Antitrypanosomal and cytotoxic activities of botanical extracts from *Murraya koenigii* (L.) and *Alpinia mutica* Roxb.

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Received 4 September 2018; received in revised form 5 November 2018; accepted 23 November 2018

Abstract. Four carbazoles (girinimbine, mahanimbine, murrayafoline and murrayanine), isolated from *Murraya koenigii*, and one kavalactone (5,6-dehydrokawain) and one flavonoid (pinostrobin) isolated, from *Alpinia mutica*, were tested for their antitrypanosomal activity using *in vitro* cultured *Trypanosoma evansi* cell lines. The cytotoxic activities of these compounds were also investigated against mammalian Vero cells using the MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide)-cell proliferation assay. Three carbazole compounds, namely mahanimbine, murrayafoline, and girinimbine, showed a potent antitrypanosomal activity, scoring a median inhibitory concentration (IC⁵₀) of 3.13, 6.35 and 10.16 µg/ml, respectively. Girinimbine was the least toxic to Vero cells, and the mean cytotoxic concentration (CC⁵₀) and the selectivity index (SI) of this compound were 745.58 ± 42.38 µg/ml and 73.38, respectively. Girinimbine and the other carbazole compounds possess potential antitrypanosomal activity with comparably low toxicity against mammalian cells. Girinimbine, in particular, is a good candidate to be further investigated as a potential antitrypanosomal agent using *in vivo* models.

INTRODUCTION

Trypanosomes comprise a diverse group of parasitic hemoflagellates that infect animals and humans, with a widespread global distribution. These pathogens are generally vectored by hematophagous invertebrates, and depending on the species, may be mechanically transmitted or undergo complex developmental stages in the dipteran vector (Sharma et al., 2008; Sharma et al., 2011). Both wild and domestic animals are susceptible to infection by *Trypanosoma evansi*, which has the widest geographical distribution among the pathogenic trypanosome species (Luckins, 1988; Desquesnes et al., 2013a; Desquesnes et al., 2013b). Chemotherapy seems to be the only available choice in the treatment of trypanosomiasis, as there is no vaccine available for immunization against the disease owing to the high antigenic variation exhibited by this protozoan parasite (Brun et al., 2010). The current therapy has been in use for a long time and encounters such problems as complexity, toxicity, high costs, and concerns over the development of resistant strains (Baker et al., 2013; Singh Grewal et al., 2016; Chappuis, 2018). This heightens the need to explore new treatment options for safer and more effective compounds.

One avenue towards this effort is to test novel botanical extracts for their antitrypanosomal activities. *Murraya koenigii* is a tropical to subtropical tree of the family Rutaceae, widely used in folk medicine (Sathaye et al., 2011; Syam et al., 2011). This plant has been reported to be rich in carbazole compounds, which have
anticancer activities against different cancer cell lines (Roy et al., 2005; Ito et al., 2006). *Alpinia mutica* is a perennial herb that belongs to the ginger family (*Zingiberaceae*) and is found in tropical areas such as Peninsular Malaysia and Thailand (Malek et al., 2011; Ghosh & Rangan, 2013). The rhizomes of *A. mutica* have been used in traditional medicine for flatulence treatment (Mustahil et al., 2013). Pinostrobin chalcone, a compound isolated from *A. mutica* rhizomes, has been reported to exert anticancer activity against different carcinoma cells *in vitro*, including KB, MCF7, and CaSki (Malek et al., 2011). These compounds with anticancer properties make good candidates to be tested against trypanosomes due to similarities in certain cell-signaling pathways in cancer and trypanosome cells (Barrett et al., 2004). The current study investigates the antitrypanosomal and cytotoxic activities of four carbazoles from *M. koenigii*, and two compounds from *Alpinia mutica*. The selection of the compounds for antitrypanosomal screening was based on previous reports on their anticancer activities (Darwanto et al., 1999; Malek et al., 2011).

**MATERIALS AND METHODS**

**Test compounds**

Six compounds were tested against cultured *T. evansi* (Table 1). Four carbazoles (girinimbine, mahanimbine, murrayafoline and murrayanine) were isolated from *M. koenigii*, and one kavalactone (5,6-dehydrokawain) and one flavonoid (pinostrobin) were isolated from *A. mutica*. Details of the extraction and isolation of these compounds have been previously described (Mustahil et al., 2013; Ahmad et al., 2014). A stock solution of 500 µg/ml in 1% dimethyl sulfoxide (DMSO, Sigma Aldrich, Steinheim, Germany) was prepared for 5,6-dehydrokawain, murrayafoline, murrayanine, and pinostrobin. For girinimbine and mahanimbine, a solution containing 200 µg/ml was prepared as it was the highest concentration of these compounds to be achieved in 1% DMSO solution.

