EVALUATION OF ANTITRYPANOSOMAL ACTIVITY OF BISINDOLE ALKALOID, OCHROLIFUANINE FROM *Dyera costulata* (Miq.) Hook.*f*

NORHAYATI BINTI ISMAIL

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

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NORHAYATI BINTI ISMAIL

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Name of Candidate : **NORHAYATI BINTI ISMAIL**

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EVALUATION OF ANTITRYPANOSOMAL ACTIVITY OF BISINDOLE ALKALOID, OCHROLIFUANINE FROM *Dyera costulata* (Miq.) Hook.*f*

ABSTRACT

Human African Trypanosomiasis (HAT) is a neglected tropical disease which can be fatal if left untreated. The search has been ongoing to explore additional or alternative remedies from natural products which have great potential to treat the disease since current drugs treatment are very limited due to resistance and side effects to patients. The aim of this study was to search for potential antitrypanosomal compound candidate from *Dyera costulata* (Miq.) Hook.*f* (locally known as jelutong) leaves. In this study, an antitrypanosomal active compound was isolated from the active methanolic extract of *D. costulata* leaves by using bioassay-guided isolation and chromatographic techniques. The compound identified as bisindole alkaloid ochrolifuanine using Nuclear Magnetic Resonance (NMR) and Mass Spectrum (MS) as well as by comparison with reported data. The *in vitro* inhibitory activity of extract and fraction samples against *Trypanosoma brucei brucei* strain BS221 was tested using Alamar Blue assay. Ochrolifuanine showed potent antitrypanosomal activity with IC_{50} value 0.05 ± 0.01 µg/ml and high selectivity towards the trypanosome cells (Selectivity Index, SI = 52). Since apoptosis plays an important role in cell density regulation in the parasite, the effect of ochrolifuanine in apoptosis induction was investigated. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to observe the effect of ochrolifuanine on apoptotic DNA fragmentation in *T. b. brucei* cells treated with ochrolifuanine. Ochrolifuanine was shown to induce an apoptotic DNA fragmentation in trypanosome cells in a dose- and time-dependent manner. Trypanosome cells treated with 0.05 µg/ml concentration of ochrolifuanine exhibited

early apoptosis and more than 50% apoptotic cells were detected in as early as 6 hours of incubation. Additional study on measurement of DNA content in trypanosome parasites was carried out using flow cytometry. In 24 hour observations, treatment of cells with 0.025 and 0.05 µg/ml of ochrolifuanine arrested the growth of *T. b. brucei* at two different phases (G_0/G_1 and in S phases). While at the highest dose treatment of 0.10 µg/ml of ochrolifuanine, blockage of cell progression at the G_2/M phase in *T. b. brucei* cell cycle was observed. These results demonstrated that ochrolifuanine displayed strong antitrypanosomal effect on *T. b. brucei* by inducing apoptosis and causing arrest of parasite cells at different growth phases. This study reports for the first time on the antitrypanosomal activity of ochrolifuanine from *D. costulata* leaves. Hence, ochrolifuanine is a potential candidate for the discovery of novel therapeutic drugs to treat trypanosomiasis disease.

Keywords: *Trypanosoma brucei brucei*, Apocynacea, alkaloid, programmed cell death

PENILAIAN AKTIVITI ANTITRIPANOSOM OLEH ALKALOID BISINDOL OKHROLIFUANINA DARI *Dyera costulata* (Miq.) Hook.*f*

ABSTRAK

Human African Trypanosomiasis (HAT) merupakan satu penyakit terabai yang mana boleh membawa kepada kematian jika tidak dirawat. Kajian diteruskan dalam usaha untuk menerokai penawar tambahan atau alternatif dari bahan semula jadi yang berpotensi tinggi untuk merawat penyakit tersebut, memandangkan pilihan rawatan semasa sangat terhad berpunca dari kesan rintangan dan kesan sampingan terhadap pesakit. Sasaran kajian ini adalah untuk mencari calon sebatian berpotensi terhadap antitripanosom dari daun *Dyera costulata* (Miq.) Hook.*f* (dikenali secara tempatan sebagai Jelutong). Dalam kajian ini, sebatian aktif antitripanosom dipencil dari ektrak aktif methanol daun *Dyera costulata* dengan menggunakan pendekatan pencerakinan berpandukan bioasai dan teknik kromatografi. Sebatian tersebut dikenalpasti sebagai okhrolifuanina dari kumpulan bisindole alkaloid oleh data spektroskopik Resonan Magnetik Nuklear (NMR) dan spektrometri jisim (MS) serta juga perbandingan dengan data yang dilaporkan. Kesan perencatan aktiviti secara *in vitro* sampel ekstrak dan fraksi terhadap sel parasit *Tryapanosoma brucei brucei* strain BS221 diuji menggunakan asai Alamar. Okhrolifuanina menunjukkan aktiviti tripanosom yang kuat dengan nilai IC_{50} 0.05 ± 0.01 µg/ml dan juga memberi kesan selektif yang tinggi terhadap sel tripanosoma (Index selektif, SI = 52). Oleh kerana apoptosis memainkan peranan penting dalam pengaturan densiti sel parasit tripanosom, kesan okhrolifuanina yang mendorong apoptosis telah dikaji. Okhrolifuanina kemudiannya dikaji lanjut untuk mekanisme tindakan melalui apoptosis. Asai pelabelan 'terminal deoxynucleotidyl transferase dUTP nick-end labelling' (TUNEL) telah digunakan untuk menilai kesan okhrolifuanina ke atas fragmentasi DNA pada sel *T. b. brucei* yang dirawat dengan

okhrolifuanina. Okhrolifuanina menunjukkan kesan perangsangan apoptosis melalui fragmentasi DNA di dalam sel parasit tripanosom secara kebergantungan pada dos dan masa rawatan. Sel tripanosom yang dirawat dengan okhrolifuanina pada kepekatan 0.05 ± 0.01 µg/ml menunjukkan kejadian apoptosis awal, iaitu lebih daripada 50% sel apoptotik dikesan seawal tempoh 6 jam inkubasi. Kajian tambahan mengenai penentuan kandungan DNA parasit dijalankan menggunakan aliran sitometri. Didapati pada 24 jam pemerhatian, rawatan dengan kepekatan okhrolifuanina 0.025 and 0.05 µg/ml, telah merencatkan sel *T. b. brucei* pada dua fasa yang berbeza (fasa G_0/G_1 dan S). Manakala, pada dos kepekatan okhrolifuanina yang paling tinggi 0.10 µg/ml, menunjukkan sekatan proses perkembangan sel pada fasa G_2/M kitaran sel *T. b. brucei.* Keputusan ini menunjukkan okhrolifuanina mempunyai kesan aktiviti antitripanosom yang kuat pada *T. b. brucei* dengan menggalakkan kematian sel secara apoptosis dan menyebabkan perencatan sel parasit pada fasa berbeza. Kajian ini melaporkan buat pertama kalinya kesan aktiviti antitripanosom okhrolifuanina dari daun *D. costulata*. Oleh itu, okhrolifuanina merupakan calon berpotensi dalam penemuan ubatan terapeutik baru untuk merawat penyakit tripanosomiasis.

Kata Kunci: *Trypanosoma brucei brucei*, Apocynacea, alkaloid, kematian sel terancang

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LIST OF SYMBOLS AND ABBREVIATIONS

^{o}C	: Degree in Celsius
µg	: Microgram
µl	: Microliter
µM	: Micromolar
ηg	: Nanogram
%	: Percentage
ANOVA	: Analysis of variance
BCECF	: 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein
BMEM	: Basal minimum essential medium
CC	: Column chromatography
cdc	: Cell division cycle
CDK	: Cyclin-dependent kinase
$CHCl_3$	: Chloroform
cm	: Centimetre
CO_2	: Carbon dioxide
ConA	: Concanavalin A
CRK	: Cyclin-Cdk related kinases
CYC	: Cyclin
ddH_2O	: Distilled deionized water
DEPT	: Distortionless enhancement by polarization transfer
DHA	: Docosahexaenoic acid
DMEM	: Dulbecco's modified Eagle's medium
DMSO	: Dimethylsulphoxide
DNA	: Deoxyribonucleic acid
DND*i*	: Drug neglected diseases initiative

dUTP	: Deoxyuridine triphosphate
EDTA	: Ethylenediaminetetraacetic acid
ESI	: Electron spray ionization
FBS	: Foetal bovine serum
g	: Gram
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMBC	: Heteronuclear multiple-bond correlation
HMQC	: Heteronuclear multiple quantum correlation
hrs	: Hour
Hz	: Hertz
IR	: Infra-red
L	: Litre
LILIT	: Long inoculation, long incubation test
M	: Molar
MEM	: Minimum essential medium
MeOD	: Deuterated methanol
MeOH	: Methanol
mg	: Milligram
mins	: Minutes
ml	: Millilitre
mM	: Millimolar
MS	: Mass spectrum
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2SO_4	: Sodium sulfate
nm	: Nanometre
NMR	: Nuclear magnetic resonance

OD : Optical density

ORC : Origin recognition complex

PBS : Phosphate buffered saline

PG : Prostaglandin

REDOX : Oxidation and reduction

R_f : Retention factor

ROS : Reactive oxygen species

rpm : Revolutions per minute

SD : Standard deviation

SPE : Solid phase extraction

SRB : Sulforhodamine B

TdT : Terminal deoxynucleotidyl transferase

TLC : Thin layer chromatography

TUNEL : Terminal deoxynucleotidyl transferase dUTP nick-end labelling

v/v : Volume over volume

WHO : World Health Organisation

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CHAPTER 1: INTRODUCTION

1.0 General Introduction

Human African Trypanosomiasis (HAT) also known as sleeping sickness is one of the Neglected Tropical Diseases (NTDs) group affected by kinetoplastid protozoa parasite from the subspecies of *Trypanosoma brucei* (Brun *et al.,* 2010). These protozoa parasites afflict both man and animals, causing major impact on health and economic problems in rural sub-Saharan Africa. Every year, nearly 20,000 cases of this infection are reported and 70 million people are at the risk (Simarro *et al.,* 2012, WHO, 2013). However, there is a decreasing trend of the HAT due to sustaining and coordinated effort over the 15 years by WHO (Bucher *et al*., 2017). In 2015, less than 3,000 cases were reported. Despite this impressive achievement, the disease is still endemic in parts of Africa and it is a considerable burden on the rural population. Besides that, HAT cases are also reported outside from endemic African countries among travelers, tourists, expatriates and migrants (Simarro *et al.,* 2012).

Five registered therapy drugs, namely, pentamidine, suramin, melarsoprol, nifurtimox, and eflornithine, are commonly used for the treatment of HAT. However, these drugs have their own limitations and have been reported to cause numerous side effects including difficulty in administration and loss of efficacy (Burri, 2010; Baker *et al.,* 2013). To date, no vaccine is accessible for this disease and its control relies on case detection, treatment and vector control. Hence, there is a crucial requirement for the development and discovery of new drugs that are safe, efficient, affordable, easy-to-apply and possess novel mechanism of actions, to treat this disease.

Trypanosoma brucei subspecies are transmitted to mammalian hosts by the bite of tsetse flies from the family of *Glossina* spp. This disease affects both psychological

changes and disruption to sleep patterns. The latter led to the development for 'sleeping sickness', which, if left untreated, will lead to coma and death (MacLean *et al.,* 2013).

Besides the human trypanosome, a number of other trypanosome species also attributed to the occurrence of animal trypanosomiasis. *Trypanosoma evansi* is the causative agent for animal trypanosomiasis (surra disease). In Malaysia, surra cases have been reported in deer, cattle, horse, buffalo and pig (Rahman *et al.,* 2012; Elshafie *et al.,* 2013; Nurulaini *et al.,* 2013). Even though animal species of trypanosome is incapable to affect humans, there are some reported cases of patients being diagnosed with *T. evansi* (Joshi *et al*., 2005) and *T. lewisi* (Dope & Kar, 2011; Verma *et al.,* 2011).

Researchers have synthesised a wide variety of drugs for the treatment of parasitic diseases. However, years down the line, some parasite strains have developed resistance against these drugs. The progression of new antiparasitic drugs has not been a preferenced choice for pharmaceutical industries because many of these parasitic diseases occurred in poor countries, whose population cannot able to pay the cost of the expensive drugs. Thus, this presented a high risk to the industries to invest in drug development against these parasitic diseases. Hence, an alternative to these synthetic drugs is to investigate natural products as potential antiparasitic drugs.

Natural products have been established as one of the most successful sources of drug compounds, not only from plants but also from microbes and marine organisms (Newman & Cragg, 2012). Over years, parasites have become immune against most of the available synthetic drugs (Wink, 2012). Therefore, there is a growing awareness to exploit the ability of natural products, which remains un-explored, for the discovery of novel antiparasitic drug.

Dyera costulata (Miq.) Hook. *f* a large tree from Apocynaceae family, is locally known as jelutong in Malaysia. Various chemical groups such as alkaloids, triterpenes and flavonoids have been isolated from *D. costulata* (Mirand *et al*., 1983; Reanmongkol *et al.,* 2002). Alkaloids have been used widely as a source of remedy to treat a broad spectrum of illnesses. Many alkaloids have also been reported to have antiparasitic effects (Freiburghaus *et al*., 1996; Kato *et al.,* 2012; Pan *et al*., 2014).

Ochrolifuanine, a bisindole alkaloid group was discovered from leaf extracts of *D. costulata* (Mirand *et al.* 1983) and is also found in root bark of *Strychnos potatorum* (Massiot *et al*., 1992). However, information on the bioactivity of ochrolifuanine is limited. Although ochrolifuanine has been reported to show antimalarial activity (Frederich *et al.,* 2002), other indole alkaloids such as staurosporine, strictosidine and acetylstrictosidine have also been reported to show antitrypanosomal properties towards *Trypanosoma brucei brucei* (del Rayo Comacho *et al.,* 2004; Pimentel-Elardo *et al*., 2010).

Trypanosome parasites are affected by programmed cell death (PCD) process known as apoptosis. Programmed cell death process in trypanosomes led to the regulation of size of parasite population (cell density) and also being done as a mechanism to control genetic stability and cell differentiation (Welburn *et al*., 2006). Even though studies on PCD in protozoa are lacking, there are reports that showed compounds such as concanavalin A and prostaglandin A induced PCD in *Trypanosoma* spp. (Welburn *et al*., 1996; Figarella *et al.,* 2006). In addition, a number of studies have shown that the bisindole alkaloid staurosporine isolated from marine actinobacteria strain induced apoptosis in trypanosome and other protozoan species (Yin *et al*., 2010; Bruges *et al.,* 2012; Foucher *et al*., 2013; Barth *et al*., 2014).

Studies have shown that Malaysian tropical forest plants can be a possible source of lead compounds in drug design and synthesis (Noor Rain *et al*., 2007). In previous study, methanol and total alkaloid extract from *D. costulata* leaves showed potent inhibitory effect against *T. b. brucei* strain BS221 and high selectivity towards the trypanosome cells compared to mammalian cells (Muhd Haffiz *et al.,* 2011; Norhayati *et al*., 2013). Hence, presence of the bisindole alkaloid ochrolifuanine reported previously in *D. costulata* should be confirmed and studied further for its antitrypanosomal activity. Therefore, the objectives of study are:

i. to isolate ochrolifuanine, a bisindole alkaloid compound from the leaves of *Dyera costulata*;
ii. to determine antitrypanosomal effect of ochrolifuanine against *Trypanosoma brucei brucei* strain BS221 and its degree of selectivity towards trypanosome cells compared to mammalian cells;
iii. to investigate if ochrolifuanine induced the cell death mechanism via apoptosis in *T. b. brucei* strain BS221.

CHAPTER 2: LITERATURE REVIEW

2.1 Neglected Tropical Disease

The term "neglected diseases" was established in 2005 and refers to a group of diseases that influence the poorest population of the world. These people are overlooked in terms of basic requirement such as medical, financial and educational aid (Marchal *et al*., 2011). This situation occurs primarily in rural regions and some poor urban countries in sub-Sahara Africa, Asia and Latin America.

The neglected tropical diseases (NTDs) are a subset of infectious diseases. The NTDs were categorised based on 17 tropical infectious groups by World Health Organization (WHO) and health society. These 17 parasitic and bacterial infections include dengue, rabies, trachoma, buruli ulcer, endemic treponematoses, leprosy, Chagas disease, human African trypanosomiasis (HAT), leishmaniasis, taeniasis/cysticercosis, dracunculiasis, echinococcosis, food-borne trematodiases, lymphatic filariasis, onchocerciasis, schistosomiasis, and soil-transmitted helminthiases (WHO, 2013; Hotez *et al.,* 2013; Mackey *et al*., 2014).These diseases are grave danger for human kind since they are the cause for life-long incapability, disfigurement, diminished economic productivity and social health (WHO, 2003). The NTDs do not receive enough international attention like the other global health threats such as HIV-AIDS, malaria and tuberculosis. Instead, they are common neglected diseases among people in the geographically isolated and difficult to explore places (Molyneux, 2013; Mackey *et al.,* 2014). The NTDs so far has affected 58 countries and several diseases have existed in 56 of these countries where one billion people had been estimated to be diagnosed with one or more neglected disease (Hotez, 2011; Molyneux, 2013; Hotez, 2017).

2.1.1 Human African Trypanosomiasis

Human African Trypanosomiasis (HAT), also known as sleeping sickness, is caused by the flagellated protozoan that belongs to the genus *Trypanosoma*. Sleeping sickness cases were estimated to be around 300,000 – 500,000 infections in the year 1998 (Lutje *et al.,* 2013). Due to the continuos effort by WHO and greater number of facility health in treatment, the new reported cases in 2009 has dropped fewer than 10, 000. The cases decreased to 9,875 cases in 2009, 7,139 cases in 2010 followed by 6,743 in 2011 (WHO, 2013) and in 2015, the cases had decreased to 3,000 cases (Buscher *et al.,* 2017). This trend represents a decreased of more than 70 % during the past 10 years recorded (WHO, 2013).

HAT is considered to be the major cause of mortality and morbidity in sub-Sahara Africa. Patients diagnosed with *T. brucei gambiense* infection results in disturbed sleeping pattern in the West and Central Africa, wheres *T. brucei rhodosiense* is the main cause of this disease in the East and Southern Africa. *Trypanosoma brucei brucei* is closely related to the first two subspecies. However, the *T. b. brucei*, which is not human infective as it is susceptible to lysis by trypanolytic factor in human serum (Alsford *et al*., 2014), is very similar to *T. b. rhodosiense and T. b. gambiense* on the cellular and metabolic level and thus ideally suited for *in vitro* experiments.

However, there are some reports of HAT occurring in the human that are caused by non-human pathogenic trypanosome species (Brun *et al*., 2010; Truc *et al.,* 2013). *T. evansi* and *T. lewisi* have been reported as potentially pathogenic for humans. Out of 15 cases of infection by non-human pathogenic species recorded between 1974 and 2010, nine cases have been reported since 2003 (Truc *et al*., 2013). Molecular diagnosis of blood samples taken from a 10-month old Ghanaian baby, who rehabilitated from a *T.*

brucei infection, showed that the parasite belongs to the *T. b. brucei* species which is normally non-infectious to human (Deborggraeve *et al*., 2008). There was also a report of a woman patient who had been infected with both of *T.brucei* and *T. congolense*. Meanwhile, the patient successfully recovered within two weeks after treatment with pentamidine drug (Truc *et al*., 2013).

Symptoms of the HAT start with pain and irregular fevers as well as swollen lymph glands and spleen. This is usually followed by a nuisance, anemia, joint cramp and tissues injury. Neurological changes include sleep disturbances, poor coordination, and personality changes, which if untreated, lead to fatality within weeks or months (Simarro *et al*., 2011; Buguet & Cespuglio, 2015; Votypka *et al*., 2015; Cheuka *et al.,* 2017).

The available drugs to treat HAT such as pentamidine, melarsoprol, suramin, eflornithine and nifurtimox (Figure 2.1) are still ineffective in many ways. They are reported with various side effects, including high toxicity, low effectiveness and drug resistance (Fairlamb, 2003; Lejon *et al.*, 2013; Stich *et al*., 2013; Stein *et al*., 2014). There are no clinical drugs in the current pipeline for the effective treatment of HAT (Buscher *et al.,* 2017) and therefore, there is an urgent necessity for the development of new drugs since no vaccines are available for the treatment of HAT (Yansouni *et al*., 2013; Ferrins *et al.,* 2013; Mackey *et al.,* 2014).

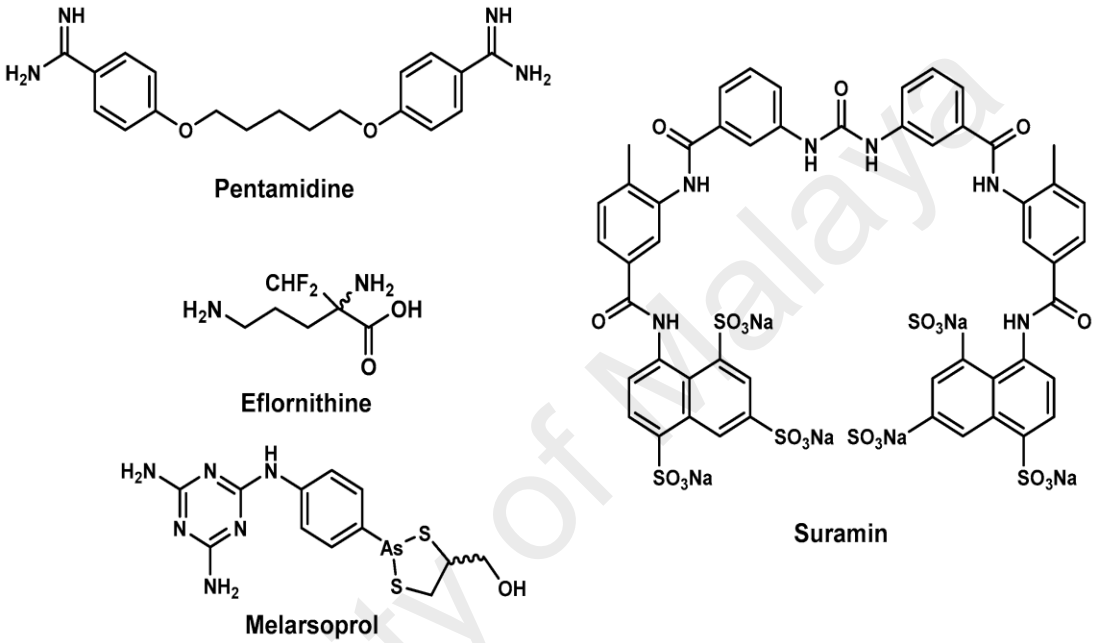


Figure 2.1: Structures of antitrypanosomal drugs in clinical use (Retrieved from Cheuka *et al*., 2016)

In Malaysia, trypanosomiasis in animal (surra disease) has been reported in cattle such as buffaloes (Abas-Mazni & Zainal Abidin, 1992; Cheah, 1999) and in rhinoceros's center (Vellayan *et al*., 2004). Meanwhile, the first outbreak of trypanosomiasis in deers was reported in a large deer breeding center in Lenggong, the state of Perak in 2006 (Nurulaini *et al*., 2007).

This surra disease caused by the haemoflagellate protozoa *Trypanosoma evansi* is a great concern for some tropical countries which involved poultry industry. The main vector for this disease is the tabanid fly (Desquesnes *et al.,* 2013). *T. evansi* is maintained in laboratory animals and is very useful for the study of trypanosomiasis. Trypanosomiasis in cattle is affected in milk production and body growth (Cheah, 1999). Mortality of pigs in a private farm in Sungai Siput Utara, Perak was shown to be due to trypanosomiasis (Nurulaini *et al*., 2013). Animal species of *Trypanosoma* is commonly non-pathogenic to human because of a trypanolytic factor protection in human serum. However, both *T. congolese* and *T. evansi* are reported to exist and survive in human blood plasma (Hawking, 1978), which one reported case described a patient with this infection in Côte d'Ivoire (also known as Ivory Coast, West Africa) harboring both *T. brucei* and *T. congolense* with associated clinical signs (Truc *et al*., 1998). In recent years, *T. evansi* has emerged as potentially pathogenic for human (Truc *et al.,* 2013). Therefore, this disease needs to be controlled to prevent an outbreak.

2.1.2 Trypanosome Parasite

Trypanosoma brucei belongs to the genus *Trypanosoma* in *Trypanosomatidae* family. It is a large family group of unicellular protozoan parasitic organisms from the order of kinetoplastida. In the human blood stream and animals, trypanosomes appear as trypomastigote form. They contained a unique organelle known as kinetoplast with

extranuclear DNA in the mitochondria which is associated with the basal body of the flagellum. It is a spindle-shaped cell (15-35 μm) form with the single flagellum. This flagellum develops from the posterior end, runs through the cell membrane and attached by an undulating membrane, until the anterior part of the cell (Figure 2.2). The trypomastigote form is the only found in the mammalian host, while the epimastigote form appears in the tsetse fly during their development phase.

Some of the common *T. brucei* strains are used as test organisms in *in vitro* antitrypanosomal assays. These include the HAT strains such as *T. b. rhodosiense* strain STIB 900, a clone of population isolated in 1982 from the patient in Tanzania (Baltz *et al*., 1985). Whereas, *T. b. brucei* strain GUTat 3.1, a clone derivative was isolated in 1996 from a naturally infected bovine in Uganda (Otoguro *et al.,* 2008).

In this study *T. b. brucei* strain BS221 was used for *in vitro* antitrypanosomal screening. This strain is a derivative of S427 also known as MiTat 1.2/221 isolated from tsetse flies (*Glossina pallidipes*) in 1960 in Uganda (Jean-robert *et al*., 2009)

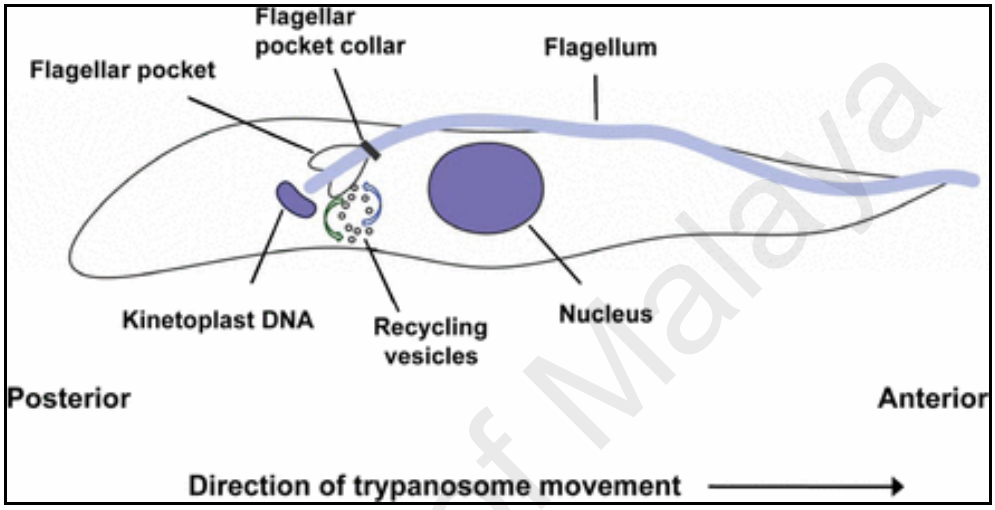


Figure 2.2: Morphological structure of a *Trypanosoma* sp. (Retrieved from Rudenko, 2011)

Figure 2.3: The bloodsucker tsetse fly from *Glossina* sp. (Retrieved from Votypka *et al*., 2015)

The parasites are transmitted among vertebrate hosts by the tsetse fly from the genus *Glossina* sp. (Figure 2.3). The trypanosome undergoes life cycle which alternates between the vertebrate and tsetse flies. In human and animal host, life cycle of trypanosomes are appeared in blood, other body fluids, and tissues whereas, in tsetse fly it appeared in gut and salivary glands. To survive in these different environments, various metabolic and morphological changes are necessary which involve the mitochondrial system and the surface membrane (Langousis & Hill, 2014). Figure 2.4 illustare the life cycle of T. brucei in human and tsetse fly.

