Original Article

In vitro Antiparasitic and Anti-severe Acute Respiratory Syndrome Coronavirus 2 Activities of Extracts and Fractions from *Pouteria alnifolia*

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Abstract

Background: Although there is widespread use of herbal medicine in many parts of the world including tropical Africa, scientific evidence to validate efficacy and safety is largely scarce. The aim of this study was to investigate the potential of *Pouteria alnifolia* as an alternative anti-infective. **Methods:** In this study, bioactivity-guided isolation of extracts from different parts of *P. alnifolia* was done, and the *in vitro* antitrypanosomal, antileishmanial, anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and cytotoxicity properties of the extracts and fractions were determined using the alamarBlue assay. Using microscopy, the growth kinetics of *Trypanosoma brucei brucei* was investigated by counting cells when parasites were exposed to different concentrations of selected extracts and fractions. **Results:** The results show that while anthracene and anthraquinone derivatives were absent in all the tested fractions, terpenoids, and cardiac glycosides were the most common phytochemicals in the fractions. Alkaloids were also detected in the dichloromethane (DCM) extracts of seeds and whole fruit (WF) compared to butanol (BuOH) fractions of the fruit pulp, stem, and roots. The IC_{50} values for *T. b. brucei* were between 07 ± 0.0 and 59.5 ± 0.3 µg/mL while that of *Leishmania donovani* ranged from 1.3 ± 0.1 to 27.8 ± 1.8 µg/mL. DCM extracts from WF, seeds, and stem inhibited the growth of *T. b. brucei*, whereas the BuOH fractions from seeds and WFs were most active against SARS-CoV-2. **Conclusion:** Extracts and fractions from *P. alnifolia* appear to have useful chemotherapeutic agents that warrant further study for a variety of infections faced by those in tropical Africa.

Keywords: Herbal medicine, *Leishmania donovani*, *Pouteria alnifolia*, severe acute respiratory syndrome coronavirus 2, *Trypanosoma brucei brucei*

Introduction

In many parts of Africa, the use of herbal medicines to treat infections is common practice and plays an important role in the healthcare system, especially in regions that have limited access to orthodox medicines. The use of many of these traditional herbal medicines is based on accumulated knowledge obtained and passed down from individuals that prepare and administer them. However, despite their frequent use, many of these plant preparations still lack in-depth scientific validation on efficacy and safety.[1,2]

Pouteria alnifolia Roberty Bak belongs to the family of plants called *Sapotaceae* family. The *Pouteria* genus is made up of over three hundred species which are primarily found in

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tropical and subtropical regions of Asia, South America, and several regions in Africa.[3,4] *P. alnifolia* is known locally in Ghana as "asamfona" with edible fruit and oil from the seeds. Several species of the *Pouteria* genus have been used in folk medicine, and *P. alnifolia* is used to treat different infectious diseases such as yaws, as well as wounds, conjunctivitis, diarrhea, and ulcers.[5] Given the rich traditional use of *P. alnifolia*, an in-depth study of its anti-infective and other *in vitro* properties is warranted.

African trypanosomiasis (sleeping sickness) and leishmaniasis are two Neglected Tropical Diseases (NTDs) caused, respectively, by *Trypanosoma brucei* and *Leishmania donovani*. Treatment for these NTDs is over-reliant on a few drugs that are associated with severe toxicity and drug resistance and run the risk of giving rise to drug-induced resistance.[6,7] In addition, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, continues to inflict people in West Africa, where full availability of SARS-CoV-2 vaccines has not been achieved.[8,9] Of particular importance is the impact of comorbidities of infections such as SARS-CoV-2 coupled with co-infections with diseases such as African trypanosomiasis and leishmaniasis, which may influence clinical outcomes and complicate management.[10,11] Thus, any natural product or medicinal plant extract/fraction with broad-spectrum anti-infective activity may have an additional benefit of targeting multiple pathogens that makeup coinfections. In this study, the *in vitro* antitrypanosomal, antileishmanial, and anti-SARS-CoV-2 activities of extracts and fractions from different parts of *P. alnifolia* including the leaves, whole fruits (WFs), seeds, fruit pulps (FPs), stem bark, and roots were investigated.

Methods

All solvents used were of analytical grade. Organic solvents used included hexane (H), petroleum ether (P), chloroform ($CHCl_3$), ethyl acetate (EtOAc), butanol (BuOH), ethanol (EtOH), and methanol (MeOH). Cold percolation was used for the extraction of the pulverized plant material. Evaporation of solvents from extracts was done under pressure using a Cole-Parmer Rotary Evaporator (Vernon Hills, Illinois), and the solvents recovered were re-used in the subsequent extractions.

Ethical considerations

In this study, extracts and fractions of *P. alnifolia* plant parts were used against the experimental microbes, *Trypanosoma brucei brucei*, *L. donovani,* and SARS-CoV-2. Ethical approval was obtained from the University of Ghana Institutional Animal Care and Use Committee (UG-IACUC) with approval number UG-IACUC 006/21-22. The study did not involve any human subjects; hence, human ethical considerations were not applicable.

Type of sampling and reasons for selection

Random sampling was used in this study. The protozoan parasites, human monocytic cell line (THP-1), and SARS-CoV-2 were cultured, and growing cells with good morphological features were used for the inhibitory and cytotoxicity assays.

Inclusion criteria

Experimental microbes and human monocytic cell line (THP-1) were appropriately cultured, and those with >90% viability were used in the assays.

Exclusion criteria

Protozoan parasites, SARS-CoV-2, and human monocytic cell line (THP-1) cultures with <90% viability were not used in the assays.

Data availability statement

The data supporting the findings of this study are available within the article and its supplementary materials.

Extraction and fractionation of *Pouteria alnifolia* plant parts

After plants collection [Supplementary File], extraction and fractionation of the six plant part materials were carried out based on the modified Kupchan method as described by Dofuor *et al*.[12] An amount of 560 g of the pulverized WFs and 565 g of seeds (S) were each repeatedly extracted with 5 L of 70% EtOH, whereas 450 g of the FP was extracted with 4 L of 70% EtOH at room temperature [Table 1]. Each plant material was filtered after 48 h and the solvent was removed *in vacuo* to give concentrates coded as 70% ethanol of *P. alnifolia* whole fruit (PAWF), 70% ethanol of *P. alnifolia* seeds (PAS), and 70% ethanol of *P. alnifolia* fruit pulp (PAFP). In a similar manner, 1.7 kg of the stems (St), 550 g of the leaves (L), and 2.4 kg of the roots (R) were also each extracted in 5 L, 2 L, and 10 L of 70% EtOH, respectively. Evaporation of each extract gave the 70% ethanol of *P. alnifolia* leaf extract (PAL), 70% ethanol of *P. alnifolia* stem extract (PASt), and 70% ethanol of *P. alnifolia* root extract (PAR).

