JBP
Journal of Biochemicals and Phytomedicine
eISSN: 2958-8561

Assessment of Antimicrobial, Antimalarial, and Antitrypanosomal Activities of Crude Extracts and Fractions from *Piliostigma thonningii* Schum. Leaves *in Vitro*

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ARTICLE INFO

Article Type:
Research

Article History:
Received: 21 Feb 2024
Revised: 09 May 2024
Accepted: 25 May 2024
Available online: 30 Jun 2024

Keywords:
Antiprotozoal Agents,
Piliostigma thonningii,
Antitrypanosomal,
Plant Extracts,
Therapeutic Uses

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ABSTRACT

Introduction: The plant *Piliostigma thonningii* (Milne-Redhead, Fabaceae) has been traditionally used as a medicinal remedy in several African countries. This study aimed to investigate and validate the antimalarial, antitrypanosomal, and antimicrobial potential of the crude methanolic extract and fractions of *Piliostigma thonningii* leaves through in vitro assays.

Methods: Cold maceration of dried *P. thonningii* leaves in methanol produced crude extracts, which were then fractionated into dichloromethane (DCM), ethyl acetate (EtOAc), and butanol (BuOH) fractions. The extracts and fractions were subsequently tested for biological activity. In vitro antimalarial activity was assessed using the plasmodial lactate dehydrogenase culture sensitivity test, while antitrypanosomal activity was evaluated using *Trypanosoma brucei* promastigote cultures.

Results: The crude methanolic extract did not exhibit observable antitrypanosomal activity. However, the EtOAc fraction demonstrated antitrypanosomal potential with an IC50 value of 13.35 µg/mL, and the BuOH fraction had an IC50 of 13.36 µg/mL. The EtOAc fraction also showed moderate antimalarial activity, with IC50 values of 33.45 µg/mL against *Plasmodium falciparum* D6 strain and 38.94 µg/mL against the W2 strain. Additionally, a sub-fraction of the EtOAc fraction, labeled PTE, exhibited enhanced antimalarial activity with IC50 values of 21.38 µg/mL against *P. falciparum* D6 and 23.21 µg/mL against *P. falciparum* W2.

Conclusion: The findings suggest that certain fractions from *P. thonningii* leaf extract have potential use in the treatment of African trypanosomiasis and could be further developed into chemotherapeutic agents for treating malaria and other ailments.

Please cite this paper as:
Michael A, Sarah A, Blessing S. Assessment of antimicrobial, antimalarial, and antitrypanosomal activities of crude extracts and fractions from *Piliostigma thonningii* schum. leaves *in vitro*. Journal of Biochemicals and Phytomedicine. 2024; 3(1): 46-52. doi: 10.34172/jbp.2024.10.

Introduction

Since ancient times, humans have relied on naturally occurring substances for medicinal purposes, and plants have been the foundation of refined traditional medicine (Afolayan et al., 2023). Most cultures throughout the world have relied heavily on plants for their medical needs over the centuries. Plant medicinal properties were passed from generation to generation orally, preventing the extinction of this knowledge (Gurib-Fakim, 2006; Okigbo et al., 2008). Throughout Asia, including China, Japan, Thailand, India, Pakistan, and Africa, including Ghana, Congo, Mali, Cameroon, South Africa, and Nigeria, medicinal plants are widely available and widely used for a variety of reasons (Mukhtar et al., 2008). In many different forms, medicinal plants can be used to treat illnesses and disorders. These forms include herbal teas, crude extracts, phytopharmaceuticals, herbal mixtures, and isolated chemicals (Rates, 2001). As chemistry advanced at the start of the 19th century, researchers started looking more closely at plants to learn about their components and how they might be used medicinally (Beutler, 2013).

