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Anti-trypanosomal and cytotoxic activity of ethanolic extracts of *Psidium guajava* leaves in Alamar Blue based assays

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ABSTRACT

Ethanolic extracts prepared from the leaves of *Psidium guajava* were evaluated for anti-trypanosoma and cytotoxicity activity in the bloodstream species of *Trypanosoma brucei brucei* (BS427) and HEK293 in 384-well Alamar Blue assays respectively. Cytotoxicity activity in HEK293 cells was subsequently used to estimate the selectivity index of the extracts. The activities of the plant extracts were determined to evaluate if further chemical and biological profiling may be warranted for potential development in early drug discovery for African Sleeping Sickness. Two trypanocides, pentamidine and diminazene, were employed as reference drugs, while puromycin was also included as control for general cell growth inhibition. The results show that the extracts inhibited growth of *T. b. brucei* with an IC_{50} of 6.3 μg/mL and 48.9 μg/mL for 80% and 20% ethanolic preparations respectively, with corresponding activity of less than 50% against HEK293 at the highest screening dose of 238.10 μg/mL. The estimated selectivity index of the extracts compares favourably with pentamidine and diminazene. Meanwhile the reference compounds were found to have activities in agreement with published sensitivities at the doses screened. The lack of cytotoxicity at the doses screened and direct activity against *T. b. brucei* whole cells, make these extracts suitable candidates for further chemical elucidation and biological profiling.

Key words: Alamar Blue assay, *Psidium guajava, Trypanosoma brucei brucei,* viability, cytotoxicity

Introduction

Human African Trypanosomosis (HAT) is a disease caused by two trypanosome species and there is an approximate incidence of around 50,000 cases annually

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(ADEYEMI et al., 2011). Current drugs used for chemotherapy against HAT have problems with toxicity, duration and cost of treatment, and the need for intravenous injection. Melarsoprol, one of the few drugs effective against the second stage of the disease, is reported to cause encephalopathy in 10 - 15% of patients, of which 40% of cases are fatal (BLUM et al., 2001). These factors highlight the need to discover new active and less toxic compounds against HAT for future drug development. Natural products are a potential source of compounds for drug discovery as they contain a large number of molecules which may be bioactive. There are a number of anti-protozoal drugs which have natural product sources, including quinine and artemisinin for the treatment of malaria (HOET et al., 2004a). Traditional medicines have also used natural products, mainly of plant origin, for the treatment of a variety of diseases, including intestinal amoebiasis (TONA et al., 1999), malaria (ASASE and OPPONG-MENSAH, 2009), helmenitic diseases and bacterial infections (OLIVER-BEVER, 1983). There have been a number of reports on the activity of various plant extracts used in herbal medicines against the causative species of HAT, or *T. b. brucei*, a related non-infective species, commonly used in early drug discovery for HAT (YABU et al., 1998; HOET et al., 2004a; ADERBAUER et al., 2008; SHUAIBU et al., 2008). *Psidium guajava* or guava, has been used for a number of traditional medicine applications (GUTIERREZ et al., 2008; ELEKWA et al., 2009), including the decoction of the leaves or bark of *P. guajava* as a cure for diarrheal diseases (TONA et al., 1999), and relief of coughs, sore throats and inflamed gums (NWINYI et al., 2008). Preparations of the plant have also been used in traditional medicines for the treatment of malaria (ASASE and OPPONG-MENSAH, 2009) and trypanosomosis in animals (ATAWODI et al., 2002). *In vitro* activity of stem bark on chloroquine resistant *Plasmodium falciparum* has been reported (NUNDKUMAR and OJEWOLE, 2002). Recent studies have shown that an ethanolic extract of *P. guajava* leaf decreased *T. b. brucei* parasitaemia in rats and increased survival time in relation to untreated controls (ADEYEMI et al., 2009). Here the activities of the *P. guajava* extracts in comparison with reference compounds were investigated to determine if further chemical and biological profiling may be warranted for potential development in early drug discovery for African Sleeping Sickness.

Materials and methods

Plant collection and extract preparation. Fresh samples of *P. guajava* leaves were collected from a local farm in Ilorin, Kwara State, Nigeria. Relevant approval was duly obtained from the local authority prior to harvesting the plant leaves for this study. The leaves were identified and authenticated at the Herbarium Unit of the Department of Plant Biology, University of Ilorin, Nigeria and a voucher specimen was deposited in the department for reference purpose, Registry number 250808.

