Review

Malaysian plants with potential *in vitro* trypanocidal activity

Abd. Latif Mohmod, Getha Krishnasamy and Mohd. Ilham Adenan*,**

Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor Darul Ehsan, Malaysia
*Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPharm), Ministry of Science, Technology and Innovation, 11700 Penang, Malaysia
**Universiti Teknologi MARA, 42300 Puncak Alam, Selangor Darul Ehsan, Malaysia

Abstract

Human African trypanosomiasis (HAT), caused by the protozoan parasite, *Trypanosoma brucei* and transmitted by the bite of tsetse flies, affects more than 60 million people in the sub-Saharan African countries. Without treatment, the disease can be fatal. Current treatment options for HAT are scarce, toxic, marginally effective, difficult to administer and compromised by the development of resistance, especially for the advanced second stage of the disease. Thus, new safe, effective, and affordable antitrypanosomal drug candidates are urgently needed. Numerous plant-derived natural products from different structural classes have been investigated for their trypanocidal activity. This review aims to provide updated information, based on published articles, on the antitrypanosomal activity of natural products from Malaysian plants investigated by researchers at the Forest Research Institute Malaysia (FRIM) and Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPharm). Extracts from a total of 1273 species of plants collected from different locations in Peninsular Malaysia were evaluated for *in vitro* growth inhibitory activity against *Trypanosoma brucei brucei* (strain BS221) and *T. b. rhodesiense* (strain STIB 900), using assays established at the respective institutes. Several plants that have demonstrated promising trypanocidal effects will be discussed using examples of other plant-derived antitrypanosomal natural products reported in the literature to compare their activity and chemical properties.

Key words: Sleeping sickness, antitrypanosomal agents, natural products, essential oils, sesquiterpene lactones, alkaloids

1. Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is caused by two morphologically identical protozoan parasites from the genus *Trypanosoma*, and is a major cause of morbidity and mortality in sub-Saharan Africa. In West Africa, HAT is characterized by a slow chronic disease caused by *Trypanosoma brucei gambiense* and the East African HAT is an acute form of the disease caused by *T. b. rhodesiense*. *Trypanosoma brucei brucei*, responsible for the cattle disease nagana, is closely related to *T. b. rhodesiense* and *T. b. gambiense* (Hoet *et al*., 2004). Infection is transmitted by the bloodsucking male and female tsetse flies (*Glossina* spp.). Wild animals and cattle are important reservoir hosts for *T. b. rhodensiense*, while humans are the main reservoir for *T. b. gambiense*. In the first stage of the disease, trypanosomes enter the bloodstream and multiply there. Often characterized by non-specific clinical symptoms for weeks or months during the first stage of HAT, the infection eventually crosses into the central nervous system (CNS) and brain bringing to the second stage

infection where the parasite is present in cerebrospinal fluid (Torreele *et al*., 2010). Without treatment, the second stage of this disease could cause mental debilitation in infected patients, leading to chronic meningo-encephalitis and encephalopathy, and eventually death (Hoet *et al*., 2004).

The rate of re-emergence and the need for intervention against HAT have led to its classification as a category1 disease by the WHO (Abdel-Sattar *et al*., 2009). It is estimated that about 30,000 cases of HAT infection occur per year (Brun *et al*., 2011). This number can increase tremendously over the years since the disease had previously shown high resurgence (Gilbert, 2014). One possible contributing factor to this trend is changes in weather patterns and climatic conditions which are known to substantially affect the risks of vector-borne diseases transmitted by arthropod species (Patz *et al*., 2005). Warming of the environment within the viable range of these vectors can increase their reproduction rate and number of blood meals, prolong their breeding season and shorten the maturation period for the microbes they transmit, thus enhancing the chances for disease transmission (McMichael *et al*., 2006). Therefore, there is always a risk that HAT prevalence can be reversed and the disease can become resurgent.

Control of trypanosomiasis in human usually relies upon treatment of patients with trypanocidal drugs. Currently, there is no single oral treatment for both the early and late stages of HAT. Available

Author for correspondence: Dr. Abd. Latif Mohmod
Director General, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor Darul Ehsan, Malaysia
E-mail: latif@frim.gov.my
Tel.: +603-62797007, **Fax:** +603-62804624, **HP:** +60192765010

trypanocidal drugs are hampered by severe side effects, requirement of lengthy parenteral administration in resource-limited settings, lack of efficacy, and treatment failure due to resistance of the parasite (Abdel-Sattar *et al.*, 2009). Additionally, prior to being treated, the stage of the disease is determined using a painful diagnostic step of spinal tap on a patient to extract cerebrospinal fluid to determine the treatment. Ideally, a better control of this disease will be to find new treatment options that are safe and effective in both stages of HAT, as well as simplified diagnosis, treatment and patient-management.

1.1 Chemotherapeutic treatment of HAT and current problems

The four drugs in use against HAT, three of which developed more than 50 years ago, are suramin, pentamidine, melarsoprol and eflornithine (Figure 1). Pentamidine, an aromatic diamidine, and suramin, a polysulphonated naphthylamine, are effective agents against early-stage *T. b. gambiense* and *T. b. rhodesiense* infection, respectively (Table 1). These drugs are not effective in the second stage of infection (Bacchi, 2009). Only melarsoprol and eflornithine are used for the second stage treatment due to their ability to cross the blood-brain barrier (Otoguro *et al.*, 2008). However, treatment with melarsoprol, an arsenical derivative, causes severe adverse reactions such as reactive encephalopathy (Gilbert, 2014). In many regions, eflornithine has replaced melarsoprol as the first-line treatment option. Complicated drug dosing regimens, however, slowed the widespread implementation of eflornithine monotherapy. Furthermore, the mechanisms of action of all these compounds remain poorly understood except for eflornithine, which selectively inhibits ornithine decarboxylase in the parasite (Barrett *et al.*, 2011). Meanwhile, there has been an upsurge in the number of patients failing to respond to melarsoprol because of the occurrence of drug resistance (Abdel-Sattar *et al.*, 2009). Additionally, reports on the occurrence of changes to, or a loss of, eflornithine transport into parasite cells indicated that genes capable of conferring resistance to this drug is in circulation (Barrett *et al.*, 2011).

