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RESEARCH ARTICLE

Antitrypanosomal and Cytotoxic Activities of Selected Medicinal Plants and Effect of *Cordyline terminalis* on Trypanosomal Nuclear and Kinetoplast Replication

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ABSTRACT

Surra is a hemoprotozoal disease affecting domesticated and wildlife animals. The causative agent is the hemoprotozoan parasite *Trypanosoma evansi*. Only few drugs are currently available for the treatment of this disease that are old and encounter the problem of resistance emergence, which punctuates the urgent demand for new drugs for the treatment of surra. In the current study, the antitrypanosomal activity of the aqueous and ethanol extracts of five selected medicinal plants namely *Acanthus ilicifolius*, *Allium sativum*, *Cordyline terminalis*, *Goniothalamus tapis* and *Maesa ramentacea* was evaluated *in vitro* against *T. evansi* strain Te7 and the cytotoxic activity of the extracts was evaluated on Vero cells using MTT-cell proliferation assay. The ethanol extract of leaves of *G. tapis* scored the most potent antitrypanosomal activity (IC_{50} of 7.61µg/ml) and a selectivity index (SI) of 11.47, while the aqueous extract of *C. terminalis* leaves exhibited an IC_{50} of 48.1 µg/ml and the highest SI of 27.21. Addition of 50 µg/ml of *C. terminalis* extract to *T. evansi* culture has led to a significant inhibition of nuclear and kinetoplast DNA replication, contributing to its *in vitro* antitrypanosomal activity. From the current study, it can be concluded that the aqueous extract of leaves of *C. terminalis* shows considerable antitrypanosomal activity and it could be a potential source of new antitrypanosomal compounds.

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INTRODUCTION

Trypanosoma evansi (Trypanosomatidae, Kinetoplastida) is the causative agent of a disease known as "surra", which infects a wide variety of domesticated animals such as camels, equines, cattle, sheep, goats, dogs, cats, pigs, as well as many species of wildlife animals (Luckins, 1988; Herrera *et al*., 2004; Da Silva *et al*., 2009). The parasite is considered endemic to Asia, Africa, South and Central America and is mechanically transmitted through the bite of blood-sucking insects, especially *Tabanus* and *Stomoxys* (OIE, 2010), in contrast to *T. brucei* that requires an intermediate host in order to complete its life cycle (Borst *et al*., 1987). In South America, vampire bats are also involved in the transmission of *T. evansi* (Hoare, 1972).

Current chemotherapy of surra is restricted to a few drugs, which have been used for a long time and there are reports from different parts of Asia and Africa indicating the emergence of resistant *T. evansi* strains to the currently available antitrypanosomal drugs (El Rayah *et al*., 1999; Zhou *et al*., 2004; Macaraeg *et al*., 2013). Therefore, searching for new antitrypanosomal compounds which are effective, cheap, and easy to administer is urgently required.

Plants have been extensively investigated for their antitrypanosomal potential and some plants have been reported to exhibit *in vitro* antitrypanosomal activity (Bizimana *et al*., 2006; Bawm *et al*., 2010; Dua *et al*., 2011; Abiodun *et al*., 2012; Iqbal *et al*., 2012; Jahan *et al*., 2012). Several plants have also been reported to have promising antitrypanosomal effect *in vivo* such as *Morinda morindiodes* and *Azadirachta indica* (Olukunle *et al*., 2010; Habila *et al*., 2011).

Compounds exerting antitumor effects make good candidates as antitrypanosomal compounds as, like cancer cells, trypanosomes are fast-growing and possess cell-signaling pathways that are similar to those present in mammalian cells. Moreover, the only drug discovered for the treatment of *T. brucei gambiense* infection was

eflornithine, which was initially used as anticancer agent and then it became a treatment of sleeping sickness (Steverding, 2010).

The current study investigates *in vitro* the antitrypanosomal and cytotoxic activities of the aqueous and ethanol extracts of five selected medicinal plants. Selection of the plants was based on the anticancer effect of these plants as mentioned in previous literature (Ooi *et al*., 1993; Pinto and Rivlin, 2001; Babu *et al*., 2002; Tantithanaporn *et al*., 2011).The study also investigates the effect of *C. terminalis* aqueous extract on the nuclear and kinetoplast DNA replication of *T. evansi*.

MATERIALS AND METHODS

Preparation of plant extracts: Fresh plant parts were collected from the Agricultural Conservatory Park at Biodiversity Unit, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). The plant species were authenticated and voucher specimens deposited at Biodiversity Unit, IBS, UPM.

