Tropical Biomedicine 39(3): 321-327 (2022)
https://doi.org/10.47665/tb.39.3.003

RESEARCH ARTICLE

Evaluation of antitrypanosomal properties and apoptotic effects of ochrolifuanine from *Dyera costulata* (Miq.) Hook.*f* against *Trypanosoma brucei brucei*

Norhayati, I.[1*], Nurhayati, Z.A.[2], Getha, K.[1], Muhd Haffiz, J.[1], Adiratna, M.R.[3]

[1]Natural Product Division, Forest Research Institute Malaysia, 52109 Kepong, Selangor, Malaysia
[2]Institute of Science Biology (ISB), University Malaya, 50603 Kuala Lumpur, Malaysia
[3]Immunology Research Centre, Institute of Medical Research (IMR), 50580, Kuala Lumpur, Malaysia
*Corresponding author: norhayati@frim.gov.my

ARTICLE HISTORY

Received: 31 January 2022
Revised: 7 June 2022
Accepted: 7 June 2022
Published: 15 August 2022

ABSTRACT

Trypanosoma brucei parasites are flagellated kinetoplastid protozoan which is responsible for Human African Trypanosomiasis (HAT). Current chemotherapy drugs have a number of side effects and drug resistance has emerged as a major issue in current treatment. Active bisindole alkaloid compound ochrolifuanine was previously isolated from the leaves of *Dyera costulata*. *In vitro* antitrypanosomal activity of ochrolifuanine against *Trypanosoma brucei brucei* strain BS221 showed strong activity with an IC_{50} value of 0.05 ± 0.01 µg/ml. We compared the effect of ochrolifuanine and reference compound staurosporine in *T. b. brucei* apoptosis. The apoptosis-inducing activity of ochrolifuanine was evaluated using TUNEL assay and cell cycle analysis. *Trypanosoma brucei brucei* was shown to undergo apoptotic cells death as demonstrated by the appearance of several conical hallmarks of apoptosis. Ochrolifuanine was found to induce apoptosis in parasites in a dose- and time-dependent manner. The cell cycle study revealed 0.025 and 0.05 µg/ml of ochrolifuanine arrested the growth of *T. b. brucei* at two different growth phases (G_0/G_1 and in S phases). While at concentration 0.10 µg/ml arrested at the G2/M phase. In conclusion, the results indicate that ochrolifuanine displayed an antitrypanosomal effect on *T. b. brucei* by inducing apoptosis cell death and causing the arrest of parasite cells at different growth phases. The results suggested that ochrolifuanine may be a promising lead compound for the development of new chemotherapies for African trypanosomiasis.

Keywords: Ochrolifuanine; *Dyera costulata*; apoptotic; *Trypanosoma brucei brucei*.

INTRODUCTION

Human African Trypanosomiasis (HAT) also known as sleeping sickness is one of the Neglected Tropical Diseases (NTDs) group affected by kinetoplastid protozoa parasites from the subspecies of *Trypanosoma brucei* (Brun *et al*., 2010). These protozoa parasites afflict both man and animals, causing a 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).

Pentamidine, suramin, melarsoprol, nifurtimox, and eflornithine, are commonly used for the treatment of HAT. However, these drugs have been reported to cause adverse drug reactions 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 need for the development and discovery of new drugs that are safe, effective, affordable, easy-to-use, and have a unique mechanism of action.

Researchers have synthesized 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. Pharmaceutical companies have not prioritized the development of new antiparasitic drugs because many of these parasitic infections occur in a poor countries where the populace cannot afford such expensive drugs.

Dyera costulata (Miq.) Hook. *f* a large tree from the Apocynaceae family is locally known as jelutong in Malaysia. 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 (Reanmongkol *et al*., 2002; Wong *et al*., 2011). Ochrolifuanine, a bisindole alkaloid group was discovered from leaf extracts of *D. costulata* (Mirand *et al*., 1983) and is also found in the 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* (Camacho *et al*., 2004; Pimentel-Elardo *et al*., 2010).

Apoptosis is a type of programmed cell death (PCD) that affects trypanosome parasites. The process of programmed cell death in trypanosomes resulted in the regulation of parasite population size (cell density) as well as a mechanism to control genetic stability and cell differentiation (Welburn *et al*., 1996). Although research on apoptosis in protozoa is limited, compounds such as concanavalin A, prostaglandin A, and staurosporine have been shown to induce PCD in *Trypanosoma* spp. (Figarella *et al*., 2006; Barth *et al*., 2014)

Staurosporine is a bisindole alkaloid, isolated from marine actinobacteria strain that has been shown to induce apoptosis in trypanosomes. The mechanism of staurosporine-induced apoptosis is unknown but it has been reported to involve alteration of phosphorylation changes, cell cycle control changes and DNA fragmentation (Barth *et al*., 2014).