Development of new drugs against HAT has been slow over the last three decades. This is mainly due to lack of interest by the pharmaceutical industry to invest into research and development of drugs for neglected diseases such as HAT (Torreele *et al.*, 2010). Drug discovery efforts, however, have been boosted by public-private partnerships such as the Drugs for Neglected Diseases initiative (DNDi) founded by the humanitarian organization 'Médecins Sans Frontières' along with public research institutions in India, Kenya, Brazil, France and Malaysia. Through its drug discovery platform partnered with various R&D institutions and pharmaceutical companies, DNDi's initiatives led to the development of nifurtimox-eflornithine combination therapy (NECT) for second stage *T. b. gambiense* infection using the oral drug nifurtimox which was used to treat *T. cruzi* (Chang and Ioset, 2011). Although the combination therapy is considered to be safer, easier to administer, affordable and more effective than treatment with eflornithine alone, the need for intravenous administration during treatment is still a limitation (Torreele *et al.*, 2010; Table 1).
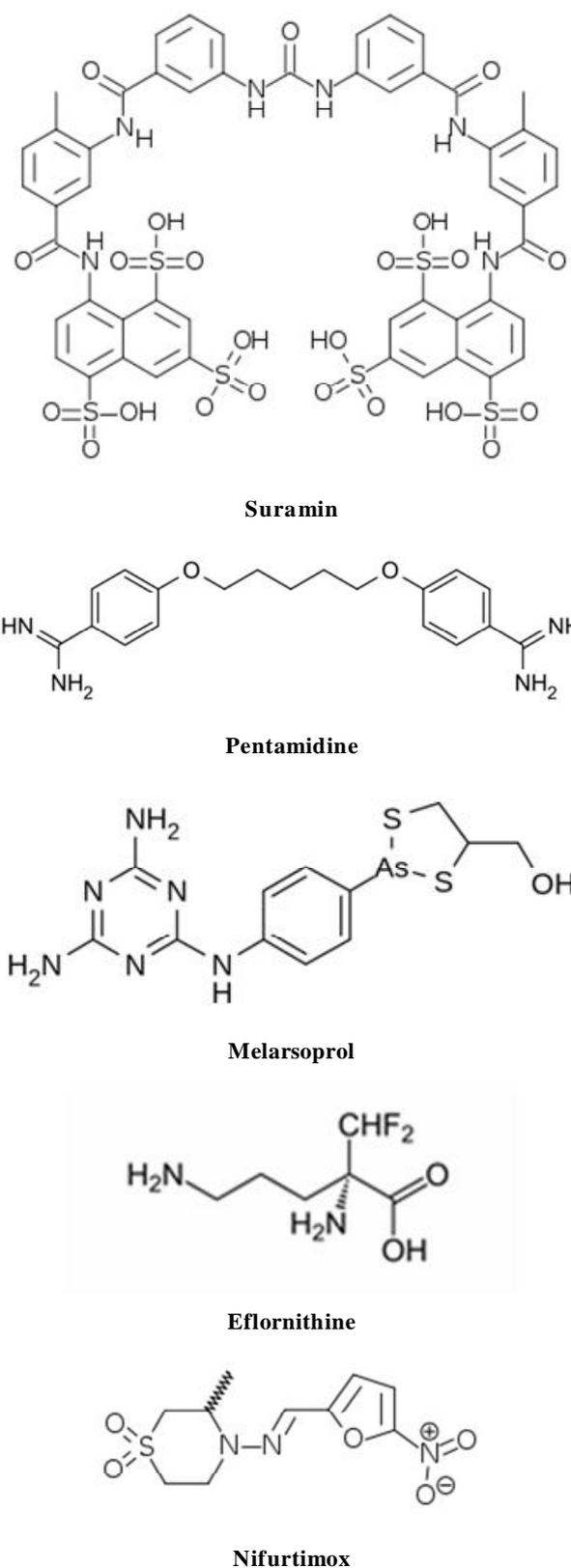

Figure 1: Structures of current antitrypanosomal drugs (Otoguro *et al.*, 2008)

Table 1: Approved trypanocidal drugs to treat first stage and second stage HAT

Trypanocidal drugs (year)	Trade name(s)	Route	Therapeutic uses	Current problems (Torreele *et al.*, 2010)
Suramin (1920's)	Naganol	IV	Stage 1 (*T.b.r*)	Not efficacious for stage 2
Pentamidine (1940)	Nebupent Pentacrinat	IM	Stage 1 (*T.b.g*)	Not efficacious for stage 2
Melarsoprol (1949)	Arsobal	IV	Stage 2 (*T.b.r* & *T.b.g*)	Ten painful daily IV injections; highly toxic; about 5% treatment related mortality; increasing number of treatment failures
Eflornithine (1981)	Ornidyl	IV	Stage 2 (*T.b.g*)	Difficult administration; only for *T. b. gambiense* stage 2 HAT
Nifurtimox-eflornithine combination therapy (2009)	NECT	Combination of O & IV	Stage 2 (*T.b.g*)	Simplified regimen; reduced toxicity and treatment duration; but not applicable to *T. b. rhodesiense* infection

IV: intravenous; IM: intramuscular; O: oral; *T.b.r*: *Trypanosoma brucei rhodesiense*; *T.b.g*: *T. b. gambiense*

The rise in drug resistance, coupled with toxicity of drugs available to treat HAT, underscores the need to discover new antitrypanosomal drug leads that are not susceptible to the same resistance mechanisms. Over the recent years, lead compounds showing promising activity against the HAT parasites have been identified for clinical trials. Namely, fexinadazole, the nitroimidazole compound which showed strong *in vitro* and *in vivo* trypanocidal activity against *T. b. rhodesiense* STIB900 and *T. b. gambiense* STIB930 with no non-specific cytotoxicity. The compound has potentials to be an effective oral treatment for both *T. brucei* strains and both stages of the disease (Kaiser *et al.*, 2011). Optimization of a series of antiparasitic benzoxaboroles active against *T. brucei* has identified the novel compound SCYX-7158 as another potential drug candidate for stage 2 HAT (Jacobs *et al.*, 2011). Despite having these lead compounds in the pipeline, limited availability and affordability of pharmaceutical medicines for HAT further emphasizes the continuous need to search for new molecules with potent and selective trypanocidal activity, and novel mechanism of action from a more comprehensive, formidable and cheaper source such as the natural products (Gilbert, 2014).