Fresh plant parts were cleaned from debris, washed and dried in a hot air oven (45-48°C). Dried parts were grinded using a laboratory grinder. About 20-40 grams of the powdered plant was soaked in sterile distilled water and absolute ethanol (1:10 w/v) for the preparation of aqueous and ethanolic extracts, respectively. The aqueous extract was then dried using freeze dryer while the ethanol was separated using rotary evaporator. The extracts were stored in tightly sealed containers and kept at -22°C until used.

Culture and maintenance of *Trypanosoma evansi*: *Trypanosoma evansi*, strain Te7, was cultured in tissue culture flasks using HMI-9 medium. The medium was supplemented with 10% fetal bovine serum (FBS), penicillin G potassium (50 U/ml) and streptomycin sulfate (60 µg/ml). Culture flasks were incubated in a CO_2 incubator under 5% CO_2, 37°C and 85% relative humidity. Trypanosomes were subcultured once every three days and were maintained for one month before commencement of the assay in order to ensure their normal growth rate.

***In vitro* antitrypanosomal activity of plant extracts:** The antitrypanosomal screening of plant extracts was performed to determine their median inhibitory concentration (IC_{50}) following the procedure previously described by Dyary *et al*. (2014).

Culture and maintenance of Vero cells: Vero cells were cultured in tissue culture flasks using RPMI-1640 medium, supplemented with 10% FBS, 100 U/ml of penicillin G potassium and 100 µg/ml of streptomycin sulfate. Culture flasks were incubated in a CO_2 incubator under 5% CO_2, 37°C and 85% relative humidity. Cells were maintained for one month before commencement of the test to ensure their consistent growth.

Cytotoxicity assay of the plant extracts: Cytotoxicity assay was performed on green monkey kidney (Vero) cells using MTT-cell proliferation assay kit (Cayman Chemical Company, USA). The procedure was performed following kit manufacturer's instructions. Negative control wells were included in each plate and diminazene aceturate was used as positive control. Median cytotoxic concentration (CC_{50}) of the extracts, which is the concentration that gives half maximal growth of Vero cells, was calculated using linear regression analysis (Microsoft excel software). Each test was conducted in triplicate.

Effect of *Cordyline terminalis* extract on the mitotic cycle of *Trypanosoma evansi*: Trypanosomes were cultured in HMI-9 medium using 25 cm^2 tissue culture flasks with the addition of 50 µg/ml of *C. terminalis* leaves aqueous extract. Negative control flasks, containing cultured *T. evansi* (2×10^5 cells/ml), and positive control flasks, containing cultured *T. evansi* with 15 ng/ml of diminazene aceturate, were also included.

After 2, 6, 12, and 24 hr of incubation, 1 ml of the trypanosome culture was taken and centrifuged at 2000 rpm and 4°C for five minutes. The supernatant was removed and the trypanosomes were then re-suspended in 0.5 ml of 4% paraformaldehyde. The suspension was poured into a 1.5 cm diameter circle on a glass slide that was made by using nail varnish and the slide was then kept in a humid chamber for 30 minutes. The slide was then dipped in phosphate buffered saline for five minutes to wash off the paraformaldehyde. After that, it was dipped in 1% triton X-100 and left for five minutes. Finally, the slide was dipped in Hoechst H33258 stain (100 ppb) and left to stain for 10 minutes. Slides were then covered with a cover slip.

Three slides were prepared from each of the negative control, positive control and *C. terminalis*-treated cultures. Slides were examined under fluorescent microscope and 400 cells were counted from each slide. Cells observed were divided into different groups according to the number of nuclei and kinetoplasts, which fluoresce under ultraviolet light. The percentage of each cell type was calculated. The difference in the percentage of different cell types was compared statistically using one-way ANOVA, followed by Duncan's test.

RESULTS

Plants used in the current study: Ten extracts from five medicinal plants were tested for antitrypanosomal activity. Plant species, their families and plant parts used in the current study are shown in Table 1.

***In vitro* antitrypanosomal activity of plant extracts:** From ten extracts tested, the ethanol extract of *G. tapis* exhibited the most potent antitrypanosomal activity with an IC_{50} value of 7.61 µg/ml, followed by the aqueous extract of *Maesa ramentacea* (IC_{50} = 40.90 µg/ml).While the aqueous extract *Acanthus ilicifolius* leaves scored 314.16 µg/ml, which was the highest IC_{50} value. The IC_{50} of diminazene aceturate was 15.3 ng/ml (Table 2).

