while higher concentrations gave 100 % inhibition. On theother hand, 100 % survival was observed in the wells without treatment (control). Crude extract of *M. flos-aquae* exhibited the highest *in vitro* antitrypanosomal activity with IC_{50} value of 0.4140 mg/mL (Figure 4.3)

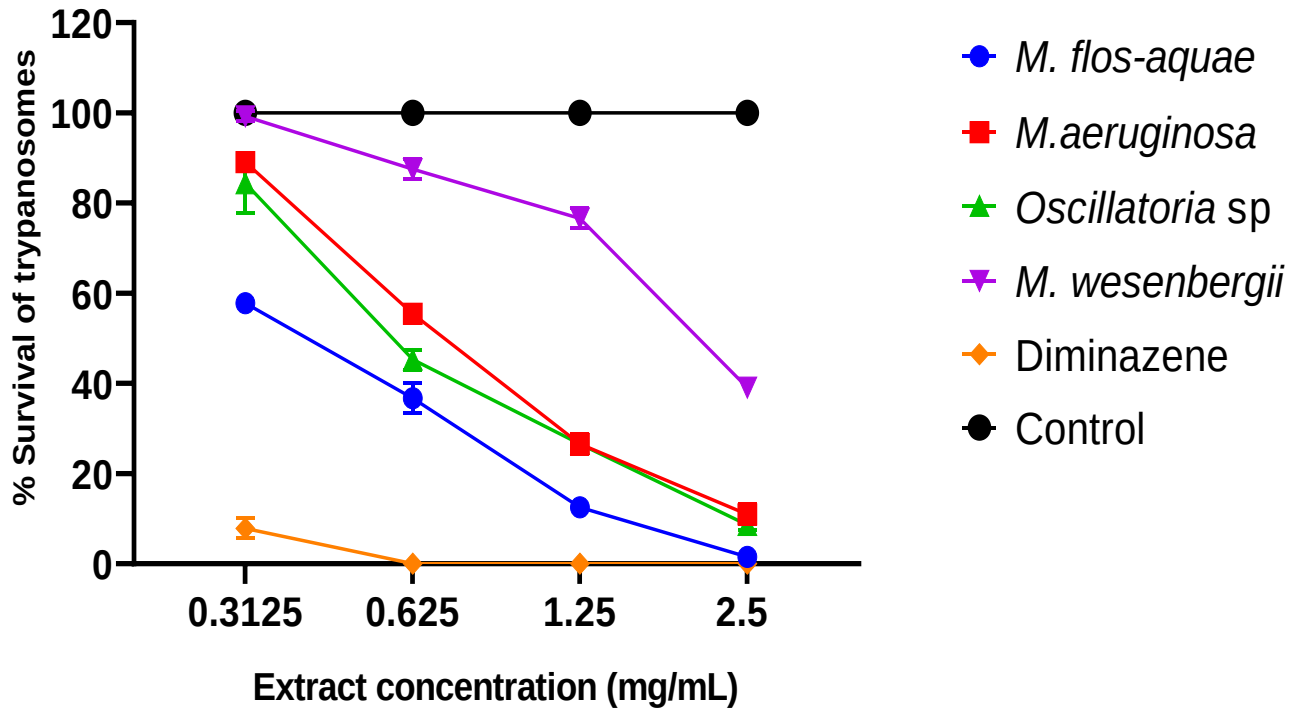


Fig.4.2: Effect of crude methanol cyanobacterial extracts on *T. b. brucei in vitro*. Results are mean ± SD of triplicate determinations. The control (negative) experiments were set up by substituting PBS for the extracts, while the standard drug Diminazene aceturate was used as a positive control and for comparing the effects of the extracts on the parasite cells.

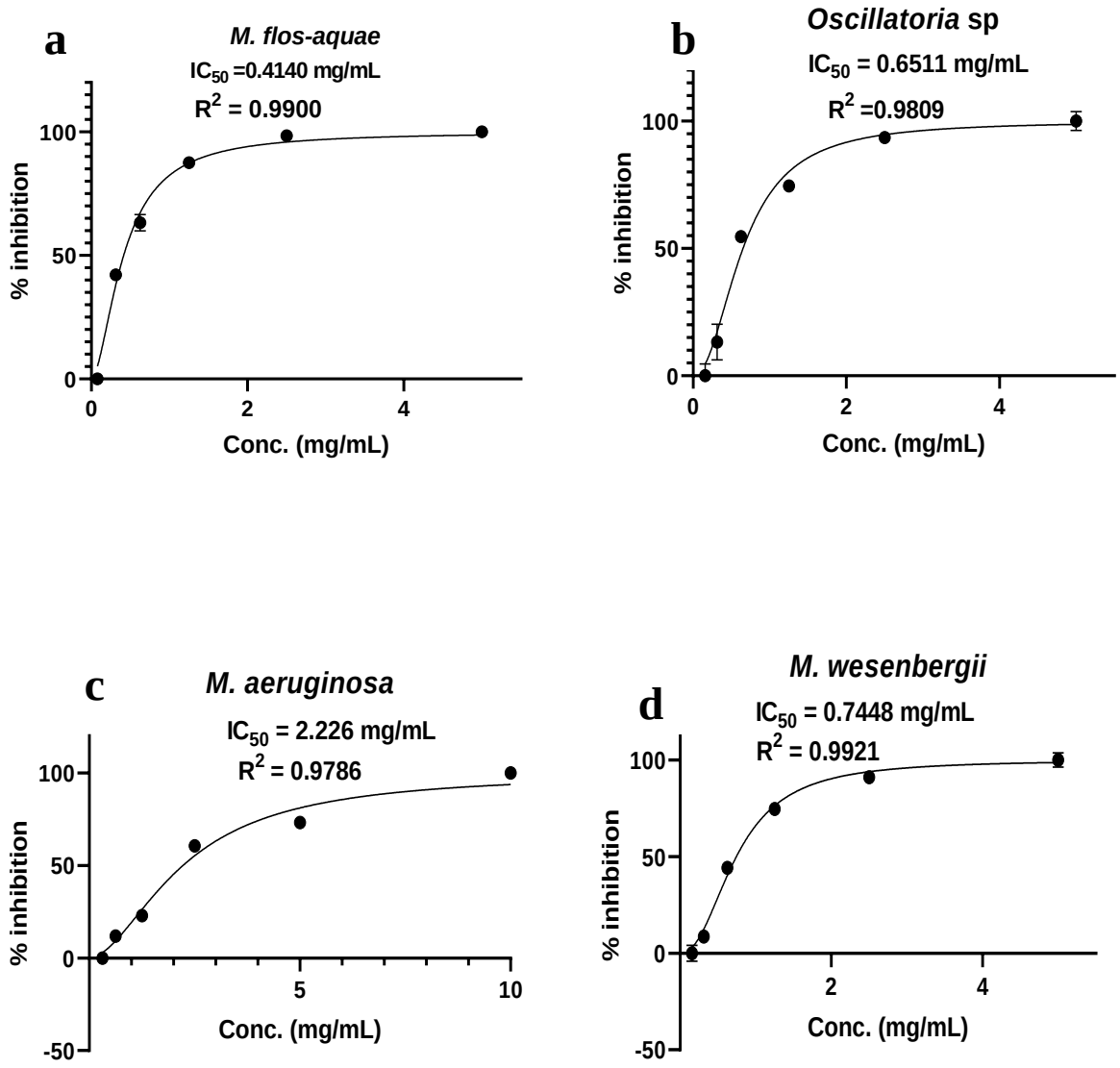


Fig.4.3: Plots for IC_{50} determination of all the cyanobacterial crude extracts at 2 hrs incubation with *T.b. brucei*. *M. flos-aquae* (**a**), *Oscillatoria* sp. (**b**), *M. aeruginosa* (**c**) and *M. wesenbergii* (**d**). The IC_{50} were calculated usig Graphpad Prism software version 8.02.

4.6. Thin Layer Chromatography of the Pooled Microcystis *flos-aquae* Fractions

Crude methanol extract of *M. flos-aquae* exhibited the highest antitrypanosomal activity. Therefore, it was subjected to fractionation. A total of 57 fractions were eluted in glass vials, each of the fraction was concentrated by evaporating the solvents. The 57 fractions were pooled into 6 fractions (Plate IV) named **A-F** based on their R_f values as determined by TLC. Fraction **A** is a sum of 11 fractions (1-11); fraction **B** is a sum of 12 fractions (12- 23); fraction **C** is a sum of 7 fractions (24-30); fraction **D** is a sum of 13 fractions (31- 43); fraction E is a sum of 7 fractions (44-50), and fraction **F** also a sum of 7 (51-57). Fraction **B**, **E** and **F** showed prominent spots with R_f values of 0.321, 0.512, 0.877. The chromatogram shown on Plates IV and VI were captured under UV lamp.