Two plant extracts were prepared with (A) 20% v/v ethanol/distilled water and (B) 80% v/v ethanol/distilled water. The leaf extract of the plant was prepared according to the method described by VIEIRA et al. (2001). Fresh samples of *P. guajava* leaves were air dried and ground. A portion of the ground sample, weighing 300 g, was soaked in 20% (v/v) ethanol/distilled water and left for 24 hours. The mixture was filtered using Whatman No. 1 filter paper and the filtrate concentrated in vacuo. The concentrate was then evaporated to dryness at 40 °C to obtain a dry extract sample, which was subsequently used to prepare the extract (A). The percentage yield was 2.46% (7.38 g). For extract B, a portion of the ground sample weighing 300 g was soaked in 80% (v/v) ethanol/distilled water and left for 24 hours. The mixture was filtered using Whatman No. 1 filter paper and the filtrate concentrated in vacuo. The concentrate was then evaporated to dryness at 40 °C to obtain a dry extract sample, which was subsequently used to prepare extract (B). The percentage yield was 3.98% (9.73g).

Extracts were vortexed in DMSO at high speed for approximately two minutes, mixed on a heated magnetic stirrer at 40 °C for 15 minutes, followed by sonication for fifteen minutes at room temperature. The highest concentration solubilised using this approach was 60 mg/mL for both extracts A and B.

T. b. brucei Alamar Blue viability assay. T. b. brucei bloodstream strain 427 (BS427) were maintained in HMI-9 media (HIRUMI and HIRUMI, 1989) at a maximum concentration of 1-2 x10^6 cells/mL. Cells were maintained in 25 cm^2 tissue culture flasks at 37 °C and 5% CO_2 in a humidified environment. The assay was performed as described previously by SYKES and AVERY (2009). Briefly, cells were diluted in HMI-9 media and 55 µL per well were inoculated into black clear bottomed 384 well plates (Biosciences, Franklin Lanes, NJ USA) as previously described. Plates were then incubated for 24 hours at 37 °C and 5% CO_2. Two separate plates were prepared: one containing triplicate serial dilutions of extracts dissolved in 100% DMSO and controls in 100% DMSO. Screening controls consisted of one column of DMSO as the positive assay control, and one column of 2 mg/mL pentamidine in DMSO for 100% inhibition of growth. The second plate contained triplicate serial dilutions of the reference compounds pentamidine (Sigma, MO, USA), diminazene (Sigma, MO, USA) and puromycin (Calbiochem, LA, USA) in DMSO. Samples in each plate were diluted 1:21 in DMEM high glucose, 1% sodium pyruvate, 1% NEAA using a MinitrackTM robotic liquid handler (PerkinElmer, MA, USA) before addition of 5 µL of this volume to the assay plate. Plates were incubated for a further 48 hours before addition of a final 10% concentration of Alamar Blue™ (Biosource, MD, USA). Incubation was continued for 2 hours at 37 °C and 5% CO_2, then at room temperature for 22 hours. Wells were read at excitation 535 nm, emission 590 nm on a Victor IITM Wallac plate reader (PerkinElmer, MA, USA). Extract and control experiments were repeated as three independent experiments and reference compounds repeated twice, with triplicates in each experiment.

HEK293 cytotoxicity assay. HEK293 cells (ATCC) were maintained in 75 cm^2 tissue culture flasks in DMEM high glucose, 1% sodium pyruvate, 1% NEAA and 10% FBS. Cells were harvested from 70-80% confluent flasks and diluted to a density of 7.27 x 10^4 cells/mL before inoculation of 55 µL into wells of black clear bottom 384 well plates (Biosciences, Franklin, NJ, USA). Extract and reference compound plates were prepared as described for the *T. b. brucei* assay, the exception was replacement of pentamidine as a negative growth control with no cell addition in one column of the plate. These plates were diluted to 1:21 in DMEM high glucose, 1% sodium pyruvate, 1% NEAA without FBS, using a MinitrackTM robotic liquid handler before addition of 5 µL of the diluted extract and reference compound samples to the cells. The additions of Alamar Blue and incubations were performed as described for the *T. b. brucei* assay, with the Alamar Blue dilution made in DMEM high glucose,1% sodium pyruvate,1% NEAA and 10% FBS. Extract and control experiments were repeated three times and reference compounds twice, with triplicates in each experiment.