We had found a pronounced antitrypanosomal activity of ochrolifuanine on *T. b. brucei* parasites (Norhayati *et al*., 2013, 2018). Determination of the effect and mechanism of antitrypanosomal actions of ochrolifuanine may lead to the development of novel drugs that can overcome the limitations of currently available antitrypanosomal drugs. Therefore, this study was undertaken to evaluate the effect of ochrolifuanine and staurosporine-induced cell death in *T. b. brucei* strain BS221 in a time- and dose-dependent manner. Ochrolifuanine significantly induced apoptosis-like cell death and resulted in a significant alteration of cell cycle and morphology of cells. Our finding indicate that ochrolifuanine has promising antitrypanosomal effects, which has implications for potential African trypanosomiasis therapeutic interversions.

MATERIAL AND METHODS

Materials and Parasite

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

The parasites *Trypanosoma brucei brucei* strain BS221, a derivative of S427 also known as MiTat 1.2/221 was obtained by the Swiss Tropical and Public Health Institute (Swiss TPHI), Basel, Switzerland. The bloodstream-form parasites were cultured in Balz Minimal Essential Medium (BMEM) containing Minimal Essential Medium (MEM) supplemented with 1 g glucose/L, 25 nM HEPES, 2.2 g $NaHO_2$/L, and 10 mL MEM non-essential amino acids/L. Additional 0.14% (v/v) mercaptoethanol dilution with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate and 0.1 mM hypoxanthine (Baltz *et al*., 1985) was added to the culture medium. The parasites were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Test Compound

Ochrolifuanine was isolated from the leaves of *Dyera costulata* based on the previous method described in Norhayati *et al*. (2018) with minor modifications. The plant species were identified by Forest Research of Malaysia (FRIM) botanist, the voucher specimen was comparable with the reference specimen of FRIM Herbarium (Voucher no: FMS 29764).

The *in vitro* viability test for trypanosome parasites

Alamar Blue assay was performed to assess cell toxicity and viability when treated with the ochrolifuanine according to the method described by Norhayati *et al*. (2013). The ochrolifuanine and staurosporine were dissolved in absolute ethanol to produce seven different final concentrations ranging from 0.001 to 0.100 µg/ml, while the standard drug (Pentamidine) was dissolved in 5% dimethyl sulfoxide (DMSO) for positive control. 5% DMSO, 25% Ethanol and deionized water were used as negative controls. All assays were performed in the flat-bottom 96-well microtiter plate. 5 µl of pre-dilution test compound, pentamidine, and negative control was added to each well of a 96-well microtiter plate. Subsequently, 95 µl of the trypanosome suspension at the density of 2.0×10^4 parasites ml^{-1} were added to the plate in triplicates. The plates were then incubated for 72 h and assayed using Alamar Blue assay (Räz *et al*., 1997) and re-incubated for 4-5 h. Optical density (OD) values were determined using the Tecan Infinite M200 fluorescent plate reader and Magellan ^{TM} data analysis software at excitation wavelength 528 nm and emission wavelength 590 nm. The IC_{50} values were determined from the dose-response curve (Räz *et al*., 1997; Otoguro *et al*., 2008).

TUNEL assay

In situ detection of DNA fragmentation following treatment of parasite cell (1×10^6 cells) with different concentration (0.025, 0.05 & 0.10 µg/ml) of ochrolifuanine and staurosporine (0.01, 0.02 & 0.04 µg/ml) for 6, 12 and 24 h was measured by TUNEL using Cell Death Detection kit (Roche) according to the manufacturer's instructions. Cells were centrifuged (1000 g, 10 min) and the supernatant was discarded. The cells pellet were fixed with paraformaldehyde (4% in PBS pH 7.4) for 1 hrs and washed with PBS. Thereafter, the cells were smeared on Poly L-lysin coated chamber slide and air-dried. The slides were washed again and permeabilized with 0.1% Triton X-100 solution for 5 min. Positive control cells were treated with DNase 1. Cells were washed twice with PBS and labeled with reaction mixture and incubated at 37°C in the dark for 1 hrs. The cells were visualized by fluorescence microscope (BX53, Olympus, Japan). Quantification of the apoptotic index was calculated based on the ratio of TUNEL positive cells over the total number of cells in the microscopic field.

Cell cycle analysis

Parasites cells (1×10^6 cells) were treated with 0.025, 0.05 and 0.10 µg/ml for 24 hrs. The cell cycle analysis was performed using CycleTEST^{TM} PLUS DNA reagent kit BD (Becton Dickson) as described in the manufacturer's protocol. The cells were centrifuged at 300 g for 5 min). The pellet was washed with buffer solution twice. Thereafter, the pellet was added with 200 µl trypsin buffer (solution A) and incubated for 10 min at 37°C to allow the reaction. Then the pellet was further re-suspended in 200 µl solution B and incubated for 10 min at 37°C and stained with PI. Data acquisition was performed using flow cytometry (FACS Calibur, BD) at scan event 10 000 cells per sample and analyzed by ModFit LV.

Data analysis

Test samples were assayed in triplicate 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 comparisons test (GraphPad Prism 7.00 software).