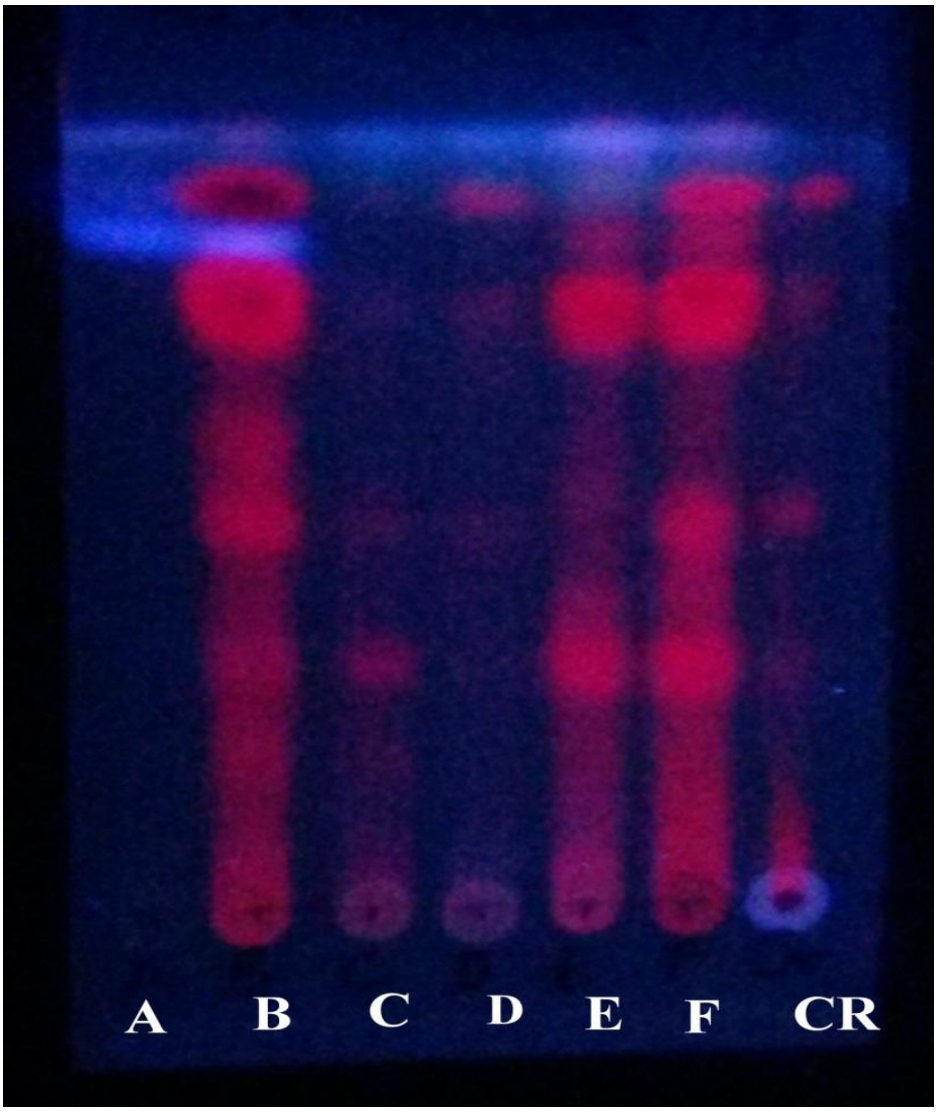


Plate IV: TLC chromatogram of the *M.flos-aquae* pooled fractions. About 2 µL of samples was spotted on the TLC plate.

NB: **A-F** represnts all the six pooled fractions, **CR**= the main crude extract

4.6.1 *In vitro* Antitrypanosomal Activity of *Microcystis flos-aquae* Fractions on *T. b. brucei*

It was observed that Fractions **B**, **E** and **F** had varying degrees of activity in a dosage-dependent manner, while fraction **A**, **C** and **D** did not show any activity (Figure 4.4). Mean parasite survival of 3.91, 25.79 and 59.25 % was observed when incubated with 1.25, 0.625 and 0.3125 mg/mL respectively of fraction **B** for 2 hours. Results also showed 3.13, 25.79 and 49.22 % survival when incubated with 1.25, 0.625 and 0.3125 mg/mL respectively of fraction **E**. As also shown in Figure 4.4, 20.32, 39.06, and 62.51% survival was observed when incubated with 1.25, 0.625 and 0.3125 mg/mL respectively fraction **F**. There was no significant diference ($P > 0.05$) in the level of activity of the fractions. An average of 0, 0 and 7.82 % survival were observed when incubated with 1.25, 0.625 and 0.3125 mg/mL respectively of Diminazene aceturate. The percentage survival recorded in control wells was 100%. Parasites were still motile in the wells without treatment and inactive fractions (**A**, **C**& **D**), while immobilized parasites were observed under the microscope in wells with the active fractions after 2 hrs incubation (Plate V). Fraction **E** exhibited the highest *in vitro* antitrypanosomal activity with IC_{50} value of 0.2991 mg/mL (Figure 4.5).

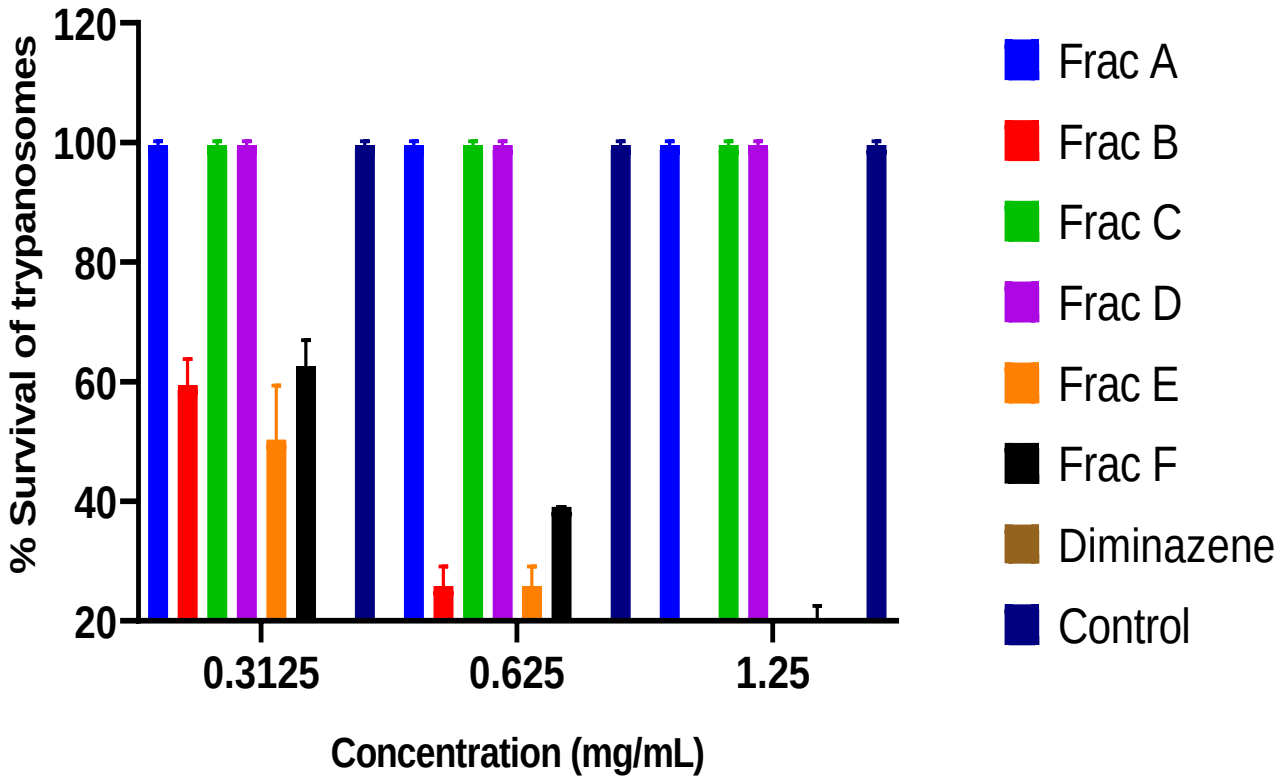


Fig.4.4: *In vitro* antitrypanosomal activity of *Microcystis flos-aquae* pooled fractions on *T. b. brucei*. Results are mean ± SD of triplicate determinations. The control (negative) experiments were set up by substituting PBS for the extracts, while the standard drug Diminazene aceturate was used as a positive control to compare the effects of the fractions on the parasite.