Table 1: Preparation of plant parts

Part of *P. alnifolia*	Mass(g)	Volume of 70% Ethanol (L)	Extract code	Code interpretation
Whole fruits	560	5	PAWF	70% ethanol of *P. alnifolia* whole fruit
Seeds	565	5	PAS	70% ethanol of *P. alnifolia* seeds
Fruit pulp	450	4	PAFP	70% ethanol of *P. alnifolia* fruit pulp
Leaves	1700	5	PAL	70% ethanol of *P. alnifolia* leaf
Stem bark	550	5	PASt	70% ethanol of *P. alnifolia* stem
Roots	2400	10	PAR	70% ethanol of *P. alnifolia* roots

P. alnifolia: *Pouteria alnifolia*

Fractionation of extracts from PAWF, PAS, PAFP, PAL, PASt, and PAR of *Pouteria alnifolia*

Each of the six 70% EtOH extracts (PAWF, PAS, PAFP, PAL, PASt, and PAR) was reconstituted in 10% aqueous EtOH and successively partitioned with 1.5 L of hexane (H) or petroleum ether (P), dichloromethane (DCM) (D) or $CHCl_3$ (C), EtOAc (E), and BuOH (B). The remaining aqueous fractions (aq) were freeze-dried. Example, the fractions obtained from the 70% EtOH extract of *P. alnifolia* whole fruit (PAWF) were coded as PWFH (hexane fraction from PAWF), PWFD (DCM fraction from PAWF), PWFE (EtOAc fraction from PAWF), PWFB (BuOH fraction from PAWF), andaqueous fractions from PAWF (PAWFaq). Fractions from the 70% EtOH extract of *P. alnifolia* seeds (PAS) extract were also coded accordingly as hexane fraction from PAS (PSH), DCM fraction from PAS (PSD), EtOAc fraction from PAS (PSE), BuOH fraction from PAS (PSB), and aqueous fraction from PAS (PASaq), whereas the fractions from the PAFP extract included PFPH, PFPD, PFPE, PFPB, and aqueous fraction from PAFP (PAFPaq). In a similar manner, fractions obtained after the partitioning of the PAL extract were coded as PLH, PLD, PLE, PLB, and PALaq, those of PASt extract are PStH, PStD, PStE, PStB, and PAStaq and finally from the PAR extract gave PRP, PRC, PRE, PRB, and aqueous fraction from PAR (PARaq) fractions [Figure 1].

In vitro antitrypanosomal and antileishmanial activities and cytotoxicity assay

The alamarBlue assay was used to determine the effect of extracts and fractions on *T. b. brucei* GUTat 3.1 bloodstream forms and *L. donovani* (MHOM/SD/62/1S) promastigotes as described previously.[12-14] For the *T. b. brucei* GUTat 3.1 parasites, the extracts and fractions were serially diluted (range 0–100 µg/mL) into a Costar flat bottom 96-well plate using HMI-9 media. A final parasite concentration of 4000 cells (100 µL) was added to each well and the plates were incubated in 5% CO_2 for 72 h at 37°C. *L. donovani* promastigotes (2×10^5 cells) were grown for 72 h at 25°C in freshly made M-199 medium either without or with varying concentrations of the extracts and fractions (0–100 µg/mL). To determine the cytotoxicity of extracts and fractions THP-1 cells were grown at 1×10^5 cells/mL in two series of passages in freshly prepared RPMI 1640 media supplemented with 10% FBS and 100 µg/mL penicillin/streptomycin. In a Costar flat bottom 96-well plate, THP-1 cells were treated with varying concentrations of the extracts or fractions (0–100 µg/mL). The plates were incubated in 5% CO_2 for 72 h at 37°C. A final concentration of resazurin sodium salt (44 µM) (Sigma-Aldrich, USA) prepared in phosphate-buffered saline was added to each well, and the different plates were incubated accordingly for a further 5 h. The Varioskan Lux Elisa plate reader (Thermo Scientific) was used to measure the absorbance at 570 nm. The absorbances obtained were extracted into GraphPad Prism (version 6) (GraphPad Software, Boston, Massachusetts, USA) and the half-maximal inhibitory concentrations (IC_{50}) values were determined. The IC_{50} reported for the extracts and fractions is the mean from three independent experiments. Diminazene aceturate, a known antitrypanosomal drug, and Amphotericin B, a known antileishmanial drug were used as positive controls. The CC_{50} reported is the mean from three independent experiments. Selectivity indices (SI) were then estimated as the ratio of the CC_{50} to IC_{50} values.

Antiviral activity of fractions of plant parts

Vero-E6 cells were obtained from the American Tissue Culture Collection and cultured in D10+ medium (Dulbecco's modified Eagle medium) with 4.5 g/L glucose and L-glutamine (Gibco, Gaithersburg, MD, USA), 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA), and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 37°C and 5% CO_2. The following reagent was deposited at the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: SARS-related coronavirus 2, isolate USA-WA1/2020, NR-52281. Virus stocks for infection studies were performed as described previously.[15]

For viral infection assays, Vero-E6 cells were first plated in D10+ at 10^5 cells/mL in 96-well format, treated with plant extracts, fractions, or remdesivir control (Sigma-Aldrich) at desired concentrations in 3-fold replicates, and incubated for 2 h. Following incubation, cells were infected with 150x TCID50 of the virus and incubated for an additional 96 h. Cells were then treated and incubated with 20 µg/mL of resazurin (Sigma-Aldrich) for 4 h and then fixed with paraformaldehyde to a final concentration of 4% at room temperature for 30 min to inactivate virus. Fluorescence intensity was measured using a ClarioStar microplate reader (BMG Lab Tech, Cary, NC, USA). Fluorescence intensities were normalized such that uninfected cell controls on each plate were denoted as 100% viability, while infected cells without extract, fractions, or drug treatment were denoted as 0% viability.