**Culture and maintenance of trypanosomes**

*Tryptosoma evansi* strain Te7, previously isolated from water buffalo (*Bubalus bubalis*) in Selangor, Malaysia, was used in the experiment. Trypanosomes were cultured in 25cm² ventilated-cap tissue culture flasks with HMI-9 medium supplemented with 10% fetal bovine serum (FBS, from Gibco, Grand Island, NY, U.S.A.), 30 µg/ml penicillin G potassium (Fluka, Buchs, Switzerland), and 100 µg/ml streptomycin sulfate (Sigma Aldrich, Steinheim, Germany). The trypanosomes were incubated under 5% CO₂, at 37°C and 85% relative humidity. Subcultures of the trypanosomes were prepared once every three days to provide the nutritional requirements for the microorganisms. The trypanosome cultures were maintained for about one month before the commencement of the antitrypanosomal screening test in order to ensure their normal and consistent growth.

**Screening for antitrypanosomal activity**

The antitrypanosomal activity of the compounds was performed *in vitro* using sterile 24-well microtiter plates. The compounds were dissolved in DMSO and the solution was diluted using sterilized water to the required concentration. The volume of DMSO did not exceed 1% of the content in each well. Control wells containing 1% DMSO were also included in the assay. The stock solution was diluted with an equal volume of double concentration HMI-9 medium (2×HMI-9) before being used in the assay in order to ensure the exact concentration of the culture medium in each well. Four hundred microliters of each compound solution (in 1×HMI-9 medium) was added to the first well and threefold serial dilution was performed covering a concentration range from 500–6.25 µg/ml for dehydrokawain, murrayafoline, murrayanine and pinostrobin, and a range from 200.00–2.47 µg/ml for girinimbine and mahanimbine. Four hundred microliters of the trypanosome culture
containing $1 \times 10^5$ trypanosomes/ml were added to each well to make a final volume of 800µl containing $5 \times 10^4$ trypanosomes/ml per well. Negative control wells containing $5 \times 10^4$ trypanosomes/ml were included in each test plate and diminazene aceturate (Berenil®), a standard antitrypanosomal agent, was used as a positive control (2000.00–2.74 ng/ml). The plates were incubated at 37°C, 5% CO$_2$ and 85% relative humidity for 72 h. The numbers of live *T. evansi* in each well was counted using a Neubauer chamber after 2, 4, 8, 24, 48 and 72 h of incubation. Each assay was conducted in triplicates. A growth curve of trypanosomes was constructed for each serial dilution and the median inhibitory concentration (IC$_{50}$), which is the concentration at which the trypanosomes number is $\frac{1}{2}$ the number achieved in the negative control well, was calculated using linear regression analysis (Microsoft excel 2010 program).

**Cytotoxicity assay**

The cytotoxic activity of the plant extracts was investigated using green monkey kidney (Vero) cell lines. The cells were cultured *in vitro* using RPMI-1640 (Roswell Park Memorial Institute) medium (Gibco, Grand Island, NY, U.S.A.) supple-
mented with 1% glutamine, 10% FBS, 60 µg/ml penicillin G potassium, and 100 µg/ml streptomycin sulfate (Sigma Aldrich, Steinheim, Germany). The Vero cells were cultured and maintained for three to five passages in 25cm² tissue culture flasks fitted with ventilated caps to ensure their consistent growth before the commencement of the cytotoxicity assay. The cytotoxicity assay of the plant extracts was performed using MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide)-cell proliferation assay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.). The assay was conducted in sterile 96-well microtiter plates according to manufacturer’s instructions. Negative control wells and wells containing 1% DMSO were included. Diminazene aceturate (1000–1.37 µg/ml) was also included in the assay. Each assay was conducted in triplicates. Dose-response curves were constructed using linear regression analysis (Microsoft excel program 2010) and the median cytotoxic concentration (CC₅₀) of each compound and diminazene aceturate was calculated from the curve. The selectivity index (SI) of the tested compounds was calculated as the median cytotoxic concentration (CC₅₀) of the compound against Vero cells divided by its median inhibitory concentration (IC₅₀) on T. evansi.

RESULTS

The rate of growth of T. evansi in the control wells (only HMI-9 media) was the highest, compared to wells containing the tested compounds. Trypanosomes in the control wells double in number after approximately 9.5 h, reaching a maximum concentration of 6 to 10×10⁶ trypanosomes/ml after 72 h. After that time, the trypanosome numbers start to decline rapidly due to depletion of the required nutrients in the culture medium. The addition of the different test compounds resulted in a dose-dependent inhibition of T. evansi growth (Fig. 1 and Fig. 2). Out of the six compounds tested, mahanimbine