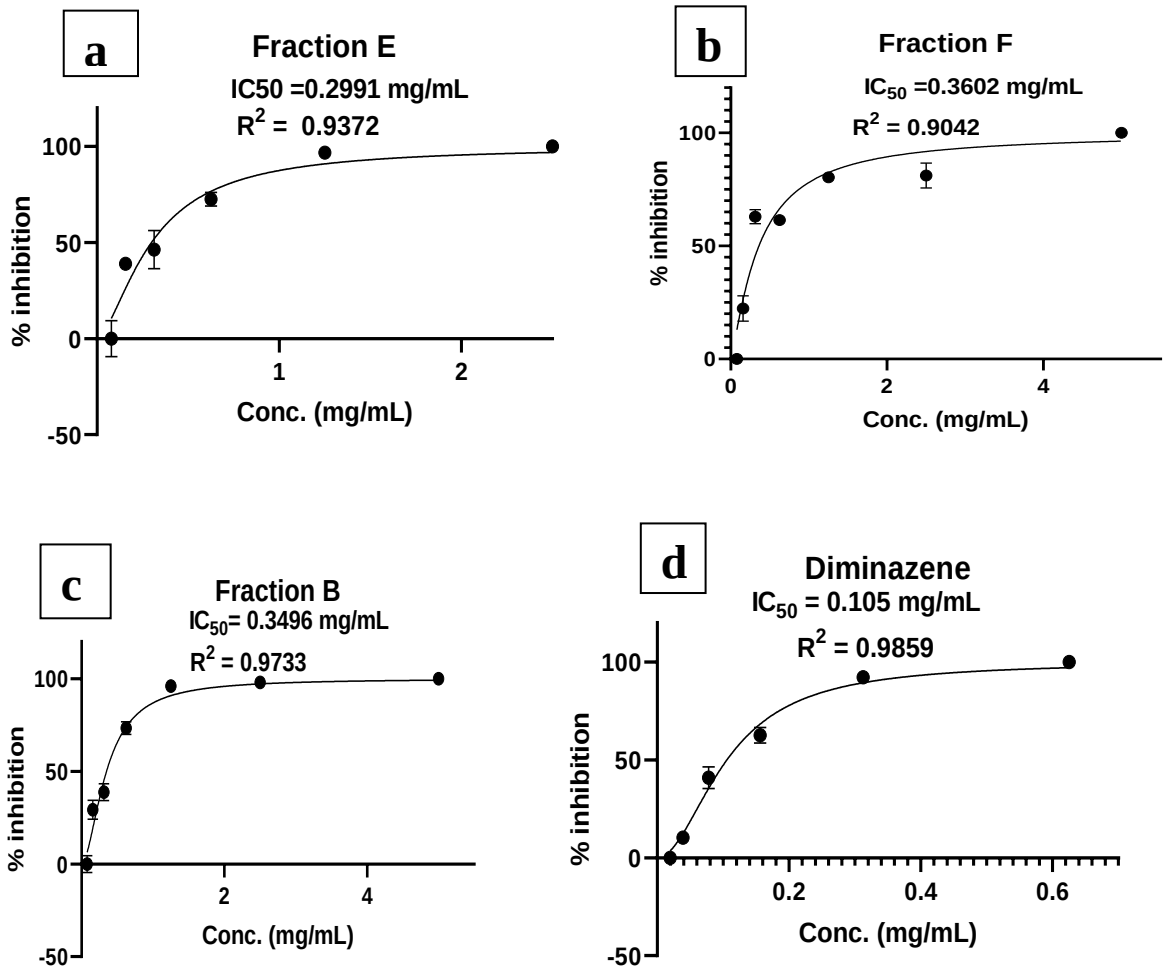
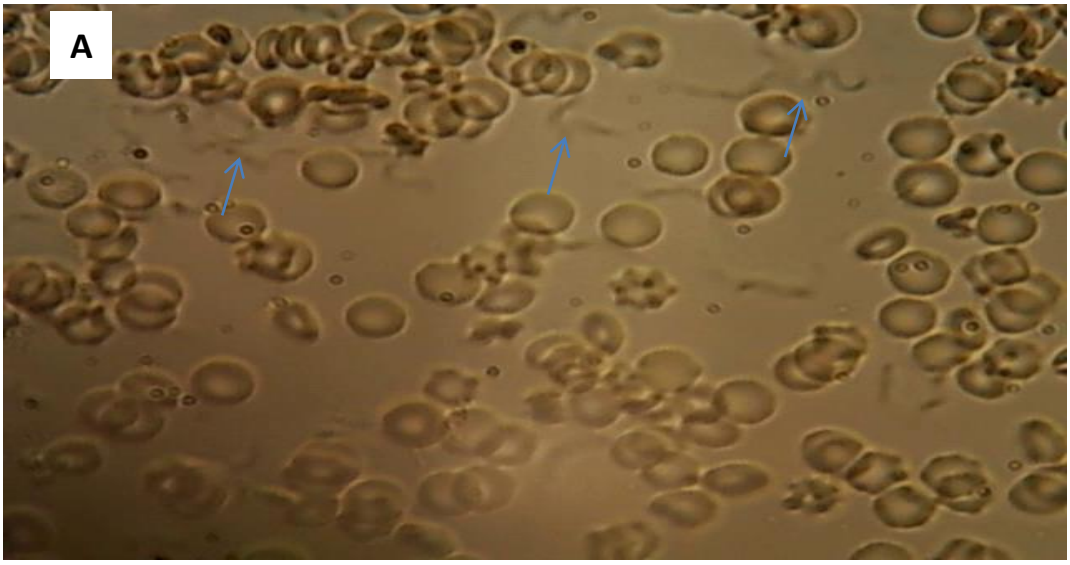


Fig.4.5: Plots for IC_{50} determination at 2 hrs incubation of *T.b. brucei* with the active fractions. Fraction E (**a**), fraction F(**b**), fraction B (**c**) and Diminazene aceturate (**d**). The IC_{50} were calculated usig Graphpad Prism sofware version 8.02.



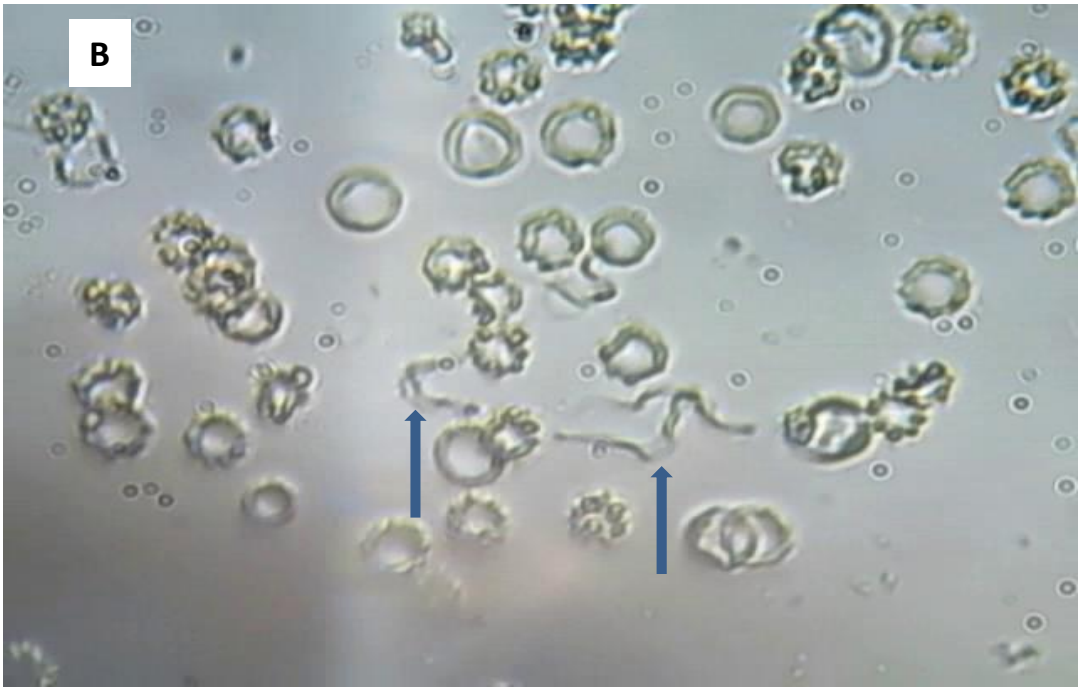


Plate V: Photomicrographs of trypanosomes (400 ×) in blood smears after *in vitro* treatments; **(A)** without extract and **(B)** with 1.25 mg/mL of fraction **E.**

NB: Motile parasites were seen as indicated by thin arrows, while immobilized parasites with distorted morphology were seen indicated by the block arrows.

4.7 Thin Layer Chromatography of the *Microcystis flos-aquae* Sub-fractions

The three active *M. flos-aquae* fractions (B, E &F) were further subjected to fractionation. Ten sub-fractions were obtained from each fraction (Plate VI), making a total of 30 fractions. Spots were observed in sub-fractions 1-5 of fraction B, sub-fractions 1-3 of fraction E and sub-fractions 1and 2 of fraction F. These spots corresponded to the spots on the main fractions (Bf, Ef & Ff) as seen in Plate VI.

4.7.1. *In vitro* Antitrypanosomal Activity of Sub-fraction from *M. flos-aquae*

Results of the *in vitro* antitrypanosomal activity with subfraction are presented on Figure 4.6. Results obtained showed varying degrees of activity. Subfraction E2 exhibited the highest antitrypanosomal activity with percentage inhibition of 76 % (24 % survival) at concentration of 0.625 mg/mL, while some of the subfractions showed mild activity against the parasite at the same concentration (Figure. 4.6). The first three collected subfractions from fraction B & F also showed varying degrees of activities at the same concentration. Subfractions eluted after the first 5 out of the 10 from each parent fraction showed less activity at the same concentration (0.625 mg/mL).