For cell viability assays, experiments were performed as described except for no virus infection. Fluorescence intensities were normalized such that untreated cell controls on each plate were denoted as 100% viability, whereas wells with D10+ medium only were denoted as 0% viability. All data are presented as the mean ± standard deviation (SD) from at least two independent experiments.

Statistical analysis

All experimental data were generated from at least two to three independent experiments, each performed in triplicate unless otherwise stated. Quantitative results are presented as mean ± SD. For the determination of inhibitory concentrations (IC_{50}) and cytotoxic concentrations (CC_{50}), dose–response curves were plotted using GraphPad Prism (version 6). Nonlinear regression analysis (log [inhibitor] vs. normalized response – Variable slope) was used to fit the data and the IC_{50} and CC_{50} values for each extract and fraction were calculated. For the anti-parasitic and cytotoxicity assays, the percentage inhibition or cell viability was calculated relative to untreated controls, which were set at 100%. For the anti-SARS-CoV-2 assays, fluorescence intensities were normalized such that

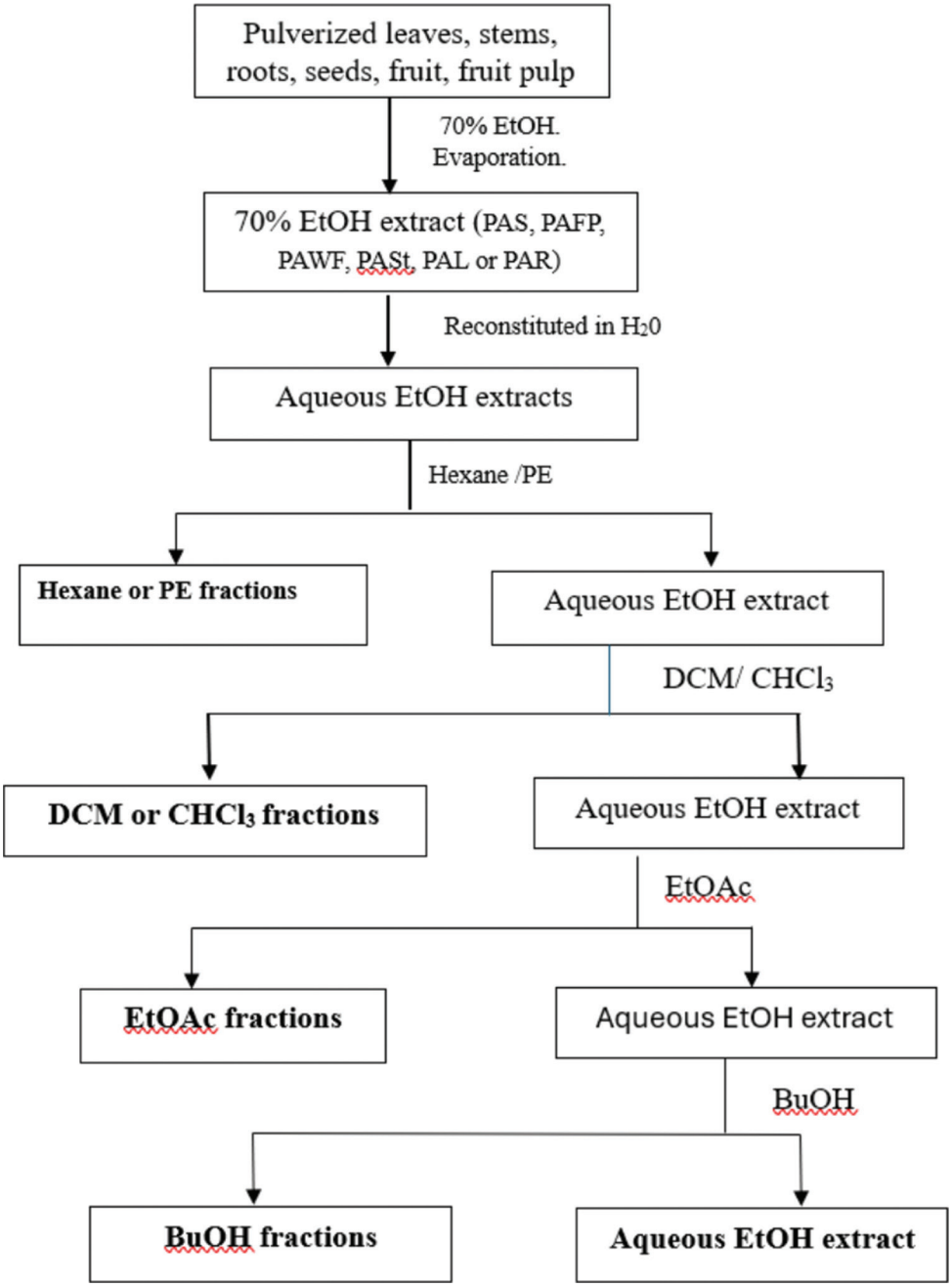

Figure 1: Scheme for the extraction and fractionation of leaves, stems, roots, and fruits of *Pouteria alnifolia* plant parts. PAL: 70% EtOH *P. alnifolia* leaf extract, PASt: 70% EtOH *P. alnifolia* stem extract, PAR: 70% EtOH *P. alnifolia* root extract, PAS: 70% EtOH *P. alnifolia* seed extract, PAFP: 70% EtOH *P. alnifolia* fruit pulp extract, PAWF: 70% EtOH *P. alnifolia* whole fruit extract

uninfected cell controls represented 100% viability and infected, untreated controls represented 0% viability. SI was calculated as the ratio of CC_{50} to IC_{50} for each extract or fraction. Where comparisons between multiple extracts or fractions were required, one-way analysis of variance was performed followed by Tukey's *post hoc* test to determine statistically significant differences between groups. A $P < 0.05$ was considered statistically significant. All statistical analyses and graphical representations were performed using GraphPad Prism (version 6). The results are reported as mean ± SD, and detailed data were available in the supplementary materials.

Data availability statement

The data are available from the corresponding author and will be shared upon reasonable request.