During blood sucking on an infected mammal, the vector ingests the trypomastigotes into their body. The parasites then multiply in the fly and go through several development stages (procyclic, epimastigotes and metacyclic trypanosomes) in the period of three to four weeks (Buscher *et al.,* 2017).

Infections to the other mammalian hosts begin with the bite of an infected tsetse fly, which injects the metacyclic trypomastigotes form of the parasite in its saliva before taking its blood meal. The metacyclic trypomastigotes transform into bloodstream trypomastigotes form in the host skin and multiply for several days, thereby, entering lymphatic system and the bloodstream. At the end, they reach all over the host body including the central nervous system (CNS). The parasites proliferate and protect themself from host's immune system by constantly changing their antigenic coat of variant surface glycoprotein (Kirchhoff, 1998; Barrett, 2003).

The patient diagnosed with trypanosomiasis had symptoms such as pain and unusual fevers as well as swelling of lymph glands and spleen, followed by anemia, headache, joint cramp and tissues injury. Some neurological disturbances also appear such as abnormal sleep pattern, poor coordination, and personality change, and if untreated lead

to fatality within weeks or month (Lutje *et al.,* 2013). The parasitic stage appearing in bloodstream and procyclic forms are reported on most of trypanosomes studies because this form can be readily cultured *in vitro* (Mathews, 2005)

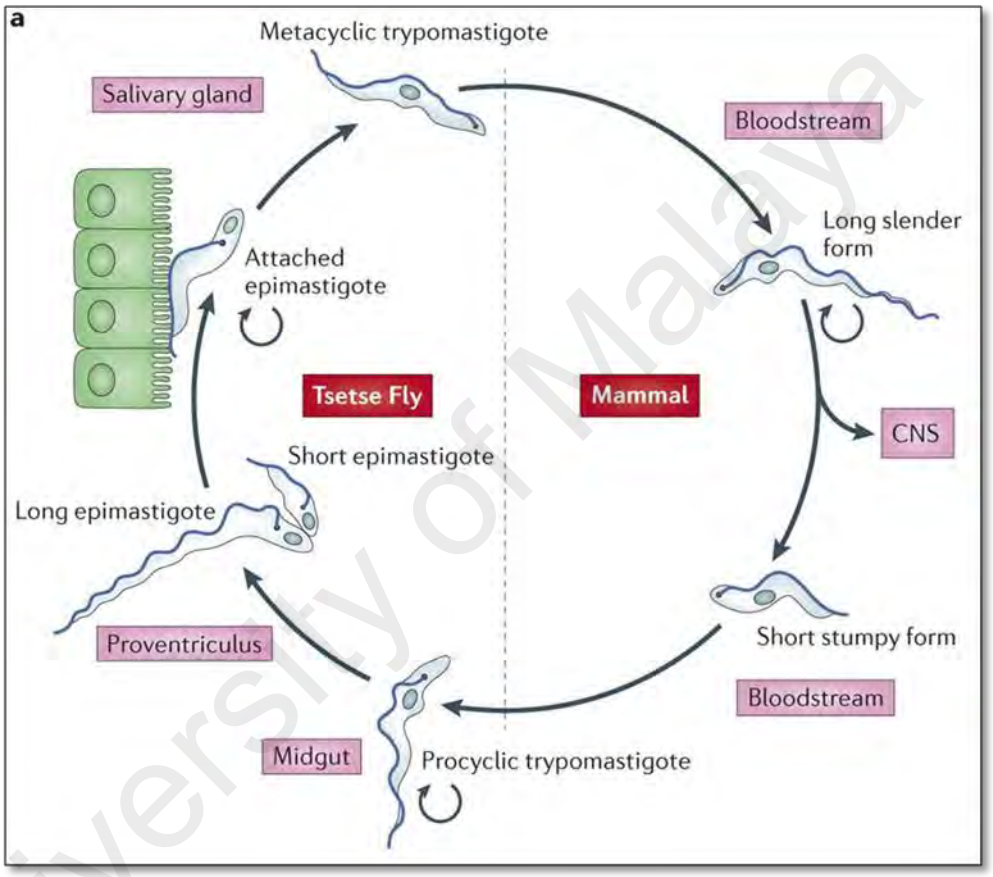


Figure 2.4: The life cycle of *T. brucei* in human and tsetse fly (Retrieved from Langousis & Hill, 2014)

2.2 Natural Product

2.2.1 Natural Product with Antitrypanosomal Activity

Plants, microorganisms and marine organisms from nature provide a wide potential source of such new drugs since these sources contain various molecules with a variety of structures and pharmacological activities (Dias *et al*., 2012). Two-thirds of the world population were estimated as still relies on traditional remedies, mainly from plants, due to restriction of availability and affordability of current medicines (Chintamunnee & Mahomoodally, 2012; Ekor, 2013).

Most of currently used drugs are natural products or derivative compounds. Several metabolites obtained from plants such as terpenes, phenolic derivatives, and alkaloids have shown to exhibit *in vitro* antiparasitic activity (Hoet *et al.,* 2004). In fact, some established drugs, such as artemisinin and quinine used for protozoal treatment are derived from nature (Kirby, 1996; Comacho *et al*., 2000; Torres *et al*., 2014; Newman & Cragg, 2016). In addition, natural products with most potent antitrypanosomal activity are listed in Table 2.1.

Screening for antitrypanosomal activity of extracts from plants was reported by Freiburgghaus *et al.* (1996). In the study, out of 178 plant extracts tested, 32 extracts (15%) showed active antitrypanosomal activity with IC_{50} < 10 µg/ml. Extract from *Annona senegalensis*, *Bussea occidentalis* and *Physalis angulata* showed high antitrypanosomal activity with IC_{50} values below 1 µg/ml.

Meanwhile, study on *in vitro* antitrypanosomal activity and cytotoxicity of 125 North American plant extracts by Jain *et al.* (2016) discovered eight plant extracts with

highest antitrypanosomal activity with IC_{50} value < 1 µg/ml. These active plant extracts are *Coccoloba pubescens, Rhus integrifolia, Nuphar luteum, Alnus rubra, Hoita macrostachya, Sabal minor, Syzygium aqueum* and *Hamamelis virginiana*.

The crude extract normaly showed a high antitrypanosomal activity IC_{50} values as compared to the standard drug used (suramin and pentamidine) (Hoet *et al.*, 2004). Hence, purification of active extracts might result in pure compounds with high biological activity need to apply to crude extracts since it's contain mixtures of compound.

Table 2.1: List of natural product with most potent antitrypanosomal activity reported in literature (Grewal *et al*., 2016; Cheuka *et al*., 2017)

Source of natural product	Compound	Remarks
Miconia lepidota (Melastomataceae)	Primin (quinine derivative)	* IC_{50} value 0.14 µM against *T. b. rhodesianse* (Butler, 2008)
Solanum congestiflorum (Solanaceae)	(1S,6S)-2,2-dimethyl-5-oxo-7,8-dioxatricyclo[7.3.1.0]tridecan-10yi-2,3-dihydro-1,4-benzodioxine-carboxylate; Merulin A derivative (solacongestidina)	* IC_{50} value of 0.043 µM against *T. b. brucei* * High selectivity over mammalian cells * Good brain permeability (Navarro *et al*., 2012)
Marine actinobacterium *Micromonospora* sp.	Labosamide A	* IC_{50} value of 0.8 µM against *T. b. brucei* (Schulze *et al.,* 2015)
Centaurea salmantica (Asteraceae)	Cynaropicrin	* IC_{50} value of 0.28 µM against *T. b. gambianse* (Schmidt *et al.,* 2009)

Table 2.1, continued.

Source of natural product	Compound	Remarks
Saussurea costus (Asteraceae)	Sesquiterpene lactone-parthenolide	∗ High activity against *T. b. rhodosiense* with IC_{50} value of 0.80 μM (Schmidt *et al.,* 2014)
Deep water fungi (*Chaetomium* species)	Chaetocin	∗ High activity against *T. b. brucei* with IC_{50} value of 0.002 μM ∗ Poor selectivity (Watts *et al.,* 2010)
Carlina acaulis (Asteraceae)	Octadec-2-ynoic acid	∗ Inhibiting *T. b. rhodosiense* with IC_{50} value of 64.5 μM (Carballeira *et al*., 2012)
Cynobacterium Nostoc 78-12A	6-chloro-2-(5-(6-chloro[3,4-b]indol-2-ium-yl)penta-1,4-diyn-1-yl)-9H-pyrido[3,4-b]indol-2-ium	∗ Derived from nostocarboline ∗ *T. b. rhodosiense* inhibitor with IC_{50} value of 0.9 μM ∗ Good selectivity (Bonazzi *et al.,* 2010)

Table 2.1, continued.

Source of natural product	Compound	Remarks
Derived from cryptolepine *Crytolepis sanguinolenta* (Apocynacea)	5-methyl-11((piperidin-4-yl)amino-10H-indolo{3,2-b}quinolin-5-ium chloride	* High activity against *T. b. brucei* with IC_{50} value of 0.01 μM * Good selectivity (Lavrado *et al.,* 2012)
Marine ascidian (*Polysyncraton echinatum)*	Ascididenim	* Potent and selective against *T. b. brucei* inhibitor with IC_{50} value of 0.032 μM (Feng *et al.,* 2010)
Carribbean sponge *(Pandaros acanthifolium)*	Pandaroside G methyl ester	* High activity against *T. b. rhodosiense* with IC_{50} value of 0.038 μM (Regalado *et al.,* 2010)
Amomum aculeatum (Zingiberaceae)	Aculeatin D	* High activity against *T. b. rhodosiense* with IC_{50} value of 0.5 μM (Heilmann *et al.,* 2000)

Table 2.1, continued.

Source of natural product	Compound	Remarks
Flindersia acuminate (Rutaceae)	Isoborreverine	* High activity against *T. b. brucei* with IC_{50} value of 2.87 µM * Low selectivity compared to mammalian cells (Scala *et al.,* 2010)
Sponge *Psammaplysilla purpurea*	Isobastadin 13	* High activity against *T. b. rhodosiense* with IC_{50} value of 1.77 µM (Calcul *et al.,* 2010)
Gossypium barbadense	6-6'-dimethoxygossypol	* High activity against *T. b. brucei* with IC_{50} value of 5.8 µM * High toxicity (Wang *et al.,* 2008)
Marchantia emarginata (Marchantiaceae)	Marchantin A	* High activity against *T. b. rhodosiense* with IC_{50} value of 2.09 µM (Ebrahimi *et al.,* 2013)

Table 2.1, continued.

Source of natural product	Compound	Remarks
Fungi- *Acremonium sclerotigenum* (Hypocreaceae)	Ascofuranone	* High activity against *T. b. brucei* with IC_{50} value of 0.13 nM * Good selectivity (Minagawa *et al.,* 1997; Yabu *et al.,* 2006)
Streptomyces grizeolus	Sinefungin	* Potent inhibitor of *T. b. rhodosiense* with IC_{50} value of 0.0004 μM * Good selectivity (Kaminsky *et al.,*1996)

2.2.2 Apocynaceae Family

The Apocynaceae family consists of more than 250 genera and 2000 species of tropical trees, shrubs and vines (Wiart, 2006; Dey *et al.,* 2017). In the Asia Pacific region, Apocynaceae species is used traditionally to treat gastrointestinal illness, fever, diabetes, malaria, injury and infectious disease, while in the South Africa, they were used to treat skin and ectoparasitic diseases (Omino & Kokwaro, 1993; Wiart, 2006; Wong *et al.,* 2013). Apocynaceae species have also been reported to display antiplasmodial and anticancer effects (Wong *et al.,* 2014; Dey *et al.,* 2017). Nevertheless, some genera of Apocynaceae are also important as timber species and almost all species produce milky sap. Apocynaceae family was reported to be the richest containing alkaloid with a total of 2664 alkaloids isolated from 400 species belonging to 76 genera (Cordell *et al*., 2001).

2.2.3 *Dyera costulata* (Miq.) Hook.*f*

Dyera costulata (Miq.) Hook.*f* locally known as jelutong belong to Apocynaceae family. It is among the largest tree in Southeast Asia and in Peninsular Malaysia, it has been recorded to reach 60.6 m in height and 7.9 m in girth with straight and cylindrical bole (Aminah & Lokmal, 2002). It's normally found in the primary evergreen lowlands or on hills in the Peninsular Malaysia, south of Thailand, Sumatra, Borneo and the intervening islands (Whitmore, 1973; Aminah & Lokmal, 2002; Middleton, 2004). The characteristic of light hardwood timber of *D. costulata* has been used in wide range of consumer industry product such as pencil, clog, picture frame and others. Beside that their latex has been used for chewing gum (Burkill, 1966; Aminah & Lokmal, 2002).

The bark and leaves of *D. costulata* have been traditionally used for the treatment of fever and inflammation (Subhadhirasakul *et al.,* 2003). Besides that, it was also reported that leaf extracts of this plant showed antiplasmodial activity and *in vivo* analgesic effect in mice (Reanmongkok *et al.,* 2002, Wong *et al*., 2011). Their sawdust also has been reported to cause allergic dermatitis (Meding et al., 1996).

Figure 2.5: *Dyera costulata* whole tree, leaves and fruit in specimen picture (From the top on the left)

2.2.4 Alkaloid

2.2.4.1 Overview of Alkaloid

Alkaloids are one of the largest chemical compounds group consisting of nitrogen atoms bases with a heterocyclic ring. According to Pelletier (2001), a "true alkaloid" possesses (1) present of a nitrogen atom, in apart of heterocyclic system; (2) a complex molecular structure; (3) important pharmacological effects; (4) originated from the plants. Most of alkaloids isolated qualified with these four parts of definition. However, some other alkaloids not from plant origin may display the commom properties of an alkaloid substance. They are alkaloid from animal origin. These examples of alkaloids from animal origin, include a steroidal alkaloid batrachotoxinin A and bufotenine from an extremely toxic skin secretions of the poison dart frogs from the genus of *Dendrobates* and *Phyllobates* from South America (Daly, 1998; Chamakura, 1994).

Alkaloids not only can act as defense compounds in plants against pathogen, predator, but also to man due to their toxicity (Matsuura & Fett-Neto, 2015). Hence they are widely used in medicine and well known by their various pharmacological effect studied (Alves de Almieda *et al*., 2017). Some important of therapeutic drugs from alkaloid include morphine to treat severe pain, emantine and cephaeline as antidotes for intoxication, quinine for its antimalarial properties. Besides that, vincristine, vinblastine and camptothecin are used for antitumor, serpentine and ajmalicine for antihypertensives, while sanguinarine for antimicrobial properties (Amirkia & Heinrich, 2014). Their biosynthetic precursors are always amino acid, other multi carbon units, e.g. acetate are also incorporated into the final structure of some alkaloids.

The majority of alkaloids which are insoluble in water but soluble in polar organic solvent (methanol, chloroform, ether) are present in crystalline, colorless, non volatile solid compounds. However, some alkaloids are water solube liquids, for examples coniniine, hygrine, nicotine (Sujata *et al.,* 2005; Jayakumar & Murugan, 2016). The presence of alkaloid in most of the substituent is detected either by precipitants or characteristic color reagents (Neelima *et al*., 2011). This is because of their reaction with solutions of molybdic acid, picric acid, chloroplatinic acid, potassium mercuric iodide and others. Various precipitant reagents are shown in Table 2.2.

Table 2.2: Reagents for detecting alkaloids

Reagent	**Composition of the reagent**	**Result**
Meyer's reagent	Potassiumercuric iodide solution	Cream precipitate
Wagner's reagent	Iodine in potasium iodide	Reddish brown precipitate
Hager's reagent	A saturated solution of picric acids	Yellow precipitate
Dragendorff's reagent	Solution of potassium bismuth iodide	Orange or reddish brown precipitate
Tannic acid reagent	A solution of 10% tannic acid	Buff color precipitate

2.2.4.2 Alkaloid as Antiprotozoal

Alkaloids are the largest group of plant secondary metabolites, already providing many drugs for human use (Cordell *et al.,* 2001; Wink, 2012). Even though no plant-derived alkaloid is currently in drug development for antiprotozoal treatment, alkaloids continue to be useful in lead discovery because of their physicochemical and biological properties.

Due to their capability to depolymerize microtubules and inhibiting cell division, plant alkaloids are widely used as therapeuticc agents. Numerous reports provide evidence that alkaloids possessing an indole moiety could display important antiprotozoal activities. The alkaloids with antiprotozoal activity according to their structure and categories were reviewed by Osorio *et al*. (2008). Over than 350 years, quinine alkaloid isolated from *Cinchona succirubra* (Rubiaceae) originated from Peru in early 17th century has been used for the treatment of malaria (Gurung & De, 2017). Quinine, quinidine, cinchonine and cinchonidine have significant antitrypanosomal activity (*T. b. brucei)* with IC_{50} values of 4.9, 0.8, 1.2 and 7.1 μM, respectively. Among them, both quinidine and cinchonine display high selectivity index (SI > 200), showing the capability of alkaloids for further drug development (Hoet *et al.,* 2004). Beside quinoline alkaloids, promising antitrypanosomal activity was found in isoquinoline alkaloids. Predicentrine, glaucine and boldine showed inhibition of the *in vitro* growth of *T. cruzi* epimastigotes with IC_{50} values of 0.08, 0.09, and 0.11 mM, respectively (Morello *et al.,* 1994).

Indole alkaloids group such as β-carboline alkaloids reported to possess antiprotozoal activity is harmaline. Harmaline, harmane, harmine, harmalol and harmol have exerted antiproliferative effects towards parasites of the genus *Trypanosoma* (Rivas *et al.,* 1999). The indolemonoterpene, camptothecin was known as anti-cancer agent was shown to have a lethal effect on *T. brucei*, *T. cruzi* and *L. donovani* (Bodley & Shapiro, 1995).

Marine alkaloids as manzamine A and other 30 manzamine- type alkaloids were reported to have a broad variety of bioactivities including anti-tumor (Pandit, 1995; Arif *et al*., 2004), insecticidal, antibacterial, antimalarial and leishmaniacidal (Franca *et al*., 2014). Other marine indole alkaloids, fascaplysin was found to show active

antitrypanosomal effects against *T. b. rhodosiense* parasites (Kirsch *et al*., 2000). Ascosalipyrrolidinone A isolated from marine fungus *Ascochyta salicorniae* was found to exhibit a more active antitrypanosomal activity against *T. cruzi* as compared to the control (benznidazole), and in addition possess active antiplasmodial activity (Osterhage *et al*., 2000).

2.2.4.3 Bisindole alkaloid

Bisindole alkaloids group is well known for their biological activities, some of them which are now in clinical use as a separate drug are vinblastine, vincristine, reserpine and taxol (Delorenzi *et al*., 2001). The structure and activity of bisindole alklaoids have been reported and a number of isolated bisindole alkaloids are increasing (Kam & Choo, 2006; Kitajima & Takayama, 2016). Twelve bisindole alkaloids of 69 alkaloids isolated from various *Strychnos* species showed antiplasmodial activity with IC_{50} values of < 2 μM against *Plasmodium falciparum* tested (Schwikkard & Van Heerden, 2002). Another antiprotozoal activity of bisindole alkaloids was reported to be against leishmanial. Those which exhibited strong leishmanial activity are conodurine, *N*-demethylconodurine and conduramine (Munoz *et al.,* 1994).

Antitrypanosomal activities have been exhibited by other bisindole alkaloids. Bruges *et al.* (2012) reported apoptosis effect by bisindole alkaloids staurosporine on *T. evansi*. Meanwhile, strictosidine and acetylstrictosidine, a group of indole alkaloids obtained from *Cephaelis dichroea* showed antitrypanosomal activity toward *T. b. brucei* (Del Rayo Comacho *et al.*, 2004).

2.2.4.4 Mechanism of Action of Alkaloids

Alkaloids cause different metabolic systems in animals, and their toxicity vary according to different mechanism of action. The toxicity may begin by enzymatic alterations affecting the physiological process, inhibition of DNA synthesis and repair mechanisms by intercalating with nuclei acids (Matsuura & Fett-Neto, 2015). The mechanism of action of alkaloids as antiprotozoal are not well known, but most of their mechanisms of action are seems to be related to DNA intercalation (Hoet *et al.,* 2004). Camptothecin, a modified indolemonoterpene alkaloid, showed a broad spectrum antiparasitic activity, probably due to its inhibitory activity against topoisomerase I. Trypanosome or leishmania treated with camptothecin demonstrated in both nuclear and mitochondrial DNA cleavage and covalently linkage to protein. Thus, it is consistent with the existence of drug-sensitive topoisomerase I activity in both compartments (Bodley and Shapiro, 1995). Liriodenine, an aporphine alkaloid was found to be a strong topoisomerase II catalytic inhibitor (likely DNA intercalator) and topoisomerase II poison in kinetoplastid DNA (Woo *et al.,* 1999). Besides that, berberine was reported by Elford (1986) as a potent *in vitro* inhibitor of both nucleic acid and protein synthesis in *P. falciparum.*

The previous study by Leung *et al.* (1996) showed the capacity of bisbenzylisoquinoline alkaloids to block Ca^{2+} uptake through the L-type Ca^{2+} channels. The changes of Ca^{2+} in tissue culture cells or parasite cultures, lead to the important mechanism for the penetration of the trypomastigote forms of *T. cruzi* into the host cell (Dorta *et al.,* 1995; Docompo & Moreno, 1996). Other report demonstrated that these alkaloids are potent inhibitor of trypanothione reductase in *T. cruzi* trypomastigote forms (Fournet *et al.,* 1998).

2.3 Apoptosis

2.3.1 Overview of Apoptosis

Apoptosis is a word of Greek origin, with the meaning of tree shedding their leaves in autumn, which described the "falling off or dropping off" petals from flower or leaves from tree. Apoptosis was first described in 1842 by Crl Vogt and the term of apoptosis was first introduced by John Foxton Ross Kerr in 1972. Apoptosis described tissues cell death by the morphological feature (Hongmei, 2012). Apoptosis is considered a vital component of a diverse biological process, including embryonic development, maintenance of immune system, hormone-dependent atrophy, chemical-induced cell death and cell turnover (Elmore, 2007; Hongmei, 2012).

The programmed cell death (PCD) also referred to apoptosis, suggesting that cell death induced by substances, following a sequence of controlled steps leading to defined self-destruction during development and not caused by accidental nature (Lockshin & Williams, 1964; Fuchs & Steller, 2015).

Apoptosis is a type of cell death regulated in a systematic way by a series of signal cascades under defined situations. This process performed an essential part in growth control, development and immune response, and diminished abnormal cells in the organism. The cooperation of the series of molecules including signal molecules, receptor, enzymes and gene-regulating proteins lead to the induction and execution of apoptosis (Launay *et al*., 2005). Therefore, the apoptotic mechanism of cell death is an active and defined process which performed an important responsibility in the cells development and in the regulation and sustainable of cell population which can be

triggered by physiological and pathological inducer (Leist & Jaattela, 2001; Hongmei, 2012).

Apoptosis has been found in tumor development, which involved alteration of apoptotic pathway leading to featured tumors. Hence molecular apoptosis signal pathway became an attractive target in cancer therapy. Besides that, apoptosis has been also found in some other diseases such as neurodegenerative diseases, stroke and diabetes (Hongmei, 2012). Two parts of ligands have been reported which can trigger or inhibit apoptosis which is extrinsic signal ligand (cytokinese, drugs, pathogen infection and native/bioactive compound) and intrinsic ligands [oxdative stress, cytocrome C, calcium iron and endoplamic reticulum (ER) stress] (Elmore, 2007; Hongmei, 2012).

Apart from apoptosis, two other forms of cell death pathways namely necrosis and autophagic cell death have been described. Necrosis cell death is morphologically characterized by cell volume increment (oncosis), swelling of organelles, disrupt of plasma membrane, and subsequent loss of intercellular content and occurring without common biochemical markers. While autophagic cell death appears in the context of autophagy and with the formation of a new double membrane-surrounded vesicle called autophagosome to engulf and digested cellular material (Kroemer *et al*., 2009).

Apoptosis was primarily considered a characteristic of the multicellular organism, but a recent report found that similar mechanism occurs in unicellular organism, including trypanosomatids from *Trypanosoma* genera (*T. cruzi and T. brucei*) and *Leishmania* (Smirlis *et al.,* 2010; Kaczanowski *et al*., 2011; Kaczanowski, 2016).

2.3.2 Apoptosis in Protozoa

Programmed cell death (PCD) is an essential feature of the life of multicellular organism for the development, growth and resistance to infection. Apoptosis has been reported in unicellular organism group and almost all groups of eukaryotes, such as yeast, kinetoplastids and other protozoan parasites (Deponte, 2008; Kaczanowski *et al.*, 2011). Understanding of PCD in parasite organism remains limited as compared to apoptosis in multicelullar organism (Kaczanowski *et al.*, 2011). Even though an apoptosis feature was first described in trypanosomatids in 1995 by Ameisen *et al*., awareness of existence PCD was discovery recently (Zandbergen *et al*., 2010; Engelbrecht *et al*., 2012). Several pieces of evidence have gained over the years showing morphological and biochemical events during the death of trypanosomatids share certain characteristics with apoptosis phenomena as a multicellular organism (Politt *et al*., 2010; Jimenez-Ruz *et al*., 2010; Smirlis *et al.,* 2010; Kaczanowski *et al.*, 2011).

PCD in protozoan parasites has developed as an attractive field of parasite cell biology. Apoptotic phenotype has been reported to show in the group of protozoans ranging from kinetoplastids to alveolates group (Jimenez-Ruz *et al.*, 2010). Certain drug targets in trypanosomatid mitochondria including electron transport chain, kDNA and topoisomerases inhibition were suggested to be involved in inducing apoptosis by impairment of mitochondrial membrane potential and/or production of reactive oxygen species (reviewed by Fidalgo & Gille, 2011). Apoptosis in protozoa was identified by several morphological and biochemical characterization such as the presented of membrane-blebs (apoptotic bodies), chromatin condensation, DNA fragmentation, cell shrinkage and phosphatidylserine (PS) exposure in membrane plasma (Jimenez-Ruiz *et al.,* 2010) shown in Table 2.3.

Besides that, the process of cell proliferation, cell cycle progression or cell death can regulate the density of parasites cell numbers in vectors and mammalian host protozoan. Hence, PCD mechanisms in trypnosomatids, which indicate the occurrence of apoptosis are associated with trypanosomatids density (Luder *et al*., 2010; Kaczanowski *et al.*, 2011).