RESULTS

Ochrolifuanine induces antitrypanosomal activity in *T. b. brucei*

The *in vitro* inhibitory effect of ochrolifuanine on *T. b. brucei* was evaluated using the Alamar Blue assay. For comparatives studies, staurosporine's *in vitro* antitrypanosomal effect was also evaluated. The findings revealed that ochrolifuanine and staurosporine reduced *T. b. brucei* growth when treated for 72 hours. The measured IC_{50} values for ochrolifuanine, staurosporine and control drug pentamidine against *T. b. brucei* are presented in Table 1.

Table 1. The IC_{50} values determined for test compound and standard drug pentamidine against *T.b. brucei* strain BS221

Test compound/ standard drug	IC_{50} value (µg/ml)
Ochrolifuanine	0.05 ± 0.01
Staurosporine	0.02 ± 0.07
Pentamidine	0.002± 0.08

Ochrolifuanine induces apoptosis in *T. b. brucei*

To investigate the possibility that ochrolifuanine induces *T. b. brucei* death through apoptosis, *T. b. brucei* were treated at various concentrations for 6, 12, and 24 hours, then labeled with TUNEL assay and fluorescence stained cells were observed under fluorescence microscopy. DNase I was used as a marker for positive control cells to indicate and validate DNA fragmentation in *T. b. brucei* which was demonstrated by the detection of green fluorescence signal. Staurosporine was used as a comparison for apoptosis study in trypanosomes. *Trypanosoma brucei brucei* treated with ochrolifuanine markedly increased DNA fragmentation in the nucleus and apoptotic body formation, observed as green fluorescent stained cells (Figure 1). Apoptosis was identified in cells as early as 6 hours after exposure to ochrolifuanine, even at the lowest concentration tested. When *T. b. brucei* was treated at a concentration of 0.05 µg/ml for 12 and 24 hours, apoptotic cells (appeared smaller and rounded shape (Figure 1C) compared to the non-treated/untreated cells (appeared in spindle shape). A similar pattern for DNA fragmentation also was observed on *T. b. brucei* treated with 0.02 µg/ml of staurosporine (Figure 1D). Both ochrolifuanine and staurosporine produced similar morphological features in response to cell apoptosis were comparable with positive control DNase I, but not untreated ones.

As shown in Figure 2, the number of apoptotic cells in *T. b. brucei* treated with ochrolifuanine were observed in a concentration- and time-dependent manner (Figure 2). After 6 hours of ochrolifuanine treatment, the percentage of TUNEL-positive cells increased considerably from 18 to 94 %. Apoptotic cells rose from 25 to 96 % in the treated cells after 12 hours and increased from 38 to 97 % at the longest incubation time (24 hours). When the concentration of ochrolifuanine was increased to 0.05 and 0.10 µg/ml, more than 50% of the cells were apoptotic. However, at the highest concentration (0.10 µg/ml) of ochrolifuanine, no significant increase of apoptotic cells was observed over time, probably because a maximal death rate was reached.

Staurosporine increased the number of apoptotic cells in *T. b. brucei* as the incubation time increased (Figure 3). Parasites cells treated with 0.02 µg/ml staurosporine demonstrated a significant increase in apoptotic cells from 61.0 to 72.8 %. Whereas, after 6 to 12 hours of incubation with 0.01 and 0.04 µg/ml staurosporine-treated cells, the percentage of apoptotic cells increased significantly from 20.5 to 26.2 % and 61.0 to 66.2 %, respectively, with no significant increase after 24 hours. Taken together, these findings revealed that ochrolifuanine induced apoptosis in a time-dependent manner but not at high concentrations (0.10 µg/ml), whereas staurosporine only induced apoptosis in a time-dependent manner at concentration 0.02 µg/ml. These findings suggested that ochrolifuanine could cause apoptotic cell death in *T. b. brucei* parasites.

Ochrolifuanine induces cell cycle arrest in *T. b. brucei*

To investigate the possibility that ochrolifuanine inhibited *T. b. brucei* growth through cell cycle arrest, cell cycle analysis was performed.