Cytotoxicity assay and selectivity index: Cytotoxicity assay of the plant extracts revealed a wide range of cytotoxicity exerted by the extracts on the mammalian Vero cells. The ethanol extract of *M. ramentacea* exhibited the highest cytotoxic effect as the median cytotoxic concentration (CC_{50}) of the extract was 70.3

Table 1: Plants used in the current study

Plant	Family	Voucher number	Plant part	Extract type	Yield (% w/w)*
Acanthus ilicifolius	Acanthaceae	Acp 0106	Leaves	Aqueous	3.03
				Ethanol	2.86
Allium sativum	Liliaceae	SK 1721/10	Bulbs	Aqueous	44.78
				Ethanol	1.2
Cordyline terminalis	Agavaceae	Acp 0016	Leaves	Aqueous	7.52
				Ethanol	6.34
Goniothalamus tapis	Annonaceae	Acp 0099	Leaves	Aqueous	5.18
				Ethanol	6.99
Maesa ramentacea	Myrsinaceae	Acp 0082	Leaves	Aqueous	13.04
				Ethanol	1.04

*Yield is calculated as the fraction of extract yield to the quantity of initial dried plant powder.

Table 2: Antitrypanosomal and cytotoxic activities of the plant extracts

Plant or drug	Extract type	IC_{50} on *T. evansi* (µg/ml)	CC_{50} on Vero cells (µg/ml)	SI*
Acanthus ilicifolius	Aqueous	314.16±37.16	6480.19±113.67	20.63
	Ethanol	163.64±3.58	> 500	>3.06
Allium sativum	Aqueous	224.97±58.02	1326.54±134.17	5.90
	Ethanol	138.24±0.44	275.96±52.52	2.00
Cordyline terminalis	Aqueous	48.17±16.79	1309.01±53.81	27.17
	Ethanol	66.68±15.82	> 500	>7.5
Goniothalamus tapis	Aqueous	42.21±16.47	90.78±2.36	2.15
	Ethanol	7.61±1.61	87.22±1.39	11.46
Maesa ramentacea	Aqueous	40.90±0.50	630.82±25.68	15.42
	Ethanol	49.97±2.63	70.30±3.89	1.41
Diminazene aceturate		0.0153±0.003	31.80±8.13	2078.4

*Selectivity index (SI) is calculated as the median cytotoxic concentration (CC_{50}) of the drug or extract on Vero cells divided by its median inhibitory concentration (IC_{50}) on *T. evansi*. Values are presented as mean±SD of three tests.

Table 3: Percentage of different cell types based on the number of nuclei and kinetoplasts in the different treatment groups

Cell type	Treatment	Time of incubation			
		2 hr	6 hr	12 hr	24 hr
N1K1	N.C.	83.6±0.38^a	83.3±0.63^a	79.8±0.25^a	80.6±0.38^a
	Diminazene	81.9±3.13^a	38.2±1.01^b	34.7±11.41^b	54.0±13.0^b
	C. terminalis	87.3±1.25^a	87.3±1.88^a	89.6±2.32^a	88.4±0.38^a
N1K2	N.C.	12.1±0.52^a	12.7±0.29^a	12.7±0.63^a	14.1±0.14^c
	Diminazene	8.1±1.42^b	2.1±0.95^c	1.0±0^c	4.0±1.0^a
	C. terminalis	8.9±0.14^b	9.4±0.63^b	7.8±1.56^b	9.3±0.38^b
N2K2	N.C.	4.3±0.38^b	4.0±0.66^c	7.5±0.90^c	5.3±0.38^c
	Diminazene	5.9±0.95^c	0.2±0.29^a	0^a	0^a
	C. terminalis	3.0±0.43^a	2.8±1.25^b	1.9±0.38^b	2.3±0.75^b
N1K0	N.C.	0^a	0^a	0^a	0^a
	Diminazene	1.4±0.38^a	52.3±1.46^b	57.9±8.53^b	29.3±11.85^b
	C. terminalis	0^a	0^a	0^a	0^a
N2K0	N.C.	0^a	0^a	0^a	0^a
	Diminazene	0.8±0.43^a	5.8±1.42^b	4.7±1.61^b	7.7±0.58^b
	C. terminalis	0^a	0^a	0^a	0^a
N2K1	N.C.	0^a	0^a	0^a	0^a
	Diminazene	1.9±0.88^b	1.4±0.38^b	1.7±0.95^b	5.0±1.0^b
	C. terminalis	0.8±0.29^a	0.5±0^a	0.7±0.29^a	0^a

Values represent the mean percentage of each cell type ± SD (three slides, 400 cells were counted from each slide). Different superscripts denote significant difference between values in the same column and for the same cell type (P<0.05); N.C.: Negative control; N1K0: One nucleus with no kinetoplasts; N1K1: One nucleus and one kinetoplast; N1K2: One nucleus and two kinetoplasts; N2K0: Two nuclei with no kinetoplasts; N2K1: Two nuclei and one kinetoplast; N2K2: Two nuclei and two kinetoplasts.