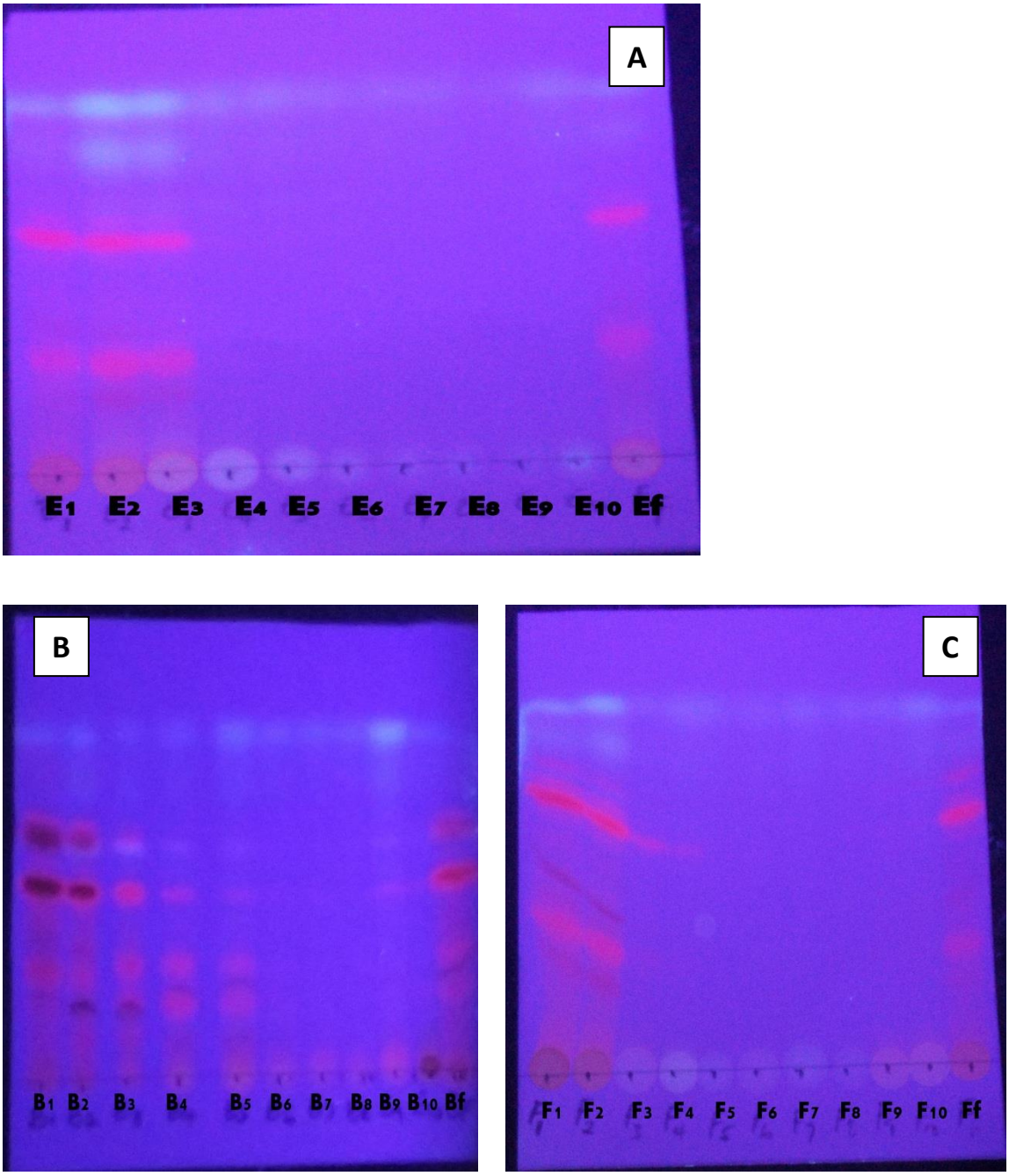


Plate VI: TLC chromatogram of the *M.flos-aquae* sub-fractions; fraction E (**A**), fraction B (**B**) and fraction F (**C**). 2 µL of the samples was spotted on the TLC plate.

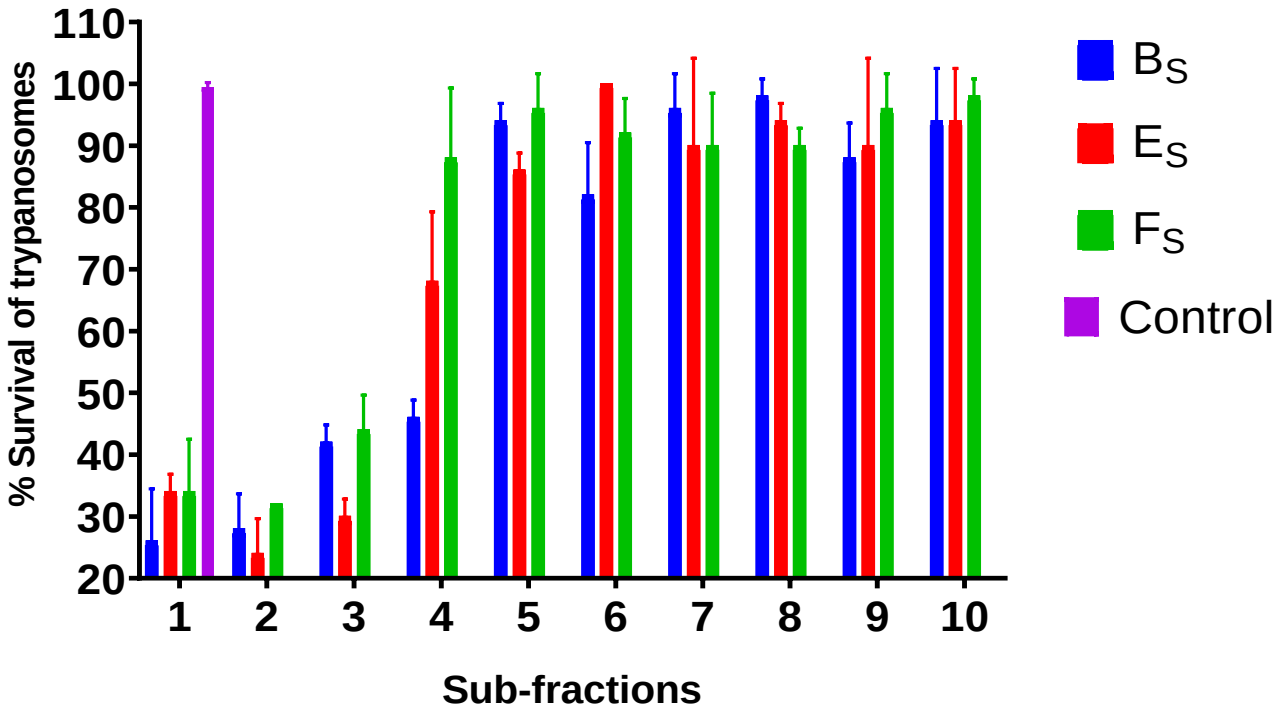
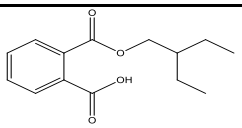
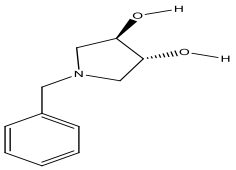
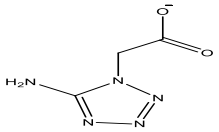
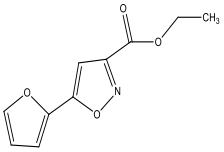
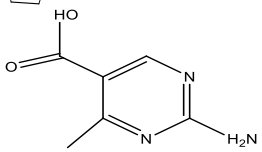
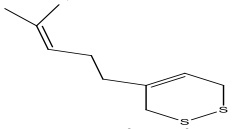
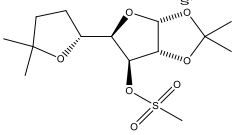
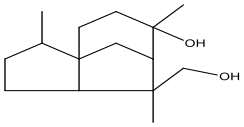
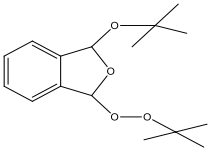


Fig. 4.6: Effect of *M. flos-aquae* sub-fractions all on *T. b. brucei in vitro.* Results are mean ± SD of triplicate determinations at concentration of 0.625 mg/mL. The control (negative) experiments were set up by substituting PBS for the fractions.**B_S**, **E_S** and **Fs** means subfractions of the corresponding parent fractions **B**, **E** and **F.**

4.8. Detection of Compounds in *Microcystis flos-aquae* Most Active Fractions by GC-MS

Identification of compounds present in the most active fractions (Fraction **E** and sub-fraction **E2**) was carried out by GC-MS analysis. Compounds such as Ethyl 5-(furan-2-yl)-1,2-oxazole-3-carboxylate,5-amino-1-tetrazolyl acetic acid, 1-Benzyl-pyrrolidin-3,4-diol, cedran-diol, 2-amino-4-(2-methylpropanyl)-pyrimidin-5-carboxylic acid, and others were identified in fraction E (Table 4.8). 2-[(2-Ethoxybutoxy) carbonyl] benzoic acid had the highest abundance with peak area of 9.38 %. GC-MS profile of the most active subfraction (E_2) also suggested compounds present in the parent fraction. These compounds have been marked with asterisk (**) (Table 4.2). The GC-MS spectrum of the fraction is shown in Figure 4.7.