![Figure 1](image_url). Effect of different girinimbine concentrations on the growth of Trypanosoma evansi after 72h of in vitro incubation. Girinimbine resulted in a dose-dependent inhibition of the growth of trypanosomes. Addition of 200 and 66.66 µg/ml of girinimbine resulted in complete inhibition of trypanosome growth after two hours of incubation. Addition of 22.22 µg/ml resulted in complete inhibition of trypanosome growth after 4 h. A girinimbine concentration of 7.40 µg/ml reduced the trypanosome numbers by 44%, compared to the control.
Figure 2. Effect of different concentrations of botanical extracts on the growth of *Trypanosoma evansi* after 72 hours of *in vitro* incubation. The kavalactone (5,6-dehydrokawain) and flavonoid (pinostrobin) were isolated from *Alpinia mutica*, while the carbazoles (mahanimbine, murrayafoline, and murrayanine) were isolated from *Murraya koenigii*. The growth rates of the trypanosomes were compared against *in vitro* incubation with varying concentrations of diminazene aceturate.

showed the most potent antitrypanosomal activity with a mean IC$_{50}$ value of 3.13 ± 0.04 µg/ml, followed by murrayafoline (6.35 ± 0.28 µg/ml) (Table 2). The kavalactone (5,6-dehydrokawain) and flavonoid (pinostrobin) were the least potent against *T. evansi* with mean IC$_{50}$ scores of 55.79 ± 0.72 µg/ml and 26.15 ± 3.58 µg/ml, respectively. Both these compounds also exhibited the lowest SI among the compounds tested (Table 2).
Although mahanimbine showed a potent antitrypanosomal activity, the CC₅₀ of this compound was 35.66 µg/ml and the SI was 11.39. Girinimbine scored a comparably less potent antitrypanosomal activity with a mean IC₅₀ of 10.16 ± 0.61 µg/ml. However, the CC₅₀ on the Vero cells was 745.58 ± 42.38 µg/ml, making it the compound with the highest SI (73.38) compared to the other botanical extracts tested. The standard anti-trypanosomal drug diminazene aceturate scored an SI of 1661.11, but it was more toxic to mammalian Vero cells (CC₅₀ of 29.90 ± 3.42 µg/ml) compared to girinimbine.

### DISCUSSION

Plants remain an important source in the development of new drugs despite the increasing role of synthetic compounds (Shah et al., 2013). The pharmaceutical potential of higher plants remains largely untapped as a vast majority have not been systematically tested for novel compounds to be utilized as therapeutic agents (Pan et al., 2012). Previous studies have demonstrated the antitrypanosomal potential of a number of botanical extracts in vitro (Dyary et al., 2014a; Dyary et al., 2014b; Maiwald et al., 2014; Molina-Garza et al., 2014; Dyary et al., 2015), substantiating the need to screen higher plants for new antitrypanosomal compounds. Girinimbine, mahanimbine, murrayanine, and murrayafoline are carbazole alkaloids highly abundant in *M. koenigii*, a plant known locally as curry leaf (Chakrabarty et al., 1997; Ramsewak et al., 1999; Bakar et al., 2007). These alkaloids are known to exert melanogenesis inhibitory effects on B16 melanoma 4A5 cells (Nakamura et al., 2013).

Girinimbine has been reported to exert antiplatelet activity through the inhibition of the cyclooxygenase enzyme and raising the level of cyclic adenosine monophosphate (Feng-Nien et al., 1994). It has also been shown to possess antioxidant effects, and a concentration of 6.0 µg/ml exerted antitumor promoting activity in vitro through the prevention of Epstein-Barr virus antigen expression in Raji cells (Kok et al., 2012).

Girinimbine can inhibit the growth and induce programmed cell death in human hepatocellular carcinoma (HepG2), HCT-15 and lung cancer (A549) cells (Wang et al., 2008; Syam et al., 2011; Mohan et al., 2013). Inhibition of the proliferation of human umbilical vein endothelial cells by girinimbine indicates its angiogenesis inhibitory potential (Iman et al., 2015). In the current study, girinimbine exerted a potent antitrypanosomal effect, combined with a comparably low toxic level on mammalian Vero cell line. The antitrypanosomal activity of girinimbine could be related to its anticancer promoting activity. Trypanosomes possess cell-signaling pathways and mitotic division cycles that are similar in design to those present in eukaryotic cells (Barrett...
et al., 2004; Gluenz et al., 2008). As such, therapeutic molecules which have shown activity against rapidly dividing neoplastic cells could be used in the treatment of trypanosomiasis. An example of these compounds is efollornithine, which was initially intended for use as an anticancer drug, but later was used for the treatment of Trypanosoma brucei gambiense infection (Steverding, 2010).

In conclusion, girinimbine possessing a potential antitrypanosomal activity against T. evansi and comparatively low cytotoxicity on mammalian cells, making this compound and other carbazoles good candidates for the development of new antitrypanosomal agents. However, further investigation on the mode of antitrypanosomal action, activity against other species of trypanosomes, and experimental animal infection models are necessary to support the in vitro antitrypanosomal effect of these plant-derived compounds.

Conflict of Interest
The authors disclose no conflict of interest.

REFERENCES