Results

Screening of *Pouteria alnifolia* stems, leaves, and roots fractions revealed the presence of various phytochemicals

Different phytochemical screening tests were used to determine the presence of phytochemicals in the different parts of *P. alnifolia* [Tables 2, 3 and Supplementary File]. Of the 24

fractions tested, terpenoids constituted the most abundant and were present in all fractions except the EtOAc fraction of the leaves (PLE) and BuOH fractions of both the whole fruit (PWFB) and fruit pulp (PFPB). Cardiac glycosides were also present in all the fractions of the leaves, stems, roots, and seeds but were absent from the FP and the hexane, and DCM fractions of the WF [Tables 2-4]. Anthracene and anthraquinone derivatives were not detected in all the fractions except in the hexane extract of the stem, PStH [Table 2]. Saponins were present in only five of the 24 fractions [Table 3]. Tannins were found in some of the stem fractions (PStD, PStE, and PStB) and the FP (PFPD, PFPE) as well as in PSE, PRE, and PWFE fractions [Tables 2 and 3].

Flavonoids were present in all fractions of the FP and some fractions of the WF, stem, and roots. Phenols were present in some fractions of the stem (PStD, PStB) and FP (PFPD and PFPE) [Tables 2 and 3] as well as in PSE, PRE, and PWFD. Alkaloids were present in the hexane and DCM extracts of the seeds and the WF as well as in the EtOAc and BuOH fractions of the FP, stem, and roots [Tables 2 and 3]. Cardiac glycosides and terpenoids were present in the PLH, PLD, and PLB. Only PStH showed the presence of anthracene and anthraquinones derivatives [Table 2]. Apart from anthracene and anthraquinone derivatives, all the other tested phytochemistry were present in PStD and PStB [Tables 2-4].

Extracts have potent antitrypanosomal and antileishmanial activities

In vitro activities of *P. alnifolia* plant parts were determined on *T. b. brucei, L. donovani, and* THP-1 cells using the alamarBlue assay. The absorbances obtained were used to calculate the half-maximal inhibitory concentrations (IC_{50}) values for each of the extracts and fractions [Tables 5 and 6]. Thirty-eight extracts and fractions were tested against *T. b. brucei* and 10 extracts and fractions were tested against *L. donovani.* The IC_{50} values for *T. b. brucei* were between 07 ± 0.0 and 59.5 ± 0.3 µg/mL, whereas that of *L. donovani* ranged from 1.3 ± 0.1 to 27.8 ± 1.8 µg/mL with PRP having the best antitrypanosomal activity [Table 5]. Ten out of 23 extracts and fractions tested (43.5%) showed SI of >10, indicating good selectivity against the trypanosome parasites.

Anti-leishmanial activity was done on 10 extracts and fractions that showed promising antitrypanosomal activity and SI. Of these PWFB, PWFD, PWFE, and PWFH of WF showed the best antileishmanial activities with IC_{50} values of 1.3 ± 0.1–1.6 ± 0.1 (µg/ml) and SI of 17.1–157.0 [Table 6]. DCM

Table 2: Phytochemical screening of *Pouteria alnifolia* stem and leaves

Phytochemicals	PASt				*P. alnifolia* leaves			
	PStH	PStD	PStE	PStB	PLH	PLD	PLE	PLB
Saponins	–	+	–	+	–	–	–	–
Tannins	–	+	+	+	–	–	–	–
Flavonoids	–	+	–	+	–	–	–	–
Phenols	–	+	–	+	–	–	–	–
Terpenoids	+	+	+	+	+	+	–	+
Alkaloids	–	+	+	+	–	–	–	–
Cardiac glycosides	+	+	+	+	+	+	+	+
Anthracene and anthraquinones	+	–	–	–	–	–	–	–

+: Means present, –: Means not present. *P. alnifolia: Pouteria alnifolia*, PASt: 70% EtOH *P. alnifolia* stem extract, PStH: Hexane fraction from PASt. PStC: $CHCl_3$ fraction from PASt, PStD: DCM fraction from PASt, PStE: EtOAc fraction from PASt, PStB: BuOH fraction from PASt, PASt aq: Aqueous fraction from PASt after partitioning, PLH: Hexane fraction from *P. alnifolia* leaf, PLD: DCM fraction from *P. alnifolia* leaf, PLE: EtOAc fraction from *P. alnifolia* leaf, PLB: BuOH fraction from *P. alnifolia* leaf, PAL*aq*: Aqueous fraction from PAL after partitioning

Table 3: Phytochemical screening of *Pouteria alnifolia* roots and fruit pulp

Phytochemicals	*P. alnifolia* roots				*P. alnifolia* fruit pulp			
	PRP	PRC	PRE	PRB	PFPH	PFPD	PFPE	PFPB
Saponins	–	–	–	–	–	–	–	+
Tannins	–	–	+	–	–	+	+	–
Flavonoids	–	+	+	+	+	+	+	+
Phenols	–	–	+	–	–	+	+	–
Terpenoids	+	+	+	+	+	+	+	–
Alkaloids	–	–	+	+	–	–	+	+
Cardiac glycosides	+	+	+	+	–	–	–	–
Anthracene and anthraquinones	–	–	–	–	–	–	–	–

+: Means present, –: Means not present. PAR: 70% EtOH *P. alnifolia* root extract. PRP: Petroleum ether fraction from PAR, PRC: $CHCl_3$ fraction from PAR, PRE: EtOAc fraction from PAR, PRB: BuOH fraction from PAR, PARaq: Aqueous fraction from PAR after partitioning, PAFP: 70% EtOH *P. alnifolia* fruit pulp extract, PFPH: Hexane fraction from PAFP, PFPD: DCM fraction from PAFP, PFPE: EtOH fraction from PAFP, PFPB: BuOH fraction from PAFP, PAFPaq: Aqueous fraction from PAFP after partitioning

Table 4: Phytochemical screening of *Pouteria alnifolia* seeds and whole fruit

Phytochemicals	*P. alnifolia* seeds				*P. alnifolia* whole fruit			
	PSH	PSD	PSE	PSB	PWFH	PWFD	PWFE	PWFB
Saponins	–	–	+	–	–	–	+	–
Tannins	–	–	+	–	–	–	+	–
Flavonoids	–	–	+	–	+	+	+	–
Phenols	–	–	+	–	–	+	–	–
Terpenoids	+	+	+	+	+	+	+	–
Alkaloids	+	+	–	–	+	+	–	–
Cardiac glycosides	+	+	+	+	–	–	+	+
Anthracene and anthraquinones	–	–	–	–	–	–	–	–