Table 4.2: List of compounds identified in fraction E of *M. flos-aquae* by GC/MS

S/N	COMPOUND	PEAK AREA (%)	CAS	STRUCTURE
1	2-((2-Ethylbutoxy) carbonyl) benzoic acid **	9.38	091401-46-6	
2	1-Benzyl-pyrrolidin-3,4-diol **	1.74	000645-88-5	
3	5-amino-1-tetrazolyl acetic acid **	2.41	1000214-57-5	
4	Ethyl5-(furan-2-yl)-1,2-oxazole-3-carboxylate **	2.66	1000411-40-9	
5	2-amino-4-(2-methylpropenyl)-pyrimidin-5-carboxylic acid **	2.66	1000411-12-4	
6	4-(4-methylpent-3-enyl)-3,6-dihydro-1,2-dithiin **	1.54	073188-23-5	
7	1,2:5,6-Di-O-isopropylidene-3-O-methanesulfonyl glucofuranose	1.54	1000214-57-5	
8	1-Heptadecanamine	0.03	1000130-89-5	
9	Cedran-diol	1.27	1000224-67-5	
10	1,3-Bis-t-butylperoxy-phthalan	1.26	097526-35-7	

NB: ** = compounds also detected in the sub-fraction (**E2**)

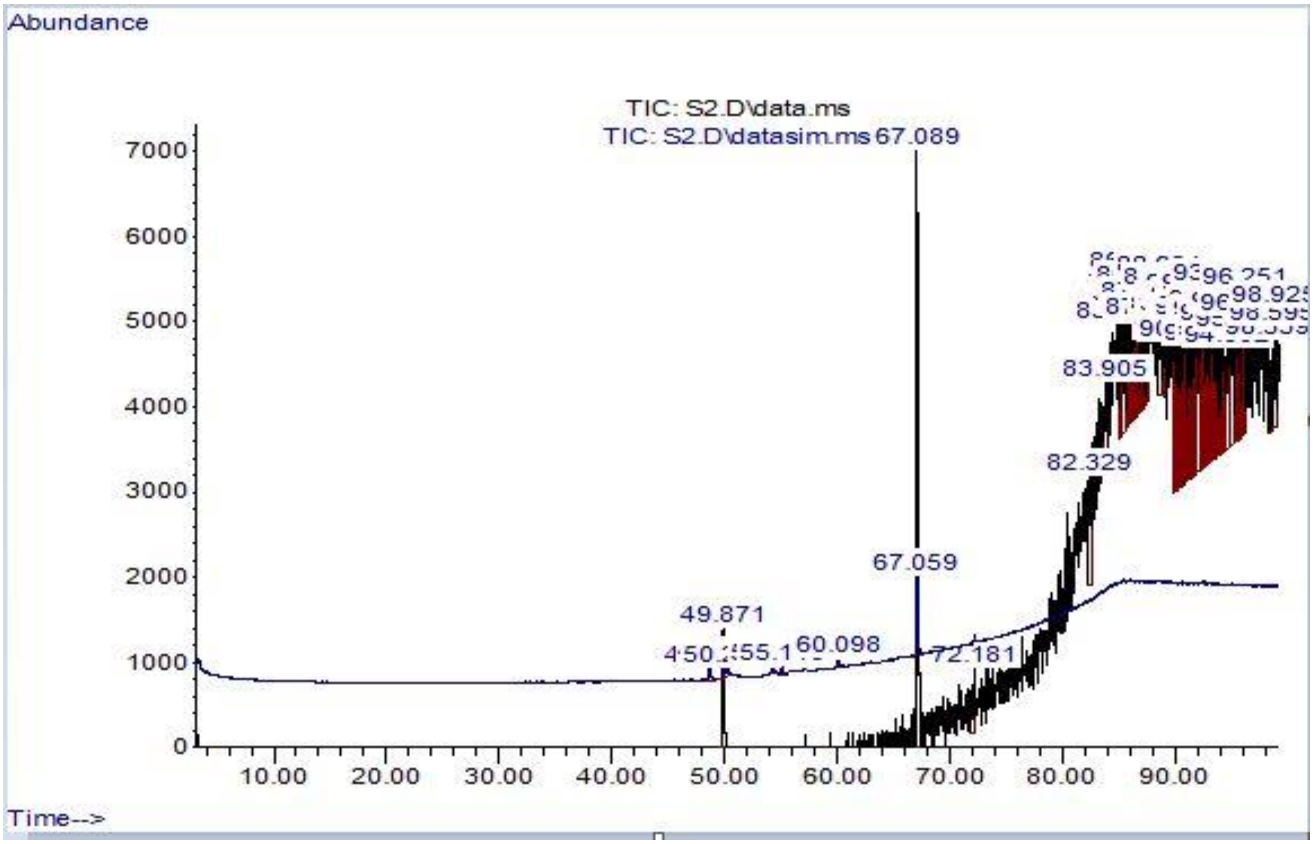


Fig.4.7: GC/MS spectrum of fraction E obtained from *M. flos-aquae* crude extract

4.9 *In vivo* Antitrypanosomal Activity of the Most Active Crude Extract (*M. flos-aquae*)

4.9.1 Acute Toxicity of the Extract

The result of acute toxicity showed that dosages ranging between 10- 1600 mg/kg body weight did not cause mortality in the animals (Table 4.3). Dosages at 2900 and 5000 mg/kg body weight produced 100 % mortality. The calculated LD_{50} was 2,154.065 mg/kg body weight.

4.9.2 Effect of Treatment with Extract on Parasitemia

Treatment of *T. b. brucei*-infected rats with three dosages of *M. flos-aquae* crude methanol extract showed suppression of parasitemia in a dosage- and time-dependent manner (Figure 4.9). Concentration of 120 mg/kg body weight significantly ($P < 0.05$) reduced the parasitemia when compared to the infected but untreated rats (INF+untreated). Dimiazene aceturate was able to clear the parasite from the blood stream at day 4 post treatment of the animals. Crude extract concentrations of 30 mg/kg and 60 mg/kg body weight did not significantly ($P > 0.05$) suppressed the level of parasitemia; however, one animal in the group treated with 30 mg/kg b.w and the untreated group died before the end of the experiment. The parasitemia in the control group, *i.e.* infected but untreated continued to rise till the end of experiment.

4.9.3 Effect of Treatment with Extract on the Weight of Rats

Animals infected with trypanosomes usually suffer great weight loss. The effect of treatment with crude extract on weight loos was assessed. Result show that there was no significant different in weight of the animals treated with the 30, 60 and 120 mg/kg body weight concentrations (Figure 4.10). Rats treated with 120 mg/kg b.w did not gain significant weight (64.86 to 65.12 g), while those infected but untreated suffered weight loss (68.29 to 64.79 g).

Animals treated with Diminazene aceturate (3.5 mg/kg) also did not gain much weight during the period of experiment. The average weight of rats in this group increased from 64.19 to 67.60 g. Animals in the control group; i.e uninfected and untreated showed a significant ($P < 0.05$) increase in weight, from 68.14 to 75.85 g.

4.9.4 Effect of Treatment with Extract on the PCV of Rats

To assess the ability of the crude extract on ameliorating anaemic condition in infected rats, PCV was estimated on the first day and last day of the experiment. Results show that crude extract concentration of 120 mg/kg significantly improved the PCV levels in infected rats, while 60 and 30 mg/kg concentrations slightly improved the PCV level (Figure 4.11). Diminazene aceturate (3.5 mg/kg body weight) which is the standard drug significantly improved the level of PCV; however, could not also ameliorate the anaemic condition. The PCV of rats reduced from 46.5 to 42.5 %, while that of rats treated with 120 mg/kg b.w reduced from 47.5 to 39.5 %. Animals left untreated significantly ($P < 0.05$) suffered from anaemia, as seen in the low level of terminal PCV; their PCV reduced from 47.5 to 32.66 %. Animals treated with 30 mg/kg b.w also significantly ($P < 0.05$) had reduction in PCV. There was no difference in the initial and terminal PCV of animals in the uninfected group.

Table 4.3: Acute toxicity of *M. flos-aquae* crude extract on Wistar rats.

S/N	Dosage (mg/kg b.w)	NO. of animals	Mortality	% Mortality
1	10	3	0	**0**
2	100	3	0	**0**
3	1000	3	0	**0**
4	1600	1	0	**0**
5	2900	1	1	**100**
6	5000	1	1	**100**