Table 2.3 Apoptosis inducement in protozoa by different inducers

Species	Live cycle stage	Signs of PCD	Induced by	Reference
Trypanosoma brucei	Procyclic form and bloodstream form	-DNA fragmentation -PS exposure -Mitochondrial membrane potential loss -Nuclear chromatin condensation -Cytoplasmic condensation -Spliced leader RNA silencing	-Drugs -Prostaglandin D_2 -Reactive oxygen species -High density culture -Endoplasmic reticulum (ER) stress	Duszenko *et al*., 2006; Michaeli, 2012, 2015
Trypanosoma cruzi	Bloodstream trypomastigotes and epimastigotes	-DNA fragmentation -Nuclear chromatin condensation -PS exposure -Mitochondrial membrane potential loss -Cytochrome *c* release	-Late stationary phase culture -*Bothrops jararaca* venom -Drugs -Aromatic diamidines -Heat shock -3-Hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile) (MBHA3)	Duszenko *et al*., 2006; Sandes *et al.,* 2014

Table 2.3, continued.

Species	Live cycle stage	Signs of PCD	Induced by	Reference
Leishmania spp.	Promastigotes	-Nuclear shrinkage -Nuclear chromatin condensation -DNA fragmentation -Membrane blebbing -Mitochondrial membrane potential lose -Cytochrome *c* release -Apoptotic bodies -PS exposure	-Reactive oxygen species -Drugs -Heat shock -Differentiation -Flavanoids -Respiratory chain inhibition -Lipopolysaccharide (LPS)	Shasha , 2006; Duszenko *et al*., 2006; Islamuddin *et al.,* 2014; Shadab *et al.,* 2017

Table 2.3, continued.

Species	Live cycle stage	Signs of PCD	Induced by	Reference
Leishmania spp.	Amastigotes	-DNA fragmentation -PS exposure -Nuclear chromatin condensation -Membrane blebbing -Protease activation	-Reactive oxygen species -Drugs -Differentiation	Shasha, 2006; Duszenko *et al*., 2006; El-Hani *et al.,* 2012; Gutierrez-Kobeh *et al*., 2013
Plasmodium spp.	Ookinetes Liver stage Blood stage	-DNA fragmentation -PS exposure -Parasite disintegration -DNA fragmentation	-Reactive oxygen species -Chloroquine -H_2O_2	Picot *et al*., Van de Sand *et al.,* 2005; Sturm *et al*., 2006; Oakley *et al.,* 2007; Ch'ng *et al*., 2010; Engelbrecht *et al.,* 2012

Table 2.3, continued.

Species	Live cycle stage	Signs of PCD	Induced by	Reference
Blastocystis hominis	Vacuolar, granular, amoeboid form	-Cellular shrinkage -PS exposure -Apoptotic bodies -Cytoplasmic vacuolization -DNA fragmentation	-Monoclonal antibody -Metronidazole	Tan & Nasirudeen, 2005; Durga *et al*., 2016
Trichomonas vaginalis	Trophozoite	-Apoptotic bodies -DNA fragmentation -Nuclear chromatin condensation	-Drugs	Chose *et al.,* 2003[a]; Kashan *et al*., 2015
Giardia lamblia	Trophozoite	-Apoptotic bodies -DNA fragmentation -Nuclear chromatin condensation -Cytoplasmic vacuolization	Drugs	Chose *et al.,* 2003[b]

Table 2.3, continued.

Species	Live cycle stage	Signs of PCD	Induced by	Reference
Dictyostelium discoideum	Amoeba	-Vacoulization -Cytoplasmic condensation -Nuclear chromatin condensation -Delayed plasma membrane disruption DNA degradation, no DNA fragmentation -Mitochondrial transmembrane potential lose -PS exposure	-Starvation - Reactive oxygen species	Cornillon *et al.,* 1994 Arnoult *et al.,* 2001; Kadam *et al*., 2017
Peridinium gatunense	Flagellate	-DNA fragmentation -Cytoplasmic condensation	-CO_2 limitation -Reactive oxygen species	Vardi *et al.,* 1999

Table 2.3, continued.

Species	Live cycle stage	Signs of PCD	Induced by	Reference
Tetrahymena thermophila	Ciliate	-Nuclear chromatin condensation -DNA fragmentation -Protease activation	-Drugs -C_2 ceramide -Fas-ligand	Kobayashi & Endoh, 2005

2.3.3 Mechanism of Apoptosis

Over the past 10 to 15 years' considerable progress has been observed in the understanding of protozoan parasites apoptotic pathway which may affect their biological and mammalian hosts. Apoptosis in multicellular organisms is a well-defined process and two apoptotic induction pathways have been established which are the extrinsic pathway and intrinsic pathway. Both of these pathways are caspase-dependent involving the effector caspases which then activate the proteases and nucleases, eventually leading to classical hallmarks of apoptosis (Duszenko *et al*., 2006; Kaczanowski, 2016).

Apoptosis in unicellular organism share some morphological features with apoptosis in multicellular organism. These features include nuclear DNA fragmentation, cell shrinkage, nuclear chromatin condensation, phosphatidylserine (PS) exposure, mitochondrial membrane potential change, cytochrome C release, protease activation and cytoplasmic condensation (Arambage *et al*., 2009; Menna-barreto & Castro, 2015). However, with limited knowledge on the molecular mechanism involved in apoptosis of a parasite, it remains unclear as to which marker plays a role and which condition contributes to apoptosis. Apoptosis in unicellular organisms may be initiated by a variety of agents which include physical stress, chemical stress and drugs (Figure 2.6) (Duszenko *et al*., 2006; Menna-Barreto & Castro, 2015). Mitochondrial in unicellular parasites play a major role in cell death pathway, leading to reactive oxygen (ROs) production (Piacenza *et al*., 2007). There is also a situation, which some protozoan parasites have been found to involve in more than one apoptotic pathway. *Blastocystis hominis*, the intestinal protozoan parasite exhibits both caspase-dependent and independent pathways of apoptosis (Tan & Nasirudeen, 2005). The detailed knowledge

of the underlying molecular mechanisms will certainly contribute to developing actions and strategies to combat protozoan parasites.

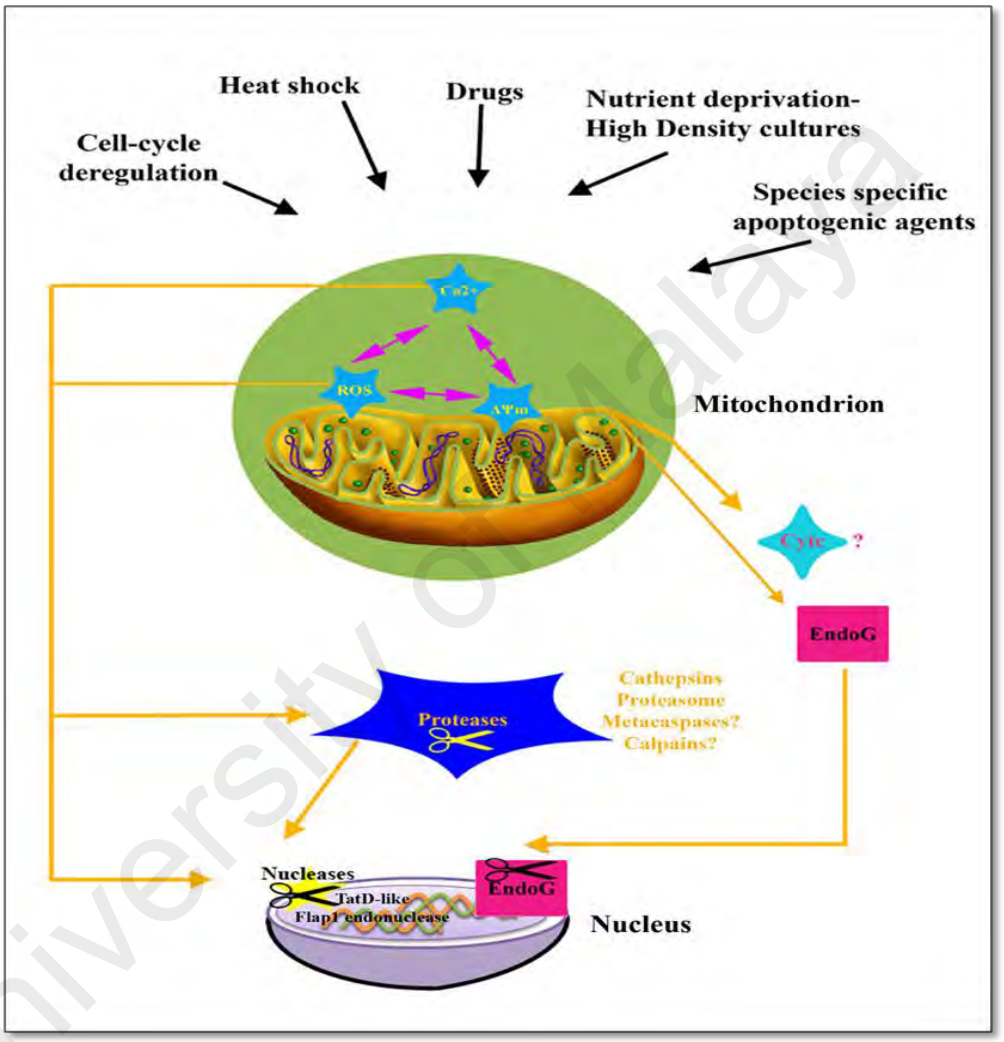


Figure 2.6: Representative of apoptotic pathways involved in trypanosomatids (Retrieved from Smirlis *et al.,* 2010)

2.3.4 Method for Detection of Apoptosis

The term of apoptosis describes a specific morphological aspect of cell death as referred to the Nomenclature Committee on Cell Death (NCCD) which showed cellular alterations during the apoptotic process. These common apoptotic cell death characterizations include cell shrinkage, DNA fragmentation, phosphatidylserine exposure, mitochondrial alterations and chromatin condensation have been observed in most unicellular organism including protozoan parasites (Jiménez-Ruiz *et al.,* 2010; Proto *et al*., 2013). Hence, methods for detecting and measuring apoptosis are based on these markers (Kaczanowski, 2016).

The series of apoptotic events was initially studied based on the morphological changes in cell structure (Kerr *et al*., 1972; 1994). Besides cellular observation, changes at molecular level affected by apoptosis can also be determined. A broad number of methods devoted to the identification of apoptotic cells and the analysis of this biological process utilize various tools and approach such as agarose gel electrophoresis, different staining method detected by light and fluorescent microscope and flow cytometry (Table 2.4).

Table 2.4: Methods for the detection of apoptosis

Methods	
Microscopic technique	Cellular features by light microscopy Fluorescence DNA stains for nuclear morphologic analysis Confocal laser microscopy Electron microscopy
Assessment of DNA fragmentation	Enzyme-linked immunosorbent assay (ELISA) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling assay (TUNEL) Comet assay DNA diffusion Gel electrophoresis
Flow cytometry and laser scanning cytometry	Cell cycle DNA content Phosphatidylserine translocation Inner mitochondrial transmembrane potential Caspase activity
Gene expression analysis	RT-PCR Northern blot RNA protection assay Immunohistochemistry
Measurement of apoptosis-associated protein	ELISA Western blot Electrophoretic mobility shift assay

2.3.5 Cell Cycle

The cell cycle is a type of mechanism which involved cell replication process in eukaryotic and prokaryotic cells. Regulation of cell division is activated by intricate and interconnected signal transduction pathways that are organized accordingly, involving the replicating and segregating the compartment of the cell (Hammarton, 2007; Hayashi & Akiyoshi, 2018). Cell proliferation and death in multicellular organisms must be regulated in order to maintain tissue development. The typical eukaryotic cells cycle set of events responsible for cell replication and consists of four growth phases – G_0/G_1, S, G_2 and M. The genetic information transform from one cell generation to the next by replication of genome in S-phase, while division into two new daughter cells present in mitosis or M-phases. In a normal cell cycle, S-phase begins first until completion and followed by M-phase. There are two gaps between S and M phases which are G_1 between M and S and G_2 between S and M (Hammarton, 2007; da Silva *et al.,* 2013).

The cell cycle event timing and order are monitored during cell cycle checkpoints at the G_1/S boundary, in S-phase, and during the G_2/M-phases (Ploubidou *et al.,* 1999). The checkpoints which are referred to as a series of the control system for the cell proliferation function in the presence of signals such as growth factors. DNA damage and misaligned chromosomes during mitotic process will also contribute to the checkpoints activation. Co-ordination of cell cycle events is important for cell survival and hence, it needs to progress in sequentially. Progressions of these events in the cell cycle are mediated by series of activation and inactivation of kinases protein namely cyclin-dependent kinases (Cdks) (Hayashi & Akiyoshi, 2018).

Trypanosome has a complex biphasic life cycle with different developmental forms displaying specific roles within hosts (Hammarton, 2007; Smith *et al*., 2017).

Trypanosome is vermiform in shape, contains a number of organelles and structures such as nucleus, mitochondrial (which contained kinetoplast DNA), golgi and basal body/flagellum complex. The cells are duplicated and segregated accurately to generate viable progeny (Hammarton, 2007).

The cell cycle of trypanosomes exhibits the same characteristics as normal eukaryotic cell cycle with the same periodic nuclear events, G_1, S, G_2 and M phases. In contrast to the normal cell cycle, trypanosome displayed a periodic S phase for their unit mitochondrial genome, the kinetoplast (Li, 2012). Prior to mitosis, the segregation of kinetoplast showed additional markers for cell position in cell cycle process (Hoffmann *et al*., 2018).

Throughout the G_1 phase, the probasal body matures (lying adjacent to the flagellar basal body), followed by daughter flagellar outgrowth and the formation of new probasal bodies. In the S phase, kinetoplast is much shorter than nucleus and commences just prior to nucleus, suggesting that inter-organelle control mechanisms coordinating DNA synthesis. Like eukaryotic cell cycle, protein kinases have been shown to be involved in the regulation of cell cycle progression in *T. brucei,* Figure 2.7 (Jones *et al*., 2014).

Cell cycle regulators in human cell are believed to be potential targets in several disorders, including cancer and neurodegenerative diseases. In protozoa, cell cycle control might be exploited in the development of novel therapeutic since the key molecule for mechanism of cell cycles such as cyclin-dependent kinases and cyclins have been identified in protozoa cells (Hammarton *et al*., 2003; Hayashi & Akiyoshi, 2018). The apoptosis and cell cycle relationship shows both mitosis and apoptosis exhibit similar morphological features. The proliferation and cell cycle control with

apoptosis indicates that an organism can regulate cell expansion and is crucial for cellular homeostasis. Hence, the deregulation of the cell cycle may result in apoptosis (Smirlis *et al*., 2010).

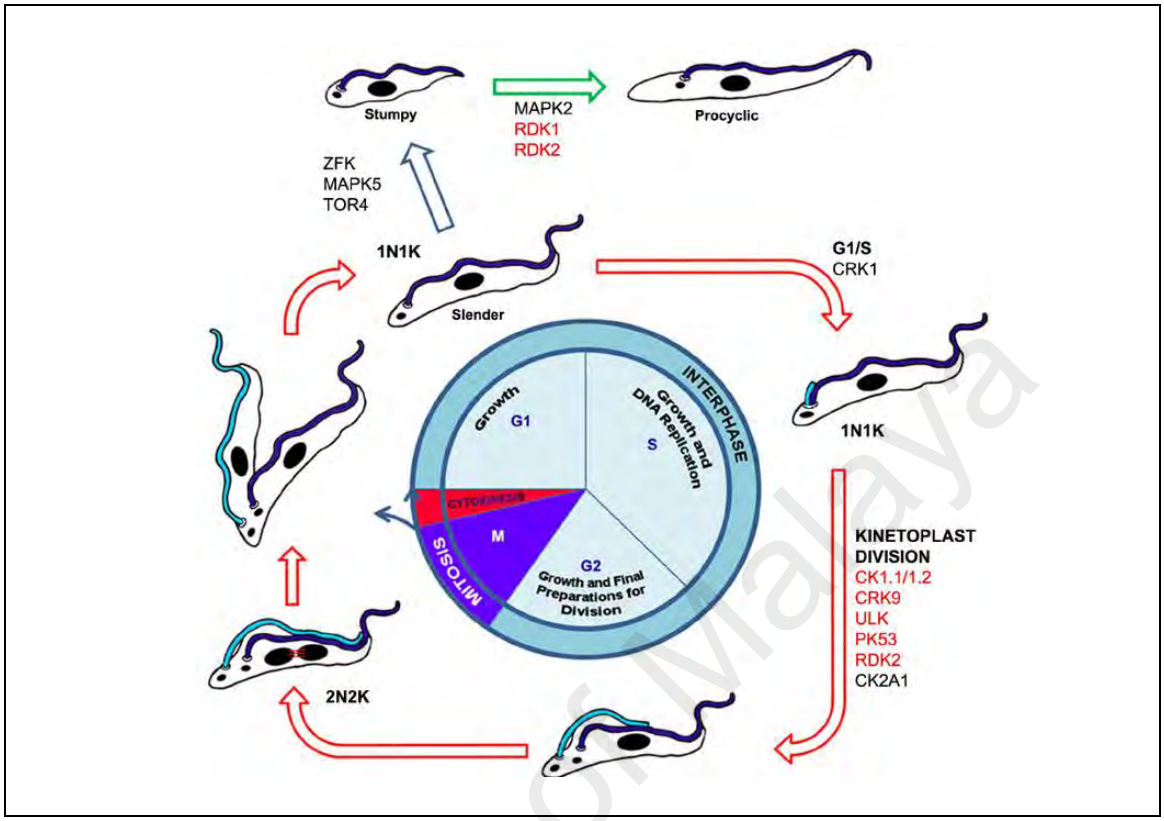


Figure 2.7: Cell cycle progression of the bloodstream form (BSF) trypanosome and differentiation from BSF to procyclic form (PCF). (Retrieved from Jones *et al*., 2014)

CHAPTER 3: MATERIALS AND METHODS

3.1 Research Outline

The current study was conducted to isolate antitrypanosomal active compound in *Dyera costulata* (Miq.) Hook.*f* leaves and investigate if the isolated compound induce apoptotic cell death in *Trypanosma brucei brucei* strain BS221. The experimental design and workflow are in Figure 3.1:

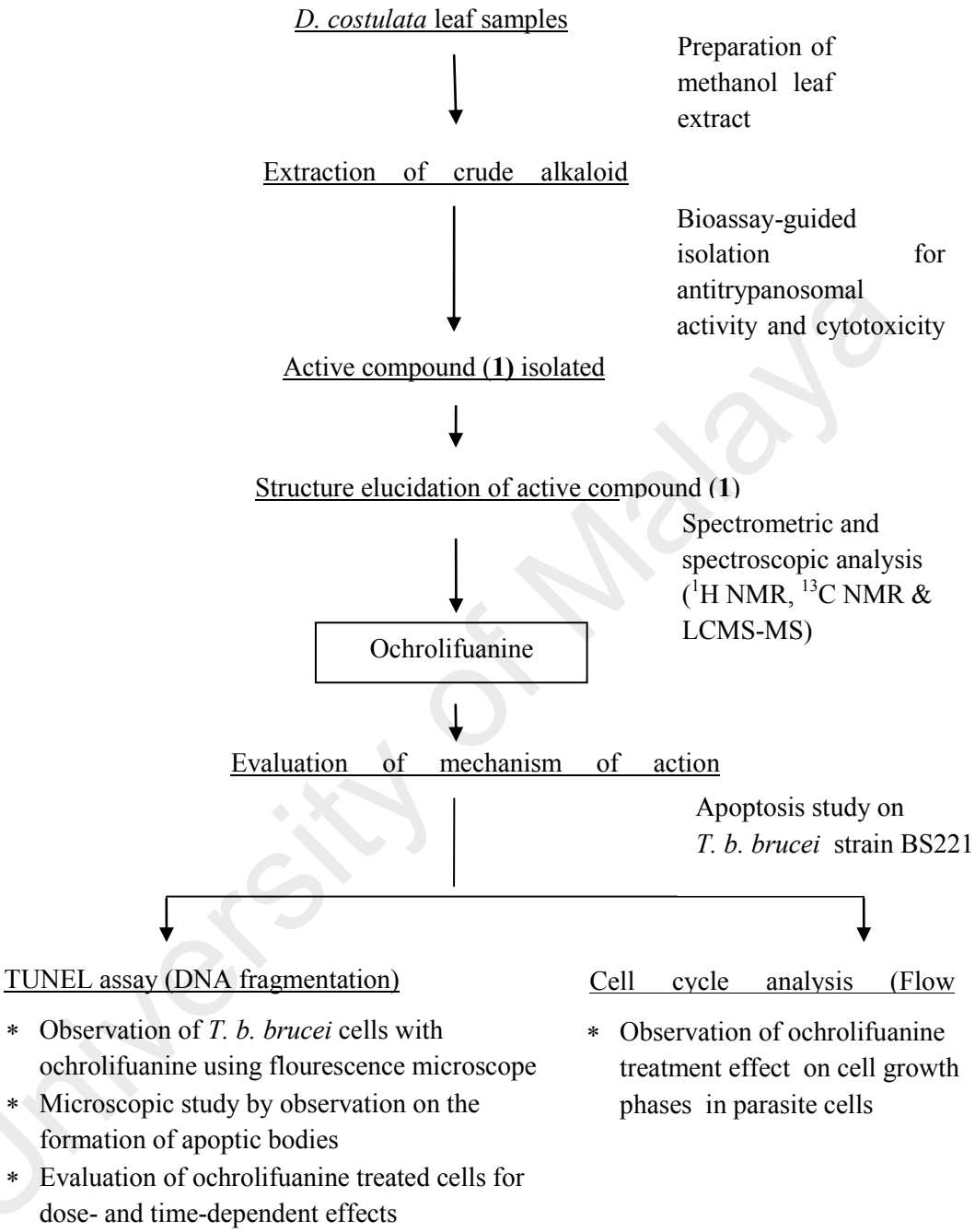


Figure 3.1: Experimental design and work flow

3.2 Plant Material

Leaf samples of *Dyera costulata* (Miq.) Hook.*f* were collected from Forest Research Institute Malaysia (FRIM), Malaysia in the month of September 2012. The taxonomic identification of plant species was authenticated by a botanist at the Forest Biodiversity Division, FRIM and was comparable with reference specimens at FRIM Herbarium (voucher no: FMS 29764).

3.3 Extraction of Crude Alkaloid Fraction from *D. costulata*

The fresh leaves of *D. costulata* (1.2 kg) were dried in the oven (Protech, USA) at 50 C for 5 days and ground into fine powder. The dried powder (91 g) was successively extracted by soxhlet extraction apparatus using 1.5 L of hexane (MERCK, Germany) for 18 hours. The mixture was then filtered through Whatman filter paper (grade no.1) and the solvent evaporated using rotary evaporator at 45 ˚C to yield hexane extract. The residue was extracted again using 1.5 L of methanol (MeOH, MERCK, Germany) using same extraction protocol to obtain MeOH extract. The crude extracts obtained were kept at 4 ˚C for further use in fractionation.

A total of 30 g crude MeOH extract was further subjected to crude alkaloid extraction using the method of Alaa *et al.* (2014) with some modifications. 600 ml of ethanol (EtOH, MERCK, Germany) was added to the extract, sonicated for 1 hour and mixed continuously in an orbital shaker at 45 ˚C, 200 rpm for 2 hours. After that, 600 ml of 10 % (w/v) lead acetate in ddH_2O was added and the mixture was shake again for another 1 hour. The defatted extract was then filtered and concentrated by rotary evaporator to remove the EtOH. The aqueous layer was then subjected to partitioning using 100 ml chloroform ($CHCl_3$, Fisher Scientific, UK) and the process was repeated

three times. The $CHCl_3$ extract was dried with anhydrous Na_2SO_4 (Sigma-Aldrich, USA) and concentrated under vacuum to yield 11.3 % (3.38 g) crude alkaloid extract.

3.4 Bioassay-Guided Isolation of Active Compound

3.4.1 Fractionation of Crude Alkaloid Fraction by Column Chromatography (CC1)

The active crude alkaloid fraction (3.38 g) was further fractionated using bioassay-guided (antitrypanosomal and cytotoxicity) silica gel column chromatography separation. A total of 38 g of silica gel 60 (70-230 mesh, MERCK, Germany) served as solid phase and was packed into glass column (30 mm id × 300 mm length). The ratio of active fraction and silica gel was approximately 1:10. The active fraction was coated with cellulose prior to the separation process. The column was packed by pouring the silica gel and hexane slurry into the column and equilibrated with hexane for at least an hour before use. A layer of sand was loaded between the extract and silica gel, followed by another layer of sand above the active fraction. The sand layers performed as a protector for the silica gel underneath from being jostled by addition of solvents.

Elution began with 100 % hexane and the solvent polarity was gradually increased using dichloromethane (DCM)/MeOH (1:10 to 0:1) to elute the chemical contituents from the column. Each fraction was collected individually and pooled based on their Thin Layer Chromatography (TLC) profiles or R_f value and tested for antitrypanosomal activity. The TLC analysis was performed routinely on each eluted fractions obtained from column chromatography, to detect the various compounds present in the active fraction.

Pre-coated TLC plates, of silica gel 60 F254, 0.25 mm thickness (MERCK, Germany) were used. The fractions and crude alkaloid fraction samples were spotted using glass capillaries on TLC plates which were then placed in the developing solvent. Solvent DCM/MeOH (9:1, v/v) was used as developing solvent. Plates were observed for separated bands by UV-light at 254 and 365 nm and sprayed with vanillin-sulphuric acid (Appendix 1.1) and dragendorff's reagent (Appendix 1.2), followed by heating on the hot plate until the spot clearly appeared. Vanillin-sulphuric acid reagent was used to detect terpenes, while Dragendorff's reagent to detect an alkaloid in test sample of TLC plate. All pooled fractions were concentrated and guided by antitrypanosomal activity and cytotoxic based on Alamar Blue assay as mentioned in section 3.7.2 and 3.8.3.

3.4.2 Fractionation of Active Fraction by Column Chromatography (CC2)

The active fractions (1.0 g) obtained from first step of bioassay-guided fractionation, column chromatography (CC1) were subjected for further bioassay-guided fractionation process. Silica column chromatography (18 mm id × 300 mm length) was packed with 40 g silica gel 60 (70-230 mesh). Elution started with 100 % hexane and the solvent polarity was gradually increased using DCM/MeOH (1:10 to 0:1). Fractions were collected and pooled based on TLC spot profile after sparayed with Dragendorff's reagent. All pooled fractions were concentrated and guided by antitrypanosomal and cytotoxic activity based on Alamar Blue assay.

3.4.3 Fractionation of Active Fraction by Solid Phase Extraction (SPE) - Reverse Phase

The SPE column was prepared in the laboratory which consist of 150 ml isolute reservoir cartridge (Biotage, Sweden) packed with 5 g of RP-C18 (particle size

25-40 µm, MERCK, Germany). Right before use, C18 column was pre-conditioned with 30 ml of 100 % water (H_2O) and H_2O/MeOH (1:1). A total of 450 mg of active fractions from second step of bioassay-guided fractionation, column chromatography (CC2) were pooled and transferred into an activated SPE column and eluted with 15 ml of a solvent mixture consisting of H_2O, MeOH and DCM. The obtained fractions were pooled based on TLC spot profile and subjected for antitrypanosomal and cytotoxicity assay.

3.4.4 Fractionation of Active Fraction by Column Chromatography (CC3)

The active fractions (250 mg) obtained from bioassay-guided fractionation, SPE separation were pooled and subjected for further bioassay-guided fractionation process using silica column chromatography (10 mm id × 300 mm length) packed with silica gel 60 (70-230 mesh). Elution started with 100 % DCM and the solvent polarity was gradually increased using DCM /MeOH. Fractions were collected and pooled based on TLC spot profile after sparayed with vanillin reagent. The obtained fractions were pooled based on TLC spot profile and subjected for antitrypanosomal and cytotoxicity assay. The most active fraction designated as compound **1** possessed a single spot on TLC plate. The compound **1** was then structurally elucidated through mass spectrometrty (MS) and nuclear magnetic resonance (NMR) spectroscopic data.