1.2 Antitrypanosomal agents from natural products

In the past, the use of microorganisms, marine organisms and plants as natural sources of novel antiprotozoal compounds for the treatment of parasitic diseases has been well documented. These compounds affect various biological targets such as protein synthesis, energy metabolism, lipid metabolism, neurotransmitters, and cellular integrity, and have selectivity against susceptible parasites (Shiomi and Omura, 2004). Thus, search for the much-needed antitrypanosomal lead compounds from untapped natural resources promises interesting discoveries, though challenging, for researchers worldwide (Hoet *et al.*, 2004). Sepulveda-Boza and Cassels (1996) suggested that many natural products exhibit their trypanocidal activity through interference with redox balance of the parasites, acting either on the respiratory chain or on the cellular defences against oxidative stress. This is because natural products possess bioactive principles capable of generating radicals that may cause peroxidative damage to the parasite enzyme trypanothione reductase which is very sensitive to alterations in redox balance. It is also known that some agents act by binding with the kinetoplast DNA of the parasite (Atawodi *et al.*, 2003).

In most reported studies, the antitrypanosomal natural products were first selected based on their *in vitro* activity on the bloodstream forms of trypanosomes expressed in IC_{50} values (concentration that caused 50% inhibition in parasite growth). Additionally, the active compounds were also tested for *in vitro* cytotoxicity to mammalian cells to determine their selectivity towards the parasite. The selectivity index (SI) was calculated based on ratio of the IC_{50} value obtained for mammalian cells divided by the IC_{50} on trypanosomes. The higher the SI value of a given extract or compound, the higher is its selective antitrypanosomal activity and safer to mammalian cells (Otoguro *et al.*, 2008). According to Hoet *et al.* (2004), active compounds that are relatively selective with SI values of more than 20 are worth investigating in animal models for their *in vivo* trypanocidal activity.

1.2.1 *Antitrypanosomal natural products from plants*

Plants have been used for ages to treat human disorders and diseases by the people in developing countries, in particular the rural population. It is estimated by the World Health Organization (WHO) that 80% of the world population still relies on plant-based traditional remedies because of limited availability or affordability of pharmaceutical medicines (Chang and Rasadah, 2004). Plant-derived antiprotozoals such as the quinoline alkaloid quinine from the bark of *Cinchona* sp., and the sesquiterpene lactone compound artemisinin from *Artemisia annua*, are well-established examples of lead compounds used to develop antiprotozoal drugs (Kayser *et al.*, 2003). Therefore, it is not surprising that many natural products of plant origin displayed potential trypanocidal and trypanostatic activities (Bala *et al.*, 2011; Mesia *et al.*, 2008).

Various natural compounds from plants, mainly the alkaloids, phenolic derivatives, quinones and terpenes, with potential antitrypanosomal activities on *T. brucei* subsp., *T. congolense* and *T.vivax* have been illustrated extensively by Hoet *et al.* (2004). Based on reports from the literature, phytochemicals from a wide array of plant species were shown to exhibit strong *in vitro* antitrypanosomal activity with IC_{50} values less than 10 µg/ml and a range of selectivity towards the parasites. The examples summarized in Table 2 are intended to be representative, but not exhaustive, of the diversity of plants active on the trypanosomes responsible for sleeping sickness.

Table 2: *In vitro* antitrypanosomal activity of methanolic extracts of different plants and their selectivity to *Trypanosoma brucei* subspecies based on cytotoxicity on different human cell lines