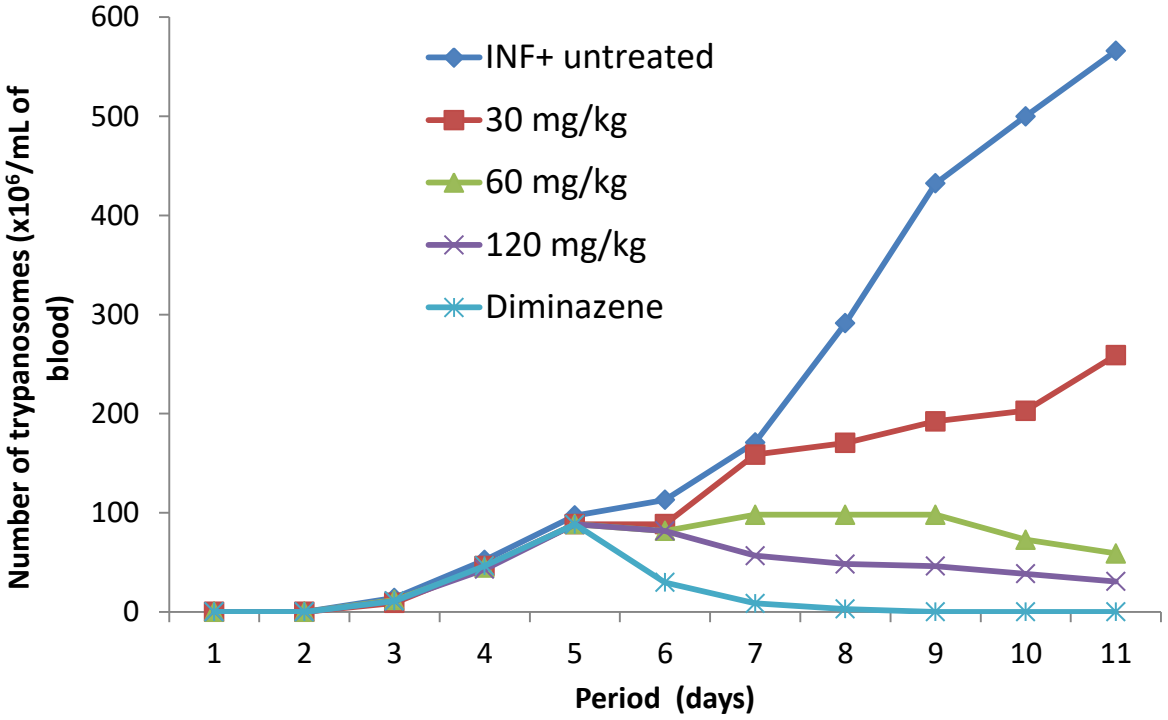


Fig. 4.9: Effect of treatment with *M. flos-aquae* extract on parasitemia in rats infected with *T. b. brucei*. Results are mean ± SD of replicate determinations (n = 4). Parasite count was done by examining wet mounts microscopically at 400×, according to the Herbert and Lumsden, (1976) matching method. Diminazene aceturate (standard drug for AAT) was used as a positive control, was given intraperitoneally at concentration of 3.5 mg/kg b.w. The negative control (INF+ untreated) experiments were set up by substituting 2 % DMSO for the extracts.

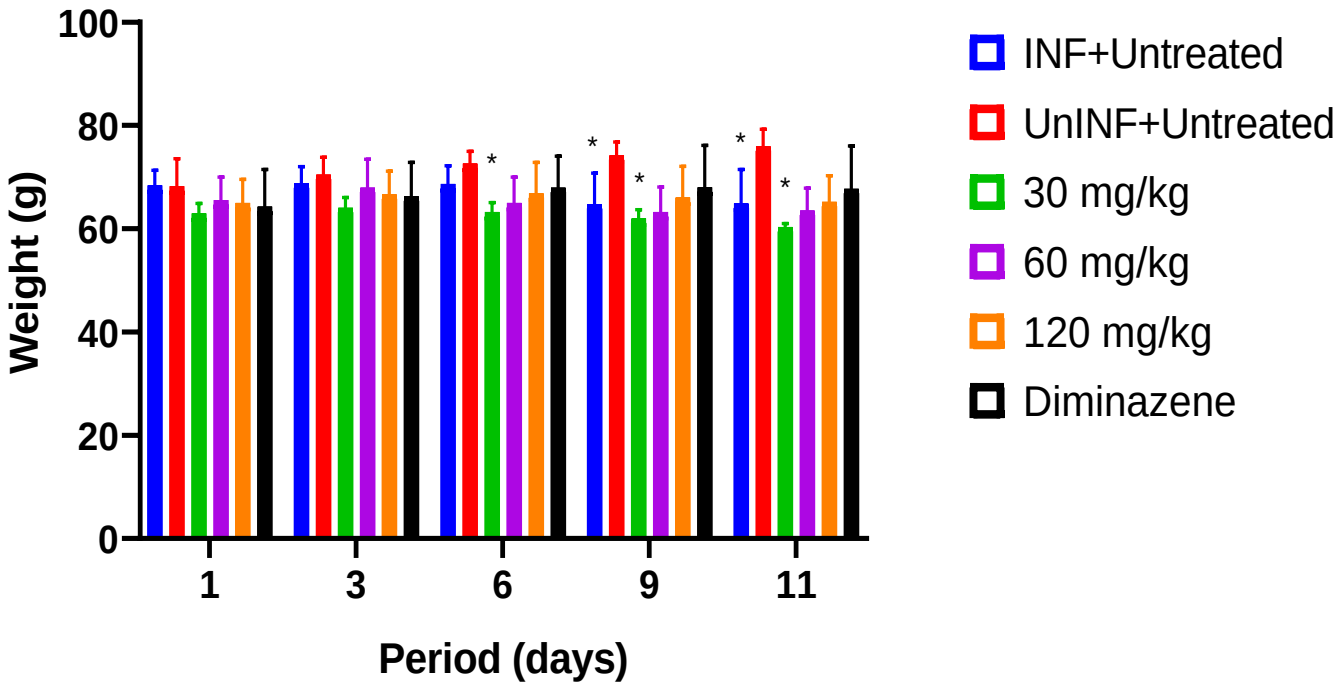


Fig.4.10: Effect of treatment with *M. flos-aquae* extract on the weight of rats infected with *T. b. brucei*. Results are mean ± SD of replicate determinations (n = 4). Values marked with * are significantly different at $p < 0.05$ using Turkey's test. Diminazene aceturate (standard drug for AAT) was used as a positive control, was given intraperitoneally at concentration of 3.5 mg/kg b.w. The negative control (INF+ untreated) experiments were set up by substituting extract with PBS, while the normal control (UnINF+untreated) were not infected with the parasite.

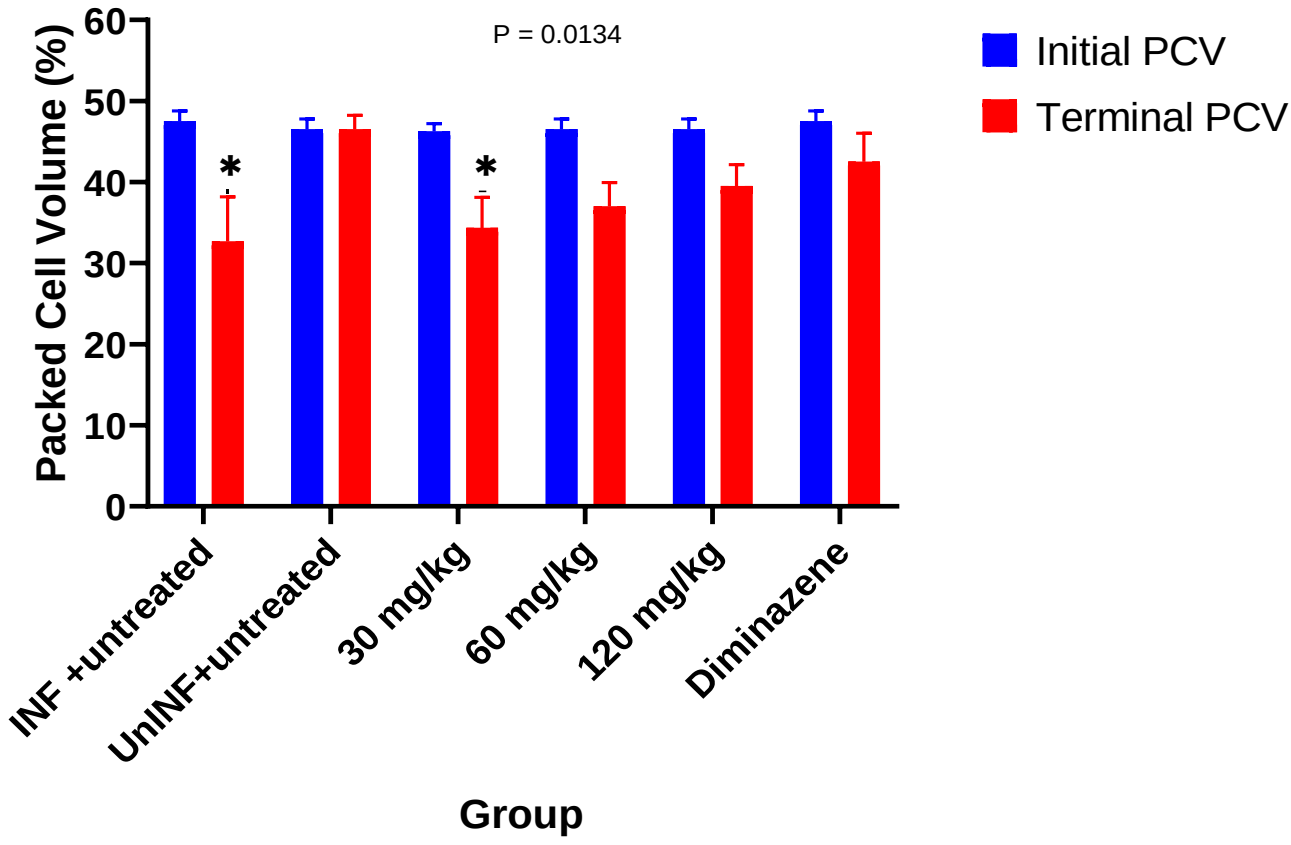


Fig.4.11: Effect of treatment with *M. flos-aquae* extract on packed cell volume (PCV) of rats infected with *T.b. brucei*. Results are mean ± SD of replicate determinations (n = 4). Values marked with * are significantly different at $p < 0.05$ using paired T-test. Diminazene aceturate (standard drug for AAT) was used as a positive control, and was given intraperitoneally at concentration of 3.5 mg/kg b.w. The negative control (INF+ untreated) experiments were infected but given only PBS instead of the extract, while the normal control (UnINF+untreated) were not infected with the parasite

CHAPTER FIVE

5.0 DISCUSSION

Due to limitations of morphological identification of microorganisms by microscopy, molecular approach comes into play, and has been shown to be more reliable than microscopy. The molecular approach to confirm the identity of the *Trypanosoma brucei brucei* stock using PCR was successful. ITS-1 of trypanosomes is variable in length; hence, it is explored in molecular identification of *Trypanosoma* spp. A single nested PCR gave an amplicon of approximately 430 bp of the ITS-1 region. This result is in agreement with previous report where an amplified DNA fragment size of 430 bp of the ITS-1 corresponded to *Trypanosoma bruce*i *brucei* (Adams *et al*., 2006).