Reference compound and extract activity analysis. The IC_{50} values of reference compounds and extracts were calculated by plotting % inhibition (100% inhibition was equal to 2 µM final pentamidine in the *T. b. brucei* assay and no cells (media only) in the HEK293 assay, against log [reference compound/ extract] in GraphPad Prism 5. The IC_{50} value was the concentration of compound estimated to result in 50% reduction in growth. One hundred % growth was calculated as an average of the DMSO control wells. IC_{50} results were expressed as a mean ± SD.

Results

Extract activity in T. b. brucei and HEK293 viability assays. The IC_{50} values obtained for extracts are shown in Table 1 and Figs. 1 - 4. Extract A had an IC_{50} value of 48.94 ± 1.52 µg/mL, based on data obtained from three biological replicates, using dilutions made from a 238.10 µg/mL final assay concentration of extract. The estimated IC_{50} value obtained for extract B was 6.33 ± 0.58 µg/mL. Cytotoxicity testing in the Alamar Blue HEK293 cell viability assay showed that extract A was 24.16 ± 5.10% active and extract B was 30.05 ± 4.89% active at 238.10 µg/mL. Both of the extracts displayed less than 50% activity at a final assay concentration of 238.10 µg/mL, with extract A exhibiting at least a 23 fold selectivity index and B at least a 5 fold selectivity index.

Table 1. Activity of *P. guajava* extracts and a panel of reference compounds in the *T. b. brucei* and HEK293 Alamar Blue assays

	IC50 T. b. brucei	IC50 HEK293, or % activity at dose	Selectivity index
Extract A (20%)	48.94 ± 1.52[a]	24.16 ± 5.10%[c]	>23x
Extract B (80%)	6.33 ± 0.58[a]	30.05 ± 4.89%[c]	>5x
Pentamidine	3.64 ± 0.21[b]	4.65 ± 9.52%[d]	>91x
Diminazene	41.12 ± 3.63[b]	0.40 ± 10.94%[e]	>936x
Puromycin	65.15 ± 14.78[b]	619.3 ± 47.65	9.5x

a = µg/mL, b = nM, c = at 238.10 µg/mL, d = 0.33 µM, e = 38.5 µM

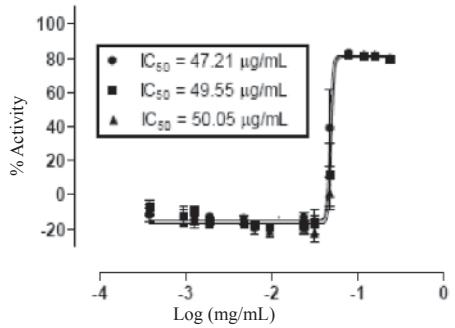

Fig. 1. Activity of *P. guajava* leaf (20%) ethanolic extract in the *T. b. brucei* Alamar Blue assay

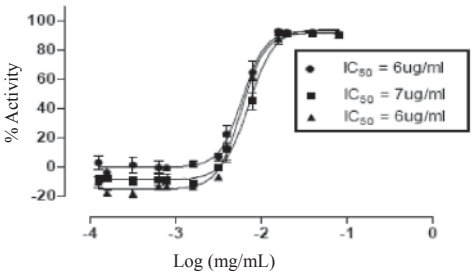

Fig. 2. Activity of *P. guajava* leaf (80%) ethanolic extract in the *T. b. brucei* Alamar Blue assay

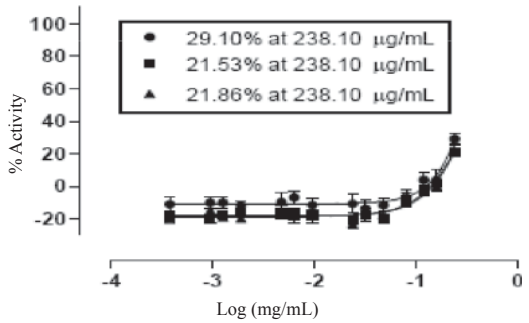

Fig. 3. Activity of *P. guajava* leaf (20%) ethanolic extract in the HEK293 Alamar Blue assay

