Effects of some natural leads on *Trypanosoma* and *Leishmania* strains

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Abstract

Well known medical herbal compounds including apigenin, daidzein, phyllanthin and tyramine were assessed against *Trypanosoma* and *Leishmania* protozoans. Two strains of the bloodstream forms of *Trypanosoma brucei*: s427-WT and TbAT1-B48, and *Leishmania major* promastigotes and *Leishmania mexicana* were utilised.. Among selected natural compounds, apigenin and daidzein displayed moderate activity against African trypanosomes with EC_{50} 16 µM for wild-type sensitive control strain. Tyramine was not found very active for trypanosomes strains while all compounds were found trivial for the inhibition of *Leishmania mexicana* strains.

Keywords: Protozoan parasites; Sleeping sickness; Plant isolates, Apigenin, Daidzein, Phyllanthin and Tyramine

1. INTRODUCTION

A leading cause of numerous tropical diseases is protozoan parasites that are members of the Trypanosomatidae family. Approximately 65 million people of African countries are at risk to become infected with sleeping sickness which can be lethal if left untreated (WHO Report, 2017). Sleeping sickness is transmitted by the tsetse fly and caused by the *Trypanosoma brucei* subspecies such as *T. b. gambiense* and *T. b. rhodesiense* (Lindner et al., 2010). In 2009, after continued control efforts, the number of cases reported dropped below 10 000 (9878) for the first time in 50 years. This turned down in number of cases has sustained with 2804 new cases reported in 2015, the lowest level since the start of systematic global data-collection 76 years ago (WHO, 2017).

Leishmaniasis refers to a collection of chronic diseases caused by protozoa of the genus *Leishmania* and naturally transmitted by sand flies (Alvar *et al.*, 2012; Stockdale *et al.*,

2013). Pentavalent antimonials and pentamidine used for the treatment of leishmaniasis, are toxic or have severe side effects, such as renal, cardiac and neural toxicity, risk of diabetes and shock (Tiuman *et al.,* 2011). No vaccine is still available for human leishmaniasis while the drugs used against the parasite are very toxic and patients are also frequently unresponsive to treatment (Koff *et al.,* 1994; Kumar *et al.,* 2014; Tiuman *et al.,* 2011).

Leishmania (L.) mexicana infects many hosts (Berzunza *et al*., 2015) and has been detected in an assortment of wild and domestic mammals, while humans are opportunistic hosts (Courtenay *et al.,* 2017; Martina *et al.,* 2017). Cutaneous leishmaniasis caused by *Leishmania (L.) mexicana* was first depicted in chicle collectors of the Yucatan peninsula in 1912 by Seidelin, therefore its name is "Chiclero´s Ulcer" (Seidelin, 1912).

The drugs currently being used for the treatment display a high level toxicity, inadequate efficacy and require a long period of treatment (Kerboeuf *et al*., 2008). Moreover, it has been reported that there is development of drug resistance by the parasites (Croft *et al.,* 2005; Kerboeuf *et al.,* 2008; Paes *et al*., 2011). Thus, it is incumbent to explore new drugs or compounds that might be allied with the conventional treatment. Natural sources such as plants, algae and micro-organisms constitute as an important source of alternative drugs (Medeiros *et al*., 2011; Rondon *et al.,* 2012; Wink, 2012; Gamboa-Leon *et al*., 2014; Sifaoui *et al*., 2014). It has been estimated that there are about 250,000 medical plant species in the world but on the other hand, the therapeutic potential of only about 6% of them has been biologically evaluated. Furthermore, only approximately 0.75% of identified medical herbal compounds have been studied in clinical trials (Jameel *et al*., 2014). The foremost virtues of herbal medicine include their low cost, the low prevalence of severe adverse effects and superior efficacy.

In the current study we selected some well-known medical herbal compounds apigenin, daidzein, phyllanthin and tyramine. Because of the reported antileishmanial and various

biological activities of these natural compounds (Wong *et al*., 2009; Ilangkovan *et al*., 2016), now we decided to further investigate their activity against various *Trypanosoma* and *Leishmania* strains including the well-characterised and multi-drug resistant *Trypanosoma brucei* clonal line B48. The clonal line B48 is highly resistant to the two main classes of trypanocides, the melaminophenyl arsenicals and the diamidines, due to the loss the TbAT1/P2 and HAPT1 drug transporters (Bridges et al., 2007).

2. MATERIALS AND METHODS

2.1 Materials

All tested pure compounds including apigenin, daidzein, phyllanthin and tyramine were purchased from Sigma (Steinheim, Germany). Stock solution in 100% DMSO for each compound was prepared and for the concentrations used in assay, the calculated amount of stock solution was taken and diluted with complete medium, ensuring that the final DMSO concentration did not exceed 1% in the final conditions. EC_{50} values were obtained using the Alamar blue assay and are given as averages in µM (±SEM), of 3 independent evaluations.