+: Means present, –: Means not present. PAS: 70% EtOH *P. alnifolia* seed extract, PSH: Hexane fraction from PAS, PSD: DCM fraction from PAS, PSE: EtOAc fraction from PAS, PSB: BuOH fraction from PAS. PASaq: Aqueous fraction from PAS after partitioning, PAWF: 70% EtOH *P. alnifolia* whole fruit extract, PWFH: Hexane fraction from PAWF, PWFD: DCM fraction from PAWF, PWFE: EtOAc fraction from PAWF. PWFB: BuOH fraction from PAWF, PAWFaq: Aqueous fraction from PAWF after partitioning

Table 5: Mean antiparasitic activities and selectivity indices of *Pouteria alnifolia* extracts and fractions of the roots, seeds, and stem against *Trypanosoma brucei brucei and Leishmania donovani* promastigotes

Plant part extract	Fraction code	IC_{50}±SD (μg/mL) for *T. b. brucei*	CC_{50}±SD (μg/mL) THP-1 cells	SI (CC_{50}/IC_{50}) *T. b. brucei*	IC50±SD (μg/mL) for *L. donovani*	SI (CC_{50}/IC_{50}) *L. donovani*
Roots (PAR)	PAR	45.0±6.5	NA	NA	NA	NA
	PRP	0.7±0.0	NA	NA	NA	NA
	PRC	18.3±1.2	345.6±28.6	18.9	NA	NA
	PLE	42.2±1.9	NA	NA	NA	NA
	PRB	26.3±1.9	NA	NA	NA	NA
	PRAaq	59.5±0.3	NA	NA	NA	NA
Seeds (PAS)	PAS	46.4±1.9	NA	NA	NA	NA
	PSH	33.2±2.8	124.3±7.0	3.7	NA	NA
	PSD	17.3±1.2	183.6±6.5	10.6	NA	NA
	PSE	28.9±1.1	163.9±9.2	5.7	NA	NA
	PSB	28.3±0.7	215.5±9.5	7.6	NA	NA
	PASaq	28.1±1.7	NA	NA	NA	NA
Stems (PASt)	PASt	6.7±0.9	137.1±21.2	20.6	NA	NA
	PStH	23.2±0.8	69.6±2.2	3.0	7.1±0.5	9.9
	PStP	21.1±5.4	126.2±7.7	6.0	NA	NA
	PStD	3.7±1.3	100.0±4.8	27.0	10.0±0.5	10.0
	PStC	4.6±0.2	85.6±3.3	18.7	NA	NA
	PStE	27.2±1.1	145.0±8.5	5.3	14.5±0.8	10.0
	PStB	33.1±6.2	157.2±21.1	4.8	15.7±2.1	10.0
	PAStaq	36.6±2.8	NA	NA	NA	NA

Diminazene Aceturate standard, IC_{50} for *T. b. brucei*=0.1±0.0 μM, Amphotericin B standard, IC_{50} for *L. donovani*=6.6±0.1 μM. Three independent experiments were performed, and data were expressed as mean±SD. Antitrypanosomal drug (positive control), amphotericin B: Antileishmanial drug (positive control). IC_{50}: 50% inhibitory concentration, SD: Standard deviation, CC_{50}: 50% cytotoxic concentration, NA: Not available, PAR: 70% EtOH *P. alnifolia* root extract, PRP: Petroleum ether fraction from PAR, PRC: $CHCl_3$ fraction from PAR, PRE: EtOAc fraction from PAR, PRB: BuOH fraction from PAR. PARaq aqueous fraction from PAR after partitioning. PAS: 70% EtOH *P. alnifolia* seed extract. PSH: hexane fraction from PAS, PSD: DCM fraction from PAS, PSE: EtOAc fraction from PAS, PSB: BuOH fraction from PAS, PAS aq: aqueous fraction from PAS after partitioning, PASt: 70% EtOH *P. alnifolia* stem extract, PStH: Hexane fraction from PASt, PStC: $CHCl_3$ fraction from PASt, PStD: DCM fraction from PASt, PStE: EtOAc fraction from PASt, PStB: BuOH fraction from PASt, PASt aq: Aqueous fraction from PASt after partitioning, PStP: Petroleum ether fraction from PASt, DA: Diminazene Aceturate, SI: Selectivity index, *L. donovani: Leishmania donovani, T. b. brucei: Trypanosoma brucei brucei, P. alnifolia: Pouteria alnifolia*

extracts of stem and WFs showed activity and good selectivity for both trypanosomes and *Leishmania* [Tables 5 and 6].

Selected extracts inhibit the growth of *Trypanosoma brucei brucei*

To determine the effect of extracts and fractions on the growth rate of the parasite, *T. b. brucei* cells were incubated in the absence or presence of different concentrations of 6 selected extracts and fractions (IC_{50} and 2x IC_{50}), and parasites were counted every 24 h for 96 h to determine the growth rate [Supplementary File]. An exponential growth was observed for untreated parasites, but there was a reduction in cell proliferation of parasites treated with increased concentration of extracts [Figure 2]. There was a drastic reduction in cell growth at 48 h for 2x IC_{50} for PStD and a

Table 6: Mean antiparasitic activities and selectivity indices of *Pouteria alnifolia* fractions of the whole fruit, fruit pulp, and leaves against *Trypanosoma brucei brucei and Leishmania donovani* promastigotes