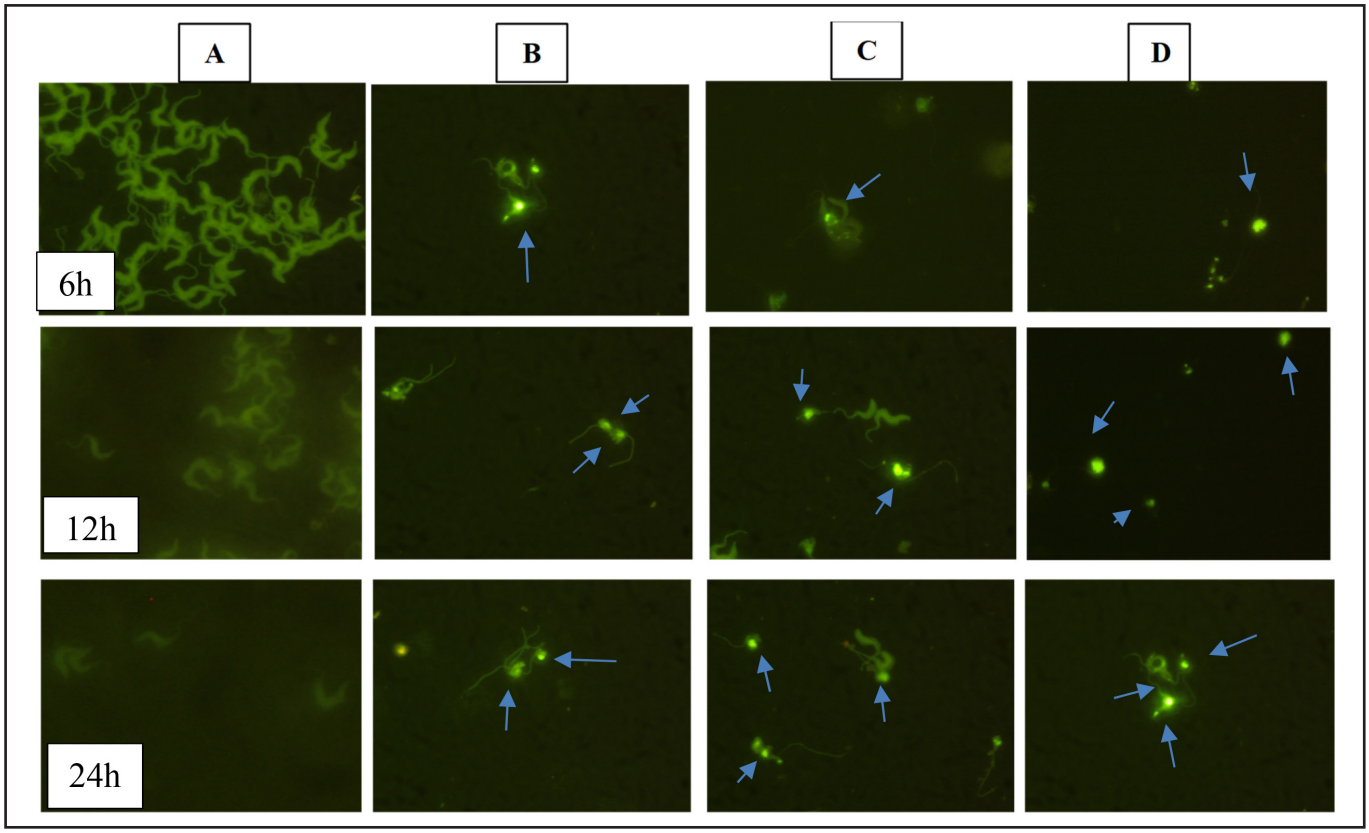
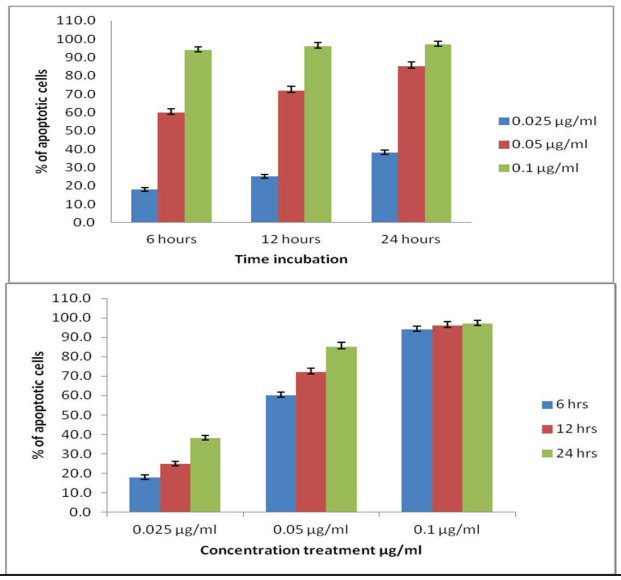
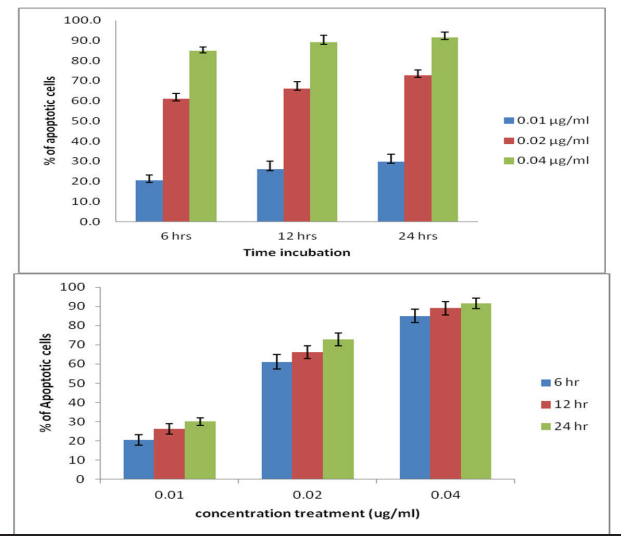


Figure 1. Apoptotic morphological changes of *T. b. brucei* strain BS221 induced by ochrolifuanine (Och) and staurosporine (STS). (A: untreated; B: DNase; C: Och; D: STS) Parasites were treated with 0.05 µg/ml Och and 0.02 µg/ml STS at 6, 12 and 24 hours incubations. The parasites morphology was observed under fluorescence microscope (400 and 1000 times magnification). Arrows showed DNA fragmentation by the green fluorescence nuclei.