µg/ml, while the aqueous extract of *A. ilicifolius* exhibited the least cytotoxic effect on Vero cells as the CC_{50} of the extract was 6480.19 µg/ml. Diminazene aceturate scored a CC_{50} value of 31.8 µg/ml (Table 2).

The aqueous extract of *C. terminalis* exhibited the highest SI of 27.17, followed by *A. ilicifolius* aqueous extract with a SI value of 20.63. Diminazene aceturate scored a SI value of 2078.4 (Table 2).

Effect of *Cordyline terminalis* extract on the mitotic cycle of *Trypanosoma evansi*: Three types of trypanosome cells were observed in the control culture of *T. evansi*, depending on the number of nuclei and kinetoplasts, which were trypanosomes containing a single nucleus and kinetoplast (N1K1), a nucleus and two kinetoplasts (N1K2) and a pair of nuclei and kinetoplasts (N2K2) (Fig. 1A, 1B and 1C, respectively). The percentage of each cell type at different time intervals is summarized in Table 3.

Addition of 50 µg/ml of *C. terminalis* aqueous extract to *T. evansi* culture medium has led to a significant reduction (P<0.05) in the percentage of N1K2 and N2K2 cells after 2hr of incubation, compared to the control cultures (Table 3), which means that the addition of *C. terminalis* extract has led to slowing of the mitotic cycle in *T. evansi*, attributing to the antitrypanosomal potential of the extract. The reduction in the percentage of N1K2 and N2K2 cells was concentration-dependent as it was observed that higher concentrations of the extract led to increased reduction in the percentage of the two mentioned cell types.

Results of the fluorochrome assay revealed that the addition of 15 ng/ml of diminazene aceturate to *T. evansi* culture resulted in a significant decrease (P<0.05) in the

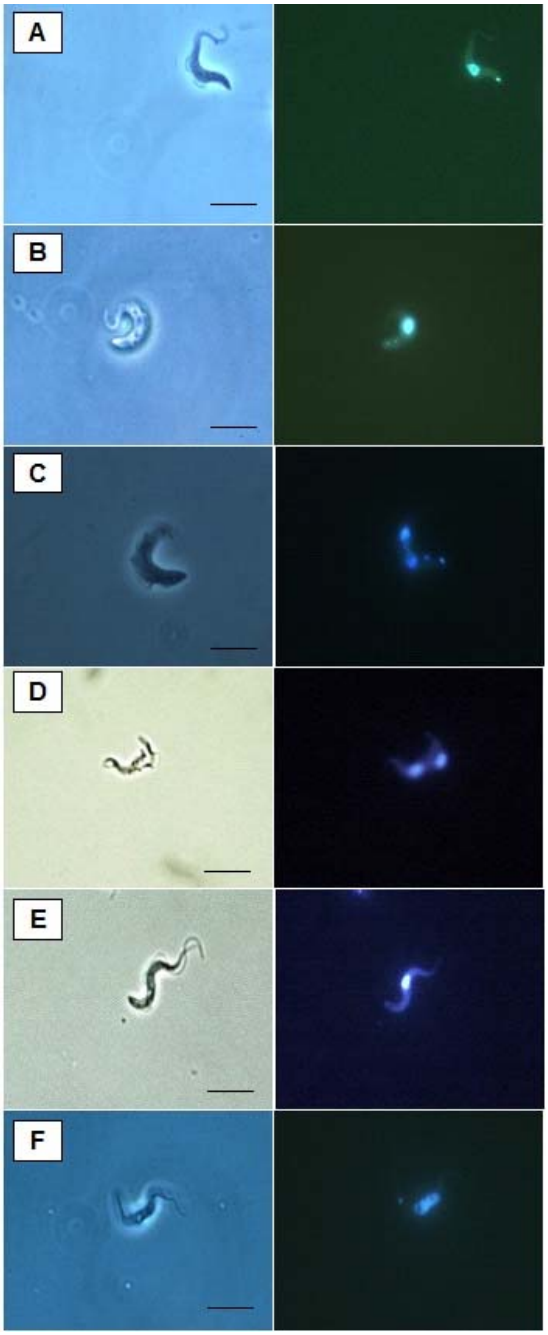

Fig. 1: *Trypanosoma evansi* cells. A, B, and C: cell types observed in a control culture of *T. evansi*; D, E, and F: trypanosome cells observed in diminazene-added culture, which are not detected in the control culture. Addition of *Cordyline terminalis* extract has led to a significant reduction in B and C cell types. Scale bar: 10 µm