3. 5 Structure Elucidation of Active Compound 1

3.5.1 Mass Spectrometry Analysis (MS)

Mass spectrometry of compound **1** was analyzed on a Thermo Finnigan LCQ Dexa XP Plus (San Diego, USA) using electrospray ionization (ESI) voltage of 4500V. The mass spectrometry analysis was performed in a positive ion mode (*m/z* M + H) for detection of compound **1**. The identity of compound **1** was obtained by referring to

previously published data on chemical constituents isolated from *Dyera costulata* (Koch *et al*., 1975; Mirand *et al.,* 1983).

3.5.2 Nuclear Magnetic Resonance Spectroscopy Analysis (NMR)

Nuclear magnetic resonance (NMR) spectroscopy was used to determine the structure of the isolated compound. Further confirmation of the compound structure was carried out by distortionless enhancement by polarization transfer (DEPT) NMR to determine the presence of primary, secondary and tertiary carbon atoms. Two dimensional (2D) NMR was also conducted to determine the correlation between proton and carbon. An amount of 1 mg of compound **1** was submitted to SIRIM Berhad for ^{1}H and ^{13}C NMR spectroscopy analysis on a Bruker NMR spectrometer. The spectral data were recorded at 400 and 100 MHz respectively in deuterated methanol (MeOD).

3.6 *In vitro* Culture of *Trypanosoma brucei brucei* BS221

Trypanosoma brucei brucei strain BS221 (a derivative of S427, also known as MiTat 1.2/221) was obtained from the Swiss Tropical and Public Health Institute (Swiss TPHI) in Basel, Switzerland.

3.6.1 Revival and Culturing of *T. b. brucei*

The frozen *T. b. brucei* BS221 stock in cryogenic vial was taken out from liquid nitrogen tank and quickly thawed at 37 °C in a water bath. The vial was then exposed to 70% (v/v) ethanol and wiped dry. A total of 100 µl of *T. b brucei* suspension were transferred from the vial into 10 wells of a sterile 24-well plate (TPP, Switzerland) containing 900 µl of Basal Minimal Essential Medium (BMEM) (Appendix 1.5) The

BMEM medium consisted of Minimal Essential Medium (MEM) (Appendix 1.3) supplemented with 15% heat activated fetal bovine serum (FBS, PAA, Austria) and Balz supplement (Appendix 1.4) (Raz *et al.,* 1997), and 0.2 mM 2-mercaptoethanol (Sigma-Aldrich, USA). The *T. b. brucei* cultures were then incubated at 37 °C in a humidified atmosphere of 5% CO_2.

After two days of incubation, culture plates were observed under an inverted microscope (BX41 Olympus, Japan) (magnification 100 ×) to select the *T. b. brucei* growing at the logarithmic growth phase which was indicated by fast and strong movement of the parasites. A routine subculturing was carried out every two days. Briefly, a dilution of 1:10 was prepared by adding 100 µl of log phase *T. b. brucei* culture into 900 µl of fresh BMEM in the first well of 24-wells sterile plate. After mixing, 100 µl of *T. b. brucei* culture from the first well was transferred into the second well which contained 900 µl of fresh BMEM and the process was repeated until the third well. A total of 100 µl of *T. b. brucei* culture was discarded from the third well to maintain the 900 µl of total volume in each well. The culture plates were incubated and maintained in a CO_2 incubator at 37 °C and supplemented with 5 % CO_2.

3.6.2 Preparation of Stock Cultures

A total of 3.6 ml of log phase *T. b. brucei* culture were transferred from 24-wells plate into a sterile 15 ml falcon tube. Falcon tubes containing *T. b. brucei* suspension was then centrifuged at 2,700 rpm and 4 °C for 10 min. The pellet was obtained after the centrifugation process and discarded of supernatant. The pellet was further added with 9 ml of freezing media (Appendix 1.6) and 1 ml of the suspension was added to many 1.5 ml cryogenic vials. The vials were kept in a freezing container

(Mr Frosty, Nalgene) and placed at -80 °C freezer for 24 hours. The frozen *T. b. brucei* cells were then transferred into liquid nitrogen (-196 °C) and stored prior to use.

3.7 Evaluation of *In vitro* Antitrypanosomal Activity

3.7.1 Preparation of Test Samples and Controls

The antitrypanosomal activity of extracts, fractions and active compound was evaluated using methods described by Otoguro *et al*. (2008) with some modifications on the concentrations of the samples. Two miligram of the test samples were dissolved in 250 µl of absolute EtOH to produce stock concentration of 8 mg/ml. Stock concentration of test smples were then further diluted with distilled deionized water (ddH_2O) to produce seven different final concentrations ranging from 0.03 to 6.25 µg/ml and 0.001 to 0.100 µg/ml, respectively (Table 3.1).

Pentamidine isothonine (Sigma-Adrich, USA), a standard trypanocidal drug was used as positive control in the *in vitro* antitrypanosomal assay. The drug sample was weighed and dissolved in 100 % dimethylsulfoxide (DMSO, Sigma-Adrich, USA) to produce a 40 mg/ml stock solution. It was further diluted in a round bottom 96-well plate to give six concentrations ranging from 0.195 to 6.25 ηg/ml (Table 3.1).

Table 3.1: Concentrations of test samples used in antitrypanosomal assay

Samples	Concentration (µg/ml) of the sample						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Extract/ Fractions	0.03	0.05	0.10	0.20	0.39	0.78	6.25
Compound	0.001	0.003	0.005	0.010	0.030	0.050	0.100
Standard drug, Pentamidine (ηg/ml)	0.195	0.390	0.780	0.1560	3.125	6.250	-

3.7.2 Alamar Blue Assay

Trypanosoma brucei brucei cultures were observed under inverted microscope (BX41 Olympus, Japan) to determine inoculum selected for cell seeding. Two wells of *T. b. brucei* culture at log phase growth were selected from the 24-well plate and pooled together. The number of *T. b. brucei* was quantified by transferring 10 µl of *T. b. brucei* suspension into 1.5 ml centrifuge tube containing 90 µl of BMEM media and mixed well as described in section 3.6.2. Then, 10 µl of the *T. b. brucei* suspension was loaded into a Neubauer hemocytometer chamber by capillary action and examined under the inverted microscope (magnification 200 ×) to count the cells. A suspension with cell density of 20,000 *T. b. brucei*/ml was prepared in BMEM media for the *in vitro* assay.

A dose-response curve was performed to determine *in vitro* antitrypanosomal activity of test samples by using Alamar Blue assay with some modification of established method by Raz *et al*. (1997). For the treatment, 5 µl of serially diluted test samples (as in section 3.7.1), positive control pentamidine (as in section 3.7.1) and 95 µl of *T. b. brucei* cell suspension was then added into 96-well microtiter plate flat bottom. Negative control-containing solvent [5 % (v/v) DMSO for drug and 25 % (v/v) ethanol

for test samples] and negative control well containing sterile MiliQ water (blank) were also include in the experiment. Plates were incubated for 72 hours at 37 °C in 5 % CO_2 incubator. An amount of 10 µl of the fluorescence dye Alamar Blue [12.5 mg resazurin (Sigma-Adrich, USA) dissolved in 100 ml phosphate buffer saline or PBS without EDTA] was added into each well, and incubation was continued for further 3-6 hours until there is a change in the fluorescent color in the wells. Plates were analyzed using the fluorescent plate reader (Tecan Infinite M200, Switzerland) at excitation wavelength 528 nm and emission wavelength 590 nm and Magellan™ data analysis software. The optical density (OD) values were transferred into the Excel graphics program to produce sigmoidal inhibition curves from which IC_{50} value for extracts, fractions and compound were calculated (Otoguro *et al*., 2008). Each assay treatments were performed in triplicate plates and in three independent experiments. The IC_{50} values are recorded as mean ± S.D.

Antitrypanosomal activities of samples were classified into three categories according to the following guideline (Otoguro *et al.,* 2008);

Antitrypanosomal activity	**IC_{50} value**
Strongly active	$IC_{50} \leq 0.78$ µg/ml
Moderately active	0.78 µg/ ml< $IC_{50} \leq$ 6.25 µg/ml
Not active	$IC_{50} > 6.25$ µg/ml

3.8 Selectivity Index Determination

3.8.1 Human Cell Culture

The WRL-68 cell lines derived from normal human liver cells were purchased from the American Tissue Culture Collection (ATCC). The routine maintenance of the cell lines was performed according to ATCC protocol.

3.8.2 Culturing of WRL-68 Cells

The frozen cryogenic vial containing WRL-68 cells was taken out from the liquid nitrogen tank. The cells were then thawed in a 37 ˚C water bath and further cultivated in 5 ml Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Adrich, USA) (Appendix 1.7), supplemented with 5 % (v/v) FBS (PAA, Austria), antibiotic iU/ ml penicillin (PAA, Austria) and 50 µg/ml of streptomycin (PAA, Austria) in T-25 cm^2 tissue culture flasks. The cell lines were then incubated in a humidified incubator of 5 % CO_2 at 37 ˚C (Siti Syarifah *et al.,* 2011). Healthy adherent cells attached and formed a single layer in the culture flask. The cultures were examined with an inverted microscope to check for growth influency and for any microbial contamination.

The cells were maintained with a routine subculture procedure. The cells layers were rinsed with 5 ml of sterile phosphate buffered saline (PBS-EDTA) (Apendix 1.8) by shaking PBS-EDTA on top layer of cells to remove all traces of serum. This step was repeated twice. Then 2 ml of 0.02 % (w/w) trypsin-ethylenediamine tetra acetic acid (EDTA) were added to the flask and incubation was carried out at 37 ˚C for 10 min to detach the cell layer. After incubation period, cells were observed under the inverted microscope to ensure all cells were completely detached. Subsequently, 5 ml of 5 %

supplemented DMEM was added into flask and mixed well. The cell suspension was then transferred into 15 ml centrifuge tube and centrifuged for 10 min at 2,000 rpm. The pellet was resuspended with 6 ml of 5 % supplemented DMEM and finally 3 ml of cells were transferred into new T-75 cm^2 culture flask which containing 7 ml of 5 % supplemented DMEM. The flask was incubated at 37 ˚C in a humidified atmosphere of 5 % CO_2 for 48 hours. Daily monitoring was performed using an inverted microscope (BX41 Olympus, Japan) to observe for any fungal and bacterial contamination. New 5 % supplemented DMEM was changed every two days.

3.8.3 Cytotoxicity Assay

The confluent, human normal liver WRL-68 cells were detached from the flask as described in section 3.8.2. Briefly, the cell pellet was obtained by centrifugation and the density of viable cells was counted using Neubauer haemocytometer chamber. A total of 100 µl cell suspension were plated in 96 well-plates at a density of 4.0×10^4 cells/well as described by Jean-Robert *et al*. (2009) . The plates were then incubated for 24 hours in a humidified incubator with 5 % CO_2 at 37 ˚C to allow cell attachment to the plate. Subsequently, the old media was discarded and 90 µl of 10 % supplemented DMEM was added to all wells. Ten microlitres of serially diluted test samples at six concentrations of 0.1, 0.39, 1.56, 6.31, 25.0 and 100 µg/ml was added into the wells. Cells without test samples but added with ethanol 5 % (v/v) and DMSO 25 % (v/v) served as negative solvent-controls. Control-blank (sterile MiliQ water) were also included in the experiment. Pentamidine was used as a positive control with a final concentration of 0.1, 0.39, 1.56, 6.25, 25.0 and 100 ηg/ml. The three replicates plates with a final volume of 100 µl/well of test sample and media were incubated for 72 hours in the CO_2 incubator. The percentage of cell viability was determined using Alamar Blue assay (Räz *et al*., 1997) as described in previous, but with an incubation period of

2 hours. The cytotoxicity effects of the test samples were then evaluated by determining the IC_{50} values of cells. The assay plate was analyzed by fluorescence plate reader (Tecan Infinite M200, Switzerland) using an excitation of 530 nm and emission wavelength 590 nm. The data were transferred into Excel program to calculate the IC_{50} values. The assay was performed in triplicates in at least three independent experiments. The IC_{50} values are given as mean ± S.D.

3.8.4 Selectivity Index

The selectivity index (SI) was used to determine the relative (selective) toxicities of the test substances against parasites compared to normal mammalian cells. Selectivity index corresponding to the ratio between IC_{50} value obtained from cytotoxicity assay on normal cell lines and IC_{50} on antitrypanosomal assay was calculated according to the following formula (Nanavaty *et al*., 2016):

$$\text{SI Value} = \frac{IC_{50} \text{ value of test sample on WRL-68 cell line}}{IC_{50} \text{ value of test sample on trypanosome parasites}}$$

The selectivity index (SI) was classified into three categories (Otoguro *et al*., 2008):

Selective	SI ≥ 100
Moderately selective	10 ≤ SI <100
Non selective	SI < 10

The SI value was determined to select test samples that showed high selectivity towards trypanosome parasites and at the same time have low toxicity effect on normal cells.

3.9 Evaluation of Apoptosis-Inducing Effects of Active compound 1 on *T. b. brucei* Strain BSS221

The DNA fragmentation revealed by the presence of multiple of DNA strand breaks is considered to be the important characteristic for identification of apoptotic cells. Since staurosporine, a bisindole alkaloid compound was shown to induce apoptosis in *Trypanosoma* spp., the current study will also investigate if ochrolifuanine from *D. costulata* induces apoptosis-like effect in *T. b. brucei* strain BS221. The ability of ochrolifuanine to induce apoptosis in trypanosome cells was observed by TUNEL assay using fluorescence microscope on the morphology cells which are undergoing apoptosis (qualitative). Further quantitative study was carried out to determine the percentage of apoptotic cells exposed to compound different concentrations treatment and time incubation, to see if there is a dose- or time-dependent effect. In addition, cell cycle analysis was perfomed to determine the effect of ochrolifuanine in cell cycle progression of parasites.

3.9.1 Determination of DNA Fragmentation by TUNEL Assay

DNA fragmentation in *T. b. brucei* strain BS221 cells was detected by using *In Situ* Cell Death Detection Kit AP (Cat No 11648795910; Roche, Germany) according to the protocols described by the manufacturer. Morphological changes in protozoa cells which are undergoing apoptosis were visualized and quantified by using a fluorescence microscope (BX53, Olympus, Japan).

Dose- and time-dependent studies were carried out to determine the effect of ochrolifuanine in parasites cells. The *T. b. brucei* cells undergoing growth at log phase were prepared as described in section 3.7.2. The *T. b. brucei* at a density of 2.0×10^6

/cells were seeded into 24-well plates and treated with the active compound (**1**) at concentrations of 0.025, 0.05 and 0.10 µg/ml in triplicate wells. The DNase I which is a standard apaoptosis enzyme that cleaves DNA and induces DNA fragmentation in *T. b. brucei*, was used as the positive control. The *T. b. brucei* cells in the negative control well were treated with 1 % (v/v) EtOH. Different staurosporine concentration treatment (0.01, 0.02 and 0.4 µg/ml) in triplicate wells on *T. b. brucei* cells also was included in the experiment as the positive control standard. The plates were then incubated at 37 ˚C in CO_2 incubator for 6, 12 and 24 hours.

At the end of treatment period, the treated *T. b. brucei* cells from triplicate wells were pooled and transferred into a 15 ml centrifuge tube (sterile) and centrifuged at 200 × g for 10 min at 4 ˚C. The supernatant was discarded and the pellets were fixed by adding 100 µl of 4 % (v/v) paraformaldehyde (Sigma-Adrich, USA) and 0.2 % (v/v) glutaraldehyde (Sigma-Adrich, USA), left for one hour and then washed with 4 ˚C cold PBS solution. The *T. b. brucei* cell suspension was then smeared on poly L-lysin coated chamber slide (Labtek® Chamber Slide- Thermo scientific, USA) and left to dry. The slides were then layered with 100 µl permeabilization buffer containing 0.1 % (v/v) Triton-X-100 (Sigma-Adrich, USA) in 0.1 % (v/v) sodium citrate (Sigma-Adrich, USA), and incubated for 2 min on ice before washing twice with 4 ˚C cold PBS. Positive control *T. b. brucei* slide cells were treated with 50 µl of DNase 1 (20 U) to induce DNA strand break whereas negative control cells were treated with the final concentration of ethanol present [0.1 % (v/v)] in the treated wells.

Subsequently, the treated *T. b. brucei* slides were labeled with 50 µl TUNEL reaction buffer and incubated for 1 hour in a moist chamber in the dark at 37 ˚C (Akiko *et al*., 2005). The reaction was terminated by washing the slides twice with 4 ˚C cold PBS. The slides were then counterstained with 1 µg/ml propidium iodide (PI)

(Sigma-Adrich, USA) for 15 min and incubated in the dark at 37 ˚C. At the end of staining, the protozoa cells were rinsed twice ice cold PBS and visualized by fluorescence microscope (BX53, Olympus, Japan) at magnifications of 400 × and 1000 ×, with an excitation wavelength in the range of 450-500 nm and detection wavelength in the range of 515-565 nm. The DNA strand breaks in treated samples were identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction and the result was shown the presence of green fluorescent apoptotic bodies were observed under microscope visualization. Eight microscopic fields were observed for each treatment and test sample. Percentage of apoptotic protozoa cells was determined by the following formula (Siti Syarifah *et al*., 2014):

$$\% \text{ of Apoptotic Protozoa Cells} = \frac{\text{No. of apoptotic protozoa cells}}{\text{No. of total protozoa cells}} \times 100$$

3.9.2 Cell Cycle Analysis by Flow Cytometry

The cell cycle analysis was performed using the CycleTEST^{TM} PLUS DNA Reagent Kit (Becton Dickson, USA) as described by the manufacturer's protocol.

3.9.2.1 *Trypanosoma brucei brucei* Seeding and Treatment Preparation.

Trypanosoma brucei brucei cells undergoing growth at log phase for treatment purpose were prepared as described in section 3.7.2. *T. b. brucei* were seeded at the density of 2.0 × 10^6/cells per well into 24-well plates and treated with 0.025, 0.05 and 0.10 µg/ml of active compound **(1)** respectively in triplicate wells. Treated and non-

treated *T. b. brucei* plates were further incubated in the CO_2 incubator for 24 hours. The *T. b. brucei* cells were then transferred into a 15 ml centrifuge tube (sterile) and centrifuged for 5 min at 300 × g at 37 ˚C. The supernatant was discarded leaving approximately 50 µl of residual fluid in the tube to avoid disturbing the pellet. The pellet was then washed 5 ml buffer solution (Appendix 1.9), followed by another wash with 1.5 ml buffer solution. During each wash the cells pellet was resuspended by gentle vortex at low speed before centrifuging at 300 × g for 5 min at 37 ˚C. The pellet was then resuspended again in 1 ml buffer solution, transferred to a 1.5 ml freezer-safe screw-capped cryogenic vial (Nalgene ®) and stored at -80 ˚C prior to flow cytometry analysis.

3.9.2.2 Staining Procedure

The frozen *T. b. brucei* cell samples were thawed rapidly in a water bath at 37 ˚C. The cells suspension was centrifuged at 400 × g for 5 min at room temperature. The supernatant was discarded and the centrifuge tube was gently tapped on tissue to wipe of the final drop. A total of 250 µl solution A (containing trypsin buffer) (Appendix 1.9) was added to each tube and gently mixed by tapping the tube by hand. The tube was left at 37 °C for 10 min to allow the reaction of solution A. A total of 200 µl solution B (trypsin inhibitor and RNase buffer) (Appendix 1.9) was then added to each tube without removing solution A and gently mixed by tapping the tube. The mixture was incubated for 10 min at room temperature. A total of 200 µl cold solution C containing propidium iodide stain solution was added to each tube. Mixtures were mixed well and incubated on ice in the dark for 10 min.

The sample mixtures were filtered into 5 ml tube with 35-µm cell strainer cap and analyzed using flow cytometry (FACS Calibur, Becton Dickson, San Jose, CA) at scan

event 10,000 *T. b. brucei* cells per sample. The percentage of cells in the cell cycle G1, S and G2-M phases were analyzed by ModFit LT (Verity Software House, Topsham, ME). All results were expressed as mean ± SD from three replicates.

3.10 Statistical Analysis

Test samples were assayed in triplicate plates and the experiment was repeated at least three times. The data are represented as means ± standard deviations (SD). Statistical analysis of data from TUNEL assay and cell cycle experiment was performed using the two-way analysis of variance (ANOVA), Turkey's and Dunnett's multiple comparison tests (GraphPad Prism 7.00 software).

CHAPTER 4: RESULTS

4.1 Preparation of Crude Alkaloid Fraction from *Dyera costulata*

The Soxhlet extraction process of *D. costulata* leaves yielded 2.29 g (2.52%) and 30 g (32.9 %) of hexane and methanol (MeOH) extracts, respectively. The methanol extract was then subjected to acid-base shake out extraction method to yield a total of 3.38 g (11.3 %) of crude alkaloid fraction. All extracts were subjected to antitrypanosomal activity assay against *Trypanosoma brucei brucei* strain BS221 and cytotoxicity assay against normal human liver WRL-68 cells (Table4.1).

Results in term of IC_{50} values (µg/ m1) were determined from the dose-response curve of the percentage of living parasites/cells versus different concentrations of the extracts. Both MeOH and crude alkaloid fraction were strongly active against *T. b. brucei*. The hexane extract was moderately active (Table 4.1). Crude alkaloid fraction exhibited the highest activity with an IC_{50} value of 0.11 ± 0.03 µg/ml. Based on cytotoxicity results against the WRL-68 cells, both MeOH and crude alkaloid fractions exhibited high selectivity towards the protozoa with selectivity index (SI) values of > 172.4 and > 909.1, respectively (Table 4.1). The crude alkaloid fraction demonstrated not only the highest antitrypanosomal activity but also a higher selectivity towards the protozoa compared to the other extracts.

Table 4.1: Antitrypanosomal activity and cytotoxicity of different extracts from *D. costulata* leaves and their selectivity index values

Samples	**IC_{50} (µg/ml) ± SD (n≥3)***		
	Antitrypanosomal	**Cytotoxicity**	**SI** values**
	T. b. brucei (BS221)	WRL-68	
Hexane extract	4.40 ± 0.05	>100	>22.7
Methanol extract	0.58 ± 0.20	>100	>172.4
Crude alkaloid fraction	0.11 ± 0.03	>100	>909.1
Standard drug			
Pentamidine	0.00339 ±0.00003	20.31 ± 0.05	>1000

* Tabulated values are mean ± standard deviation (SD) of at least three replicates.
** SI value IC_{50} cytotoxicity/ IC_{50} antitrypanosomal

4.2 Bioassay-Guided Isolation of Bioactive Compound from Crude Alkaloid Fraction of *D. costulata*

The crude alkaloid fraction of *D. costulata* was subjected to bioassay-guided isolation to isolate and identify the active compound responsible for the antitrypanosomal activity. A schematic diagram of the bioassay-guided isolation protocol used in this study is shown in Figure 4.1

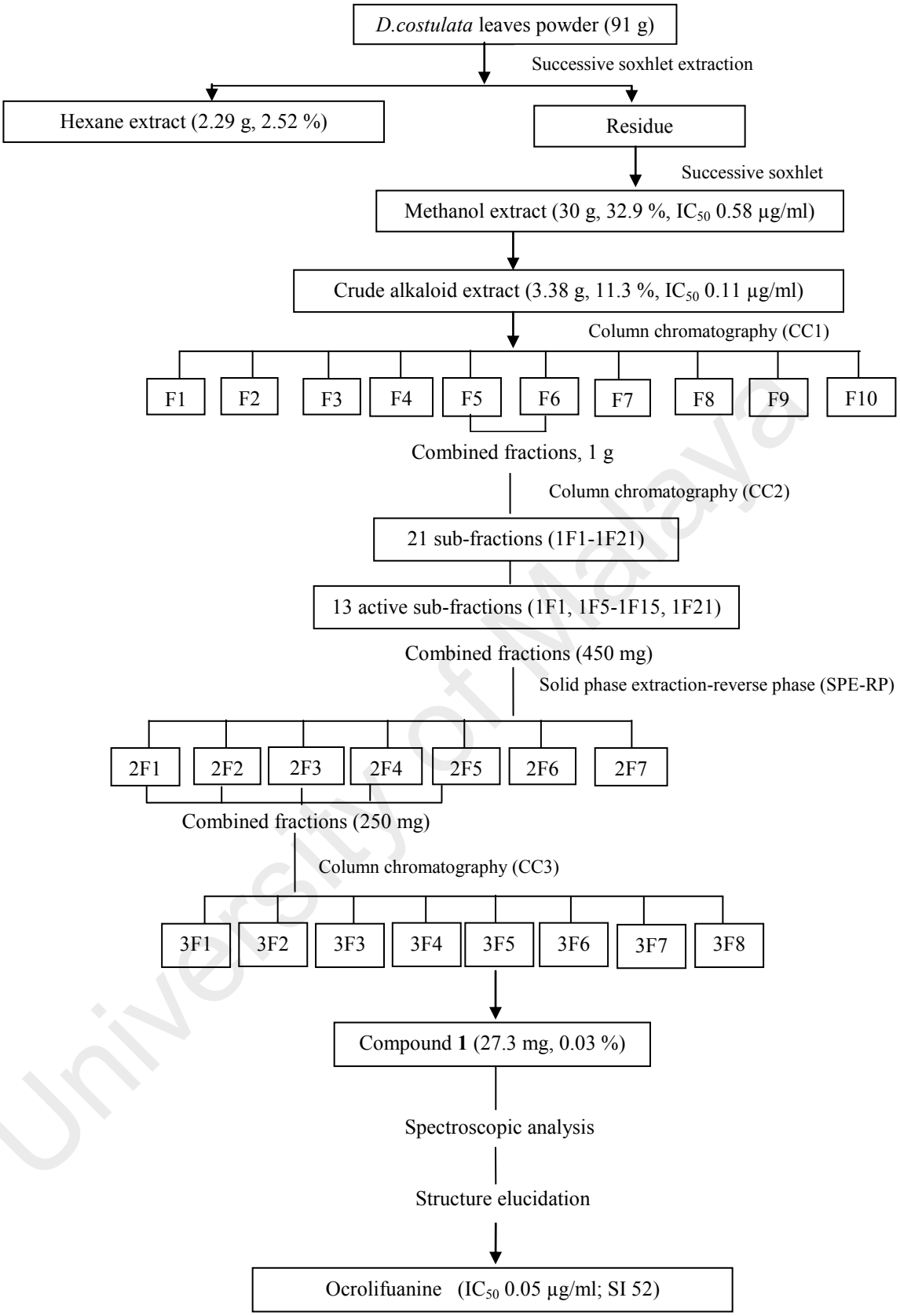


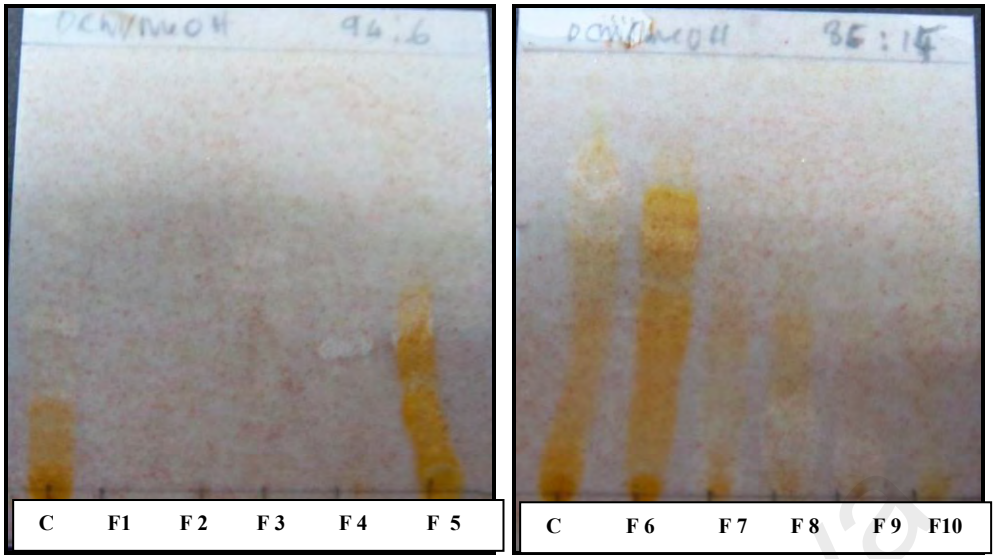
Figure 4.1 Bioassay-guided isolation of ochrolifuanine from *Dyera costulata* leaf extract.