Plant species	Family	Parts used	Antitrypanosomal IC$_{50}$ (µg/ml)	SI	Reference
Albizia gummifera C. A. Smith	Mimosaceae	RB	0.2 [x]	2.9 [a]	Freiburghaus
Ehretia amoena Klotsch.	Boraginaceae	SB	9.6 [x]	7.0 [a]	*et al.*, 1996
Entada abyssinica Stud. Ex. A. Rich.	Fabaceae	R	3.3 [x]	0.8 [a]	
		SB	1.3 [x]	3.8 [a]	
Securinega virosa Baill.	Euphorbiaceae	R	5.9 [x]	0.8 [a]	
Echolium viride (Forsk.) Alston	Acanthaceae	L	9.37 [y]	6.4 [b]	Abdel-Sattar
Adenium obesum (Forssk.) Roem & Schult	Apocynaceae	L	9.30 [y]	0.01 [b]	*et al.*, 2009
Periploca somaliensis Browicz	Asclepiadaceae	L	7.10 [y]	6.4 [b]	
Achillea biebersteinii Afan.	Asteraceae	L	6.45 [y]	9.5 [b]	
Kleinia odora	Asteraceae	L	7.03 [y]	1.9 [b]	
Psiadia punctulata DC.	Asteraceae	L	7.49 [y]	6.5 [b]	
Vernonia schimperi	Asteraceae	L	7.06 [y]	0.6 [b]	
Echium arabicum R. Mill	Boraginaceae	L	7.00 [y]	9.1 [b]	
Heliotropium zeylanicum (Burm.f.) Lam	Boraginaceae	L	4.60 [y]	2.8 [b]	
Trichodesma trichodesmoides var. *tormentosum*	Boraginaceae	L	7.05 [y]	14.1 [b]	
Cleome paradoxa DC.	Capparaceae	L	7.00 [y]	8.9 [b]	
Cleome ramosissima Webb ex Par	Capparaceae	L	7.03 [y]	13.5 [b]	
Chenopodium schraderianum Schult.	Chenopodiaceae	L	7.03 [y]	11.7 [b]	
Dipterygium glaucum Decne	Cruciferae	L	7.05 [y]	> 14.2 [b]	
Cucumis prophetarum L.	Cucurbitaceae	L	7.03 [y]	2.1 [b]	
Chrozophora oblongifolia	Euphorbiaceae	L	7.78 [y]	> 12.9 [b]	
Euphorbia schimperiana	Euphorbiaceae	L	7.10 [y]	1.8 [b]	
Ricinus communis	Euphorbiaceae	L	7.04 [y]	> 14.2 [b]	
Crotalaria emarginella	Fabaceae	L	6.88 [y]	> 14.5 [b]	
Indigofera spinosa Forssk.	Fabaceae	L	7.70 [y]	8.3 [b]	
Tephrosia nubica (Boiss.) Bak.	Fabaceae	L	7.07 [y]	6.8 [b]	
Lanvandula dentate L.	Lamiaceae	L	7.06 [y]	> 14.2 [b]	
Lanvandula pubescens Decne	Lamiaceae	L	7.08 [y]	> 14.1 [b]	
Marrubium vulgare	Lamiaceae	L	7.03 [y]	8.4 [b]	
Teucrium yemense Defl.	Lamiaceae	L	7.10 [y]	14.2 [b]	
Psidium guajava L.	Myrtaceae	L	7.00 [y]	8.1 [b]	
Olea europaea L. subsp. *africana* (Burm. F.) Green	Oleaceae	L	7.20 [y]	9.8 [b]	
Lycium shawii Roem. and Schult.	Solanaceae	L	7.20 [y]	10.2 [b]	
Solanum incanum L.	Solanaceae	L	7.00 [y]	10.2 [b]	
Solanum schimperianum Hochst. ex. A.Rich.	Solanaceae	L	0.061 [y]	898.9 [b]	
Triumfetta flavescens Hochst. ex. A.Rich.	Tiliaceae	L	7.20 [y]	8.4 [b]	
Cissus quadrangularis L.	Vitacceae	L	8.30 [y]	8.9 [b]	
		L	2.5 [x]	12.0 [c]	Malebo *et al.*,
Annickia kummeriae	Annonaceae	SB	2.5 [x]	21.5 [c]	2009
		RB	2.3 [x]	26.7 [c]	
Caralluma penicillata (Deflers) N.E.Br.	Asclepiadaceae	L	8.50 [y]	> 7.6 [b]	Mothana *et al.*,
Hypoestes forskalei (Vahl) R.Br.	Acanthaceae	L	8.10 [y]	> 1.4 [b]	2014
Leucas virgate	Labiatae	L, T	8.30 [y]	> 7.7 [b]	
Loranthus regularis Steud. ex Sprague	Loranthaceae	R	9.50 [y]	4.3 [b]	
Verbascum bottae (Deflers) Huber-Mor.	Scrophulariaceae	L, F	2.30 [y]	14.1 [b]	

L: leaves; T: fruits, R: roots or rhizomes; F: flowers; RB: root bark; SB: stem bark; [x] *T. brucei rhodesiense*; [y] *T. b. brucei;* SI: Selectivity Index (IC$_{50}$ Cytotoxicity/IC$_{50}$ Antitrypanosomal); [a] WI-38 (human fetal lung cells); [b] MRC-5 (human diploid embryonic cells); [c] L-6 (rat skeletal myoblast cells).

Other examples of plants exhibiting strong trypanocidal activity was reported by Atawodi *et al.* (2003) who tested methanolic extracts of 23 Nigerian savannah plants for *in vitro* activity against *T. b. brucei* and *T. congolense*. Extracts of *Securidaca longepedunculata* and *Terminalia avicennioides* caused complete cessation in motility of both parasites after 30 - 55 min of incubation at the lowest extract concentration of 0.4 mg/ml. Wurochekke and Nok (2004) screened a total of 13 medicinal plants for activity against *T. b. brucei* and observed that aqueous extract of the bark of *Khaya senegalensis* exhibited the highest activity. Whereas the methanolic leaf extract of *Hypoestes pubescens* was reported to exhibit strong *in vitro* trypanocidal activity with selectivity or specific efficacy against *T. b. brucei* (IC_{50} 2.0 µg/ml; SI 16.3; Mothana *et al.*, 2012). Their results also showed that although extracts from *Ballochia atrovirgata* and *Euphorbia socotrana* displayed high antitrypanosomal activity (IC_{50} 1.9 - 2.1 µg/ml), noticeable cytotoxicity showed the activities being non-specific and hence not considered for further evaluations. Various medicinal plants with strong *in vitro* antitrypanosomal activity reviewed by Mbaya and Ibrahim (2011) and Ibrahim *et al.* (2014) further confirmed the importance of plants as potential sources of trypanocidal compounds.

1.3 Evaluation of Malaysian plants for *in vitro* antitrypanosomal activity

Malaysia, known for its rich mega-diversity, is reported to have around 15,000 species of vascular plants. Medicinal plants play a unique part in this mega-biodiversity, and one of the earliest records by Burkill (1935) also reported on the high number of Malaysian plants used in traditional medicine. Thus, the tropical rain forest plants are sources of chemically diverse phytochemicals with potential to be lead compounds in drug design and synthesis (Ibrahim, 2004; Noor Rain *et al.*, 2007). In the search for potential antitrypanosomal active compounds, a collaborative study was carried out by the Forest Research Institute Malaysia (FRIM) and Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPharm) to screen natural products from Malaysian plants and soil microorganisms using a cell-based assay approach that has been proven effective for discovering potential inhibitors with high selectivity to *T. brucei*. The study was mainly aimed at establishing a natural substances library to aid in antitrypanosomal drug discovery. This review is hoped to be an updated source on the progress achieved so far in the investigation of trypanocidal activity of the different Malaysian plant species, and also to be relevant to researchers to carry out *in vivo* studies in future using the active antitrypanosomal extracts or compounds to understand their toxicity and efficacy profiles.