percentage of N1K1, N1K2 and N2K2 cells, compared to the control, after 6 hr of incubation (Table 3). While a significant increase in the proportion of trypanosome cells containing two nuclei and a kinetoplast (N2K1), two nuclei and no kinetoplasts (N2K0) and one nucleus with no kinetoplasts (N1K0) was observed (Fig. 1D, 1E and 1F). These results showed that the addition of diminazene to *T. evansi* culture resulted in the inhibition of kinetoplast division, while nuclear division continued, and this attributes to the cells' death as trypanosome cells lacking kinetoplast are not viable.

DISCUSSION

A trypanosome cell contains a single nucleus and a mitochondrion that is known as the kinetoplast and contains the mitochondrial DNA (Sherwin and Gull, 1989). During trypanosomal cell cycle, the existing organelles, including the nucleus and kinetoplast, are divided in a tightly controlled program to ensure that each of the resulting daughter cells receives a single nucleus and mitochondrion with fidelity (Tyler *et al*., 2001).

The process of DNA replication in the trypanosomes starts with the kinetoplast division, which is preceded by G_1, S and G_2 phases of the kinetoplast and G_1, S and part of the G_2 phases of the nucleus (Woodward and Gull, 1990). After approximately 30 minutes of kinetoplast division, mitosis of the nucleus starts and the predivision cell, which is in the cytokinesis phase, contains a pair of nuclei and kinetoplasts (N2K2 cells, Figure 1 C) before it is divided into two daughter cells (Woodward and Gull, 1990). Hence, three cell types are observed in a trypanosome culture, based on the number of nuclei and kinetoplasts, which are N1K1, N1K2 and N2K2 cells. The percentage of each cell type can vary in different culture media depending on many factors such as the culture conditions and the availability of nutrients in the medium.

In the current study, the antitrypanosomal and cytotoxic activities of the aqueous and ethanol extracts of five medicinal plants were investigated. The aqueous extract of leaves of *C. terminalis* scored the highest SI of 27.17. Selection of *C. terminalis* aqueous extract for the fluorochrome assay was based on the antitrypanosomal screening and cytotoxicity assay results, as this extract scored the highest SI among the ten aqueous and ethanolic extracts prepared from the plants tested in the current study. *Cordyline terminalis*, which is an evergreen tropical perennial plant, has been reported to have antibacterial potentials (Ahmed *et al*., 2003) and the aqueous extract of the plant's leaves was reported to have antitumor effects against murine lymphoma (EL4) and human breast cancer (MCF-7) cell lines (Ooi *et al*., 1993). The antitrypanosomal activity of *C. terminalis* could be related to its antineoplastic potential. Trypanosomes have cell-signaling pathways that resemble those present in mammalian cells and there are suggestions that some compounds, which have shown activity against neoplastic cells, make good candidates to be tested for the treatment of trypanosomiasis (Barrett *et al*., 2004). Results of the fluorochrome assay have shown that when *C. terminalis* was added to the trypanosome culture the percentage of trypanosome cells undergoing replication decreased, compared to the control culture. This means that the *C. terminalis* caused an inhibition of trypanosomal nuclear mitosis and DNA replication.

Diminazene aceturate is an aromatic diamidine compound used for the treatment of trpan osomiasis in domesticated animals such as cattle, buffalos, sheep, goats, cats and pigs (Peregrine and Mamman, 1993; Da Silva *et al*., 2009). The drug binds to the adenine-thymine binding sites in the trypanosomal kinetoplast DNA (kDNA) and inhibits kinetoplast replication, thereby inhibiting trypanosomal mitosis (Wilson *et al*., 2008). The kinetoplast DNA inhibitory activity of diminazene aceturate shown in the current study by using the fluorochrome assay

agrees with the previously known mechanism of antitrypanosomal activity of this compound.

It can be concluded from the current study that *C. terminalis* extract possesses antitrypanosomal activity and it can be considered for further investigation as a potential source of new antitrypanosomal compounds.

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