Plant part extracts	Fraction codes	IC_{50}±SD (μg/mL) for *T. b. brucei*	CC50±SD (μg/mL) THP-1 cells	SI (CC_{50}/IC_{50}) *T. b. brucei*	IC_{50}±SD (μg/mL) for *L. donovani*	SI CC_{50}/IC_{50}) *L. donovani*
Whole fruit (PAWF)	PAWF	37.4±1.2	NA	NA	NA	NA
	PWFH	5.2±0.6	24.5±4.2	4.7	1.4±0.1	17.5
	PWFD	4.4±0.3	59.1±6.4	13.6	1.3±0.1	44.2
	PWFE	7.7±0.7	96.3±2.6	12.4	1.4±0.1	70.3
	PWFB	29.3±1.8	244.5±11.8	8.4	1.6±0.1	157.0
	PAWFaq	45.6±5.3	NA	NA	NA	NA
Fruit pulp (PAFP)	PAFP	38.6±23.9	NA	NA	NA	NA
	PFPH	14.8±0.5	49.0±4.3	3.3	NA	NA
	PFPD	8.1±1.7	40.1±4.5	5.0	NA	NA
	PFPE	28.5±7.1	104.8±9.1	3.7	NA	NA
	PFPB	23.1±2.9	278.3±18.4	12.0	27.8±1.8	10.0
	PFPaq	47.9±8.6	NA	NA	NA	NA
Leaves (PAL)	PAL	18.2±0.7	221.5±24.8	12.2	NA	NA
	PLH	13.3±0.9	33.6±1.3	2.5	21.7±1.0	1.6
	PLD	10.8±0.8	117.3±4.3	10.8	NA	NA
	PLE	47.9±3.3	NA	NA	NA	NA
	PLB	53.1±1.6	NA	NA	NA	NA
	PALaq	43.1±4.4	NA	NA	NA	NA

Diminazene Aceturate standard, IC_{50} for *T. b. brucei*=0.1±0.0 μM, amphotericin B standard, IC_{50} for *L. donovani*=6.6±0.1 μM. Three independent experiments were performed, and data were expressed as mean±SD. DA: Antitrypanosomal drug (positive control), amphotericin B: antileishmanial drug (positive control). DA: Diminazene aceturate, IC_{50}: 50% inhibitory concentration, SD: Standard deviation, CC_{50}: 50% cytotoxic concentration, NA: Not available, PAWF: 70% EtOH *P. alnifolia* whole fruit extract, PWFH: Hexane fraction from PAWF, PWFD: DCM fraction from PAWF, PWFE: EtOAc fraction from PAWF, PWFB BuOH fraction from PAWF, PAWFaq: Aqueous fraction from PAWF after partitioning, PAFP: 70% EtOH *P. alnifolia* fruit pulp extract, PFPH: hexane fraction from PAFP, PFPD: DCM fraction from PAFP, PFPE: EtOH fraction from PAFP, PFPB: BuOH fraction from PAFP, PAFPaq: Aqueous fraction from PAFP after partitioning, PAL: 70% EtOH *P. alnifolia* leaf extract, PLH: hexane fraction from PAL. PLD: DCM fraction from PAL, PLE: EtOAc fraction from PAL, PLB: BuOH fraction from PAL, PAL*aq*: aqueous fraction from PAL after partitioning, *L. donovani*: *Leishmania donovani, T. b. brucei: Trypanosoma brucei brucei, P. alnifolia: Pouteria alnifolia*

relatively more drastic reduction in cell densities at 72 h at 2x IC_{50} for PWFD. Treatment with IC_{50} concentrations of PWFE, PFPB, and PStC did not have any strong effect on cell proliferation [Figure 2].

Antiviral activities of fractions of *Pouteria alnifolia*

To assess whether plant fractions and extracts of *P. alnifolia* plant parts have antiviral properties, their inhibition of SARS-CoV-2 replication using an established assay that measures restoration of infected cell viability was done.[15] SARS-CoV-2 infection in the absence of treatment reduced cell viability by 63.4 ± 7.0% (mean ± SD), whereas co-treatment with positive control 10 μM remdesivir restored cell viability (i.e. inhibited virus replication) by 87.0 ± 15.3%. Overall, 38 primary extracts and fractions from *P. alnifolia* plant parts were screened and six (15.8%) which restored at least 10% cell viability at 25 μg/mL were identified. These extracts included PWFB, aqueous (PASaq), and BuOH (PSB) fractions of seeds, hexane (PStH) and PE fractions of the stem (PStP), and PE fraction of roots (PRP). The most active agent was PSB, which inhibited 73.7 ± 17.7% of SARS-CoV-2 replication at 12.5 μg/mL, followed by PWFB, which inhibited 67.2 ± 17.3% virus replication at 50 μg/mL [Figure 3a]. In uninfected Vero-E6 cells treated with fractions for 96 h in parallel, no major effects on viability (i.e., >25% reduction relative to untreated cells) at up to 50 μg/mL were observed, except for 50 μg/mL of PSB, which reduced cell viability by 50.0 ± 7.2% [Figure 3b].

Discussion

In this study, the antiparasitic and anti-viral potencies of extracts and fractions obtained from a medicinal plant, *P. alnifolia,* commonly used to treat illnesses across many areas in Africa including Ghana are reported. When the kind of phytochemicals that were present in the different parts of the plant were analyzed, it was observed that while anthracene and anthraquinone derivatives were absent in all the tested fractions, terpenoids and cardiac glycosides were the most present phytochemicals in the fractions. The triterpenoids betulin and lupeol have been isolated from the stem of *P. alnifoli*a, whereas the fatty acids tridecanoic acid, pentadecanoic acid octadecenoic (oleic) acid, heptadecanolide, and an unsaturated monoglyceride have been reported from the roots of *P. alnifolia*.[4] Furthermore, triterpenoids such as betulinic acid and ursolic acid have been reported from some species of *Pouteria* including *P. campechiana* and *P. gardneri.*[3,4,16,17] The secondary metabolites obtained from the plant extracts might explain some of the reported biological activities of this plant. For example, while the phenolic compounds found in *Pouteria* extracts can explain the antioxidant and free radical scavenging activities, the