Antitrypanosomal screenings were initiated at FRIM and IPharm through technology transfer activities carried out in collaboration with the Drugs for Neglected Diseases initiative (DNDi). Using screening protocols developed by DNDi partner institutes, *in vitro* assays to determine the antitrypanosomal activities of extracts against *T. b. brucei* strain BS221 was optimized and validated at FRIM, and against *T. b. rhodesience* strain STIB 900 was established at IPharm. The *Trypanosoma* strains were grown in supplemented standard media according to Baltz *et al.* (1985) and the *in vitro* antitrypanosomal activity was evaluated by culturing standard cell density of the parasites in serial dilutions of extract samples in 96-well microtitre plate for 72 h. at 37°C under a 5% CO_2 atmosphere.

The standard drug pentamidine was used as a positive control. Estimation of the percent surviving trypanosomes in extract-treated cultures was done colorimetrically using the fluorochrome Alamar blue dye and dose-response curve generated from the Alamar blue assay was used to calculate IC_{50} values according to Raz *et al.* (1997). The resulting antitrypanosomal activity was scored into three categories: Score 1 (weak activity; $IC_{50} > 12.5$ µg/ml), Score 2 (moderate activity; $1.56 < IC_{50} \leq 12.5$ µg/ml) and Score 3 (strong activity; $IC_{50} \leq 1.56$ µg/ml) according to the methods of Lili Sahira *et al.* (2011).

One major problem in many antiparasitic natural products is the high cytotoxicity and accordingly low selectivity towards the parasites (Kayser *et al.*, 2003). Thus, the priority was to select active extracts displaying strong antitrypanosomal activity and high selectivity towards the parasite. This was done by testing extracts which showed strong and moderately active antitrypanosomal activity for cytotoxicity in rat skeletal myoblast (L-6) or monkey kidney epithelial (Vero) cells using Alamar blue assay to determine the cell viability. Based on the cytotoxicity results, calculation of selectivity index (SI) value was done to select extracts with low cytotoxicity and high selectivity to the trypanosome parasites (Zuriati *et al.*, 2014). Screening results of some of the plants showing strong and moderate antitrypanosomal activity with good selectivity will be reviewed here.

1.4 Antitrypanosomal activity and selectivity of some Malaysian plants

A total of 1273 plants were collected in the course of the study from identified sites in various states throughout Peninsular Malaysia. Some of these locations include Kepong in the state of Selangor; Sungai Menyala Forest Reserve, Port Dickson and Berembun Virgin Forest Reserve, Jelebu in the state of Negeri Sembilan; Cameron Highlands Forest Reserve and Lanchang Forest Reserve in Pahang; Royal Belum Rainforest, Grik in Perak; and Gunung Jerai Forest in Kedah (Mohd Ilham *et al.*, 2013). Taxonomic identities of the collected plants were confirmed by a FRIM botanist and the voucher specimens were deposited at the herbarium in FRIM and IPharm. Different parts of the plants were collected and extracted with methanol for the *in vitro* antitrypanosomal and cytotoxicity assays.

Screening of these plants resulted in 10 extracts exhibiting strong and selective *in vitro* trypanocidal activity with IC_{50} values below 1.56 µg/ml (Score 3) against *T. b. brucei* BS221 or *T. b. rhodesience* STIB 900. While 15 plant extracts were moderately active against the parasites (Score 2; $1.56 < IC_{50} \leq 12.5$ µg/ml), with a SI value of more than 20 as illustrated in Table 3. Out of the 10 potential antitrypanosomal plants, three species of particular interest were *Cymbopogon nardus*, *Elephantopus scaber* and *Dyera costulata* which showed strong activity and also very high selectivity to the parasites. Interestingly, three out of the 10 strongly active plants (*Baccaurea parviflora*, *Antidesma tomentosum* and *Aporosa aurea*) belonged to the family of Euphorbiaceae. Moreover, *Mallotus paniculatus* and *Croton argyratus*, two plants which showed moderate activity against *T. b. brucei* BS221, were also from the same family suggesting that more antitrypanosomal active plants may be found from this family (Table 3). This observation supported findings of Noor Rain *et al.* (2007) who showed that plants from the Euphorbiaceae family were most commonly

reported to demonstrate strong antiprotozoal activity. Their study showed that *C. argyratus* leaf extracts exhibited very strong activity against *Plasmodium falciparum* with $IC_{50} < 0.03$ µg/ml. Similarly, *C. gratissimus*, also from this family, was reported to demonstrate good antiplasmodial activity (Noor Rain *et al.*, 2007). Therefore, evaluation of targeted plant families known for their potential antiprotozoal activity as candidates for antitrypanosomal screening may prove advantageous.

Table 3: *In vitro* antitrypanosomal activity of methanolic extracts of different plant species that exhibited Score 3 ($IC_{50} \leq 1.56$ µg/ml) and Score 2 ($1.56 < IC_{50} \leq 12.5$ µg/ml) activities against the parasites