In this study the partial 16S rRNA-region was also targeted to confirm the identity of the isolated cyanobacterial strain identified by microscopy as *Oscillatoria* sp. The attempt to amplify the 16S rRNA of the cyanobacteria DNA yielded an amplicon of approximately 700 bp long. Amplification of the partial 16S rRNA region of *Oscillatoria* species has also been previously reported (Hameed, 2008).

DNA analysis is one of the most reliable methods of identifying cyanobacteria strains by using oligonucleotide primers, and it also helps in identification of bloom forming cyanobacteria (Neilan *et al.*, 1995; 1997; Keshari *et al*., 2015). In some cases the classical morphological features of the corresponding taxa may be adequate for identification but not up to species level. Turner (1997) suggested that, although cyanobacterial 16S rDNA sequence comparisons are not suitable for resolution below the genus level, generic assignments appear to correlate with sequence data.

The cyanobacterial biomasses were subjected to extraction (cold maceration method) using absolute methanol as a solvent. Generally, natural products obtained from microorganisms are limited in availability, inconsistent in product quality and and low yield; and all these are greatly influence by the process employed for extraction (Zhang and Furusaki, 1999; Kylegh, 2005; Kwei, 2012). The percentage yield of the crude extract obtained for all the species was generally low. This could be attributed to the low availability of metabolites in the cyanobacteria or the method employed for the extraction.

The results obtained shows that crude extracts from all the species exhibited mild to strong antitrypanosomal activity in a time- and dosage-dependent manner when compared to the control (wells without extracts). This difference in the degree of activity displayed by extracts of the screened cyanobacterial species could be due to variation in their metabolite profile.

Variations in the degree of *in vitro* antitrypanosomal activity exhibited by the methanolic crude extracts of *M. flos-aquae, M. aeuruginosa, M. wesenbergii and Oscillatoria* sp. is in agreement with the previous findings of other researchers who have established that the extracts of different cyanobacteria (Broniatowska *et al*., 2011) or other microalgae (Leon-Deniz *et al*., 2009), may exhibit *in vitro* antitrypanosomal activity differently when compared with the control as a measure of *in vitro* trypanocidal activity of the extracts. *In vitro* trypanocidal activity of different crude extracts and fractions of two cyanobacteria *Nostoc commune* and *Rivularia biasolettiana* isolated from Ireland exhibited mild to strong antitrypanosomal activity against *T. b. rhodesiense* and *T. cruzi*, with no cytotoxic effect when tested on L6 cells (Broniatowska *et al*., 2011).

A difference was also observed in the mode of inhibition of the fractions; fraction **B** exhibited a trypanolytic effect, where the parasites were lysed when observed after 20-30 minutes exposure to 1.25 mg/mL concentration. On the other hand, fractions **E** and **F** caused the immobilization of the trypanosomes, which were still visible when observed under the microscope at the same dosage and period. This could possibly be that the principles responsible for the inhibitory activity in the fractions are different. From the results obtained, the tested sub-fractions exhibited mild to moderate activity. The metabolites seemed to have exhibited a synergistic effect rather than individual inhibition, because lower percentage inhibition was observed in the sub-fractions. The *in vitro* antitrypanosomal effect exhibited by these extracts may be attributed to the presence of some secondary metabolites like alkaloids, tannins, flavonoids, saponins, cyanide, glycosides, steroids and terpenoids which have already been identified in cyanobacteria. This suggestion is supported by the lack of activity observed in fractions without visible spots on TLC as seen on Plates 4.6 and 4.7.

The results from this study also showed that the crude extract of *M. flos-aquae* exhibited mild to moderate antitrypanosomal activity *in vivo* in a dosage-dependent manner, by suppressing the proliferation of the parasite. However, all the tested dosages did not completely clear the parasite in the blood stream. On the other hand, the standard drug (diminazene aceturate) cleared the parasites from bloodstream at day four post commencement of treatment without relapse of infection throughout the period of study. To the best of our knowledge, there is currently no report in literature on the *in vivo* antitryapanosomal activity of any cyanobacterial crude extract. On the other hand, several reports on the *in vivo* antitrypanosomal activity of Plant extracts on

various *Trypanosoma* species are available (Ogoti *et al*., 2009; Umar *et al*., 2010; Mergia *et al.*, 2014).

Total clearance of parasitaemia in rats infected and treated with diminazene aceturate 4 days post-treatment is in agreement with previous findings (Ezeokonkwo *et al.*, 2007; Antia *et al*., 2009; Umar *et al*., 2010, Ene *et al*., 2014). The positive effect of the cyanobacterial extract against the disease can further be deduced from the weight status of the animals as the body weight improvement was consistent with the observation made on parasitemia; although, there was no significant difference in the weight gain of the untreated animals and those treated with the extract, as compared to the uninfected group. This could be that the animals with better physical state were able to feed more than those in the other groups.

It has been shown that the measurement of anaemia gives an indication of the severity of trypanosomiasis (Umar *et al.*, 2000). The decrease in PCV of the infected untreated rats confirms earlier reports of the anaemic condition in trypanosomiasis. The observed antitrypanosomal effect of the crude extract in this study was accompanied by corresponding improvement in PCV when compared to the untreated group, suggesting that it has a potential to ameliorate anaemic condition. This effect could possibly be by suppressing parasite proliferation, neutralizing the toxic metabolites produced by the trypanosomes or scavenging the associated free radicals associated with the diseases (Mpiana *et al.*, 2007; Ekanem *et al*., 2008; Ogoti *et al.*, 2009). The infected rats treated with the diminazine aceturate at 3.5 mg/kg showed significant improvement in PCV more than the extract. Perhaps, because the drug was able to successfully eliminate trypanosomes from the blood stream to levels undetectable by microscopy. Failure of the extract and diminazene aceturate to completely ameliorate anaemia could be that the eatiological factors

involved in blood cells lysis were already established before treatment was commenced (Umar *et al*., 2010).

The inability of the extract to clear the parasites from the blood stream could be as a result of their failure to reach the action site due to poor absorption in the gut (Dwivedi, 1997; Wurochekke *et al*., 2005). Since the route of administration was orally, low/lack of activity could also be due to biotransformation of the active principle within the gut and liver (Wurochekke *et al*., 2005). Nevertheless, the extract contains active metabolites which might be acting individually or in synergy to cause the strong antitrypanosomal activity observed in the *in vitro* assays.

There is no report in the literature on the antitrypanosomal activity of the compounds identified by GC-MS. However, previous reports have shown that these compounds or related compounds possess antimicrobial and inhibitory activities on key cellular targets. Compounds with substituted tetrazoles and pyrimidines moieties have been acknowledged for antimicrobial activities (Soliman *et al*., 2018), and occupies a central position in drug design. 5-benzyl-2, 4-diaminopyrimidine has been shown to be an inhibitor of Trypanosomal dihydrofolate reductase (Chandra *et al*., 2005). Compounds belonging to imidazole and indolinone have also been previously acknowledged for their good pharmacological properties such as antibacterial, antifungal and anticancer activities (Bharat *et al*., 2013). A series of de-substituted pyrrolidine sulphonamides containing imidazole mannich bases have been synthesized and tested for antimicrobial activity (Kumar *et al*., 2012).

The use of GC/MS technique is helpful in identifying the bioactive metabolites present in extract with proven activity. However, its ability to detect only volatile compounds is a big limitation, due to the fact that many components in the extract cannot go into vapour phase within the range of the operating conditions. Non-volatile compounds from cyanobacteria, belonging to the chemical class such as peptides, lipopeptide, polyketides have been commonly reported for antimicrobial activity (Demay *et al*., 2019). Such compounds could be present in the extracts and responsible for the activity observed; however, GC/MS is limited in detecting such compounds.

Majority of the bioactive metabolites isolated from cyanobacteria are within the chemical classes including phenolics, polyphenols, alkaloids, terpenes, peptides and lipids (Demay *et al*., 2019), and majority of these have OH and COOH as their functional groups. Phenolics and polyphenols have been previously reported in literature for antitrypanosomal activity. One of such compound is ascofuranone, a prenylphenol antibiotic isolated from a phytopathogenic fungus, *Ascochyta visiae*, which was found to be effective against *T.b. brucei* and *T. vivax* in infected mice (Yabu *et al*., 2003; 2006). Earlier studies on the mechanism of inhibition showed that ascofuranone interfered with the Trypanosomes mitochondrion electron transport system, which is composed of glycerol-3-phospate dehydrogenase and Trypanosome Alternative Oxidase (TAO) enzymes (Minagawa *et al*., 1997). A trypanocidal lactam named Hoshinolactam, isolated from a marine cyanobacterium in Okinawa, Japan has been reported for its antitrypanosomal activity against *T. brucei* (Ogawa *et al*., 2017).

The exact mechanism for the *in vitro* and *in vivo* activity of these cyanobacterial extracts is unknown since the active principles were not individually isolated and tested. However, previous reports with pure compounds from some cyanobacteria have shed light on their mode of action. Almiramide isolated from *Lyngbya majuscula* exhibited activity through interference with the

trypanosome's glycosomal function by disruption of membrane assembly machinery (Sanchez *et al*., 2013). Dibenzodiazepine alkaloid isolated from a cyanobacterium was shown to inhibit *T. brucei* trypmastigotes by interfering with protease rhodesain (Abdelmohsen *et al*., 2012).