The significant choice of plant derived natural products is premised on ethnobotanical knowledge, the ease of extraction and cultivation, combined with ease of accessibility. Plants create numerous structurally distinctive compounds; extraordinarily, over a hundred thousand low molecular mass natural products have been revealed from plants (Dixon, 2001). Estimates propose that only 5 – 10% of plant species have been evaluated for bioactivity and reinvestigation of previously studied sources may provide even more leads (Cragg and Newman, 2001). One of such plants with immense medicinal values and which needs evaluation and reinvestigation is *Piliostigma thonningii*.

One of the species of flowering plant belonging to the Fabaceae family of legumes is *P. thonningii* (Milne-Redhead). It is a member of the Caesalpinioideae subfamily. *P. thonningii* is a woody tree or plant that grows between 4 and 15 metres tall. It has a rounded crown and a short, frequently crooked bole (Daniyan et al., 2010). Except for Somalia, *P. thonningii* is distributed across Africa. It is consistently linked to Grewia mollis, Annona senegalensis, and Combretum spp. (Farnsworth, 2008). Local names for it include kidakpam in Obudu, omepa in Igede, ejei-jei in Igala, kalgo in Hausa, okpoatu in Igbo, nyihar in Tiv, and abefe in Yoruba (Igoli et al., 2005).

P. thonningii is utilised medicinally in several African nations by the usage of its fruits, barks, roots, seeds, and leaves (Silva et al., 1997). The leaves and bark are decocted and used to cure wounds, ulcers, pyrexia, leprosy, heart pain, cough, arthritis, diarrhoea, bronchitis, gingivitis, and toothache (Ighodaro & Omole, 2012). It has been reported that twigs and roots of the plant are utilized in the management of skin diseases, dysentery, cough, fever and wound infections (Afolayan et al., 2018).

This present study intends to determine the in vitro antitrypanasomal, antimicrobial, and antimalarial activities of methanolic crude extract and some fractions of *P. thonningii* leaves and to justify the claim of traditional healers which could possibly lead to drug development.

Materials and Methods

Collection and Identification of Samples

In June 2016, during the rainy season, leaves of *P. thonningii* were gathered from the medicinal plants garden of Sheda Science and Technology Complex (SHESTCO), located in Abuja, Nigeria. With voucher reference number FHI 110688, the plant sample was identified and verified at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state, Nigeria. The sample was placed at FRIN as a voucher specimen for the herbarium section's future use.

Plant Samples Preparation

The plant's leaves were allowed to air dry for seven days at room temperature in the shade. Afterwards, they were ground into a powder using an electric hammer mill (Model TRAPP TRF 80 Hammer mill foliage), and then they were kept dry until needed for additional usage.

Extraction

The process of extracting the powdered leaves involved using aqueous methanol. In a glass jar with a stopper, 1.5 kg of powdered leaves were combined with exactly 7 L of 10% aqueous methanol and sealed tightly. After the mixture was stirred occasionally each day for three days at room temperature, the extract was finally filtered using cotton wool and Whatman 125 mm filter paper No. 1. A Stuart RE 300B W13 rotary evaporator was used to condense the resulting methanol extract at low pressure. To acquire the sample's crude methanolic extract, the extract was dried out by evaporating it completely and then distilling out the methanol at 40 degrees Celsius. Afterwards, the dry extract was stored in a refrigerator in bottles with tight stoppers until it was needed for additional analysis (Afolayan et al., 2023)

Fractionation of Crude Extracts

About 80 g the methanolic crude extract of the sample gotten above was dispersed in about 100 cm3 of water – methanol mixture in equal proportions; the mixture was put into a separating funnel and equal volume of DCM was added to it before separating the two different layers in a separating funnel. Two more times, the procedure was carried out followed by gathering the DCM layers (lower layer) in a beaker. To obtain the total DCM soluble fraction (PTA), the gathered DCM layers