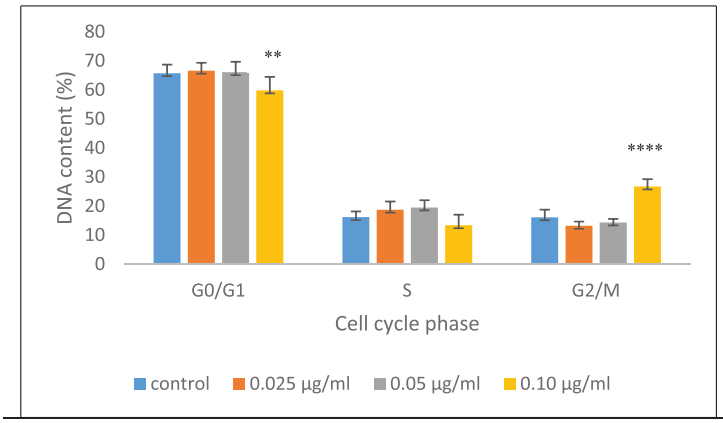
Ochrolifuanine concentration	% of apoptotic cells		
	6 hours	12 hours	24 hours
0.025 µg/ml	$18.0^{a, x}$	$25.0^{a, y}$	$38.0^{a, z}$
0.05 µg/ml	$60.0^{b, x}$	$72.0^{b, y}$	$85.0^{b, z}$
0.10 µg/ml	$94.0^{c, x}$	$96.0^{c, xy}$	$97.0^{c, yz}$

Figure 2. 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 represent significant differences ($p < 0.05$) within the groups while the $^{x, y, z}$ letter represents 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 3. 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 represent significant differences ($p < 0.05$) within the groups while the $^{x, y, z}$ letter represents significant differences ($p < 0.05$) between the groups as analyzed by two-way ANOVA, Tukey's multiple comparisons test.



	% of DNA content		
	G0/G1	**S**	**G2/M**
control	65.7	16.17	16.1
0.025 µg/ml	66.58	18.72	13.16
0.05 µg/ml	66.05	19.47	14.32
0.10 µg/ml	59.77	13.36	26.71

Figure 4. 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).

Our data revealed that treatment with 0.025 µg/ml ochrolifuanine resulted in 66.58 ± 2.74 % cellular DNA content in the G_0/G_1 phase, 18.72 ± 2.84 % in the S phase and 13.16 ± 1.49 % in the G_2/M phase (Figure 4). Treatment with 0.05 µg/ml ochrolifuanine showed that 66.05 ± 3.61 % of cells were in the G_0/G_1 phase, 19.47 ± 2.54 % in the S phase and 14.32 ± 1.25 % in the G_2/M phase. Treatment with 0.10 µg/ml showed that 59.77 ± 4.70 % of the cells were in the G_0/G_1 phase, 13.36 ± 3.65 % in the S phase and 26.71 ± 2.54 % in the G_2/M phase. The percentage of cells in the 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 %). Our data indicated that ochrolifuanine led to cell accumulation in the G_0/G_1 phase with a concomitant decrease in the number of cells in the S phase and G_2/M phases in a dose-dependent manner except for 0.10 µg/ml. These data indicated that the mechanism of ochrolifuanine in suppressing *T. b. brucei* viability is involved in arresting the cell cycle at the G_0/G_1 phase.

DISCUSSION

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 aminomethyl-benzoxaboroles have potent antitrypanosomal against *T. brucei.*

The isolated compound identified as ochrolifuanine from the bisindole alkaloid group from previous studies showed a 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 Koch *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 the root bark of *Aspidosperma excelsum* (Verpoorte *et al*., 1983).

Generally, the observed activity of ochrolifuanine from *D. costulata* may be due to the presence of alkaloids. 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 being developed as a possible new trypanocidal.

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 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 a 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 defense 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.

Morphological differences in nuclear are 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 recognized as one of the distinguishing characteristics for identifying 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 a 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 anti-parasitic agents need a good understanding of the complex interaction that 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 preclinical studies by establishing a dose-response relationship. The correlation between drug concentrations, time-course, therapeutic strength and adverse effects, are known as pharmacodynamics (Kang & Lee, 2009; White, 2013). To achieve the admired therapeutic effect, the safety and efficacy of any drug must be optimized to determine the administered dosage presents minimal adverse effects (White, 2013).

The results presented herein provide evidence that ochrolifuanine induced biochemical and morphological alterations in *T. b. brucei* leading to the parasite's death. Since, ochrolifuanine had been shown to cause DNA fragmentation, indicated by the appearance of apoptotic cells in *T. b. brucei*, further studies need to be performed to support these findings. In addition to a better understanding of the molecular mechanism of apoptosis in trypanosome parasites, a series of signaling pathways involved in apoptotic cell death needs to be investigated 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.