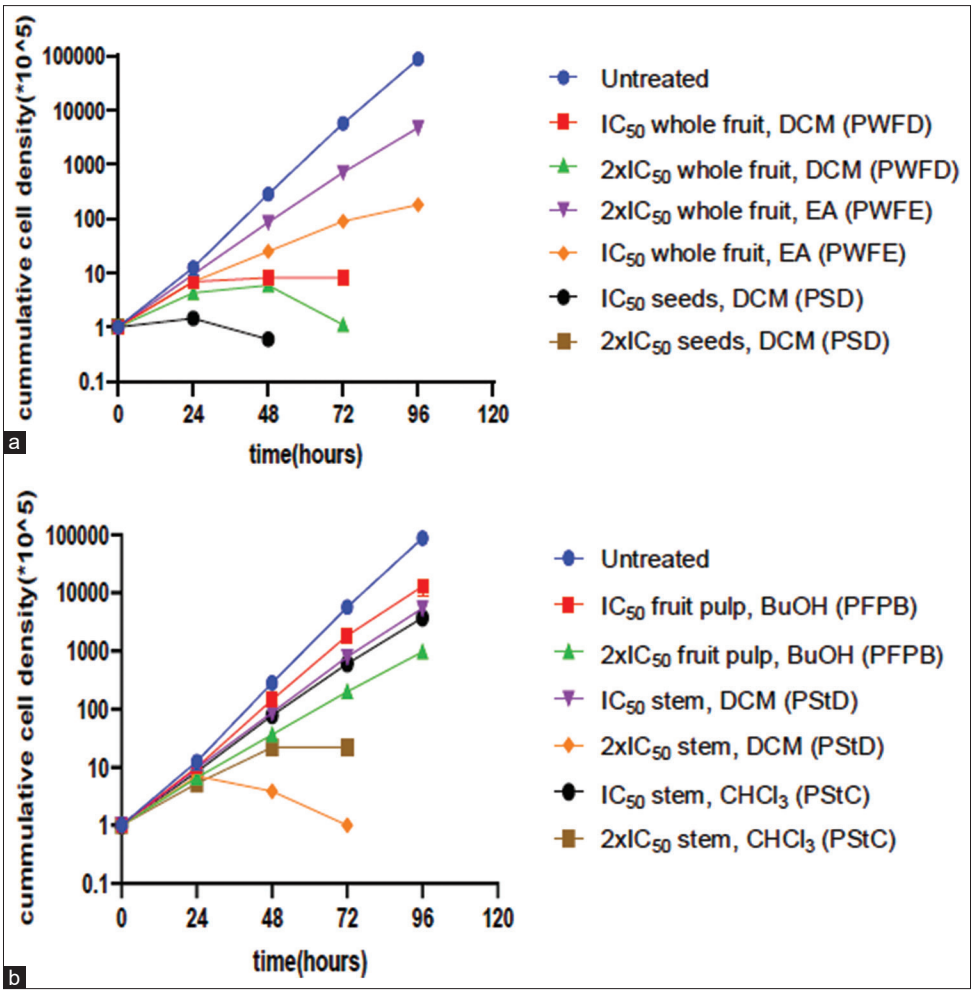

Figure 2: Selected extracts inhibit growth of *Trypanosoma brucei brucei* bloodstream forms. Trypanosomes were treated with individual extracts at IC_{50} and 2x IC_{50}. The growth kinetic curve was determined by counting parasites every 24 h for 120 h. Data from three independent experiments are represented as mean ± standard deviation. The extracts shown were selected based on their good inhibitory activity and selectivity indices. NC: Untreated negative control, BuOH: Butanol, DCM: Dicloromethane, EA: Ethyl acetate, $CHCl_3$: Chloroform, PWFD: DCM fraction from PAWF, PWFE: EtOAc fraction from PAWF, PSD: DCM fraction from PAS, PFPB: BuOH fraction from PAFP, PStD: DCM fraction from PASt, PStC: CHCl3 fraction from PASt

presence of triterpenes has been shown to exhibit a protective effect by efficiently intercepting free radicals before they reach their biological targets.[18,19] Alkaloids were detected in the dichloromethane fractions of seeds and WF compared to BuOH fractions of the FP, stem, and roots. Furthermore, the dichloromethane fractions tested were less toxic to normal cells than the BuOH fractions indicating that the dichloromethane fraction is better tolerated by normal cells than the BuOH fraction.

When extracts were tested for their activity against the selected pathogens, it was observed that some of the extracts and fractions showed growth inhibition properties and their presence in culture resulted in eventual cell death of *T. b. brucei*, *L. donovani*, and SARS-CoV-2. The results indicate good antitrypanosomal and antileishmanial activities which are comparable to the activities of known antitrypanosomal and antileishmanial compounds.[12-14,20] While the dichloromethane fractions obtained from WF, seeds, and stem were potent in reducing cell densities and resulting in the death of trypanosomes parasites, the BuOH fraction from seeds and WFs were the most active against SARS-CoV-2 replication. These differences in activities observed against trypanosomes and SARS-CoV-2 could be due to the possible structural variations of the active principles found in the different extracts and/or the mode of action in the pathogens[15,21] – which remains to be elucidated.

Conclusions

Our findings demonstrate that several fractions of *P. alnifolia* show moderate to good activities against multiple pathogens and can be further investigated toward chemotherapy for the diseases caused by these microbes.

Outcomes of the study

Extracts and fractions from different parts of *P. alnifolia* showed good antiparasitic and anti-SARS-CoV-2 activities indicating potential for further investigations for future chemotherapeutic drugs.

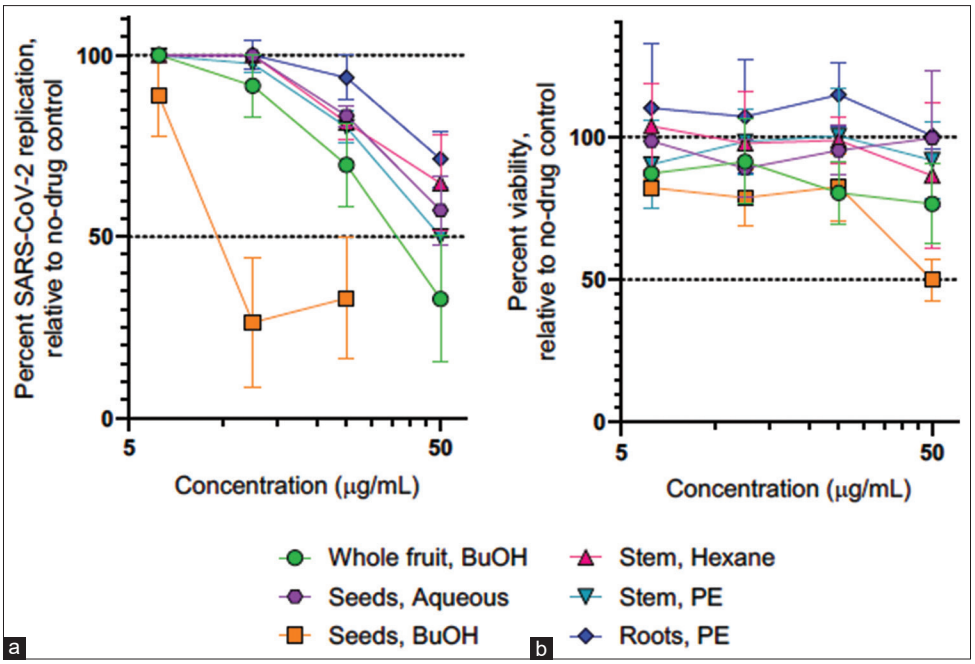

Figure 3: Effects of extracts on severe acute respiratory syndrome coronavirus 2 replication in infected Vero-E6 cells (a) and uninfected Vero-E6 cell viability (b). All data are presented as the mean ± standard deviation from at least 2 independent experiments. BuOH: Butanol, PE: Petroleum ether