4.2.1 Fractionation of Crude Alkaloid Fraction of *D. costulata* by Column Chromatography (CC1)

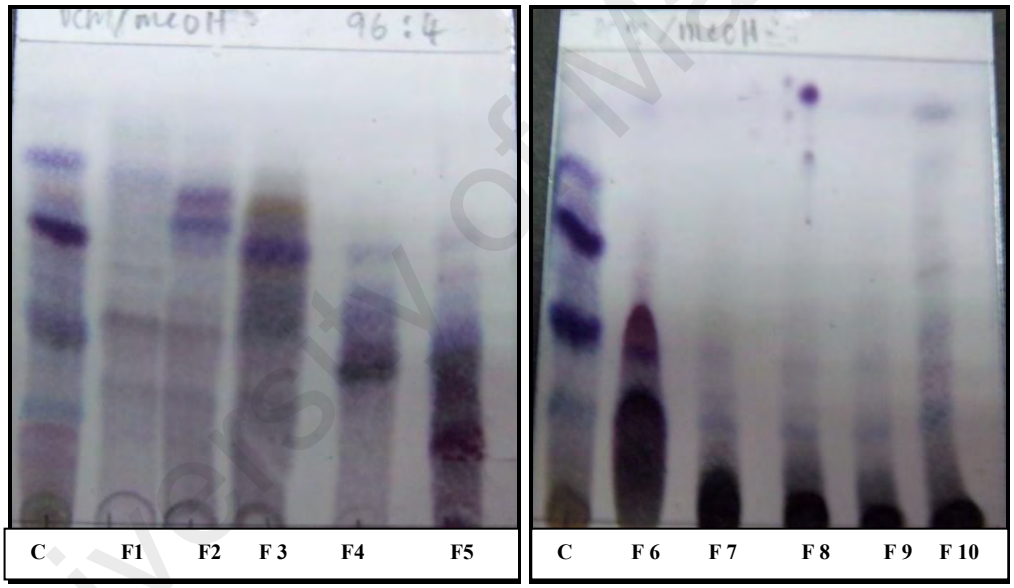
The crude alkaloid fraction was further fractionated using silica gel chromatography (CC1) with a solvent mixture of increasing polarity gradient [*n*-hexane, dichloromethane (DCM) and methanol (MeOH)]. A total of 314 fractions were collected from the column chromatography separation. The fractions were organised based on the similarity of spots detected on TLC plates developed in a solvent system of DCM/ MeOH (9:1, v/v) with vanillin and Dragendorff's reagents used for spot detection. The vanillin reagent was used to detect the appearance of terpenes, while Dragendorff's reagent to detect an alkaloid fraction on TLC plate. A total of 10 fractions were pooled based on the TLC analysis (Table 4.2). All fractions were subjected to TLC analysis as shown in the Figure 4.2. Results showed that the TLC plates yielded a majority of yellowish/orange, purple and red spots with different intensities after spraying with Dragendorff's and vanillin reagents. Fractions F5 and F6 developed clear and well-separated yellowish/orange spot as compared to F7, F8, F9 and F10, after spraying with Dragendorff's reagent followed by heating on a hot plate. It can be concluded that high concentration of alkaloids is present in the isolated fractions of F5 to F6.

Table 4.2: Fractions (F1-F10) collected from different solvent systems using column chromatography (CC1)

Fraction no.	Fraction code	Solvent system	Solvent ratio	Fraction weight (mg)	Fraction yield (%, w/w)
1	F1	DCM 100%	100	9.4	0.3
2	F2	DCM/MeOH	98:2	40.6	1.1
3	F3	DCM/MeOH	97:3	278.8	7.3
4	F4	DCM/MeOH	97:3	264.6	6.9
5	F5	DCM/MeOH	97:3	286.6	7.5
6	F6	DCM/MeOH	88:12	918.8	24.9
7	F7	DCM/MeOH	82:18	124.9	3.2
8	F8	DCM/MeOH	80:20	43.2	1.1
9	F9	DCM/MeOH	76:24	27.0	0.7
10	F10	DCM/MeOH	72:28	108.9	2.8



a) Detection by Dragendorff's reagent



b) Detection by Vanillin reagent

Figure 4.2: Thin layer chromatography (TLC) profiles of 10 fractions F1-F10 in a a) Dragendorff's and b) Vanillin reagent. (C: crude alkaloid extract)

4.2.2 Antitrypanosomal Activity and Cytotoxicity of Fractions against *Trypanosoma brucei brucei* Parasites.

Fractions F1 – F10 were evaluated for antitrypanosomal activity using Alamar blue assay against *T. b. brucei*. Three fractions exhibited IC_{50} values of less than ≤ 0.78 µg/ml. Fractions F5 and F6 showed the most potent activity with the lowest IC_{50} values (0.04 - 0.09 µg/ml). Fraction F10 also exhibited strong antitrypanosomal activity with IC_{50} values 0.52 µg/ml (Table 4.3). Meanwhile, the remaining fractions either showed moderate activity (0.78 µg/ml < IC_{50} ≤ 6.25 µg/ml) with IC_{50} or not active (IC_{50} > 6.25 µg/ml) against the trypanosome. Fractions F5 and F6 showed high intensity of alkaloid based on TLC profile sprying with dragendorff's reagent and showed high antitrypanosomal activity (Figure 4.2 a).

The active fractions F5, F6, and F10 were tested for cytotoxicity on WRL-68 cell lines. Fractions F6 and F10 showed highest toxicity towards WRL-68 cell with IC_{50} values of 1.49 and 20.61 µg/ml, respectively. Whereas, fraction F5 did not display cytotoxic effect on the normal cell lines as IC_{50} values showed > 100 µg/ml. Hence, fraction F5 have the most potent antitrypanosomal activity and was the most selective (SI ≥ 100) towards the parasites (IC_{50} = 0.52 ± 0.06 µg/ml; SI > 1111.1). The IC_{50} values of antitrypanosomal activity, cytotoxicity and selectivity index values of fractions F1 – F10 were tabulated in Table 4.3.

The bioassay-guided fractionation of crude alkaloid fraction of *D. costulata* revealed the presence of noncytotoxic fractions at the semi-polar phases. Fractions F5 and F6 were obtained from the early fractionation of semi-polar phase in silica gel column at a solvent ratio (DCM/ MeOH) of 97:3 and 88:12. On the other hand, fraction F10 has obtained a polar portion phase of column chromatography with the ratio of solvent

(DCM/ MeOH) used was 72: 28. Based on the similarity in TLC bands profile, fractions F5 and F6 were further pooled and subjected to the second step of bioassay-guided fractionation.

Table 4.3: Antitrypanosomal activity and cytotoxicity of fractions of F1 – F10 from crude alkaloid fraction of *D. costulata* and their selectivity index values

Fractions	IC_{50} µg/ml ± SD (n≥3)*		SI values
	Antitrypanosomal activity *T. b. brucei*	**Cytotoxicity** WRL-68	
F1	2.28 ± 0.60	>100	>43.9
F2	5.82 ± 0.40	>100	>17.2
F3	2.72 ± 0.50	>100	>36.8
F4	> 6.25	ND	ND
F5	0.09 ± 0.05	>100	> 1111.1
F6	0.04 ± 0.02	1.49 ± 0.50	37.3
F7	0.97 ± 0.08	13.80 ± 2.00	14.2
F8	> 6.25	ND	ND
F9	> 6.25	ND	ND
F10	0.52 ± 0.09	20.61 ± 1.40	39.6
Standard drug Pentamidine	0.00339 ±0.00003	20.31 ± 0.05	>1000

[Fractions F1 – F10 were obtained from column chromtograhy (CC1) fractionation of crude alkaloid fraction]

* Highlighted values were considered as high antitrypanosomal activity and selectivity. Tabulated values are mean ± standard deviation (SD) of at least three replicates. (ND: not determined).

4.2.3 Fractionation of Combined Active Fractions (F5 and F6) of *D. costulata* by Column Chromatography (CC2)

Fractions F5 and F6 which contained high intensity of alkaloids, were pooled (total weight 1 g) and subjected to further fractionation using silica gel column chromatography (CC2). The fractions obtained were collected and organised based on the similarity of spots detected on TLC plates using DCM/MeOH (9:1, v/v) as the developing solvent system.

A total of 160 fractions obtained were pooled into 21 sub-fractions based on similarity of TLC profiles. Yield and the solvent system used in the separation process of each sub-fraction were tabulated in Table 4.4. Fraction 1F1 was discarded due to very low yield obtained. The TLC profiles of the sub-fractions, 1F2 - 1F21 were shown in Figure 4.3. All 20 sub-fractions were subjected to antitrypanosomal activity and cytotoxicity assays as described previously.

As alkaloids have been reported as one of the most abundant group of compounds determined in *D. costulata* (Mirand *et al.,* 1982), detection of alkaloids sub-fractions 1F2 - 1F21 was done using Dragendorff's spraying reagents followed by heating of TLC plates on a hot plate. As mentioned above, orange/dark brown color showed the presence of alkaloid after spraying with the reagent.

Table 4.4: Sub-fractions (1F1 - 1F21) collected from different solvent systems in column chromatography (CC2)

Sub-fraction no.	Sub-fraction code	Solvent system	Solvent ratio	Sub-fraction weight (mg)	Sub-fraction yield (%, w/w)
1	1F1	DCM/MeOH	98/2	0.3	0.03
2	1F2	DCM/MeOH	97/3	77.8	7.78
3	1F3	DCM/MeOH	97/3	123.2	12.32
4	1F4	DCM/MeOH	96/4	118.8	11.80
5	1F5	DCM/MeOH	96/4	25.4	2.54
6	1F6	DCM/MeOH	96/4	77.9	7.79
7	1F7	DCM/MeOH	95/5	3.8	0.38
8	1F8	DCM/MeOH	95/5	11.1	1.11
9	1F9	DCM/MeOH	95/5	20.3	2.03
10	1F10	DCM/MeOH	94/6	73.3	7.33
11	1F11	DCM/MeOH	94/6	45.1	4.51
12	1F12	DCM/MeOH	93/7	43.1	4.31
13	1F13	DCM/MeOH	92/8	89.8	8.98
14	1F14	DCM/MeOH	92/8	89.3	8.93
15	1F15	DCM/MeOH	91/9	32.2	3.22
16	1F16	DCM/MeOH	89/11	37.2	3.72
17	1F17	DCM/ MeOH	89/11	26.3	2.63
18	1F18	DCM/ MeOH	87/13	12.8	1.28
19	1F19	DCM/ MeOH	87/13	10.8	1.08
20	1F20	DCM/ MeOH	85/15	6.2	0.62
21	1F21	DCM/ MeOH	83/17	14.2	1.42

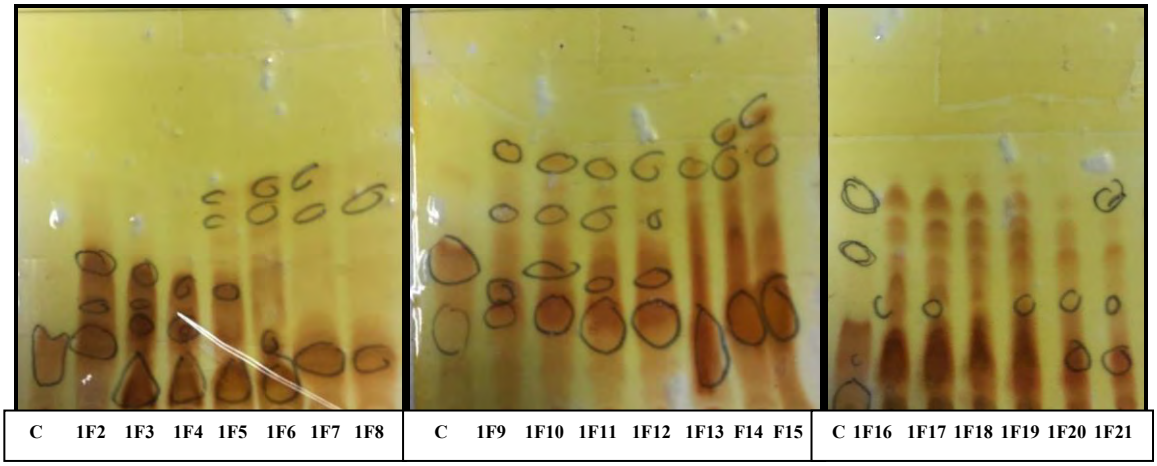


Figure 4.3: Thin layer chromatography (TLC) profiles of sub-fractions 1F2 - 1F21 sprayed with Dragendorffs reagent. (C: Crude alkaloid extract).

4.2.4 Antitrypanosomal Activity and Cytotoxicity of Sub-fractions (1F2 - 1F21) from Combined Active Fractions (F5 and F6) against *T. b. brucei*.

Antitrypanosomal activity of sub-fractions (1F2 – 1F21) were tested using *in vitro* assay system of growth inhibition against *T. b. brucei*. The IC_{50} values of sub-fractions 1F2 - 1F21 were summarized in Table 4.5. Results showed that most of the sub-fractions tested displayed potent antitrypanosomal activity. Out of the 21 sub-fractions tested, 13 sub-fractions showed active antitrypanosomal activity with IC_{50} values ranging from 0.04 to 0.54 µg/ml.

The active antitrypanosomal sub-fractions were further tested for cytotoxicity and the IC_{50} values were shown in Table 4.5. Overall, those sub-fractions with high antitrypanosomal activity also showed high cytotoxicity effect on normal mammalian WRL-68 cell lines. However, two sub-fractions (1F5 and 1F6) appeared to be most selective ($SI \geq 100$) towards the parasite with SI values of 117.1 and 163, respectively. While the rest showed moderately selective ($10 \leq SI < 100$), except for sub-fraction 1F2 which showed selectivity index lower than 10 and considered as not selective towards

the parasite. All the active 13 sub-fractions were then combined and subjected to the third step of bioassay-guided fractionation.

Table 4.5: Antitrypanosomal activity and cytotoxicity activity of sub-fractions (1F2 - 1F21) from the combined active fractions F5 and F6 of *D. costulata* and their selectivity index values

Sub-fractions	IC_{50} µg/ml ± SD (n≥3)*		SI values
	Antitrypanosomal activity (*T. b. brucei*)	**Cytotoxicity** WRL-68	
1F2	0.54 ± 0.03	4.52 ± 4.52	8.4
1F3	> 6.25	ND	ND
1F4	> 6.25	ND	ND
1F5	0.14 ± 0.01	16.4 ± 2.1	117.1
1F6	0.08 ± 0.04	13.04 ± 2.05	163.0
1F7	0.08 ± 0.05	3.88 ± 1.4	48.5
1F8	0.14 ± 0.06	11.97 ± 0.9	85.5
1F9	0.14 ± 0.06	3.62 ± 0.2	25.9
1F10	0.06 ± 0.08	3.37 ± 0.4	56.2
1F11	0.09 ± 0.08	7.23 ± 1.0	80.3
1F12	0.07 ± 0.07	3.31 ± 1.3	47.3
1F13	0.05 ± 0.02	2.1 ± 0.2	42.0
1F14	0.04 ± 0.02	1.39 ± 0.21	34.8
1F15	0.07 ± 0.05	3.33 ± 0.6	47.6
1F16	1.91 ± 0.06	ND	ND
1F17	> 6.25	ND	ND
1F18	1.99 ± 0.20	ND	ND
1F19	2.22 ± 0.40	ND	ND
1F20	2.00 ± 0.57	ND	ND
1F21	0.29 ± 0.18	14.04 ± 0.1	48.0
Standard drug Pentamidine	0.00119 ± 0.00006	20.31 ± 0.05	>1000

[Sub-fractions 1F2 – 1F21 were obtained from (CC2) fractionation of combined active sub-fractions (F5- F6) from column chromtograhy (CC1) fractionation]

* Highlighted values of 13 fractions with high antitrypanosomal activity and selectivity. Tabulated values are mean ± standard deviation (SD) of at least three replicates. (ND: not determined).

4.2.5 Fractionation of Combined 13 Active Sub-fractions of *D. costulata* by Solid Phase Extraction (SPE)-Reversed Phase (RP) Chromatography

Thirteen sub-fractions showed strong antitrypanosomal activity against *T. b. brucei*. These sub-fractions were pooled according to their similarity in TLC profile. A total of 450 mg of this combined sub-fraction was subjected to further bioassay-guided fractionation. The pooled sub-fractions were subjected to SPE-RP chromatographic technique with mobile phase of H_2O/MeOH/DCM (60:40:0, 10:90:0, 0:100:0, 0:90:10, 0:50:50) gradient. These sub-fractions collected were organised by similarity of TLC spots as described previously.

The chromatography fractionation of pooled active sub-fraction of *D. costulata* yielded a total of 28 sub-fractions. Based on TLC profile analysis, the sub-fractions were combined into 7 sub-fractions (2F1 - 2F7). The yield obtained for each sub-fraction and solvent systems used in the fractionation process were shown in Table 4.6. The TLC profiles of all sub-fractions (2F1 - 2F7) were shown in Figure 4.4. All sub-fractions were subjected again to antitrypanosomal activity and cytotoxicity assay.

Table 4.6: Sub-fractions (2F1 - 2F7) collected from different solvent systems in SPE-RP chromatography

Sub-fraction no.	Sub-fraction code	Solvent system	Solvent ratio	Sub-fraction weight (mg)	Sub-fraction yield (%, w/w)
1	2F1	H_2O/MeOH	60:40	20.1	4.02
2	2F2	H_2O/MeOH	50:50	68.4	13.68
3	2F3	H_2O/MeOH	40:60	120.8	24.16
4	2F4	H_2O/MeOH	20:80	140.2	28.04
5	2F5	MeOH	100	75.1	15.02
6	2F6	MeOH/DCM	90:10 80:20	32.5	6.50
7	2F7	MeOH/DCM	50:50	36.2	7.24

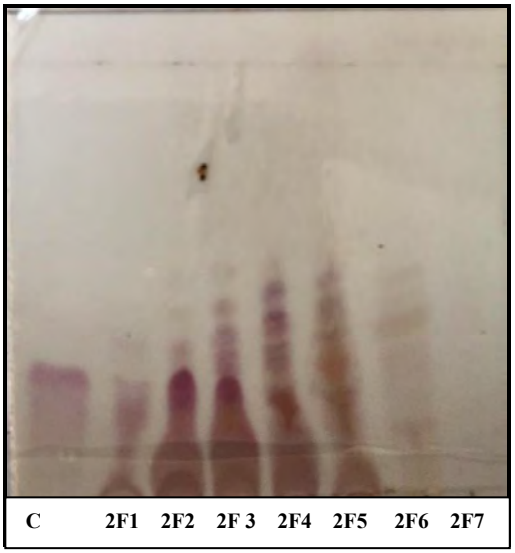


Figure 4.4: Thin layer chromatography (TLC) profile of sub-fractions 2F1-2F7 in vanillin reagent.

4.2.6 Antitrypanosomal Activity and Cytotoxicity of Sub-fractions 2F1 - 2F7 from Combined Active 13 Sub-fractions against *T. b. brucei*.

Antitrypanosomal activity and cytotoxicity effect of sub-fractions 2F1 - 2F6 were summarized in Table 4.7. Sub-fraction 2F7 was discarded because it did not present any bands/spots on TLC plate (Figure 4.4). Interestingly, all six sub-fractions tested showed strong antitrypanosomal activity with IC_{50} values ranging from 0.04 to 0.60 µg /ml, respectively (Table 4.7).

Even though all sub-fractions showed active antitrypanosomal activity, the next step for active compound isolation was based on TLC profile analysis. Five sub-fractions, 2F1 to 2F5 which showed the most intense bands/spots on TLC, were pooled and forwarded to the fourth step of bioassay-guided fractionation.

Table 4.7: Antitrypanosomal activity and cytotoxicity of sub-fractions (2F1 - 2F6) from combined active 13 sub-fractions and their selectivity index values

Sub-fractions	IC_{50} µg/ml ± SD (n≥3)*		SI values
	Antitrypanosomal activity (*T. b. brucei)*	**Cytotoxicity** (WRL-68)	
2F1	0.08 ± 0.02	11.5 ± 2.45	143.8
2F2	0.09 ± 0.03	13.2 ± 1.15	147.7
2F3	0.04 ± 0.02	5.6 ± 0.8	140
2F4	0.06 ± 0.01	3.8 ± 0.17	63.3
2F5	0.15 ± 0.03	15.1 ± 1.54	100.1
2F6	0.60 ± 0.08	7.9 ± 1.48	13.2
Standard drug Pentamidine	0.00445 ±0.00004	20.31 ± 0.05	>1000

[Sub-fractions 2F1 – 2F6 were obtained from solid phase extraction-reversed phase (SPE) fractionation of combined active 13 sub-fractions from column chromatography (CC2) fractionation]

4.2.7 Fractionation of Combined Five Active Sub-fractions (2F1 - 2F5) of *D. costulata* by Column Chromatography (CC3)

As the TLC profiles of sub-fractions 2F1 - 2F5 obtained from SPE fractionation were similar, they were pooled and subjected to further bioassay-guided fractionation. A total 250 mg of pooled sub-fractions were fractionated on silica gel column (CC3) and eluted in a gradient manner with increased polarity. A total of 200 sub-fractions collected were organised by the similarity of bands/spots detected in TLC plates as described previously.

Silica column chromatography of pooled active sub-fractions *D. costulata* resulted in the fractionation of eight sub-fractions (3F1 - 3F8). The solvent system used in the

fractionation process and yields of each sub-fractions obtained were tabulated in Table 4.8 and their TLC profiles were shown in Figure 4.5. All the sub-fractions (3F1- 3F8) were subjected to antitrypanosomal activity and cytotoxicity assays as described before.

Table 4.8: Sub-fractions (3F1 - 3F8) collected from different solvent systems in column chromatography (CC3)

Sub-fraction no.	Sub-fraction code	Solvent system	Solvent ratio	Sub-fraction weight (mg)	Sub-fraction yield (%, w/w)
1	3F1	DCM/MeOH	99:1	1.7	0.07
2	3F2	DCM/MeOH	98:2	1.5	0.01
3	3F3	DCM/MeOH	98.2	3.5	0.01
4	3F4	DCM/MeOH	97:3	17.1	0.07
5	3F5	DCM/MeOH	96:4	27.3	0.11
6	3F6	DCM/MeOH	95:5	35.0	0.14
7	3F7	DCM/MeOH l	94:6	22.3	0.09
8	3F8	DCM/MeOH	93:7	37.9	0.15

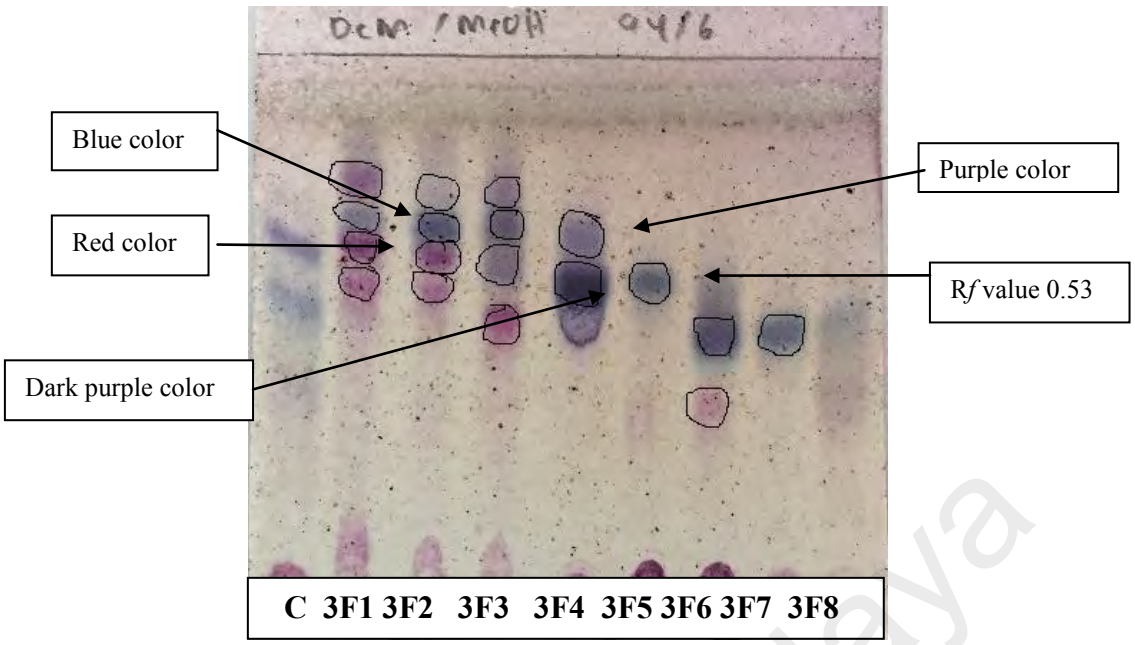


Figure 4.5: Thin layer chromatography (TLC) profiles of sub-fractions (3F1-3F8) sprayed with vanillin reagent

It is clearly shown in Figure 4.5 that intense purple, red and blue spots were detected on TLC plate of sub-fractions 3F1 to 3F7. The purple spots were seen on the plate with sub- fractions 3F3 and 3F4, while sub-fractions 3F1, 3F2 and 3F3 showed a similarity of chromatogram bands with the appearance of red spots. However, sub-fraction 3F4 showed different TLC profile with a high-intensity dark purple band was seen in the fraction compared to the other sub-fractions. The presence of purple, red and blue spots indicated that the isolated sub-fractions contained compounds which have the terpenes group.

4.2.8 Antitrypanosomal Activity and Cytotoxicity of Sub-fractions (3F1 - 3F8) from Combined Active (2F1 - 2F5) Sub-fractions against *T. b. brucei* Parasites.

Sub-fractions 3F1 - 3F8 obtained from CC3 fractionation were evaluated for antitrypanosomal activity and cytotoxicity. The IC_{50} values of sub-fractions against *T. b. brucei* were summarised in Table 4.9.