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*., 1999). 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 was involved in the inhibition of cell cycle progression at the G_2/M phase. Recently, clomipramine as an antidepressant drug has been described as a 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).

In the present study, treatment of 0.10 µg/ml of ochrolifuanine significantly ($p < 0.05$) arrested the 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 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 the parasite cell cycle can be exploited in the development of potential novel therapeutics.

CONCLUSION

The mechanisms of action of ochrolifuanine warrant further investigation because it was a potent apoptosis inducer and caused G2/M phase arrest in *T. b. brucei*. Exploration of unusual pathways and their novel regulators in trypanosomes will undoubtedly not only advance our understanding of the therapeutic target's mechanism but will also provide novel drug targets for trypanosomiasis disease treatment. 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, which 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 studies. In addition, the isomer of ochrolifuanine which has been reported in the past should be investigated in order to find compounds with a better cytotoxic/trypanocidal ratio.

ACKNOWLEDGEMENT

The authors wish to thank the Ministry of Science and Technology (MOSTI), Malaysia for financial assistance and support through the grant (09-05-IFN-BPH-003), Drugs for Neglected Diseases initiatives (DNDi), Swiss Tropical and Public Health Institute (Switzerland), Kitasato Institute for Life Sciences (Japan) and Institute of Pharmaceuticals and Nutraceuticals (IPharm, Malaysia) for guidance and assistance in the bioassay. The authors also thank late En Kamarudin Salleh (FRIM) for plant identification and staff at Biomolecules Research Lab, FRIM for technical assistance.

Conflict of interests

The authors declare that is no conflict of interest in this study.

REFERENCES

Baker, N., de Koning, H.P., Maser, P. & Horn, D. (2013). Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. *Trends in Parasitology* **29**: 110-118. https://doi.org/10.1016/j.pt.2012.12.005

Baltz, T., Baltz, D., Giroud, C. & Crockett, J. (1985). Cultivation in a semi-defined medium of animal infective forms of Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense, T. gambianse. *The EMBO Journal*, **4**, 1273-1277. https://doi.org/doi: 10.1002/j.1460-2075.1985.tb03772.x.

Barth, T., Bruges, G., Meiwes, A., Mogk, S., Mudogo, C.N. & Duszenko, M. (2014). Staurosporine-induced cell death in Trypanosoma brucei and the role of endonuclease G during apoptosis. *Open Journal of Apoptosis*, **3**, 16-31. https://doi.org/10.4236/ojapo.2014.32003

Brun, R., Blum, J., Chappuis, F. & Burri, C. (2010). Human African trypanosomiasis. *The Lancet* **375**: 148-159. https://doi.org/10.1016/S0140-6736(09)60829-1

Burri, C. (2010). Chemotherapy against human African trypanosomiasis: is there a road to success? *Parasitology* **137**: 1987-1994. https://doi.org/10.1017/S0031182010001137

Camacho, M.D.R., Phillipson, J.D., Croft, S.L., Yardley, V. & Solis, P.N. (2004). In vitro antiprotozoal and cytotoxic activities of some alkaloids, quinones, flavonoids and coumarins. *Planta Medica* **70**: 70-72. https://doi.org/10.1055/s-2004-815460

Debrabant, A., Lee, N., Bertholet, S., Duncan, R. & Nakhasi, H.L. (2003). Programmed cell death in trypanosomatids and other unicellular organism. *International Journal for Parasitology* **33**: 257-267. https://doi.org/10.1016/s0020-7519(03)00008-0

Duszenko, M., Figarella, K., Macleod, E.T. & Welburn, S.C. (2006). Death of trypanosome: a selfish altruism. *Trends in Parasitology* **22**: 536-542. https:// doi.org/10.1016/j.pt.2006.08.010

Fernandez-Presas, A.M., Tato, P., Becker, I., Solano, S., Kopitin, N., Berzunza, M., Willms, K., Hernandez, J. & Molinari, J.L. (2010). Specific antibodies induce apoptosis in *Trypanosoma cruzi* epimastigotes. *Parasitology Research* **106**: 1327-1337. https://doi.org/10.1007/s00436-010-1803-4

Figarella, K., Uzcategui, N.L., Beck, A., Schoenfeld, C., Kubata, B.K., Lang, F. & Duszenko, M. (2006). Prostaglandin-induced programmed cell death in *Trypanosoma brucei* involves oxidative stress. *Cell Death and Differentiation* **13**: 1802-1814. https:// doi.org/10.1038/sj.cdd.4401862

Frederich, M., Jaccquier, M.J., Thépenier, P., De Mol, P., Tits, M., Philippe, G., Delaude, C., Angenot, L. & Zèches-Hanrot, M. (2002). Antiplasmodial activity of alkaloids from various *Strychnos* species. *Journal of Natural Product* **65**: 1381-1386. https://doi.org/10.1021/np020070e