Plant species	Family	Parts used	IC$_{50}$ (µg/ml)*		SI
			Antitrypanosomal	Cytotoxicity	
Cymbopogon nardus	Poaceae	W P	0.31 [x]	> 100.00 [a]	> 323
Elephantopus scaber	Asteraceae	L	0.22 [y]	45.00 [b]	205
Dyera costulata	Apocynaceae	L	0.58 [x]	> 100.00 [a]	> 172
Reinwardtiodendron cinereum	Meliaceae	L	< 0.20 [y]	31.64 [b]	> 158
Baccaurea parviflora	Euphorbiaceae	F	0.38 [y]	59.09 [b]	156
Thottea corymbosa	Aristolochiae	L	0.72 [y]	> 90.00 [b]	> 125
Antidesma tomentosum	Euphorbiaceae	L	0.42 [y]	52.68 [b]	125
Archidendron clyperia	Fabacea	L	0.41 [y]	47.23 [b]	115
Aporosa aurea	Euphorbiaceae	L	0.77 [y]	81.44 [b]	106
Rinorea anguifera	Violaceae	L	< 0.20 [y]	19.00 [b]	> 95
Diplazium esculentum	Dryopteridaceae	L	4.32 [x]	> 100.00 [a]	> 23
Hibiscus rosa sinensis	Malvaceae	L	4.34 [x]	> 100.00 [a]	> 23
Malastoma malabathricum	Melastomataceae	L	4.40 [x]	> 100.00 [a]	> 23
Cymbopogon citratus	Poaceae	R	4.44 [x]	> 100.00 [a]	> 23
Murraya koenigii	Rutaceae	L	4.38 [x]	> 100.00 [a]	> 23
Aglaia exstipulate	Meliaceae	L	2.70 [x]	60.79 [a]	23
		SB	> 12.5 [x]	ND	ND
Clidemia hirta	Melastomataceae	L	4.41 [x]	99.31 [a]	23
Blumea balsamifera	Compositae	L	4.62 [x]	> 100.00 [a]	> 22
Xylopia malayana	Annonaceae	L	4.71 [x]	> 100.00 [a]	> 21
Xylopia ferruginea	Annonaceae	L	4.81 [x]	> 100.00 [a]	> 21
Parkia speciose	Fabaceae	F	4.77 [x]	> 100.00 [a]	> 21
Cleome gynandra	Capparaceae	L	4.93 [x]	> 100.00 [a]	> 20
Alseodaphne peduncularis	Lauraceae	L	5.04 [x]	> 100.00 [a]	> 20
		SB	> 12.5 [x]	ND	ND
Cinnamomum iners	Lauraceae	L	5.02 [x]	> 100.00 [a]	> 20
Murraya paniculata	Rutaceae	L	5.10 [x]	> 100.00 [a]	> 20
Polyalthia cauliflora	Annonaceae	L	5.40 [x]	> 100.00 [a]	> 19
Mallotus paniculatus	Euphorbiaceae	L	5.14 [x]	95.59 [a]	18
Piper betle	Piperaceae	L	4.61 [x]	76.24 [a]	17
Baeckea frutescens	Myrtaceae	L	3.94 [x]	63.89 [a]	16
Algaia sp.	Meliaceae	L	4.75 [x]	59.17 [a]	12
		SB	> 12.5 [x]	ND	ND
Croton argyratus	Euphorbiaceae	L	4.85 [x]	54.85 [a]	11
		SB	> 12.5 [x]	ND	ND
Lithocarpus ewyckii	Fagaceae	L	5.36 [x]	54.16 [a]	10
Andrographis paniculata	Acanthaceae	L	4.71 [x]	37.68 [a]	8
Litsea machilifolia	Lauraceae	L	5.13 [x]	18.97 [a]	4
Alpina galangal	Zingiberaceae	R	5.22 [x]	18.19 [a]	3
Curcuma longa	Zingiberaceae	R	5.48 [x]	11.71 [a]	2

L: leaves; F: fruits, R: rhizomes; SB: stem bark; WP: whole plant; [x] *T. brucei brucei* BS221; [y] *T. b. rhodesiense* STIB 900; [a] Vero cells; [b] L-6 cells; SI: Selectivity Index (IC_{50} Cytotoxicity/IC_{50} Antitrypanosomal).

*IC_{50} data reported in Norhayati *et al.* (2013), Mohd Ilham *et al.* (2013) and Zuriati *et al.* (2014).

All of the plant extracts screened in this study were evaluated for *in vitro* activities against either *T. b. brucei* or *T. b. rhodesiense*, based on the investigating teams. None of the plants were tested against both subspecies of the parasite. Two out of the 10 strong trypanocidal plants, namely *C. nardus* and *D. costulata,* were active against *T. b. brucei* and the rest were active against *T. b. rhodesiense*. There is a possibility that the plants that showed potential activity against one subspecies may also have activity against the other subspecies of the parasite. However, Wurochekke and Nok (2004) reported that plant extracts that did not show activity to one *T. brucei* subspecies may have activity against other subspecies of the parasite. Similarly, Iten *et al.* (1995) reported that *T. b. gambiense* and *T. b. rhodesiense* showed different susceptibilities towards the commercial drug eflornithine used in HAT treatment. In addition, the observation that some plant species which showed strong trypanocidal activity to *T. b. brucei* but only weak activity to *T. congolense* suggested that species-dependent factors may play a role in susceptibility (Atawodi *et al.*, 2003). Therefore, the 10 active extracts from this study should be tested for trypanocidal activity on both subspecies to reveal their true potential as agents against the different HAT parasites.

Studies have shown that different parts of the same plant could exhibit varying levels of antitrypanosomal activity. This is consistent with the findings of this study where methanol extract of some plants were inactive against *T. b. brucei* (IC_{50} > 12.5 μg/ml), but extracts from other parts of the same species have been reported elsewhere as active against the parasite. For example, methanolic leaf extracts of *Aloe vera* and *Allium sativum* screened for *in vitro* trypanocidal activity were not active against *T. b. brucei* BS221 in this study. In addition, the leaf extract of *Azadirachta indica* was also not active against *T. b. brucei* BS221 (Norhayati *et al.*, 2013). However, Mbaya and Ibrahim (2011) reported that extracts of the pulp from *A. vera* and *A. sativum* exhibited strong trypanocidal activity against *T. b. brucei*. Similarly, the stem bark methanolic extract of *A. indica* was also reported to show remarkable *in vitro* trypanocidal effect on *T. b. brucei*. Antia *et al.* (2009) recorded similar observations when root bark and leaf extracts of *Afzelia africana* caused complete cessation of *T. b. brucei* motility at concentrations of 6.3 and 3.1 mg/ml, respectively while the stem bark extract was not active at all. These findings corroborated with the observations by Atawodi *et al.* (2003) where root extracts of *Adansonia digitata* eliminated motility in *T. congolense* and significantly reduced motility in *T. brucei*. On the other hand, leaf extracts of this plant had little or no effect on the parasites. Therefore, these findings highlighted the importance to study all plant parts individually in order to increase the number of active candidates from plant screening programs. The investigations carried out by FRIM and IPharm should have considered this point very carefully and evaluated extracts from many different parts of the same plant in order to increase the number of screening hits. The status of a plant being trypanocidal or not should be taken within the context of the parts investigated.