The sub-fraction 3F5 showed the most potent antitrypanosomal activity with IC_{50} value 0.05 ± 0.01 µg/ml and selectivity index (SI) of 52. This sub-fraction 3F5 was obtained as light yellowish fraction (27.3 mg; 0.03 % yield). Sub-fraction 3F5 possessed only a single spot with Rf value of 0.53 on TLC plate and was designated as compound (**1**) (Figure 4.5). Compound (**1**) was then subjected to spectrometry and spectroscopic analysis for chemical structure identification.

Table 4.9: Antitrypanosomal activity and cytotoxicity of sub-fractions 3F1 – 3F8 from combined active (2F1 - 2F5) sub-fractions and their selectivity index values

Sub-fractions	IC_{50} µg/ml ± SD (n≥3)*		SI values
	Antitrypanosomal activity (*T. b. brucei)*	**Cytotoxicity** (WRL-68)	
3F1	1.20 ± 0.32	40.20 ± 3.07	33.5
3F2	0.98 ± 0.10	54.20 ± 4.57	55.4
3F3	1.57 ± 0.08	14.90 ± 0.94	9.5
3F4	1.38 ± 0.20	10.10 ± 1.65	7.3
3F5	0.05 ± 0.01	2.60 ± 0.45	52
3F6	0.07 ± 0.02	3.80 ± 0.60	54.3
3F7	1.94 ± 0.09	51.70 ± 1.57	26.6
3F8	2.06 ± 0.08	48.20 ± 2.57	23.2
Standard drug Pentamidine	0.00210 ± 0.00003	20.31 ± 0.05	>1000

[Sub-fractions 3F1 – 3F8 were obtained from column chromtograhy (CC3) fractionation of combined active sub-fractions (2F1-2F5) from SPE fractionation]

*Highlighted values were considered as high antitrypanosomal activity and selectivity. Tabulated values are mean ± standard deviation (SD) of at least three replicates.

4.3 Structural Elucidation of Compound (1)

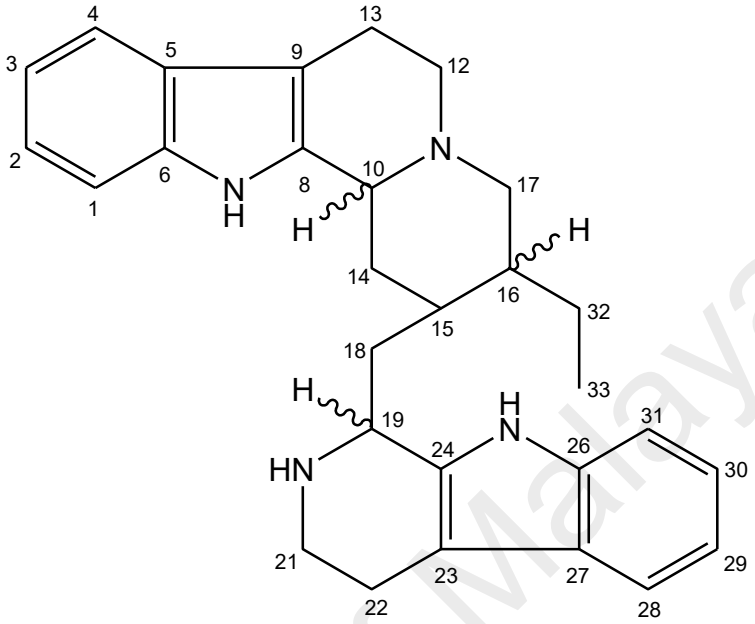
Compound (**1**) was isolated and characterised as described in the methodology section. Compound (**1**) was obtained as a white solid with a molecular formula $C_{29}H_{34}N_4$ on the basis of ESI-MS data ($[M+H]^+$ *m/z* 439.5 calcd 438.619). This compound was analyzed spectroscopically using ^1H NMR (Appendix 2.3), ^{13}C NMR (Appendix 2.4), as well as 2D NMR spectral techniques namely ^1H and ^{13}C HQMC and DEPT (Appendix 2.5), and HMBC (Appendix 2.6). The ^1H chemical shifts for isolated compound **1** are presented in Table 4.10. The ^{13}C NMR assignments of compound **1** and that reported by Koch *et al.* (1975) are presented in Table 4.11. By correlating spectral data (^1H, ^{13}C NMR and MS) from reported data (Koch *et al.,* 1975; Mirand *et al*., 1983), compound **1** was identified as ochrolifuanine as illustrated in Figure 4.6.

Table 4.10: ^{1}H NMR chemical shifts (δ) values of compound (1) (400,100Mhz in MeOD)

Position	^{1}H
1	7.51, dd, *J* = 2.4, 11.2 Hz
2	7.16, t, *J* = 7.6 Hz
3	7.36, dd, *J* = 4.4, 8 Hz
4	7.51, dd, *J* = 1.6, 7.6 Hz
5	-
6	-
7	-
8	-
9	-
10	4.74, m
11	-
12	3.55, m
13	2.99, m
14	3.16, m
15	1.76, m
16	2.56, m
17	3.65, m
18	3.08, m
19	4.82, m
20	-
21	3.89, m
22	3.38, m
23	-
24	-
25	-
26	-
27	-
28	7.54, dd, *J* = 2.4, 11.2 Hz
29	7.11, dd, *J* = 0.8, 7.6 Hz
30	7.22, t, *J* = 1.6, 7.6 Hz 7.6
31	7.42, dd, *J* = *J* = 4.8, 8 Hz
32	3.29, m
33	1.04, t, *J* = 6.8 Hz

Table 4.11: ^{13}C NMR chemical shifts (δ) values of compound (1)

Position	Compound 1 (400 Mhz, MeOD)	Ochrolifuanine (Koch *et al*., 1975), (100 Mhz, $CDCl_3$)
1	110.97	110.0
2	122.35	118.9
3	119.38	121.0
4	117.80	117.7
5	126.16	127.0
6	136.98	135.9
7	-	-
8	134.88	134.7
9	106.35	107.3
10	60.81	59.3
11	-	-
12	52.83	52.6
13	18.14	21.5
14	32.15	34.2
15	33.86	35.8
16	45.33	42.2
17	56.92	59.9
18	34.83	38.1
19	50.53	48.8
20	-	-
21	42.66	42.2
22	18.88	22.4
23	106.37	108.1
24	134.91	135.5
25	-	-
26	137.30	136.1
27	126.15	127.2
28	117.91	117.9
29	119.52	121.3
30	122.52	119.0
31	111.16	110.9
32	31.43	23.2
33	9.12	11.0



Active compound	IC_{50} µg/ml ± SD (n≥3)		SI value
	Antitrypanosomal activity *(T. b. brucei)*	**Cytotoxicity** (WRL-68)	
Ochrolifuanine	0.05 ± 0.01	2.60 ± 0.3	52
Pentamidine	0.00210 ± 0.00	20.31 ± 0.05	>1000

Figure 4.6: Chemical structure, IC_{50} values of antitrypanosomal activity, cytotoxicity and selectivity index of ochrolifuanine.

4.4 Evaluation of Apoptosis-Inducing Effects of Ochrolifuanine on *T. b. brucei* Strain BS221.

4.4.1 Detection of DNA Fragmentation by TUNEL Assay (Qualitative and Quantitative Analysis).

Different morphological features including DNA fragmentation and formation of apoptotic bodies were observed in *T. b. brucei* strain BS221 after being exposed to different concentrations of ochrolifuanine and staurosporine at three different incubation periods, as shown are visualised in Figures 4.7, 4.8, 4.9 and 4.10.

The parasite cells were treated with DNase 1 which represented the positive control for DNA fragmentation marker and the resulting DNA fragments were labelled as yellow fluorescence signal with propidium iodide (PI) staining, and as green fluorescence signal without PI staining (Figures 4.7 B,4.8 B, 4.9 B and 4.10 B). The treated DNase 1 cells showed that apoptotic cells were observed in reduction of size and rounded cells (Figures, 4.8 B and 4.10 B). Wheres the non-treated control cells displayed an ordinary spindale-shape with red (PI stain) and green (without PI stain) non-fluorescent intensity background color, indicating the absence of apoptotic cells (Figures 4.7 A, 4.8 A, 4.9 A and 4.10 A).

The parasites treated with ochrolifuanine (**1**) and staurosporine gave the yellow fluorescence (PI stain) and green fluorescence color (without PI stain) indicating the occurrence of DNA fragmentation. Even at 6 hours post incubation after treatment with ochrolifuanine (**1**) and staurosporine, fluorescence stained nuclei were already detected in parasites cells as shown in Figures 4.7, 4.8, 4.9 and 4.10. The parasites that being treated with ochrolifuanine (**1**) at the concentrations of 0.025, 0.05 and 0.10 μg/ml for 6 hours showed the emergence of yellow fluorescence (PI stain) and green fluorescence (without PI stain) stained nuclei, indicating that DNA fragmentation occurred in the

parasites cells. However, when cells were treated with ochrolifuanine (**1**) at concentrations 0.05 and 0.10 µg/ml for 12 and 24 hours incubation periods, the apoptotic cells (with and without PI stain) were clearly observed having smaller and rounded shape (Figures 4.7 D E, F, G and 4.8 D, E, F, G) as compared to the control non-treated cells. Whereas when treated with 0.025 µg/ml ocrolifuanine for 12 and 24 hours, the apoptotic cells remained in spindle shape and contained fluorescent stained nuclei (Figures 4.7 C and 4.8 C).

The fragmentation of DNA can be observed in parasite cells treated with staurosporine at 0.01, 0.02 and 0.04 µg/ml for 6, 12 and 24 hours incubation periods as yellow (PI stain) and green (without PI stain) fluorescence stained nuclei (Figures 4.9 and 4.10). The apoptotic cells were observed in smaller and rounded size after treatment with staurosporine concentrations of 0.01, 0.02 and 0.04 µg/ml for 12 and 24 hours of incubation period (Figures 4.9 C, D, E, F, G and 4.10 C, D, E, F, G).

From the results, it is clearly evident that the apoptotic nuclei were fragmented as the cells were positive to TUNEL assay (yellow and green fluorescence stained nuclei) detected in ochrolifuanine and staurosporine treated cells. In addition, apoptotic cells were also observed in smaller rounded shape. These findings suggested that ochrolifuanine has the potential of inducing apoptotic cell death in *T. b. brucei* parasites.

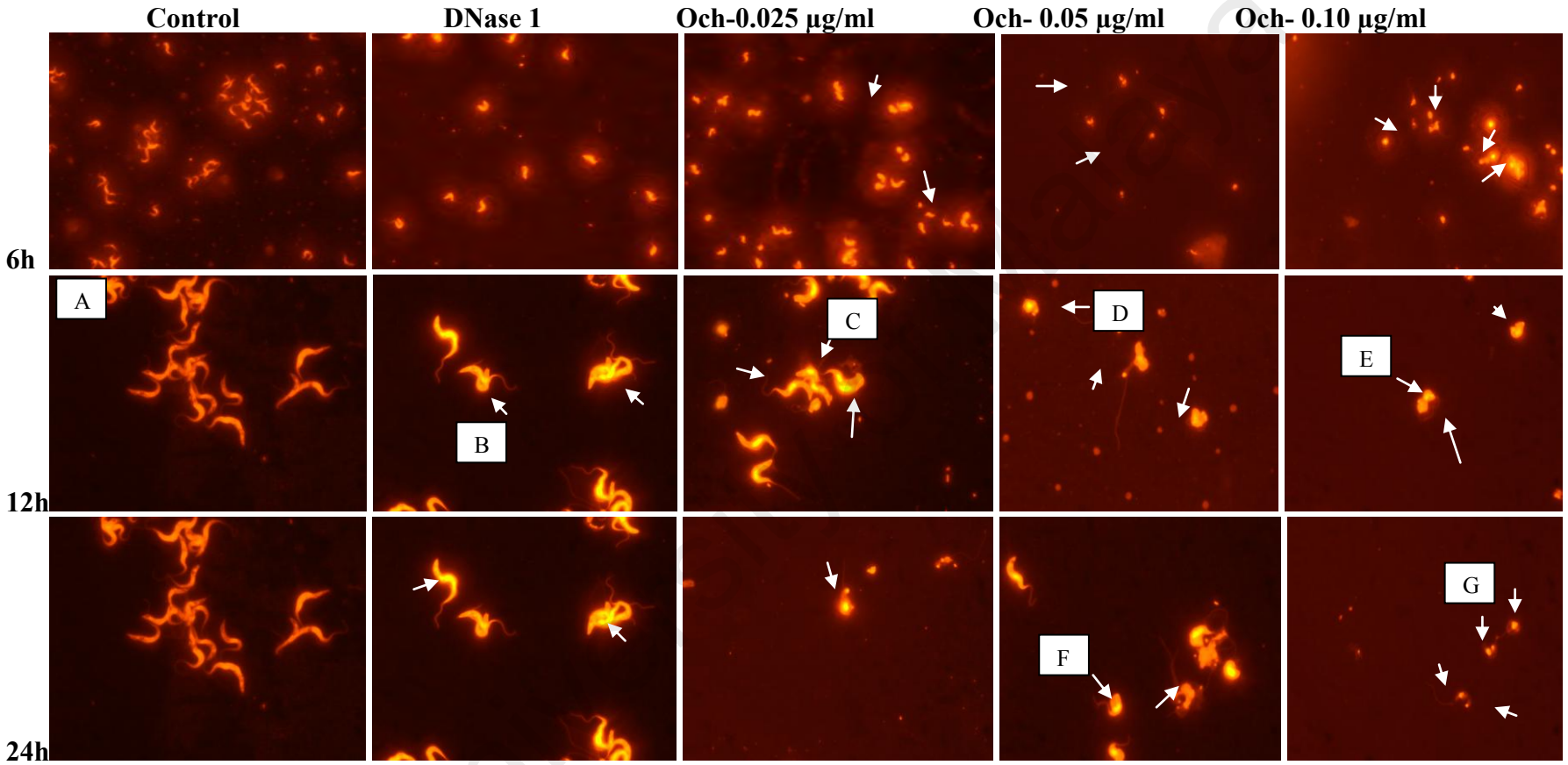


Figure 4.7: Apoptotic morphological changes of *T. b. brucei* strain BS221 induced by ochrolifuanine (Och) with Propidium iodide (PI) staining. Parasites were treated with 0.025, 0.05 and 0.10 µg/ml at 6, 12 and 24 hours time incubations. The parasites morphology was observed under fluorescence microscope (400 and 1000 times magnification). Arrows showed DNA fragmentation by the yellow fluorescence nuclei.

(A- Control, cells in spindle shape without florescence stained nuclei, B- DNase 1, apoptotic cells in spindle shape with florescence stained nuclei, C- treated Och, apoptotic cells in spindle shape with florescence stained nuclei, D, E, F, G- treated Och, apoptotic cells in smaller and rounded shape).

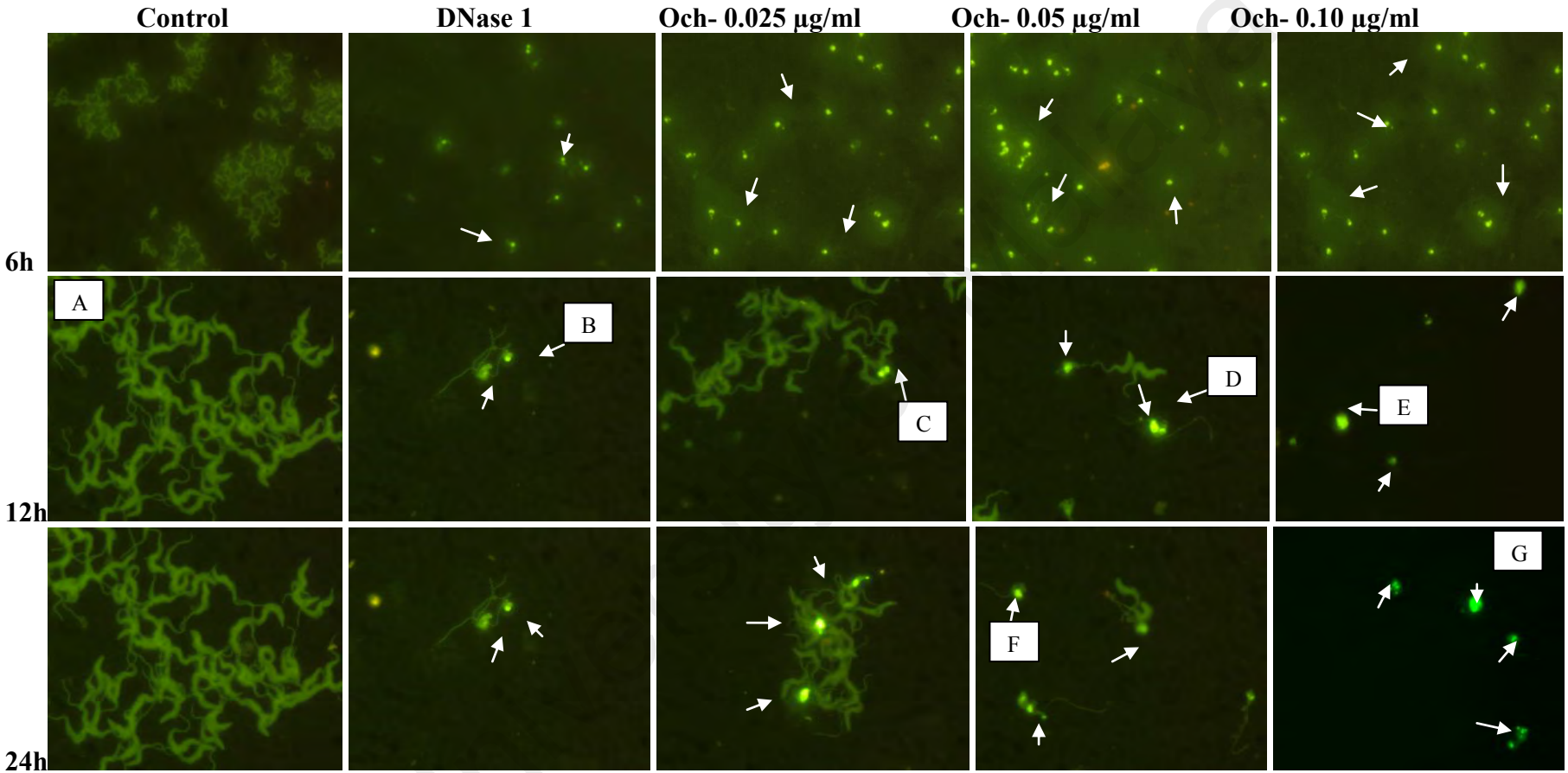


Figure 4.8: Apoptotic morphological changes of *T. b. brucei* strain BS221 induced by ochrolifuanine (Och) without of Propidium iodide (PI) staining. Parasites were treated with 0.025, 0.05 and 0.10 µg/ml at 6, 12 and 24 hours time incubations. The parasites morphology was observed under fluorescence microscope (400 and 1000 times magnification). Arrows showed DNA fragmentation by the green florescence nuclei stained.

(A- Control, cells in spindle shape without florescence stained nuclei, B- DNase 1, apoptotic cells in smaller and rounded shape with florescence stained nuclei, C- treated Och, apoptotic cells in spindle shape with florescence stained nuclei, D, E, F, G- treated Och, apoptotic cells in smaller and rounded shape).

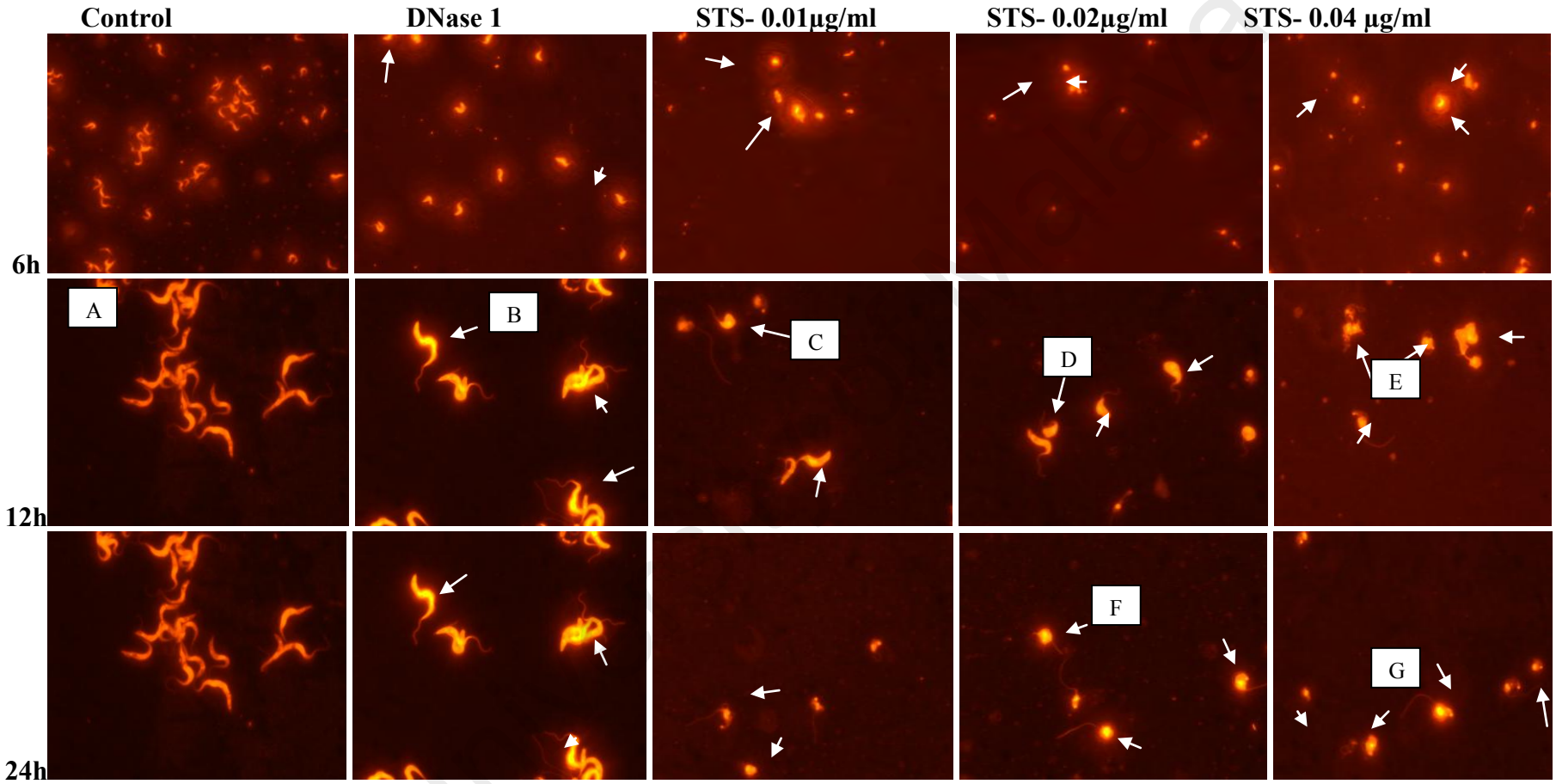


Figure 4.9: Apoptotic morphological changes of *T. b. brucei* strain BS221 induced by staurosporine (STS) with Propidium iodide (PI) staining. Parasites were treated with 0.01, 0.02 and 0.04 µg/ml at 6, 12 and 24 hours time incubations. The parasites morphology was observed under fluorescence microscope (400 and 1000 times magnification). Arrows showed DNA fragmentation by the yellow flourescence nuclei stained.

(A- Control, cells in spindle shape without florescence stained nuclei, B- DNase 1, apoptotic cells in spindle shape with florescence stained nuclei, C, D, E, F, G- treated STS, apoptotic cells in smaller and rounded shape)

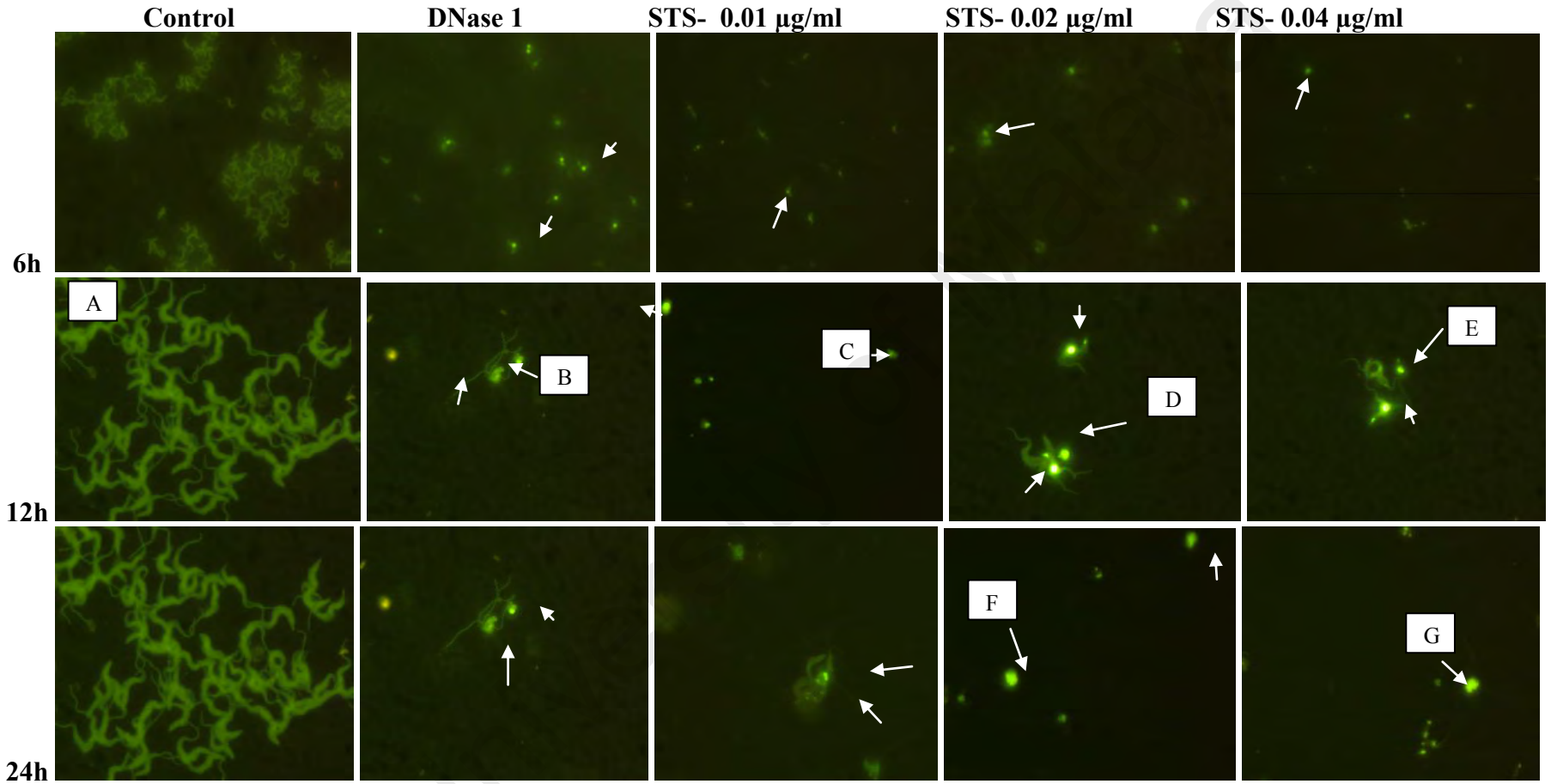


Figure 4.10: Apoptotic morphological changes of *T. b. brucei* strain BS221 induced by staurosporine without stained of Propidium iodide (PI) staining. Parasites were treated with 0.01, 0.02 and 0.04 µg/ml at 6, 12 and 24 hours time incubations. The parasites morphology was observed under fluorescence microscope (400 and 1000 times magnification). Arrows showed DNA fragmentation by the green nuclei stained flourescence.