Hoet, S., Opperdoes, F., Brun, R. & Quetin-Leclercq, J. (2004). Natural products active against African trypanosomes: a step towards new drugs. *Natural Product Reports* **21**: 353-364. https://doi.org/10.1039/B311021B

Jimenez, V., Kemmerling, U., Paredes, R., Maya, J.D., Sosa, M.A. & Galanti, N. (2014). Natural sesquiterpene lactones induce programmed cell death in *Trypanosoma cruzi*: a new therapeutic target? *Phytomedicine* **21**: 1411-1418. https://doi.org/10.1016/j.phymed.2014.06.005

Kang, J.S. & Lee, M.H. (2009). Overview of therapeutic drug monitoring. *The Korean Journal of Internal Medicine* **24**: 1-10. https://doi.org/10.3904/kjim.2009.24.1.1

Koch, M.C., Plat, M.M., Preaux, N., Gottlieb, H.E., Hagaman, E.W., Schell, F.M. & Wenkert, E. (1975). Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances. XXXIII. The ochrolifuanines and emetine. *Journal of Organic Chemistry* **40**: 2836-2838. https://doi.org/10.1021/jo00907a030

Massiot, G., Thepenier, P., Jacquier, M.J., Men-Oliver, L.L. & Delaude, C. (1992). Alkaloids from roots of *Strychnos potatorum*. *Phytochemistry* **31**: 2873-2876. https://doi.org/10.1016/0031-9422(92)83650-N

Menna-Barreto, R.F.S. & Castro, S.L. (2015). Between armour and weapons-cell death mechanisms in trypanosomatid parasites. In T. M. Ntuli (Ed.), Biochemistry, genetics and molecular biology - cell death – autophagy, apoptosis and necrosis. (pp 1-10). InTech. https://doi.org/ 10.5772/61196

Mirand, C., Le Men-Oliver, L., Le Men, J. & Delaude, C. (1983). Alkaloids of *Dyera costulata*. *Phytochemistry* **22**: 577-579. https://doi.org/10.1016/0031-9422(83)83050-7

Mutomba, M.C. & Wang, C.C. (1996). Effects ofapihidicolin and hydroxyurea on the cell cycle and differentiation of *Trypanosoma cruzi* bloodstream forms. *Molecular and Biochemical Parasitology* **80**: 89-102. https://doi.org/10.1016/0166-6851(96)02675-8

Nguewa, P.A., Fuertes, M.A., Valladares, B., Alonso, C. & Perez, J.M. (2004). Programmed cell death in trypanosomatids: a way to maximize their biological fitness? *Trends in Parasitology* **20**: 375-380. https://doi.org/10.1016/j.pt.2004.05.006

Nnadi, C.O., Nwodo, N.J., Kaiser, M., Brun, R. & Schmidt, T.J. (2017). Steroid alkaloids from *Holarrhena africana* with strong activity *against Trypanosoma brucei rhodosiense. Molecules* **22**: 1129. https://doi.org/10.3390/molecules22071129

Norhayati, I., Getha, K., Haffiz J.M., Ilham, A.M., Sahira, H.L., Syarifah, M.S. & Syamil, A.M. (2013). *In vitro* antitrypanosomal activity of Malaysian plants. *Journal of Tropical Forest Science* **25**: 52-59.

Norhayati, I., Muhd Haffiz, J., Getha, K., Mohd Ilham, A., Nurhayati, Z. A., Lili Sahira, H., Muhd Syamil, A. & Mohd Hafidz Hadi, A. (2018). Bioassay-guided isolation of ochrolifuanine, a bisindole alkaloid from *Dyera costulata* active on *Trypanosoma brucei brucei*. *Journal of Tropical Forest Science* **30**: 560-569.

Otoguro, K., Ishiyama, A., Namatame, M., Nishihara,A., Furusawa, T., Masuma, R., Shiomi, K., Takashashi, Y., Yamada, H. & Omuro, S. (2008). Selective and potent *in vitro* antitrypanosomal activities of ten microbial metabolites. *The Journal of Antibiotics* **61**: 372-378. https://doi.org/10.1038/ja.2008.52

Pearson, T.W., Beecroft, R.P., Welburn, S.C., Ruepp, S., Roditi, I., Hwa, K.Y., Englund, P.T., Wells, C.W. & Murphy, N.B. (2000). The major cell surface glycoprotein procyclin is a receptor for induction of a novel form of cell death in African trypanosomes *in vitro*. *Molecular and Biochemical Parasitology* **111**: 333-349. https://doi.org/10.1016/S0166-6851(00)00327-3

Peube-Locou, N., Koch, M., Plat, M. & Potier, P. (1972). [Plants of New Caledonia. X. Alkaloids of the leaves of *Ochrosia lifuana* Guillaumin (Apocynaceae)]. *Annales Pharmaceutiques Francaises* **30**: 775-779.