2.2 Cell culture

2.2.1 Trypanosoma brucei bloodstream forms (BSF) in-vitro

In this research, two strains of the bloodstream forms of *Trypanosoma brucei* were utilised. The first was the wild type strain of *Trypanosoma brucei brucei* (s427-WT) and the other was TbAT1-B48 that was acquired from the clone TbAT1-KO (Matovu et al., 2003) by exposure to pentamidine thus causing more resistance to pentamidine, diminazene as well as melaminophenyl arsenicals. Consequently, these cells have neither TbAT1/P2 transporter nor the high affinity-pentamidine transporter genes (Bridges et al., 2007;Munday et al., 2014). Both strains were cultured in HMI-9 medium (pH 7.4) supplemented with 10% Fetal Calf

Serum and 14 µl/ Litre of 13.4M β-mercaptoethanol, (FCS) as described by Hirumi and Hirumi(Hirumi et al., 1989). The medium was sterilized by filtration (0.22 µm, Millipore) inside a flow cabinet using. The *T. b. brucei* cultures were incubated at 37ºC and 5% CO_2 and passaged invented flasks three times a week. All

2.1.2. Leishmania major Promastigotes and Leishmania mexicana

Leishmania major strain Friedlin (LmjF) and strains of *Leishmania mexicana* (MNYC/BZ/62/M379) were propagated in essential medium (HOMEM) with a PH value of 7.4 and 10% Fetal Calf Serum (FCS) heat-inactivation using plastic flasks at the temperature of 25ºC. The resultant cultures were then passed through fresh medium thrice per week.

2.3 Alamar blue assay to determine the sensitivity totest compounds

Resazurin sodium salt (Alamar Blue) is commonly used as a cell metabolic function indicator. It is a non-fluorescent, blue dye that is mixed with cell cultures containing various drug concentrations, in order to determine the sensitivity of African trypanosomes or *Leishmania* culturesto the test compounds in vitro (Fumarola et al., 2004;Raz et al., 1997). In case there are no toxic effects caused by the drug, the color of the living cells changes to red and fluorescent from blue. Preparation of Alamar Blue involves the dissolution of 12.5 milligrams of Resazurin sodium salt (Sigma) in 100 mL of phosphate-buffered saline (PBS) of pH 7.4, which was then filter-sterilized and stored in the dark at 4ºC.

2.3.1 Drug sensitivity using Alamar Blue assay in T. b. brucei BSF

For each test compound a solution of 200 µM in HMI-9 medium + 10%FCS is prepared using a 20 mM stock solution in DMSO; 200 µL of this is added to a first well of a 96-well plate. Of this, 100 µL is transferred to the next well, containing 100 µL of the same medium, achieving a 1:1 dilution, initiating a doubling dilution series across 2 rows of the plate; each experiment was positively controlled using pentamidine and the final well for each compound

received 100 microliters of HMI-9 medium as negative control. To each well, 100 µL of a suspension of 2 × 10^5 cells/ml was added, amounting to 1×10^5 cells/ml as the final cell density. The plate is then incubatedfor 48 hours at 37 ºC/5% CO_2 after which 20 µL of the Alamar Blue solution is added, followed by another incubation of the plate for 24 hours. A fluorimeter (FluoStar Optima) is used to read the fluorescence of the plate at the wavelengths of 590 nm for emission and 530 nm for excitation and the data are analysed using the GraphPad Prism5 software package, fitting the fluorescence to a sigmoid curve with variable slope to determine the EC_{50}value.

3. RESULTS AND DISCUSSION

In the present study, we demonstrated the effect of natural compounds on the growth of promastigotes of *L. major*, *L. mexican* and different strains of *Trypanosoma brucei brucei in vitro*. The antitrypanosomal and leishmanicidal activities of natural compounds are summarized in Table 1.

3.1 Apigenin and daidzein

Apigenin belongs to the flavone subclass of flavonoids (Kim, 2003) and is abundant in a variety of fruits, vegetables, and herbs. It frequently exists in food sources as a glycoside, which improves its solubility and bioavailability (Ross et al., 2002). An array of biological activities has been reported for apigenin, including the ability to inhibit proliferation and induce apoptosis in several cancer cell lines, as well as a capacity to inhibit angiogenesis (Zhou et al., 2017). Daidzein is a naturally occurring isoflavonic phytoestrogen, belonging to the non-steroidal estrogens, and is mainly derived from leguminous plants such assoybean and mung bean. Traditional Chinese medicine *Gegen* contains Daidzein as its main bioactive component is used often in the treatment of fever, acute dysentery, diarrhoea, diabetes, cardiac dysfunctions, liver injury etc (Knight et al., 1996).