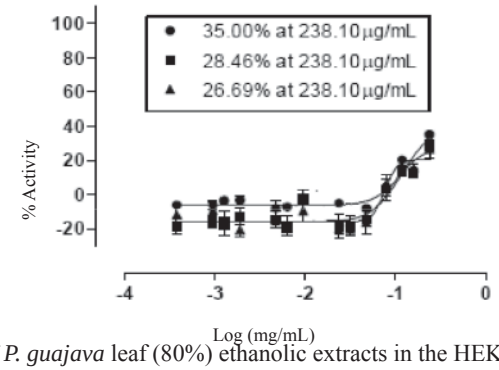

Fig. 4. Activity of *P. guajava* leaf (80%) ethanolic extracts in the HEK293 Alamar Blue assay

Reference compound activities in T. b. brucei and HEK293 viability assays. The activities of the reference drugs are shown in Table 1 and Figs. 5 - 6. The reference compounds did not exhibit activity greater than 50% in the HEK293 assay at the highest doses screened (0.33 µM and 38.5 µM for pentamidine and diminazene respectively). Therefore the selectivity index was at least 91 fold for pentamidine and at least 936 fold for diminazene. However for puromycin, activity in the HEK293 assay was greater than 50% with a lower selectivity index.

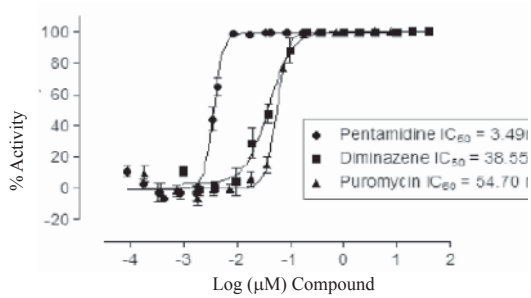

Fig. 5. Reference compound activity in the *T. b. brucei* Alamar Blue assays

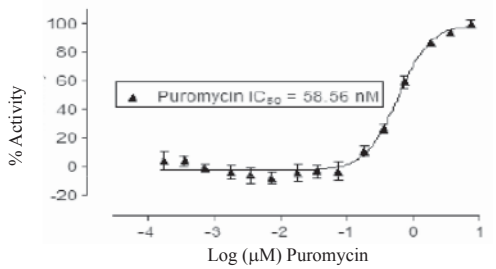

Fig. 6. Reference compound activity in the HEK293 Alamar Blue assay

Discussion

It has been reported that *P. guajava* leaf extracts demonstrated no cytotoxicity in clinical trials with humans (GUTIERREZ et al., 2008). Moreover a previous study detailing the cytotoxic and immune-modulatory NF-kappa B activities of *P. guajava* extract against MDA-MB231 and MCF7 cells was demonstrated (KAILEH et al., 2007). In a separate study, LING et al. (2010) reported that some ethanolic extracts including that of *P. guajava* lack cytotoxicity in assays involving 3T3 and 4T1 cells. However, to our knowledge, this is the first report of direct *in vitro* anti-*T. b. brucei* activity of *P. guajava* extracts and supports previous observations of *in vivo* activity of a leaf extract to reduce parasitaemia in *T. b. brucei* infected rats (ADEYEMI et al., 2009). As a measure of the *T. b. brucei* assay performance, a series of reference compounds were screened alongside the assay to determine whether the trypanosome culture was being inhibited by known compounds, as previously characterised in this and other assay systems (SYKES and AVERY, 2009). Extract A had an IC_{50} value of 48.94 ± 1.52 μg/mL, based on data obtained from three biological replicates, using dilutions made from a 238.10 μg/mL final assay