Although *in vitro* screening of bioactives from plants is generally regarded as a useful method to pre-select candidates for bioassay-guided isolation of active compounds, this approach should not be the only criterion used. It was suggested that *in vivo* studies should be carried out on plant extracts which lack *in vitro* activity to obtain additional evidence for the presence of bioactive principles.

The inactive extracts may show antitrypanosomal activity after oral administration in an animal model where biotransformation of plant materials may convert inactive precursor molecules to active ones (Wurochekke and Nok, 2004). This may also agree with the findings of Abedo *et al.* (2013) who observed that a plant with high *in vivo* antitrypanosomal activity may not have *in vitro* activity and vice versa due to the peculiarities in the metabolic disposition of the plant chemical constituents. Plant extracts which failed to show good *in vitro* activities in this study were not tested further *in vivo* because of the approach used where only a small amount of plant materials were collected for the initial evaluation of *in vitro* antitrypanosomal activity. Moreover, the study was aimed at identifying active extracts rapidly based on an established *in vitro* screening strategy before proceeding to hit compound isolation and efficacy studies.

Cymbopogon nardus (L.) Rendle

One of the most active antitrypanosomal plant identified in the investigation is *Cymbopogon nardus* or locally known as serai wangi. *Cymbopogon* species are commonly used in folk medicine to treat many diseases. Essential oils of these species are known for various bioactivities ranging from antimicrobial, antifungal, antioxidant, analgesic, antinociceptive, neurobehavioral, insecticidal and as insect repellents (Kpoviessi *et al.*, 2014). However, there was no direct activity shown for *C. nardus* essential oils against *T. brucei* prior to this study. Anthony *et al.* (2005) reported the potential use of plant essential oils for treating parasitic infections because of their immunomodulatory properties and parasiticidal effects. The authors reported that essential oil from *Melaleuca alternifolia*, with terpinen-4-ol as the major constituent, showed an ED_{50} value of 0.02 μg/ml against *T. b. brucei* and was more than 1000-fold more selective to the parasite than to human lymphocytic cells. This was also confirmed by Mothana *et al.* (2014) who demonstrated that the strong antitrypanosomal activity of *Leucas virgata* leaf extracts against *T. brucei* (IC_{50} 8.8 μg/ml) was attributed to the presence of essential oil constituents.

The crude essential oil extract from *C. nardus* showed strong antitrypanosomal activity with IC_{50} value of 0.31 μg/ml against *T. b. brucei* BS221 and scored the highest selectivity index towards the parasite (SI > 323; Table 3). Further, investigations on the *C. nardus* crude essential oil by Muhd Haffiz *et al.* (2013) determined the presence of α-eudesmol, γ-eudesmol and eugenol as the constituents contributing to the strong *in vitro* trypanocidal activity against *T. b. brucei* BS221. Selective and potent antitrypanosomal activity exhibited by terpenes such as α-eudesmol from plant sources have been reported by Otoguro *et al.* (2011). Recently, these findings were supported by Kpoviessi *et al.* (2014) who investigated the *in vitro* antitrypanosomal and antiplasmodial activity of essential oils from *Cymbopogon* species. However, the essential oils of *C. nardus* from the Malaysian studies displayed much stronger *in vitro* trypanocidal activity compared to findings from Kpoviessi *et al.* (2014) who reported an IC_{50} value of 5.71 μg/ml against *T. b. brucei* strain 427. Investigations on the crude essential oil extracts from the roots of another species of *Cymbopogon* from Malaysia, *Cymbopogon citratus* (DC.) Stapf., showed moderate activity and selectivity against *T. b. brucei* BS221 (IC_{50} 4.44 μg/ml; SI > 23; Table 3). On the other hand, studies by Kpoviessi *et al.* (2014) showed that the *C. citratus* essential oils exhibited stronger activity (IC_{50} 1.83 μg/ml) against *T. b. brucei*

strain 427 and revealed the presence of citral as its major compound. The differences in IC_{50} values observed in the essential oil extracts from *C. nardus* and *C. citratus* collected from Malaysia to findings by Kpoviessi *et al.* (2014), may be due to the differences in the origin and composition of essential oils of these *Cymbopogon* species and in the strain of *T. b. brucei* tested. Nevertheless, these studies signify the antitrypanosomal effectiveness of essential oils of *C. nardus* which warrants further toxicity and *in vivo* studies to investigate its potentials in HAT treatment.

Elephantopus scaber Linn.

Another plant that showed high *in vitro* antitrypanosomal activity is *Elephantopus scaber*, or locally known as tutup bumi. Although the plant is known for its medicinal properties, there were no reports on the role of neither the plant nor its active compound/s against *Trypanosoma*. Findings from the screening studies showed that the methanolic leaf extract potently reduced *in vitro* growth of *T. b. rhodesiense* STIB 900 with IC_{50} value of 0.22 µg/ml (Table 3). Based on the cytotoxicity effects on L-6 mouse skeletal cells, the plant extract showed high selectivity index (SI) of 205. In bioassay-guided isolation of antiprotozoal principle from the ethyl acetate fraction of *E. scaber* methanolic leaf extracts, Zahari *et al.* (2014) isolated the known sesquiterpene lactone compound deoxyelephantopin (Figure 2). With an IC_{50} value of 0.024 µg/ml and SI value of 65, the compound showed potent trypanocidal activity although its activity is lower than that of the standard HAT drugs suramine and pentamidine (Zahari *et al.*, 2014). The researchers attributed the strong antitrypanosomal activity of this compound to the presence of a lactone ring with an α-methylene group in the ring. Their results are in agreement with findings from other studies on sequiterpene lactones from different plant species which showed strong trypanocidal activity. One example is pseudoguaianolides which have been reported to show antiprotozoal activities (Otoguro *et al.*, 2011; Cogo *et al.*, 2012).