Pimentel-Elardo, S.M., Kozytska, S., Bugni, T.S., Ireland, C.M., Moll, H. & Hentschel, U. (2010). Anti-parasitic compounds from *Streptomyces* sp. strain isolated from Mediterranean sponge. *Marine Drugs* **8**: 373-380. https://doi.org/10.3390/md8020373

Ploubidou, A., Robinson, D.R., Docherty, R.C., Ogbadoy, E.O. & Gull, K. (1999). Evidence for novel cell cycle checkpoints in trypanosomes: kinetoplast segregation and cytokinesis in the absence of mitosis. *Journal of Cell Science* **112**: 4641-4650. https://doi.org/10.1242/jcs.112.24.4641

Räz, B., Iten, M., Grether-bühler, Y., Kaminsky, R. & Brum, R. (1997). The Alamar Blue® assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. *Acta Tropica* **68**: 139-147. https://doi.org/10.1016/S0001-706X(97)00079-X

Reanmongkol, W., Subhadhirasakul, S., Pairat, C., Poungsawai, C. & Choochare, W. (2002). Antinociceptive activity of *Dyera costulata* extract in experimental animals. *Songklanakarin Journal of Science Technology* **24**: 227-234.

Rodrigues, J.H.D.S., Stein, J., Strauss, M., Rivola, H.W., Ueda-Nakamura, T., Nakamura, C.V. & Duszenko, M. (2016). Clomipramine kills *Trypanosoma brucei* by apoptosis. *International Journal of Medical Microbiology* **306**: 196-205. https://doi.org/10.1016/j.ijmm.2016.03.009

Simarro, P.P., Cecchi, G., Franco, J.R., Paone, M., Diarra, A., Ruiz-Postigo, J.A., Fevre, E.M. & Mattioli, R.C. & Jannin, J.G. (2012) Estimating and mapping the population at risk of sleeping sickness. *PLoS Neglected Tropical Diseases* **6**: e1859. https://doi.org/10.1371/journal.pntd.0001859

Subhadhirasakul, S., Jankeaw, B. & Malinee, A. (2003). Chemical constituents and antioxidative activity of extracts from *Dyera costulata* leaves. *Songklanakarin Journal of Sciences and Technology* **25**: 351-357.

Uzcategui, N.L., Carmona-Gutierrez, D., Derhinger, V., Schuenfeld, C., Lang, F., Figarella, K. & Duszenko, M. (2007). Antiproliferative effect of dihyroxyacetone on *Trypanosoma brucei* bloodstream forms: cell cycle progression, subcellular alterations and cell death. *Antimicrobial Agents and Chemotheraphy* **51**: 3960-3968. https://doi.org/10.1128/AAC.00423-07

Van Zandberge, G., Luder, C.G., Heussler, V. & Duszenko, M. (2010). Programmed cell death in unicellular parasites: a prerequisite for sustained infection? *Trends in Parasitology* **26**: 477-483. https://doi.org/10.1016/j.pt.2010.06.008

Verpoorte, R., Kos-Kuyck, E., Tsoi, T.A., Ruigrok, C.L., de Jong, G. & Baerheim Svendsen, A. (1983). Medicinal plants of surinma. III: Antimicrobially active alkaloids from *Aspidosperma excelsum*. *Planta Medica* **48**: 283-289. https://doi.org/10.1055/s-2007-969935

Welburn, S.C., Dale, C., Ellis, D., Beeccroft, R. & Pearson, T.W. (1996). Apoptosis in procyclic *Trypanosoma brucei rhodesianse in vitro*. *Cell Death and Diffrentiation* **3**: 229-236.

Welburn, S.C., Lillico, S. & Murphy N.B. (1999). Programmed cell death in procyclic form *Trypanosoma brucei rhodosiense* – Identification of differentially expressed genes during con A induced death. *Memorias do Instituto Aswaldo Cruz* **94**(2): 229-234. https://doi.org/ 10.1590/s0074-02761999000200020

White, N.J. (2013). Pharmacokinetic and pharmacodynamic considerations in antimalarial dose optimization. *Antimicrobial Agents and Chemotheraphy* **57**: 5792-5807. https://doi.org/10.1128/AAC.00287-13

White, N.J. (2017). Malaria parasite clearance. *Malaria Journal* **16**: 1-14. https://doi.org/10.1186/s12936-017-1731-1

WHO (World Health Organization) (2013). Trypanosomiasis, Human African (sleeping sickness). https://www.who.int//mediacentre/factsheets/fs259/en/. Accessed December 2013.

Wong, S.K., Lim, Y.Y., Noor Rain, A. & Fariza Juliana, N. (2011). Assessment of antiproliferative and antiplasmodial activities of five selected Apocynaceae species. *BMC Complementary and Alternative Medicine* **11**: 1472-6882. https://doi.org/10.1186/1472-6882-11-3

Zhang, N., Zoltner, M., Leung, K-F., Scullion, P., Hutchinson, S., Del Pino, R.C., Vincent, I.M., Zhang, Y-K., freund, Y.R., Alley M.R.K. *et al.* (2018). Host-parasite co-metabolic activation of antitrypanosomal aminomethyl-benzoxaboroles. *PLoS Pathogens* **14**: e1006850. https://doi.org/10.1371/journal.ppat.1006850