concentration of extract. The estimated IC_{50} value obtained for extract B was 6.33 ± 0.58 μg/mL. Both of the extracts displayed less than 50% activity at a final assay concentration of 238.10 μg/mL, with extract A exhibiting at least a 23 fold selectivity index and B at least a 5 fold selectivity index which compare favourably with the reference drugs, except for puromycin, which had cytotoxicity activity greater than 50%. Pentamidine and diminazene did not exhibit activity greater than 50% in the HEK293 assay at the highest doses screened (0.33 μM and 38.5 μM for pentamidine and diminazene respectively). The selectivity index was at least 91 fold for pentamidine and at least 936 fold for diminazene. Puromycin is a general cell growth inhibitor of both eukaryotic and prokaryotic cells, with a mode of action via disruption of protein synthesis. Puromycin was found to be active on both cell lines, with less than 10 times the selectivity index estimated, which would be expected for such a compound with a non-specific mode of action. Pentamidine is a trypanocide used to treat HAT and diminazene is a veterinary drug used to treat *T. b. brucei* disease in animals. Both of these known drugs had IC_{50} values in agreement with relatively low nM sensitivities as previously observed in the Alamar Blue assay in 384 well format, (29.8 nM and 91.8 nM, respectively; SYKES and AVERY, 2009) and also reported in 96 well formats (STEWART et al., 2005; LANTERI et al., 2006; MERSCHJOHANN and STEVERDING, 2006). Diminazene has been reported to have activities in various mammalian cancer cell lines, which have been commonly reported in the literature to determine cytotoxicity, including: a 3670 x selectivity index between HL-60 cells and *T. b. brucei* cells in an Alamar Blue assay (MERSCHJOHANN et al., 2001); an MIC of greater than 20 μM in an Alamar Blue assay estimating J774 macrophage cell viability (HOET et al., 2004); greater than 10-100 μM in a panel of carcinoma cell lines in an MTT assay (GONZALEZ et al., 1999) and 93.5 μM for L6 cells in an Alamar Blue assay (STEWART et al, 2005). Pentamidine has reported activities including an IC_{50} of 162 μM against empty vector transfected CHO cells (MING et al., 2009) and an IC_{50} value of 46.6 μM (BAKUNOV et al., 2008) and 2.53 μM (STEWART et al., 2005) against L6 cells in Alamar Blue based assays. These results are in agreement with the level of activity at the screened doses of these known drugs exhibited in the HEK293 assay. The assays reported here agree with previous reports of *T. b. brucei* and some mammalian cell line sensitivities at the screened doses for the series of compounds. This supports the validity of the estimated extract activity in these assays. This preliminary study suggests that the *P. guajava* leaf contains compounds with a synergistic effect that may have anti trypanosomal/ specific anti- HAT activity. Based upon the data presented here, further studies are warranted to include chemical isolation and biological profiling of the compounds in these extracts for potential development in early HAT drug discovery.

Conclusion

The lack of cytotoxicity at the doses screened and direct activity against *T. b. brucei* whole cells make these extracts suitable candidates for further chemical elucidation and biological profiling. Based upon the anti-*T. b. brucei* activity and selectivity of the extracts on *T. b. brucei* cells compared with HEK293, we recommend these extracts for further chemical analysis to identify the active principles that may be responsible for this activity.

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Declaration of Interests: The authors report no conflict of interest.

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SAŽETAK

Istraženo je tripanocidno djelovanje etanolskog iscrpka lišća *Psidium guajava* na vrstu *Trypanosoma brucei brucei* (BS427) i njegova citotoksičnost na stanice HEK293 bojanjem alamarskim plavilom u 384 jažice. Citotoksični učinak na stanice HEK293 rabljen je za procjenu indeksa selektivnosti. Učinkovitost biljnih iscrpaka određivana je da bi se procijenila svrhovitost budućih kemijskih i bioloških istraživanja potencijalnoga lijeka za afričku bolest spavanja. U istraživanju su rabljena dva tripanocida, pentamidin i diminazen, te puromicin kao sredstvo koje usporava rast stanica. Rezultati su pokazali da 80% etanolskih pripravaka s IC_{50} od 6,3 μg/mL koči rast i razvoj tripanosoma, a samo 20% onih s IC_{50} od 48,9 μg/mL, s odgovarajućom aktivnosti manjom od 50% na stanice HEK293 u najvećoj dozi od 238,10 μg/mL. Indeks selektivnosti iscrpaka bio je sukladan s aktivnošću pentamidina i diminazena. Aktivnost istraživanih sastojaka bila je sukladna s razinom prije objavljene osjetljivosti. Izostanak citotoksičnosti na razini rabljenih koncentracija i izravna djelotvornost na stanice *T. b. brucei* daju osnovu za daljnja kemijska i biološka istraživanja predmetnih pripravaka.

Ključne riječi: alamarsko plavilo, *Psidium guajava, Trypanosoma brucei brucei,* vitalnost, HEK293, citotoksičnost