Based upon the findings presented in this review, the sesquiterpene lactone compound deoxyelephantopin which has been previously isolated from the *E. scaber* plant may have the potential to be developed further as lead compounds against trypanosomes. Further studies, however, are needed to include a comprehensive structure-activity relationship investigation and evaluation of its mechanism of action against the parasite.
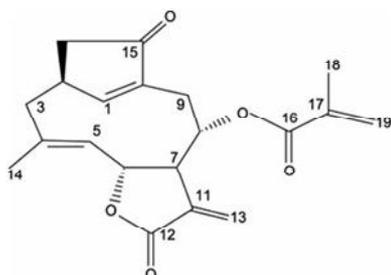

Figure 2: Structure of deoxyelephantopin

Dyera costulata (Miq.) Hook.f.

The methanolic leaf extract from *Dyera costulata* showed potent inhibitory effects to *T. b. brucei* BS221 (IC_{50} 0.58 µg/ml; Table 3). The selectivity index (SI) value of more than 172 placed *D. costulata* among the top three active and selective plant extracts identified in

the study. *Dyera costulata*, also known as jelutong, belongs to the Apocynaceae family and is an important timber species naturally found in southern Thailand, Malaysia and Sumatra. Its medicinal uses have been reported for analgesic (Reanmongkol *et al.*, 2002) and anti-inflammatory effects (Subhadhirasakul *et al.,* 2003), and antiplasmodial activity (Wong *et al.*, 2011). Phytochemical analysis of the leaf extract of *D. costulata* showed the presence of a number of chemical constituents, namely the bisindole alkaloids ochrolifuanines A, E, F and 18-dehydroochrolifuanines A, E, F; flavonoids such as rhamnazin and quercetin-3-O-α-L-rhamnopyranoside; and the pentacyclic triterpenoid β-amyrin (Wong *et al.,* 2013). However, antitrypanosomal activity has not been documented for the extract or compounds reported from this species.

Based on these findings, Muhd Haffiz *et al.* (2011) showed that the total alkaloids from the methanolic leaf extracts of *D. costulata* and their chromatographic fractions revealed strong trypanocidal activity in the *in vitro* assays with IC_{50} values of < 0.5 µg/ml against *T. b. brucei* BS221. The results were promising and further purification and characterisation of indole alkaloids of *D. costulata* are being carried out to provide evidences on their potential activity against the trypanosomes. Previous studies have reported on potential antiprotozoal properties of some plant-derived indole alkaloids such as strictosidine and acetylstrictosidine obtained from *Cephaelis dichroea* which showed good antitrypanosomal activity towards *T. b. brucei* (IC_{50} 6.1 and 17 µM, respectively) and low toxicity against KB cells (del Rayo Comacho *et al.*, 2004). While indole alkaloid tryptanthrin from *Strobilanthese cusia* exhibited IC_{50} values of 23 µM against the bloodstream form *T. b. brucei* (Scovill *et al.*, 2002). Understanding that the major stumbling block to work further on active compounds is the lack of mechanistic rationale for their activity, ongoing studies are also aimed at looking to resolve this issue for the potential development of this interesting group of antitrypanosomal compounds.

Other plants

A number of other Malaysian plants investigated for *in vitro* antitrypanosomal activity have also demonstrated promising trypanocidal activity with IC_{50} values below 1.56 µg/ml (Score 3) against *T. b. rhodesience* STIB 900 and high selectivity with SI values more than 95. These include *Reinwardtiodendron cinereum*, *Baccaurea parviflora*, *Thottea corymbosa*, *Antidesma tomentosum*, *Archidendron clyperia*, *Aporosa aurea* and *Rinorea anguifera* (Table 3). No reports on the antiprotozoal or antitrypanosomal activity of these plant species were found in the literature. However, anti-inflammatory properties observed in the methanolic extracts of the medicinal plant *A. clyperia* Jack. have been attributed to the presence of the flavonoid quercetin (Yang *et al.*, 2013). While the flavonol glycoside mauritianin, lignan (+)-syringaresinol and camptothecin compounds isolated from *R. anguifera* (Lour.) Kuntze extracts showed the ability to inhibit topoisomerase I enzyme (Ma *et al.*, 2005). Based upon the antitrypanosomal activity and selectivity of these plant extracts on *T. b. rhodesiense* STIB 900 compared with L-6 cells, further chemical analysis to confirm the active principles that may be responsible for this activity is warranted. Phytochemical studies of some of the active plants have been conducted and the results will be published in future. It is noteworthy that the lack of *in vivo* antitrypanosomal activity for all the active plants identified in this study prevents a more positive conclusion to be drawn on their trypanocidal efficacy.

2. Conclusion

Natural products present a potentially rich source of lead compounds with promising trypanocidal activity that could give impetus for further studies towards their development as antitrypanosomal drug candidates. To the best of our knowledge, the screening study reported in this review has presented the first report on *in vitro* antitrypanosomal activity for most of the investigated plants. Although it will take many years to further develop the screening hits through lead optimization process, efforts in building the HAT hit compound pipeline is vital. With this as the main objective, the study carried out by FRIM and IPharm formed an important research platform where a natural substances library from Malaysian plants and other sources of natural products was established successfully to serve as a basis in the search for potential antitrypanosomal candidates.

This review has highlighted ten promising plants for further antitrypanosomal investigations and the determination of their active constituents, with a view to optimize their utilization. This review also highlights that the Malaysian plants have a multitude of chemical constituents that could provide leads for the development of new trypanocidal compounds. Some of the active compounds identified with strong activity deserve further *in vivo* studies. The availability of new lead structures showing sufficiently active antitrypanosomal activity and few or no side effects is much needed in HAT therapy (Hotez and Pecoul, 2010). However, many of these compounds have restricted use in human due to high toxicity, or low bioavailability, and/or poor solubility. Overcoming these pharmaceutical problems through medicinal chemistry research where a mechanistically-based, structural modification of chemical leads from nature will lead to the development of new safe and effective drugs (Kayser *et al.*, 2003). Additionally, knowledge of the molecular target of compounds can greatly facilitate lead optimisation and development, and also reduce the risk of unexpected toxicity and allows synergism and resistance mechanisms to be predicted. Therefore, studies focused in the search for new antitrypanosomal compounds, particularly new lead structures from natural products, must pay special attention to these issues.

Acknowledgement

Conflict of interest

We declare that we have no conflict of interest.

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