were combined and dried over a water bath at 40 oC. Further, 100 cm3 of EtOAc and additional H2O were added to the remaining H2O layer. After giving it a good shake, the top layer, or EtOAc layer was collected and allowed to settle. After DOIng this procedure twice, the collected EtOAc layers were combined and dried over a water bath at 40 oC to get the EtOAc soluble fraction (PTB). To obtain the BuOH soluble fraction (PTC), BuOH was additionally employed as a fractionating solvent. The residue from the fractionation process was preserved as the aqueous soluble fraction (PTD) by drying it in a freeze dryer. In order to produce four subfractions (PTE–PTG), the EtOAc fraction (PTB), 8 g, was loaded onto a 200 g silica gel column and eluted with 1 L of the solvent mixture consisting of DCM, EtOAc, MeOH, and H2O in different proportions. The ratios of solvents were obtained using TLC. PTE, PTF, and PTG were eluted with 8: 15: 4: 1, 6: 10: 4: 1, and 4: 6: 4: 1 of DCM, EtOAc, MeOH, and H2O, respectively.

Antitrypanosomal Assay

The samples' antitrypanosomal activity was evaluated in vitro using a culture of promastigotes of Trypanosoma brucei. They were raised at 26 oC in RPMI 1640 medium with 10% foetal calf serum added. 5 X 105 promastigotes / mL was the dilution of a culture that was 3 days old. In 96-well plates, drug dilutions were made directly in cell suspension. Plates were incubated at 26 oC for 48 hours, and the proliferation of trypanaosomal promastigotes was assessed using the Alamar blue assay. Using a Fluostar Galaxy plate reader (BMG Lab Technologies) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm, standard fluorescence was recorded. Amphotericin B, pentamidine, and alpha-difluoromethylornithine were the common antitrypanosomal drugs used as standards. By graphing the growth against drug concentration, dose-response curves were generated, from which IC50 and IC90 values were determined (Bharate et al., 2007).

Antimalarial Assay

The assay's foundation is the measurement of the activity of plasmodial lactate dehydrogenase (pLDH). The pLDH culture sensitivity assay suggests field applicability because it is rapid, inexpensive, easy to interpret, and repeatable. In order to conduct the assay, a 96-well plate containing 10 µL of test samples diluted in medium at different concentrations was filled with a suspension of red blood cells infected with D6 and W2 strains of P. falciparum (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin). The plate was incubated for 72 hours at 37 oC in a modular incubation chamber (Billups-Rothenberg, CA) that had been flushed with a gas combination of 5% carbon dioxide, 5% oxygen, and 90% nitrogen. MalstatTM reagent (Flow Inc., Portland, OR) was used to measure pLDH activity in accordance with Makler and Hinrich's (1993) protocol. In summary, 30 minutes were spent incubating at room temperature after mixing 20 µL of the incubation mixture with 100 µL of the MalstatTM reagent. After adding 20 µL of a 1:1 NBT/PES combination (Sigma, St. Louis, MO), the plate was further incubated for one hour in the dark. The addition of 100 µL of a 5% acetic acid solution terminated the process. The EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) was used to read the plate at 650 nm. We computed the IC50 values using the dose-response curves. As the drug controls, chloroquine and artemisinin were added to each experiment 0.25% dimethyl sulphoxide was used as the vehicle control. (Bharate et al., 2007)

Antimicrobial Assay

The methicillin-resistant bacteria, Staphylococcus aureus ATCC 43300 (MRS), Mycobacterium intracellulare ATCC 23068, Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC35218 as well as the fungi Cryptococcus neoformans ATCC 90113, Aspergillus fumigatus ATCC 90906 and Candida albicans ATCC 90028 were organisms used for the assay and all obtained from the American Type Culture Collection (Manassas, VA). Testing for susceptibility was carried out using a modified version of the CLSI (formerly NCCLS) method (NCCLS, 2002a–d). Serial dilution of Samples were done in 20% DMSO/saline solution and transferred to 96-well flat bottom microplates in duplicates. Microbial inocula were created by correcting the OD630 of microbe suspensions in incubation broth to yield final target inocula. Each assay had drug controls, which were Fluconazole & Amphotericin B for fungi and Meropenem & Ciprofloxacin [all manufactured by ICN Biomedicals, Ohio] for bacteria. Prior to and following incubation, all organisms were read at either 630 nm using the Bio-Tek Instruments, Vermont-based EL-340 Biokinetics Reader, or 544ex/590em (M. intracellulare, A. fumigatus) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany). To find the Minimum Inhibitory concentrations, 5µL was taken out of each clear well, transferred to agar, and then allowed to incubate. The lowest test concentration at which the organism is killed and no growth is allowed on agar is defined as the MIC. To determine the IC50, the percent increase was plotted against the test concentration (Radwan et al., 2007).