The apigenin dimer amen to flavone, the only bioflavonoid investigated here, also showed some antileishmanial effect (IC_{50}, 6.0 µg/ml) (Tasdemir et al., 2006). In the current evaluation two different types of *Ttrypanosoma brucei brucei* were used including wild type (WT) and the highly resistant *Trypanosoma brucei* clonal line B48. Regarding possible mechanism of action, apigenin and daidzein showed highly similar effects on both strains, as presented in Table 1. EC_{50} values for both compounds for these compounds were found between 13.4 to 16.9 µM and the results indicate that the TbAT1/P2 and HAPT1 drug transporters are not utilized by either compound. Considering the virtually identical antitrypanosomal effects, the position of the 4-hydroxybenzene moiety on the main pharmacophore did not seem to be critical.

Table 1: Effect of selected natural compounds on *Trypanosoma brucei brucei* and *Leishmania mexicana* strains.

No.	**Compounds**	***Trypanosoma brucei brucei*** EC_{50} µM			***Leishmania promastigotes*** EC_{50} µM	
		(WT)	**(B48)**	**RF**	***L. major***	***L. mexicana***
1.	Apigenin	16.9±1.2	13.4±0.6	0.79	70.2±10.7	>100
2.	Daidzein	16.6±1.8	16.4±1.0	0.99	>100	>100
3.	Phyllanthin	66.6±2.3	64.5±3.6	0.97	>100	>100
4.	Tyramine	>100	>100	-	>100	>100
	Pentamidine	0.005±0.00	0.37±0.01	74.0	4.3±0.2	1.2±0.1

All EC_{50} values were obtained using the Alamar blue assay and are given as averages in µM (±SEM), of 3 independent evaluations. WT = wild-type sensitive control strain; B48 is a multi-drug resistant clone; Resistance Factor = EC_{50} (resistant clone)/EC_{50} (WT).

On the other hand, only apigenin showed some effect on *Leishmania major* promastigotes and there where as at concentrations up to 100 µM no antileishmanial activity was observed for daidzein; neither substance displayed activity against *L. mexicana* promastigotes.

3.2 Phyllanthin

Phyllanthin, found in many *Phyllanthus* species, has various biochemical and pharmacological properties especially hepatoprotective effects. Indeed, we have previously reported that among the bioactive constituents of *Phyllanthus amarus* Schum and Thonn (Euphorbiaceae), phyllanthin has been well examined for its biochemical and pharmacological properties, and particularly for its protective effects on the liver (Ilangkovan *et al*., 2016). Phyllanthin has also been much investigated for its antitumor effects on various cancer cell lines (Krithika *et al*., 2011). In the present research, phyllanthin was also examined for effects on *Trypanosoma brucei brucei* and *Leishmania mexicana*. Results are shown in table 1 and it was observed that this natural compound has similar if moderate effect on both strains of *Trypanosoma brucei brucei*, showing that phyllanthin is also not using specified drug transporters and that compounds of this class would not display cross-resistance with diamidines and melaminophenyl arsenical drugs.

3.3 Tyramine

Tyramine is a substance generally found in nature, as it can be formed from the amino acid tyrosine by the action of the ubiquitous enzyme aromatic amino acid decarboxylase. Tyramine is a vasoactive amine that promotes blood pressure elevation, resulting in pain. Tyramine leads to cerebral vasoconstriction and subsequent rebound vasodilatation that causes a migraine attack in susceptible persons (Costa *et al*., 2003). This compound was not found active against *Trypanosoma* and *Leishmania* strains and showed >100 µM EC_{50}.

4. Conclusion

In this study, some new natural compounds were selected for determination of their activity against various *Trypanosoma* strains and *Leishmania* species including multi-drug resistant *Trypanosoma brucei* clonal line B48.The results obtained herein revealed that the two related natural compounds, apigenin and daidzein displayed moderate activity against African

trypanosomes and are not cross-resistant with current dugs for this infection. A systematic investigation of this scaffold is likely to result in the identification of compounds with substantially improved activity against kinetoplastid parasites, particularly the African trypanosomes, which are not only the etiological agents of human sleeping sickness but also of the livestock disease nagana, which causes agricultural losses in Africa that measure in the billions of dollar (Giordani et al., 2016). For both conditions, new drugs are urgently needed.

Conflicts of Interest

The authors declare no conflict of interests.

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