(A- Control, cells in spindle shape without stained fluorescence stain nuclei, B- DNase 1, apoptotic cell in smaller and rounded cells, C, D, E, F, G- treated STS, apoptotic cells in smaller and rounded shape).

Quantification of cells with fragmented nuclei induced by ochrolifuanine and staurosporine in *T. b. brucei* strain BS221 was evaluated by the percentage of apoptotic index from the ratio between TUNEL positive cells and total of cells in microscopy field observed. The effects of ochrolifuanine and staurosporine in dose concentration and time incubation effects in inducing apoptosis was determined at two events, first on different concentration treatment of ochrolifuanine and staurosporine and secondly by different incubation periods. The percentage of apoptotic index was calculated for each event. This observation was carried out on the treated cells and non treated cells without staining as it gave clear green fluorescence intensity toward TUNEL positive cells (apoptotic cells).

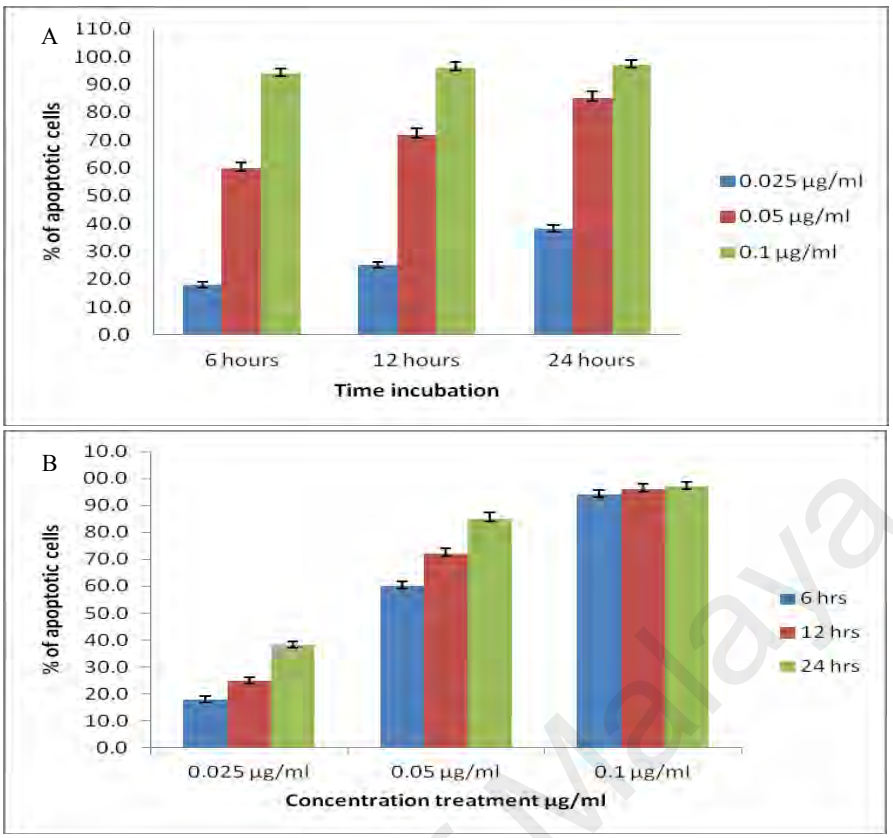
The number of apoptotic cells in *T. b. brucei* significantly increased as the concentration of ochrolifuanine increased (0.025, 0.05 and 0.10 µg/ml) (Figure 4.11 A). The percentage of TUNEL positive cells was significantly increased from 18 to 94 % after treatment of ochrolifuanine at 6 hours. While in the treated cells for 12 hours, apoptotic cells increased from 25 to 96 %. Wheres, at the highest incubation period (24 hours) the apoptosis cells increased from 38 to 97 %. More than 50 % apoptotic cells were observed when the concentration of ochrolifuanine increased to 0.05 and 0.10 µg/ml (Figure 4.11 A).

The similar pattern increments of percentage apoptotic cells were observed on *T. b. brucei* treated with staurosporine as the concentration increased (0.01, 0.02 and 0.04 µg/ml) (Figure 4.12 A). After the treatment with staurosporine for 6 hours, the percentage of cells was significantly increased from 20.5 to 85.0 %. While, after staurosporine treatment for 12 and 24 hours , the percentage of apoptotic cells increased from 26.2 to 89.1 % and 29.9 to 91.5 %, respectively, as shown in Figure 4.12 A. Result showed that there is a significant ($p < 0.05$) increment of apoptosis cells induced by

ochrolifuanine and staurosporine as the increased concentrations compound used. These results obtained suggest that ochrolifuanine and staurosporine induced apoptosis in cells in dose-dependent manner.

In time-dependent study, different incubation times of ochrolifuanine and staurosporine treatment were observed to evaluate the inducing effects parsites cells. The percentage of apoptotic cells was increased as the time of incubation increased from 6, 12 and 24 hours (Figure 4.11 B). The parasites cells treated with 0.025 and 0.05 µg/ml of ocrolifuanine, showed TUNEL positive cells significantly increased from 18 to 38 % and 60 to 85 % respectively. However, at the highest concentration (0.10 µg/ml) of ochrolifuanine, no significant increased of apoptotic cells were observed over time, probably because a maximal death rate was reached.

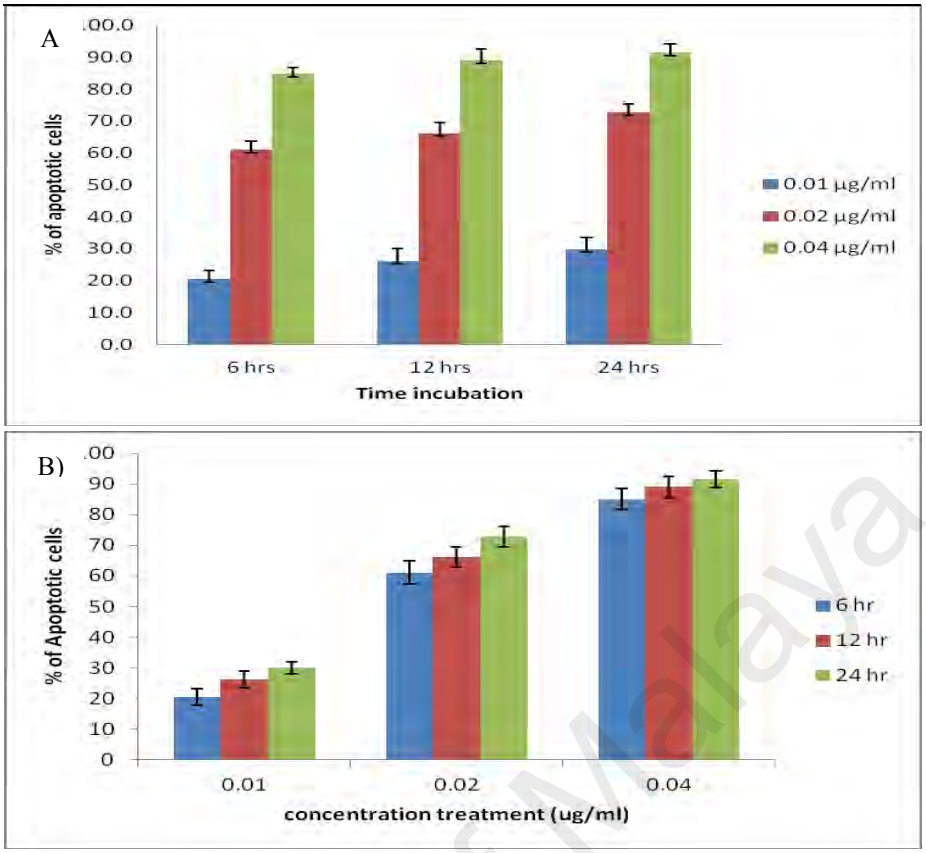
As shown in Figure 4.12 B, staurosporine had caused an increment of apoptotic cells in *T. b. brucei* as the time of incubation increases. Parasites cells treated at 0.02 µg/ml of staurosporine showed significantly increased of apoptotic cells from 61.0 % to 72.8 %. Wheres, at 0.01 and 0.04 µg/ml of staurosporine treated cells for 6 to 12 hours, the percentage of apoptotic cells significantly increased from 20.5 to 26.2 % and 61.0 to 66.2 %, with no significant increment after 24 hours incubation. Taken together, these result showed that ochrolifuanine induced apoptosis in a time-dependent manner but not at a high concentration (0.10 µg/ml), while staurosporine only showed inducing apoptosis effect in time-dependent manner at concentration 0.02 µg/ml.



Ochrolifuanine concentration	% of apoptotic cells		
	6 hours	12 hours	24 hours
0.025 µg/ml	$18.0^{a, x}$	$25.0^{a, y}$	$38.0^{a, z}$
0.05 µg/ml	$60.0^{b, x}$	$72.0^{b, y}$	$85.0^{b, z}$
0.10 µg/ml	$94.0^{c, x}$	$96.0^{c, xy}$	$97.0^{c, yz}$

Figure 4.11: The effect of dose- (A) and time- (B) dependent pattern of treated *T. b. brucei* cells with ochrolifuanine as observed by TUNEL assay.

$^{a, b, c}$ letters represents significant differences ($p < 0.05$) within the groups while the $^{x, y, z}$ letter represent significant differences ($p < 0.05$) between the groups as analyzed by two-way ANOVA, Tukey's multiple comparisons test.



Staurosporine concentration	% of apoptotic cells		
	6 hours	12 hours	24 hours
0.01 µg/ml	$20.5^{a, x}$	$26.2^{a, y}$	$29.9^{a, yz}$
0.02 µg/ml	$61.0^{b, x}$	$66.2^{b, y}$	$72.8^{b, z}$
0.04 µg/ml	$85.0^{c, x}$	$89.1^{c, y}$	$91.5^{c, yz}$

Figure 4.12: The effect of dose- (A) and time- (B) dependent pattern of treated *T. b. brucei* cells with staurosporine as observed by TUNEL assay.

$^{a, b, c}$ letters represents significant differences ($p < 0.05$) within the groups while the $^{x, y, z}$ letter represent significant differences ($p < 0.05$) between the groups as analyzed by two-way ANOVA, Tukey's multiple comparisons test.

4.2.2 Cell Cycle Analysis by Flow Cytometry

Inhibition of parasite proliferation also led to cell cycle arrest, which was mostly caused by nuclear DNA interruption. Thus, in order to assess the interference of ochrolifuanine on cell growth by blocking the cell cycle in *T. b. brucei* parasite, flow cytometry assay was performed to evaluate cell distribution in the different phases of cell cycle after treatment with different concentration of compound for 24 hours. Figure 4.13 showed the effect of ochrolifuanine in growth phase of *T. b. brucei* at different dose concentrations.

The results showed that stained DNA cells after treatment with 0.025 µg/ml of ochrolifuanine demonstrated 66.58 ± 2.74 % DNA content of cells in G_0/G_1 phase, 18.72 ± 2.84 % in S phase and 13.16 ± 1.49 % in the G_2/M phase. While stained DNA treated with 0.05 µg/ml ochrolifuanine demonstrated 66.05 ± 3.61 % of cells were in the G_0/G_1 phase, 19.47 ± 2.54 % in S phase and 14.32 ± 1.25 % in the G_2/M phase. However, parasites treated with the highest dose of ochrolifuanine, at 0.10 µg/ml showed that 59.77 ± 4.70 % of cells were in the G_0/G_1 phase, 13.36 ± 3.65 % in S phase and 26.71 ± 2.54 % in the G_2/M phase. The percentage of cells in late G_2/M phase significantly increased to 26.71 % after treatment with 0.10 µg/ml of ochrolifuanine for 24 hours as compared to control cells (16.10 %). These results suggested treatment of 0.025 and 0.05 µg/ml of ochrolifuanine caused the G_0/G_1 and in S phase arrest of *T. b. brucei* and those 0.10 µg/ml concentrations significantly induced the G_2/M phase arrest of *T. b. brucei* ($p < 0.0001$).

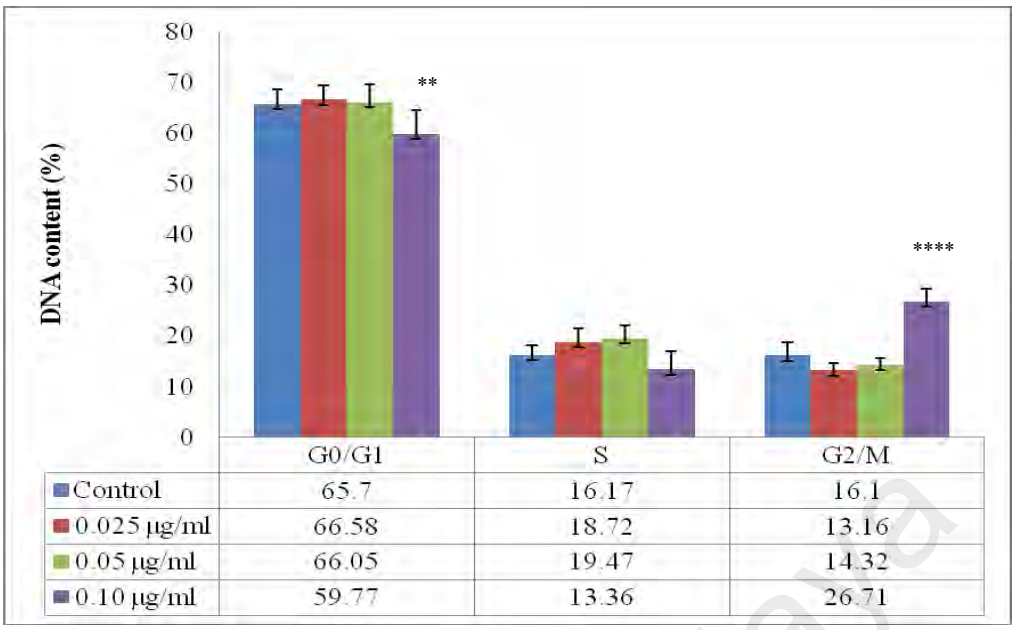


Figure 4.13: Effect of different concentrations of ochrolifuanine on cell cycle phase distribution in *T. b. brucei*.

Parasite cells at density 1 x 10^6 cells/ml were incubated in the absence (control) or presence of different dose concentration of ochrolifuanine for 24 hours and then analyzed by flow cytometry. The asterisk (*) indicates a significant difference as analyzed by two-way ANOVA, Dunnett's multiple comparisons test (**p 0.0021, ***p 0.0002, ****$p < 0.0001$)

CHAPTER 5: DISCUSSION

Current treatment of Human African Trypanosomiasis (HAT) is based on synthetic drugs, pentamidine and suramin which were developed over than 50 years ago. Few other drugs were developed later including melarsoprol and eflornithine due to the previous drug resistance and undesirable side effects. However, the potency of these drugs against HAT are compromised due to severe side effects (Lutje *et al*., 2013; Andrews *et al*., 2014). Hence, there is a need for new drug discoveries that are safe and effective (Renslo & McKerrow, 2006; Phillips, 2012; Bobokhov *et al.,* 2013; Mackey *et al.,* 2013; Yansouni *et al*., 2013; Ferrins *et al.,* 2013).

For over thousands of years, medicinal plants have been a source to treat illnesses and health disorders (Wink, 2012). Many drugs today are derived from natural products based or their derivatives (Kinghorn *et al.,* 2011; Cragg & Newman, 2013). As an alternative to synthetic drugs, natural products are sources of promising drug candidates mainly for treating of infectious and neglected diseases (Butler, 2008; Hannaert, 2011; Zimmermann *et al*., 2012). Earlier studies discovered many medicinal plant species as potential sources of novel trypanocidal compounds (Mann *et al*., 2011; Wink, 2012; Nwodo *et al.,* 2015; McKerrow *et al.,* 2015).

In the present study, crude alkaloid fraction of *Dyera costulata* leaf extract was evaluated for antitrypanosomal activity followed by bioassay-guided isolation of active compound. The isolated compound was then evaluated for its mechanism of action through apoptosis studies.

5.1 Preparation of Crude Alkaloid Fraction from *Dyera costulata*

Plants comprise of a complex mixture of secondary metabolites with different functional groups and polarities. Commonly secondary metabolite classes found in plants are fatty acids, polyacethylenes, terpenoids, steroids, essential oils, phenolics, alkaloids and glycosidic derivatives (Seidel, 1998). Therefore extraction procedure plays a critical role in the analysis of medicinal plants to obtain the desired outcome before the consequent separation and characterisation.

The search for new biologically active compounds begins with testing of crude extracts for bioactivity followed by isolation and characterisation of active constituent(s) responsible for the bioactivity. The extraction methods applied is strongly affected by the chemical structure, and biological activity of the extract. Therefore, the solvent selection and extraction method for isolation of any bioactive compound depends upon its solubility and characteristic in order to obtain higher product recovery during the preparation of extract.

In a previous study by Norhayati *et al.* (2013), out 119 plants Malaysian plant species tested, two extracts from *Dyera costulata* leaf and whole plant of *Cymbopogon nardus* showed strong antitrypanosomal activities with good selectivity index (IC_{50} values 0.58 ± 0.01 µg/ml; SI > 169 and 0.31 ± 0.03 µg/ml ; SI > 323), respectively. Hence, *D. costulata* was chosen in this study to isolate active compound/s against *T. b. brucei* and evaluate their mechanism of action. Initial crude extract was prepared from ground powder of *D. costulata* leaves with hexane solvent using 'solid-liquid" soxhlet extraction method to remove chlorophyll from the sample as reported by Cos *et al*. (2006). Hexane, a nonpolar solvent, was chosen because of its ability to solubilize most lipophilic compounds (e.g., alkenes, fatty acids, pigments, waxes, sterols, some

terpenoids, alkaloid and coumarins). Soxhlet extraction used in this study is a widely used method because of its convenience in terms of less time- and less solvent used as compared to other methods. The extraction process is continuous and this may reduce the organic solvent consumption. Nevertheless, one weakness of this extraction method is that the extract is constantly heated at the solvent boiling point, which can damage thermolabile compounds of interest (Seidel, 1998).

D. costulata leaves were then extracted using solvent with increasing polarity to yield the methanol extract. Polar solvents such as water, methanol, ethanol or ethyl acetate are employed to extract polar compound. This is based on the capability of alcoholic solvents to permeate the cell wall and efficiently extract polar to medium-to-low polarity of compounds (Seidel, 1998).

Phytochemical studies on *D. costulata* leaves have shown that this species contained bisindole alkaloids constituents such as ochrolifuanines A, E, F and 18-dehydroochrolifuanines A, E, F (Koch *et al*., 1975; Mirand *et al*., 1983). However, it has not yet been investigated for *in vitro* antitrypanosomal activity. In the present study, extraction of alkaloids was performed on methanolic extract of *D. costulata* by using acid-base shake out technique (Alaa *et al.,* 2014) with some modifications. Alkaloids in plant materials in nature occur partially as free bases and another part as salts which are insoluble in organic solvents and they might be also insoluble or slightly soluble in water. Nonetheless, they are soluble in the less polar solvent such as hexane, ether or chloroform, (Rahman *et al*., 2011).

In this study, the high yield of crude alkaloids obtained (11.3 %) revealed high extraction rate of alkaloids when acid and water were used in the extraction and this was agreed by Congai *et al.* (2013). This is due to acid contained in lead acetate and with

water and alcohol, allowed for alkaloids to separate from the plant material and form salts which precipitated at the bottom of flasks. Repeating the extraction process for three times in chloroform also permitted optimum alkaloids to be extracted out from the plant material (Alaa *et al*., 2014).

5.2 Bioassay-guided Isolation and Identification of the Bioactive Compound from *D. costulata*

5.2.1 Isolation of Active Compound

Plants remain as one of the main sources secondary metabolites with biological activities such as cytotoxicity, antiprotozoal and anti-microbial (Wink, 2012). New drug candidates for HAT have been explored by many researchers since the current drugs have shown several side effects. In line with that, two new synthetic drug candidates namely, fexinidazole and oxaborole SCYX-7158 are currently undergoing clinical trials (Nagle *et al.,* 2014).

Quinine was first isolated in 1820 from the tree of *Cinchona succiruba* (Rubiaceae) to treat malaria. This compound was the first antiparasitic drug originating from nature to treat parasitic diseases caused by *Plasmodium*, *Leishmania* and *Trypanosoma* spp. (Kayser *et al*., 2003; Gurung & De, 2017). Synthetic drugs were introduced later that led to the development of synthetic antiprotozoal drugs such as melarsoprol, sodium stibogluconate, pentamidine and metronidazole. However, efforts to discover novel antiprotozoal compounds from natural resources such as plants still continue.

Bioactivity-guided isolation is an ideal strategy to identify bioactive compound/s from the active extracts identified from plant samples. Since crude extracts consist of a

high abundance of complex mixtures, bioactivity-guided isolation is the best approach to guide the isolation of active compounds toward drug discovery.

The fractions obtained during isolation process are tested for bioactivity and the active fractions are further separated, most often by column chromatography (CC) and thin column chromatography (TLC). Several chromatography steps are also involved to obtain the pure active compounds, which were then identified by using different spectroscopic techniques (Monteiro, 2016).

In the present work, the crude alkaloid fraction from *D. costulata* was subjected to column chromatography technique by using bioassay-guided isolation approach to yield active fractions. The active fractions were further re-fractionated by using two-steps column chromatography and solid phase extraction (SPE) to yield active compound identified as ochrolifuanine. The SPE method was used for both removing the impurities and concentrating samples to purify the bioactive compounds (Shankland *et al*., 1998). The bioassay-guided fractionation of active fractions from *D. costulata* revealed that ochrolifuanine was found mainly in the non-polar phase of solvent system used (dichloromethane/ methanol). A strong inhibition effect of ochrolifuanine suggests the potential of this compound to be developed as antitrypanosomal therapeutic agent.

5.2.2 *In vitro* Antitrypanosomal Assay on Extract, Fractions and Isolated Compound of *D. costulata* on *T. b. brucei*

5.2.2.1 Alamar Blue Assay to Determine Cell Viability of *T. b. brucei*

In this study, the cultivation of *T. b. brucei* strain BS221 under axenic conditions was carried out prior to determination of the *in vitro* antitrypanosomal activity of crude alkaloid fraction, the column fractions and isolated compound. *Trypanosoma brucei brucei* is commonly used in research laboratories for initial identification of antitrypanosomal compounds (Pink *et al*., 2005; Bonnet *et al*., 2017). Extracellular bloodstreams form of trypomastigotes from other subspecies of *Trypanosoma brucei* sp. such as *T. b. rhodesianse* and *T. b. gambianse* are also used for *in vitro* studies.

Various assays have been adopted to assess the viability of trypanosome cells for screening activity towards identifying novel drug candidates. Kaminsky and Brun (1993) have described several assays which used different parameters including enumeration of cultured trypanosome and incorporation of radioactive nucleotides which involved [^{3}H]hypoxanthine (Brun & Kunz, 1989), cleavage of the tetrazolium salt MTT [3-(4,5-dimethylethiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Ellis *et al*., 1993), colorimetric measurement of pH change due to pyruvate production (Zinsstag *et al*., 1991), fluorometric measurement of BCECF (Obexer *et al*., 1995) and Alamar Blue assay (Raz *et al*., 1997), detection of ATP assay, luciferase based assay (Mackey *et al*., 2006;) and lastly SYBR green assay (Faria *et al.,* 2014). Nevertheless, some of these assays have their respective advantages and disadvantages (Raz *et al*., 1997).

Many biological assays are aimed at measuring survival and proliferation of mammalian cells. Since 1993, Alamar Blue assay was found to be the ideal *in vitro* test

for cell proliferation and cytotoxicity. It is efficient, reliable, simple, rapid, sensitive, safe and cost-effective for measuring of cell viability (Fields & Lancaster, 1993; Raz *et al*., 1997; Sykes & Avery, 2009; Sykes *et al.,* 2012). Among calorimetric methods, Alamar Blue is non-toxic to the test cells and has not required to killing the cells to obtain measurements, as with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (O'Brien *et al*., 2000).

Alamar Blue assay is simple assay, which does not require the process of cell lysis, washing or extraction process in the determination of cell viability (Ahmed *et al.,* 1994; Zachari *et al*., 2014). Viability measurement with Alamar Blue uses spectrophotometer microtiter plate reader. The cell viability and proliferation are observed at the endpoint of assay. Therefore, as published by Raz *et al.* (1997), the Alamar Blue assay showed great potential for the determination of drug sensitivities of antitrypanosomal activity and *in vitro* cytotoxicity.

In the study, antitrypanosomal activity of methanol, crude alkaloid, fractions and isolated compound ochrolifuanine, as well as standard drug pentamidine were evaluated using Alamar Blue assay. This assay (containing resazurin) is a fluorometric REDOX indicator that reacts to chemical reduction of growth medium by visible cells. This chemical reduction resulted from the metabolism activity within mammalian cells, which converted resazurin to resorufin, a fluorescent end product (O'Brien *et al*., 2000). However, the reduction of Alamar Blue in the bloodstream form trypanosome has been postulated to occur by the alternative oxidase pathway (Bienen *et al*., 1991). It has been reported that the intensity of the dye that fluoresced provides direct indication to the number of *T. b. brucei* cells in well (Skyes & Avery, 2009). In addition, this assay is homogenous and provides higher sensitivity in which it detects as low as 200 cells per well (Page *et al*., 1993).

5.2.2.2 *In vitro* Antitrypanosomal Activity of Extract, Fractions and Isolated Compound of *D. costulata* on *T. b. brucei*

In the present study, strong antitrypanosomal activity was detected in methanolic extract of *D. costulata* with IC_{50} 0.58 ± 0.2 µg/ml (SI > 172.4). The activity was much higher as compared to the hexane extract (IC_{50} 4.4 ± 0.05 µg/ml, SI > 22.7). Literature survey revealed that there is limited information available on the bioactivity of *D. costulata,* particularly on antiprotozoal activity. Wong *et al*. (2011) reported on the antiplasmodial activity of methanolic, dichloromethane/methanol and dichloromethane extracts of *D. costulata* against *Plasmodium falciparum* strain 3D7 with IC_{50} values ranging from 2.13 to 8.31 µg /ml. While study by Muhd Haffiz *et al*. (2011), reported the potent antitrypanosomal activity of crude alkaloid of *D. costulata* leaves extract with IC_{50} value of < 0.5 µg /ml. Besides that, the chemical constituents reported in *D. costulata* plant leaves are bisindole alkaloids: ochrolifuanines A, E, F and 18-dehydroochrolifuanines A, E, F (Mirand *et al.,* 1983). The present study confirmed the presence of bisindole alkaloid ochrolifuanine in *D. costulata* and was therefore evaluated further for its antitrypanosomal activity.