Rationale of the study

The need for more effective and safer chemotherapy for parasitic and viral diseases drives the search for new and more efficacious drugs from the medicinal plant *P. alnifolia*.

Limitations of the study

The data obtained from this study are from *in vitro* studies which may differ from experiments performed in a living host.

Author contribution statement

Authors contributed to the conception, design, data collection, analysis, and writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary File

Methods

Plant collection and preparation

All plant parts including the leaves, whole fruits, seeds, fruit pulp stem bark, and roots, of *Pouteria alnifolia* tree were collected from the University of Ghana Botanical Garden (GA 541-7506) on the 17th of February 2023 and authenticated by the taxonomist Mr. Patrick Epke. A voucher specimen coded PAG/002 was deposited at the University of Ghana Herbarium, Department of Plant and Environmental Biology. Seeds were obtained by separating them from the fruit pulp of fresh whole fruits. In total six plant parts of *P. alnifolia* were obtained for this study; namely the leaves, whole fruits, seeds, fruit pulps, stem, and roots. All the plant parts obtained were air-dried for 4 weeks after which they were pulverized with a miller.

Phytochemical screening

To determine the presence of the chemical diversity in *P. alnifolia*, phytochemical screening of the fractions of the various parts of *P. alnifolia* (stem, leaves, seeds, roots, whole fruit, and fruit pulp) was carried out based on previous protocols for terpenoids, cardiac glycosides, tannins, saponins, alkaloids, flavonoids, and leucoanthocyanins as well as anthraquinones and anthracene derivatives.[1]

Test for cardiac glycosides, anthraquinones, and anthracene derivatives

The Salkowski's Test was used to determine the presence of aglycone moiety of cardiac glycosides. Approximately 2 mL of chloroform was added to 0.10 g of each of the four fractions (hexane or petroleum ether, dichloromethane or chloroform, ethyl acetate, and butanol) of the six extracts in separate test tubes. Concentrated sulphuric acid was added slowly to the sides of each test tube to form a layer. The presence of a reddish-brown color at the interface indicated the presence of the aglycone portion of cardiac glycosides in the fraction. Further, the Borntrager's test was used to test for anthraquinones and anthracene derivatives. Approximately 6.6 mL of distilled water was added to 0.10 g of each fraction in different boiling tubes. The tubes were heated in a water bath for 5–10 min at below 40°C. The test solution was then transferred into a separating funnel shaken vigorously with 2.2 mL of benzene and allowed to stand for equilibration. The benzene layer in each separating funnel was decanted into a test tube, and 0.5 mL of concentrated ammonia solution was added and allowed to stand. The presence of red color in the ammonia layers of the extracts inferred the presence of anthraquinones and anthracene derivatives in the fractions.

Test for tannins, saponins, and terpenoids

The presence of tannins was determined using the protocol previously described by Mann and Saunders.[2] A mass of 0.10 g of each of the four fractions (hexane or petroleum ether, dichloromethane or chloroform, ethyl acetate, and butanol) of the six extracts was dissolved in 3 mL of methanol in separate test tubes. Fresh iron (III) chloride solution (10%) was then added to each of the methanol solution. Dark greenish coloration was observed for fractions with the indicated presence of tannins in the fractions. In addition, saponins were tested by adding distilled water to 0.10 g of each fraction in separate test tubes and shaken vigorously. The presence of a persistent foam which remained after the solution was left to stand for an hour indicated the presence of saponins. Test for terpenoids was done according to the Liebermann-Burchard method.[3] Thin layer chromatography (TLC) analyses of each fraction were developed in 7:3 and 8:2 petroleum ether/acetone solvent systems. The plates were left to dry and then sprayed with anisaldehyde reagent. They were then heated in an oven at 110°C for 15 min. The presence of purple spots on the TLC plate indicated the presence of terpenoids.

Test for alkaloids, flavonoids, and leucoanthocyanins

Test for alkaloids was done as described by.[4] Firstly, 0.10 g of each fraction was put in different test tubes and 5 mL of concentrated HCl was added. The acidified fractions were stirred while warming in a water bath at a temperature below 40°C and then filtered. The filtrate from each fraction was then divided into three test tubes labeled A, B, and C. To portion A of a particular fraction, Mayer's reagent was added, and to portions B and C, Dragendoff's reagent and Wagner's reagent were added, respectively. The presence of a yellowish or reddish-brown precipitate in the test tubes indicated the presence of alkaloids. The presence of flavonoids and leucoanthocyanins was investigated by dissolving 0.10 g of each fraction in 30 mL ethanol and filtering it. Each filtrate of a fraction was divided into three portions labeled A, B, and C in separate test tubes. Test tube A served as a blank or control. To test tube B of the fractions, three pieces of magnesium turnings (3.5 mg) were added followed by 0.5 mL of concentrated hydrochloric acid. The final solution was then observed for any color change. A volume of 0.5 mL of concentrated hydrochloric acid was added to test tube C and warmed for 5 min in a water bath at a temperature below 40°C. The presence of light pink color in test tubes B and C indicates the presence of flavonoids and leucoanthocyanins, respectively.

Growth kinetics

Bloodstream forms *Trypanosoma brucei brucei* were seeded at an initial density of 1.0×10^5 parasites/mL. The parasites were then incubated without or with the IC_{50} concentration and 2x IC_{50} concentration of extracts and fractions and subcultured every 24 h for a period of 120 h. A cumulative growth curve was plotted using Microsoft excel 2010. Three independent experiments were performed, and data were expressed as mean ± standard deviation.

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