Previous reports with plant extracts attributed the trypanocidal activity of a number of tropical plants to tetracyclic iridoids (mulucidin), flavonoids (azaanthraquinone) and quaternary alkaloids (Hopp *et al*., 1976; Nok, 2001; Kwofie *et al*, 2016). Mechanism of action showed that mulucidin mediated death by induced apoptosis (Kwofie *et al*., 2016). Published reports also indicate that several natural products such as alkaloids, polyphenols, terpenoids and saponins primarily interact with important molecular targets such as DNA, microtubules, biomembranes, receptors and may induce cytotoxicity and death in trypanosomes (Rosenkranz and Wink, 2008).

The antitrypanosomal activity observed in this study might be attributed to the presence of one or more of these metabolites which, unlike synthetic pharmaceuticals, may exert their effects through a synergistic action of several compounds acting at a single or multiple target sites that play vital role in the physiological processes of trypanosome.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Molecular characterization of the isolated indigenous cyanobacterium (*Oscillatoria* sp.) yielded a PCR amplicon of approximately 700 bp, corresponding to the expected amplicon lenght for *Oscillatoria* sp.

According to this study, the cyanobacteria *Microcystis flos-aquae, Microcystis wesenbergii, M. aeruginosa* and *Oscillatoria* sp. exhibited *in vitro* antitrypanosomal activity agiaints *T. b. brucei* with IC_{50} values of 0.4140, 2.226, 0.7448 and 0.6511 mg/mL respectively.

Three fractions and sub-fractions obtained from the most active crude cyanobacterial extract (*M. flos-aquae*) also exhibited *in vitro* antitrypanosomal activity against *T. b. brucei*, with the three fractions named **B**, **E** and **F** showing IC_{50} values of 0.34960, 0.2991 and 0.3602 mg/mL.respectively.

Futhermore, the most active cyanobacterial crude extract (*M. flos-aquae*) exhibited *in vivo* antitrypanosomal activity in rats infected with *T. b. brucei*, by suppression of parasitemia and improvement of PCV at 60 and 120 mg/kg b.w dosages.

Compounds such as Ethyl 5-(furan-2-yl)-1,2-oxazole-3-carboxylate, 5-amino-1-tetrazolyl acetic acid, 1-Benzyl-pyrrolidin-3,4-diol, cedran-diol and 2-amino-4-(2-methylpropanyl)-pyrimidin-5-carboxylic acid have been detected in the most active fractions of *M. flos-aquae*.

6.2 RECOMMENDATIONS

This study has shown promising therapeutic potentials of the screened cyanobacterial extracts, especially the *M. flos-aquae*. However, isolation of the individual compounds was not achieved. It is therefore recommended that:

1. Further studies should be carried out to isolate and test the individual compound(s) that could be responsible for the antitrypanosomal activity observed.

2. Structural elucidation of the isolated compound(s) should be carried out in order to gain insight into the chemistry of the structures.

3. The toxicity of the isolated compounds should be carried out to establish the safety of the compounds; as such, they could be further considered as lead for development of trypanocidal drug.

4. Mode of action of the isolated compound(s) on the trypanosomes should also be investigated in further studies.

6.3 CONTRIBUTION TO KNOWLEDGE

1. This study has shown the *in vitro* antytrypanosomal activity of cyanobacterial strains (*Microcystis flos-aquae, Microcystis wesenbergii, M. aeruginosa* and *Oscillatoria* sp.) whose such biological activity has not been reported in the literature before.

2. The *in vitro* antitrypanosomal activity of Nigeria cyanobacterial isolate (*Oscillatoria* sp.) has been reported here for the first time.

3. To the best of my knowledge, this study is the first investigation reporting the *in vivo* antitrypanosomal activity of *M. flos-aquae* extract. This shows that cyanobacteria have potentials to be considered for ethnomedicinal purposes.

4. This study has also provided insight into the biochemical profile of the cyanobacteria *M. flos-aquae*.

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APPENDICES

APPENDIX I: Composition and Preparation of BG-11 medium

Stock number	Stock solution	Amount in 200 mL	Volume for 1Litre media
1	$NaNO_3$	30g	10
2	K_2HPO_4	8g	1mL
3	$MgSO4.7H_2O$	15g	1mL
4	$CaCl_2.2H_2O$	7.2g	1mL
5	Citric Acid	1.2g	1mL
6	Ammonium ferric citrate	1.2g	1mL
7	EDTA NA_2	0.2g	1mL
8	$NaCO_3$	4g	1mL
9	Trace metals		1mL
i.	H_3BO_3	1.43g	
ii.	$MnCl_2.4H_2O$	0.923g	
iii.	$ZnSO4.7H_20$	0.11g	
iv.	$Na_2MoO4.2H_20$	0.193g	
v.	$CuSO4.5H_2O$	0.04g	
vi	$Co(NO3)_2.6H_2O$	0.023g	

APPENDIX II: ANOVA Table for the effect of treatments with crude extracts on the Parasitemia *in vitro.*

Treatment	**Concentration (mg/mL)**				
	2.5	1.25	0.625	0.3125	P-value
M. flos-aquae	1.56±0.00d	12.50±0.00c	36.72±2.34b	57.82±1.57a	0.000
M. wesenbergii	10.94±1.56d	26.57±1.57c	55.47±0.78b	89.06±0.00a	0.000
M. aeruginosa	39.06±0.00d	76.57±1.57c	87.50±1.56b	99.22±0.78a	0.000
Oscillatoria sp.	8.59±0.78d	26.56±1.56c	45.32±1.56b	84.38±4.69a	0.000
D. aceturate	0.00±0.00b	0.00±0.00b	0.00±0.00b	7.82±1.57a	0.005
Control	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	NA

APPENDIX III: ANOVA table for the effect of treatments with *M. flos-aquae* fractions on the parasitemia *in vitro*.

Fraction	**Concentration (mg/mL)**			
	1.25	0.625	0.3125	P-value
Fraction A	100.00±0.00	100.00±0.00	100.00±0.00	NA
Fraction B	3.91±0.78c	25.79±2.35b	59.38±3.13a	0.001
Fraction C	100.00±0.00	100.00±0.00	100.00±0.00	NA
Fraction D	100.00±0.00	100.00±0.00	100.00±0.00	NA
Fraction E	3.13±0.00c	25.79±2.35b	49.22±5.47a	0.006
Fraction F	20.32±1.57c	39.06±0.00b	62.51±3.13a	0.002

APPENDIX IV: ANOVA table for effect of treatment with extract on the parasitaemia in rats

	Treatment				
Period (days)	INF+ untreated	INF +30 mg/kg	INF+60mg/kg	INF+120mg/kg	INF+DA
D1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
D2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
D3	14.00±2.58a	9.00±2.52a	12.00±2.31a	11.00±3.00a	11.00±3.00a
D4	52.25±7.46a	46.25±6.69a	44.50±7.76a	43.00±4.36a	46.50±6.90a
D5	97.25±12.96a	88.50±15.11a	88.50±15.11a	88.25±15.24a	88.25±15.24a
D6	113.00±7.51a	88.50±15.11a	88.50±15.11a	81.75±10.54a	29.75±1.65b
D7	171.00±17.97a	158.75±32.96a	98.00±13.12b	56.75±3.90bc	8.50±2.87c
D8	291.25±42.77a	170.50±35.51b	98.00±13.11bc	48.25±5.45cd	3.00±0.58d
D9	432.33±34.33a	192.33±36.29b	98.00±13.11c	46.25±6.69cd	0.00±0.00d
D10	501.00±0.00a	203.00±26.89b	72.75±9.09c	38.50±4.27c	0.00±0.00d
D11	566.00±65.00a	259.00±78.05b	52.25±7.47c	22.75±8.32c	0.00±0.00c

APPENDIX V: ANOVA table for effect of treatment with extract on the weight of rats

Days	**Groups**					
	INF+ untreated	30 mg/kg	60 mg/kg	120mg/kg	D. aceturate	Control
D1	62.88±1.01a	65.46±2.26a	64.86±2.33a	64.20±3.65a	68.29±1.50a	65.19±5.06a
D3	63.98±1.04a	67.84±2.81a	66.53±2.31a	66.13±3.36a	68.67±1.66a	68.99±3.73a
D6	63.16±0.95b	64.89±2.55ab	66.77±3.05ab	67.84±3.11ab	68.56±1.81ab	72.71±2.91a
D9	61.86±0.92b	63.15±2.47b	65.93±3.07ab	67.93±4.09ab	64.65±3.05b	75.31±2.79a
D11	60.25±0.43b	63.42±2.55b	65.12±2.57b	67.62±4.21b	64.79±4.72b	78.25±2.35a

APPENDIX VI: Table showing IC_{50} values of the crude cyanobacterial extract and the active fractions of *M. flos-aquae* against *T. b. brucei* after 2 hrs.

Extract	**IC_{50} (mg/mL)**
M. flos-aquae	0.02648
M. wesenbergii	0.0643
Oscillatoria sp	0.02941
M. aeruginosa	0.2035
Fraction B	0.0218
Fraction E	0.0217
Fraction F	0.0264