Alkaloids with potential therapeutic effects against trypanosomes have been studied extensively. Emetine, an isoquinoline alkaloid from *Cephaelis ipecacuanha* was found to have potent activity with an IC_{50} value of 0.21 µM against *T. b. brucei.* Besides that, quinoline alkaloids from *Cinchona* sp. bark (Rubiaceae) such as quinidine, cinchonine, quinine and cinchonidine were reported to have significant activity against *T. b. brucei* with IC_{50} values of 0.8, 1.2, 4.9 and 7.1 µM respectively (Hoet *et al.,* 2004). A recent study by Nnadi *et al.* (2017) found that 3β-holaphyllamine, 3α- holaphyllamine, 3β-dihydroholaphyllamine, *N*-methylholaphyllamine, conessimine, conessine, conessimine and holarrhesine alkaloids from *Holarrhena africana* showed significant activities with

IC_{50} values ranging from 0.08 – 0.67 µM against *T. b. rhodesiense,* and selectivity indices ranging from 13 to 302. While, Zhang *et al*. (2018), reported that amminomethyl-benzoxaboroles have potent antitrypanosomal against *T. brucei.*

In this study, the crude alkaloid fraction obtained from methanol extract of *D. costulata* was found to exhibit a strong antitrypanosomal effect (IC_{50} value of 0.11 ± 0.03 µg/ml), about 5-fold higher than methanol extract (IC_{50} value of 0.58 ± 0.2 µg/ml). The alkaloid fraction also presented a higher selectivity towards the protozoa compared to methanol extract. These finding suggest that the active component in *D. costulata* leaves may correspond to the alkaloids present and were responsible for the antitrypanosomal activity observed. These observations supported the findings from a previous study which reported on the wide spectrum of antiprotozoal activity were observed in alkaloids (Hoet *et al*., 2004; Feng *et al.,* 2010; Kato *et al.,* 2012; Nnadi *et al., 2*017).

As mentioned earlier, natural products play a major role in disease therapy. Between 1981 to 2014, more than 50 % of the drugs prescribed worldwide originated from natural products (Newman & Cragg, 2016). Discoveries on the potential therapeutic role of alkaloids in various antiparasitic activities have been highlighted by many researchers. However, only few studies were based on *in vivo* model (Wink, 2012).

The isolated compound identified as ochrolifuanine from the bisindole alkaloid group showed strong antitrypanosomal effect with IC_{50} value 0.05 ± 0.01 µg/ml (0.114 µM) (SI: 52). Previously ochrolifuanine was isolated from *Ochrosia lifuana* Guillaumin (Apocynaceae) by Peube-Locou *et al*. (1972) and Kotch *et al.* (1975). It was later discovered in the leaves of *D. costulata* by Mirand *et al.* (1983). Besides that, ochrolifuanine has also been reported in other species such as in the root bark of

Strychnos potatorum (Massiot *et al.,* 1992) and in root bark of *Aspidosperma excelsum* (Verpoorte *et al.,* 1983).

Frederich (2002) reported on antiplasmodial activity of ochrolifuanine against *Plasmodium falciparum* (IC_{50} 0.5 μM). Even though ochrolifuanine is a known compound, there is no report prior to this study on its antitrypanosomal activity of against *T. b. brucei*. The significant antitrypanosomal activity and high selectivity exhibited by ochrolifuanine warrants further investigation into its mode of action which inhibited growth of parasite.

To our knowledge, this is the first study which described the antitrypanosomal activity of ochrolifuanine on *T. b. brucei*. Indole alkaloids including ochrolifuanine are the group of alkaloids that can be found distributed in the plant of families of Apocynaceae, Loganiaceae, Rubiaceae and Nyssacea (Hamid *et al*., 2017). Indole alkaloids are recognized by their bicyclic structure, benzene ring and nitrogen-containing pyrrole ring. The pyrrole ring consisting of nitrogen atom contributed to the basic properties of indole alkaloids which present their active pharmacology activity (El-Sayed & Verpoorte, 2007). Rosekranz and Wink (2008) demonstrated the sensitivity of *T. brucei* to berberine an indole alkaloid, at IC_{50} value 0.5 μM. Other monomeric indole derivatives like ellipticine and olivacine have also shown antitrypanosomal activity on *T. cruzi* (Fournet *et al.,* 1996; *Gantier et al.,* 1996).

In 2012, Wink reported that plant-derived drug from bisindole alkaloid group, namely vinblastine isolated from *Catharanthus roseus,* exhibited potent antitrypanosomal effects with an IC_{50} value of 0.21 μM against *T. b. brucei*. Pimentel-Elardo (2010) also found that staurosporine (also a group of bisindole alkaloid), from *Streptomyces staurosporeus,* showed high antitrypanosomal activity with an IC_{50} value

of 0.022 μM. Based on the findings from the present study, the antitrypanosomal effect of ochrolifuanine (IC_{50} value of 0.114 μM) was found to be comparable with that of other reported bisindole alkaloid compounds (Note: to compare, IC_{50} unit not in μg/ml). In addition, ochrolifuanine was showed to be selective towards the parasite compared to mammalian cells.

New strategies to treat trypanosomiasis have been studied throughout, where as an alternative to single drugs treatment, the use of combination therapy (multi drugs) were applied as the treatment. One such example is nifurtimox-eflornithine (NECT) combination therapy which was developed in 2009 and is considered as one of the safest therapies (Horn & Duraisingh, 2017). In studies on synergism, combinations of bioactive alkaloids have been shown to enhance trypanocidal effect (Kristin *et al*., 2015). Their study showed that combination of digitonin (a steroidal saponin obtained *Digatalis purpurea* flower) and vinblastine exhibited higher activity against *T. b. brucei*. In addition, the combination of more than one drug with different mechanism of action might enhance the effective effects on target disease (Chou, 2006). The approach is an interesting alternative to be future studied with the ochrolifuanine isolated in this study.

Generally, the observed activity of ochrolifuanine from *D. costulata* may be due to present of alkaloid. The high levels of antitrypanosomal activity observed in ochrolifuanine and the other reported bisindole alkaloid compounds suggested that these compounds may share the same target site in the parasites. Ochrolifuanine may be worth to be developed as a possible new trypanocidal.

5.2.3 *In vitro* Cytotoxicity of Extract, Fractions and Isolated Compound of *D. costulata* on Normal Human Cell Lines and Selectivity Index (SI)

Cytotoxicity assay was performed to obtain an indication of degree of selectivity of samples towards the normal mammalian cell lines compared to protozoan cells. According to the Badisa *et al.* (2009), a good drug candidate must exhibit less side-effect on normal mammalian cells. Minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role. Various normal cell lines were used in cytotoxicity study of antitrypanosomal active constituents such as MRC5 (human diploid embryonic), THP1 (transformed human monocytic), L6 (rat skeletal myoblast) and Vero (monkey kidney) (Otoguro *et al*., 2008; Norhayati *et al*., 2013; Nwodo *et al*., 2015 & Jain *et al*., 2016). Cytotoxicity screening models provide an important data for selection of plant extract with no cytotoxicity effect on normal cell lines for further selectivity index determination (SI) (Hoet *et al*., 2004).

Calculation of selectivity index (SI) was performed based on the ratio of IC_{50} on normal cell lines (cytotoxicity) to the IC_{50} of antitrypanosomal activity (Nanavaty *et al*., 2016). An extract/fraction/ active compound that has low toxicity effects on mammalian cell lines but strong activity against trypanosome parasite were selected based on SI value. According to Kaminsky *et al.* (1996), the *in vitro* selectivity index result cannot be used to extrapolate the dosages for *in vivo* study. However, this parameter is valuable to identify compounds that are toxic to parasite but not to host cells and are worth investigating in animal model (Hoet *et al*., 2004). The criterion of selectivity index (SI) in this study was based on 3 categories according to Otuguro *et al*. (2011); selective (SI > 100), moderate selective (100 < SI < 10) and not selective (SI < 10).

In this study, of methanol extract and crude alkaloid fraction of *D. costulata* leaves were found to be selective against *T. b. brucei*. There have been limited reports on the potential extract derivative from *D. costulata* in bioactivity studies. Wong *et al.* (2011) reported that methanol extract of leaves of *D. costulata* showed high antioxidant activity, while dichloromethane/methanol and dichloromethane extracts showed potent antiplasmodial activity.

The SI value of ochrolifuanine was found to be 52, which fall under the moderate selectivity criteria based on Otoguro *et al.* (2011). However, both Hoet *et al.* (2004a) and Skyes *et al*. (2012) have shown that compounds with SI $\geq$ 20 were considered as selective towards the parasite.

5.3 Evaluation of Apoptosis-Inducing Effects of Ochrolifuanine on *T. b. brucei*

5.3.1 Determination of DNA Fragmentation by TUNEL Assay

Analysis of DNA fragmentation in agarose gel are the early method applied to determine apoptosis cell death and is widely used for metazoans cells. However, detection of DNA ladders in parasites such *Leishmania* spp. and *T. brucei* were found to be restricted as compared to the detection in metazoan cells (Jimenez-Ruiz *et al*., 2010). Over the past years, the use of terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay which is based on fluorochrome labelling to determine the DNA strand breaks are widely used in apoptosis studies. This assay was first described for detection of DNA strand break in a cancer cell by Gorczyca *et al*. (1992). In this method, labelled cells can be analysed by fluorescence microscopy and flow cytometry.

Several studies reported that DNA fragmentation was detected in trypanosome parasite by TUNEL assay in response to stimuli such as ROs (Ridgley *et al.,* 1999), incubation with prostaglandin D_2 (Jimenez-Ruiz *et al*., 2010) and lectin ConA (Pearson *et al.,* 2000). Besides that, Rosenkranz & Wink (2008) observed DNA fragmentation induced by several alkaloids such as steroidal alkaloid, piperine, cinchonidine, cinchonine, berberine, emetine in *T. b. brucei* by using flow cytometry. The formation of fragmented nuclei was also reported in *T. b. brucei* to be induced by the bisindole alkaloid staurosporine (Barth *et al*., 2014). The molecular mechanisms associated with programmed cellular death (PCD) or apoptosis have been widely explored in mammalian cells. However, the molecular details of PCD in protozoan parasites have still remained limited. Many researchers considered induction of apoptosis in protozoa may be enabling the regulation of parasite densities in distinct host compartments and aid in avoiding inflammatory responses which can thereby facilitate a sustained infection (Zandbergen *et al.,* 2010).

In this present study, ochrolifuanine was evaluated for its potential apoptotic activity on *T. b. brucei.* Detection of DNA fragmentation in *T. b. brucei* treated with ochrolifuanine was performed using TUNEL assay (qualitative & quantitative) by: i) Observation on morphological changes in *T. b. brucei* induced by ochrolifuanine and ii) quantification of percentage of apoptotic cells formed at different compound doses and incubation time. In addition, cell cycle analysis was performed to determine the growth inhibition of ochrolifuanine in *T. b. brucei* cell cycle.

5.3.2 DNA Fragmentation Effect in *T. b. brucei* Induced by Ochrolifuanine

Over the years, much evidence has been accumulated to describe morphological and biochemical events displayed during the death of trypanosomatids which share certain characteristics with mammalian apoptosis phenomena (Debrabant *et al.,* 2003; Fernandez-Presas *et al*., 2010; Menna-Barreto & Castro, 2015). The apoptotic features shown by these kinetoplastid parasites include the DNA fragmentation, depolarization of mitochondrial membrane potential, protease activation, membrane blebbing, the exposure of phosphatidylserine in the outer leaflet of the plasma membrane, chromatin condensation and cytochrome c release.

Apoptosis or programmed cell death (PCD) in multicellular organism such as protozoa mainly kinetoplastid parasites, have been described in *T. brucei, T. cruzi* and *Leishmania* spp. It was reported that PCD in kinetoplastid parasites could serve as a mechanism of adaption and defence against the host and might be used to maximize their (parasites) biological fitness (Nguewa *et al*., 2004). Besides that, PCD also plays a role in the regulation of cell density in parasitic protozoa. Therefore, PCD could be a potential pharmacological target for protozoan control.

Apoptosis is triggered in trypanosome parasite in response to diverse stimuli such as heat shock (Alzate *et al.,* 2006; Raina & Kaur, 2006; Abaza, 2015), reactive oxygen species (ROs) (Figarella *et al*., 2006; Deolindo *et al*., 2010; Paes *et al*., 2011; He *et al*., 2012), antiparasitic drugs (Nguewa *et al.,* 2005; Urbina, 2006 ; De Sauza *et al.,* 2006; Uzcategui *et al*., 2007; Rosenkranz *et al*., 2008; Menna-Bareto *et al*., 2009; Dos Anjos *et al.,* 2016;), prostaglandins (Figarella *et al*., 2006), cell starvation (Zangger *et al.,* 2002; Alvarez *et al*., 2008; Jimenez *et al*., 2008). Additionally, antimicrobial peptides, antibodies and mutation in cell cycle regulation genes were found to be an

inducer for apoptosis in trypanosome (Selvapandiyan *et al*., 2004; Kulkarni *et al.,* 2009; Luque-Ortega *et al*., 2010; Fernandez-Presas *et al*., 2010).

In the present study, appearance of fluorescence stained nuclei (TUNEL positive cells) in *T. b. brucei* parasite treated with different concentrations (0.025, 0.05 and 0.10 µg/ml) of ochrolifuanine and staurosporine for 6, 12 and 24 hours incubation period have confirmed the apoptosis-inducing capability of both compounds. The appearance of yellowish (with PI stain) and green (without PI stain) fluorescence stained nuclei in ochrolifuanine and staurosporine treated *T. b. brucei* within 6 hours incubation showed that early apoptosis occurred in cells; whilst, non-stained nuclei were observed in non-treated cells. This is agreeable with the appearance of fluorescence stained nuclei reported in other DNA fragmentation studies carried out on trypanosome parasites induced by different inducer (Uzcategui *et al*., 2007; Castillo-Acosta *et al*., 2008; Deolindo *et al*., 2010; de Silva Rodrigues *et al.,* 2016).

Morphological differences in nuclear is commonly considered as the most excellent indicator of an apoptotic process. Currently, DNA fragmentation displayed by the formation of a multitude of DNA strand breaks is granted as one of the hallmark characteristic markers for identification of apoptosis in protozoa (Duszenko *et al.,* 2006; van Zandbergen *et al.,* 2010). In the present study, the morphology of parasites cells was affected by treatment with ochrolifuanine and staurosporine. The apoptotic cells were observed to be smaller and rounded shape as compared to untreated cells, similar to what has been reported as morphological features of apoptosis (Jimenez *et al*., 2014).

Determination of therapeutic dose and success of an anti-parasitic agents need a good understanding of the complex interaction existed between the potency of an agent and exposure time within the host body (White, 2013; 2017). Thus, the effective dose

and time for a given drug can be accomplished in pre-clinical studies by establishing a dose-response relationship. The correlation between drug concentrations, time-course, therapeutic strength and adverse effects, are known as pharmacodynamics (Kang and Lee, 2009; White, 2013). In order to achieve the admired therapeutic effect, safety and efficacy of any drug must be optimized to determine the administered dosage present minimal adverse effect (White, 2013).

Time-kill assays are carried out to determine the pharmacodynamics of a compound concentration versus the total time of exposure needed to achieve it effective action (Nare *et al.,* 2010; Jacobs *et al.,* 2011). In this study, ochrolifuanine was found to induce apoptosis in *T. b. brucei*. The percentage of apoptotic significantly increased as compound concentration (0.025 - 0.10 µg/ml) and incubation period increased (6 -24 hours). Hence it can be concluded that ofochrolifuanine induced apoptosis in a dose- and time-dependent manner. Treatment with staurosporine showed significant increasing of apoptotic cells when concentration increased, but not in time-dependent manner.

Recently, a number of efficacy studies have been reported on the treatment of trypanosome parasite infections. Clomipramine, drug originally used as antipsychotic exhibited a dose-dependent effect on growth of *T. brucei* (de Silva Rodrigues *et al*., 2016). Moraes *et al.* (2014) showed that trypanocidal drugs benznidazole, nifurtimox and fexinidazole sulfone showed concentration-dependent effects and therefore were more efficacious at higher doses for Chagas disease treatment.

The results presented herein provide evidence that ochrolifuanine induced biochemical and morphological alterations in *T. b. brucei* leading to parasites death. Since, ochrolifuanine had been shown to cause DNA fragmentation, indicated by the

appearance of apoptotic cell in *T. b. brucei*, further studies need to be performed to support these findings. In addition to a better understanding of molecular mechanism of apoptosis in trypanosome parasites, a series of signalling pathways involved in apoptotic cell death needs to be investigated in order to determine the role of ochrolifuanine in apoptosis of trypanosome parasites. The ability of ochrolifuanine to induce apoptosis in *T. b. brucei* warrants further investigation to establish its potential as a candidate compound for developing new antitrypanosomal drugs.

5.3.3 Effects of Ochrolifuanine on Cell Cycle Arrest in *T. b. brucei*

Cell cycle progression is well known for the mediated of cell growth and proliferation in mammalian cells (Gerald & Goldbeter, 2016). The induction of apoptosis in mammalian cells is frequently associated with changing of the vital biological process. The link between proliferation and cell cycle control with apoptosis bring indication that organism can extend their cell regulation and cellular homeostasis. Therefore, the distraction of cell-cycle may cause apoptosis in mammalian cells (Kasten & Giardano, 1998; Allan & Clarke, 2009). The presence of key cell cycle regulators such as cyclin-dependent kinases and cyclins in protozoa suggest that protozoa utilize cell cycle machinery just as mammalian cells (Hammarton *et al*., 2003: 2007; Li, 2012; Silvester *et al.,* 2017).

A simple and quicker staining method for analysis of DNA per cell by flow microfluorometry has been described by Crissman and Steinkamp (1973). The prospect of rapid quantification of a multitude of cell attributes' in the cell population by cytometry led to the wide use of this method in cell proliferation (cell cycle) and cell death studies (Darzynkiewicz *et al*., 2001). The functions of cytometry in the field of cell cycle analysis provide two purposes: (i) to reveal the distribution of cells in

particular phases of the cycle or to determine the kinetics of progression through these phases; (ii) to explain the molecular and functional mechanisms associated with the cell cycle (Blasi *et al.,* 2016).

In this study, the DNA content in *T. b. brucei* parasites were stained by propidium iodide staining followed by flow cytometry, to determine whether the growth inhibition of *T. b. brucei* by ochrolifuanine may result in cell cycle arrest. Ochrolifuanine showed antitrypanosomal effect against trypanosomes with an IC_{50} value of 0.05 µg/ml. Parasites treated with ochrolifuanine were not able to progress through the cell cycle. It was observed that in 24 hours treatment with 0.025 and 0.05 µg/ml of ochrolifuanine, the growth of *T. b. brucei* was arrested at two different degrees (G_0/G_1 and in S phases). While in the highest concentration treatment of 0.10 µg/ml of ochrolifuanine, there were significantly more G_2/M phase cells in the population. This suggested that *T. b. brucei* cells spending a longer time during G_2/M because of the effect of ochrolifuanine in cells growth which caused the delayed of mitosis progression.

The relationship between cell cycle control and apoptosis is evident in trypanosome parasites. Pearson *et al.* (2000) reported that lectin concanavalin A (ConA) induced cell cycle arrested in *T. brucei* parasites. In addition, ConA also has been reported to induce apoptosis in the same parasites (Welburn *et al.,* 1996). In different studies, the anti-microtubule drug, Rhizoxin, isolated from *Rhizopus chinensis* and the nuclear DNA synthesis inhibitor drug, aphidicolin an antibiotic isolated from *Cephalosporum aphidicola* were shown to affect cell cycle arrest in *T. brucei* bloodstream form at G_0/G_1 and G_2/M phases (Ploubidou *et al.*, 1999). Whereas, hydroxyurea drug also has been reported to inhibit cell cycle progression at G_0/G_1 and G_2/M phases in *T. brucei* parasites (Mutomba & Wang, 1996). Besides that, Uzcategui *et al.* (2007) found that *T. brucei* treated with dihydroxyacetone involved in inhibition of cell cycle progression at G_2/M

phase. Recently, clomipramine as an antidepressant drug has been described as trypanothione inhibitor found to be involved in cell cycle inhibition by arrested G_0/G_1 phase which led to apoptosis in *T. b. brucei* (Rodrigues *et al*., 2016).

The contribution of cyclin-dependent kinases (CDKs) in the control of the cell cycle progression in yeast and higher eukaryotes is well established (Pines, 1995; Lui & Kipreos, 2000; Walker *et al.,* 2011). In contrast to the availability of information on other species of kinetoplastid parasites, there are few studies dealing with cell cycle control and apoptosis in *Trypanosoma* spp. However, a number of putative cells cycle regulator, cyclins molecule has been successfully identified in kinetoplastid parasites. Cyclins (CYCs) are a family of proteins that have been involved in the control of progression of cell cycle by activating the CDK enzymes (Walker *et al*., 2011; Li, 2012).

In trypanosome parasites, cyclin CYC2 plays a major role in promoting G_1/S phase of cell cycle (Hammarton *et al*., 2004; Li & Wang, 2003; Li, 2012). Cyclin-related kinases (CRK), CRK1 and CRK2, have also arrested trypanosome cells at G_1 phase (Tu & Wang, 2004; 2005; Li, 2012). DNA replication and regulation of S phase in *T. brucei* appeared to be different from other eukaryotes. The origin recognition complex (Orc) which consists of Orc1 to Orc10, and cell division cycle (Cdc), are proteins involved in initial DNA replication. The Orc1/Cdc6-like protein and Orc4 are reported to be important for DNA replication in the trypanosome (Dang & Li, 2011; Tiengwe *et al*., 2012). Whereas, G_2/M phase in trypanosome is controlled by CRK3 and CYC6 pair (Hammarton *et al*., 2004; Li & Wang, 2003; Li, 2012). However, CYC8 and CRK9 are also reported to be involved in trypanosome G_2/M transmission (Li, 2012). Walker *et al.* (2011) reported that CYC6 and CRK3 were involved in G_2/M in *Leishmania mexicana*. Besides that, CYC6 also has been reported to cause the nucleus to arrest in a metaphase-

like state without preventing cytokinesis (Hayashi & Akiyoshi, 2018). Thus, the importance of kinases in cell cycle progression and apoptosis are recognized and inhibition of homologous kinases may induce apoptosis in both trypanosome and mammals (Li, 2012).

Several studies had been performed to elucidate the mechanism of action of compounds on trypanosome cell cycle. Studies by Rodrigues *et al.* (2016) found that treatment of *T. cruzi* with clomipramine after 24 hours resulted in cell cycle arrest at sub G_0 phase. In a different study, dihydroxyacetone induced up to 70% of cells which indicated that the apoptotic cell death in *T. brucei* was due to cell cycle arrest in G_2/M phase (Uzcategui *et al*, 2007). On the other hand, treatment with aphidicolin and hydroxyurea affected the growth differentiation of *T. brucei* and cell cycle arrest at G_1/S phase (Mutomba & Wang, 1996). Zuma *et al*. (2014) reported that treatment of *T. cruzi* with campthotecin, a topoisomerase I inhibitor was found to induce cell cycle arrest at the late stage of S/G_2/M.

In the present study, treatment of 0.10 µg/ml of ochrolifuanine significantly ($p < 0.05$) arrested G_2/M growth phase in *T. b. brucei*, after indicating possible down-regulation of CRK3 and CYC6. In addition, the accumulation of cells in G_0/G_1 and in S phases induced after treatment with 0.025 and 0.05 µg/ml of ochrolifuanine may be affected by regulation of CYC2 and both CRK1 and CRK2 in G_0/G_1 and Orc1/Cdc6-like protein in S phase. The essential molecules regulating parasite cell cycle can be exploited in the development of potential novel therapeutics.

CHAPTER 6: CONCLUSION

6.1 Conclusion

The present study was carried out to search for potential compound candidates from Malaysian natural product and to enhance drug discovery in treating for promising activity in the therapy of trypanosomiasis diseases. Even though the disease does not exist in Malaysia, the awareness of contribution to public health became as one of the consideration.

The aim of this study was to isolate ochrolifuanine, a bisindole alkaloid group from the leaves of *Dyera costulata* (Miq.) Hook.*f* and to evaluate its antitrypanosomal effect and mode of action as well as their therapeutic target at the cellular level by apoptosis studies.

The methanolic and crude alkaloid fractions from the leaves of *D. costulata* (Apocynacea) were evaluated for antitrypanosomal effect against *T. b. brucei* strain BS221. The antitrypanosomal effects of extracts were found to exhibit strong antitrypanosomal activity against trypanosome cells. Further bioassay-guided isolation of alkaloid extract has led to identified of one bioactive compound, ochrolifuanie which are comparable with the reported ochrolifuanine presence of in *D. costulata*. The results demonstrated that ochrolifuanine has strong antitrypanosomal effect with high selectivity index, which also indicated a low toxicity level on normal liver mammalian cells lines (WRL-68). Since alkaloids have been reported to induce killing activity in trypanosome cells, the present study was confirmed that the antitrypanosomal effect in *T. b. brucei* was contributed from the alkaloid present in *D. costulata*.

Even though ochrolifuanine is a known compound, but this is a first report which explained the antitrypanosomal activity of ochrolifuanine against *T. b. brucei*. The effect of ochrolifuanine and staurosporine in inducing apoptosis via DNA fragmentation was evaluated using different dose concentrations treatment and time incubation. The appearance of apoptotic bodies and morphology change observed indicating that the cells undergo apoptosis cell death.

The pharmacodynamic effects of ochrolifuanine were evaluated by dose- and time-dependent in *T. b. brucei*. Ochrolifuanine was found to induce apoptosis in *T. b. brucei* as the percentage of apoptotic cells were increased in line with the increment of dose concentration and time incubation. Hence, it can conclude that ochrolifuanie inducing apoptosis in *T. b. brucei* in time- and dose-dependent manner. Besides that, the ochrolifuanine was also shown to affect the cell cycle growth in *T. b. brucei* by causing G_2/M phases arrest.

The mechanisms of action of ochrolifuanine are worth to be further studied as it showed potent apoptosis inducer and causing G_2/M phases arrest in *T. b. brucei*. Undoubtedly, exploration of the unusual pathways and their novel regulators in trypanosome not only will further our understanding of the mechanism of the therapeutic target but also provide novel drug targets for trypanosomiasis disease treatment. Hence, ochrolifuanine has a potential lead for new therapies against trypanosomiasis.

6.2 Recommendation for Future Study

As the current study was focused on *in vitro* evaluation of antitrypanosomal effects of total alkaloid extracts, fractions and active compound, ochrolifuanine on *T. b. brucei*. There are a number of additional studies that need to be further performed toward a discovery of the drug development process of ochrolifuanine. The present finding suggested that the antitrypanosomal effect of the isolated compound, ochrolifuanine is mediated by induction of apoptosis, but the exact pathway involved is still unclear, means further investigations on the mode of action need to be done. Exploration of ochrolifuanine effects *in vivo* study also should be considered as one of the target study. In addition, the isomer of ochrolifuanine which have been reported in the past should be investigated in order to find compounds with a better cytotoxic/trypanocidal ratio.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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PAPERS PRESENTED

Norhayati, I., Getha, K., Nurhayati, Z. A., Muhd Haffiz, J., Adiratna, M. R., &Lili Sahira, H. (2018, Oct). *Bisindole alkaloid ochrolifuanine isolated from Dyera costulata (Miq.) Hook.f induced cell death effect in Trypanosoma brucei brucei*. Poster presented at 15th Seminar on Medicinal and Aromatic Plants: Unravelling nature's treasures & secrets - current species of interest, FRIM, Kepong, Selangor, Malaysia.

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Norhayati, I., Getha, K., Nurhayati, Z. A., Muhd Haffiz, J., & Lili Sahira, H. (2016, Mar). *In-vitro antitrypanosomal of bisindole alkalid isolated from Dyera costulata leaves and its apoptosis effects*. Poster presented at 52nd Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine: Toward impactful collaboration in parasitology and tropical medicine, Grand Season Hotel Kuala Lumpur, Malaysia.

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