Statistical Analysis

The findings were presented in the form of mean ± SD.

Results

Antimalarial Properties of the Fractions and Crude Extract

Two plasmodium strains (*P. falciparum* D6 and *P. falciparum* W2) were used to assess the antimalarial properties of the crude extracts and fractions. The assay's results, which are shown in Table 1, showed that the crude extracts had a negligible antimalarial effect. Nevertheless, fractionation and additional fractional partitioning produced fractions and sub-fractions with notable antimalarial activity. The result of this assay is presented in Table 1.

With an IC50 value of 38.94 µg/mL against *P. falciparum W2* and 33.45 µg/mL against *P. falciparum D6*, only the fraction PTB demonstrated moderate antimalarial activity. In contrast, the sub-fraction PTE, which was derived from fraction PTB, demonstrated more promising antimalarial activity, showing an IC50 value of 23.21 µg/mL against P. falciparum W2 and 21.38 µg/mL against P. falciparum D6. The antimalarial activity of the extract is seen to be enhanced by fractionation and additional partitioning; although, the crude extract and all of the fractions exhibited lower levels of activity when compared to the reference medications (chloroquine and artemisinin).

Antitrypanosomal Properties of the Fractions and Crude Extract

The antitrypanosomal activity of the fractions and crude extracts was assessed using Trypanasoma brucei Promastigotes, with the outcomes displayed below. Table 2 displays the results of the main assay indicating percent inhibition, while Table 3 records and presents the results of the secondary and tertiary assays indicating IC50 and IC90 values.

For the primary assay, the crude extract and fraction PTA only showed very weak antitrypanosomal activity, but interestingly the sub – fractions PTF and PTG had excellent antitrypanosomal activity of 100 and 99 percent inhibition respectively. *P. thonningii* crude extracts and fractions had no observable antitrypanosomal activity for the secondary assay with IC50 values above the standard value acceptable. However, PTF as well as PTG sub – fractions (obtained in fraction PTB) showed moderate antitrypanosomal secondary activity giving values of 13.35 µg/mL and 13.36 µg/mL for IC50 respectively. All fractions and crude methanolic extracts tested were not as active as the standard drug (pentamidine).

Antimicrobial Properties of the Fractions and Crude Extract

Results of the primary antibacterial activity is shown in Table 4.

Table 1: Antimalarial properties of *Piliostigma thonningii* fractions and crude extract

Samples	Concentration (µg/mL)	*P. falciparum D6* IC50 (µg/mL)	*P. falciparum W2* IC50 (µg/mL)
PT	47 – 5.29	43.50	32.46
PTA	47 – 5.29	>47	>47
PTB	47 – 5.29	33.45	38.94
PTC	47 – 5.29	>47	>47
PTE	47 – 5.29	21.38	23.21
PTF	47 – 5.29	>47	>47
PTG	47 – 5.29	>47	>47
Artemisinin	2.38 – 0.026	<0.026	<0.026
Chloroquine	2.38 – 0.026	<0.026	0.15

PT: *Piliostigma thonningii* crude methanol extract; PTA: DCM fraction; PTB: EtOAc fraction; PTC: BuOH fraction; PTE: DCM 8: EtOAc 15: MeOH 4: H2O 1 subfraction; PTF: DCM 6: EtOAc 10: MeOH 4: H2O 1 subfraction; PTG: DCM 4: EtOAc

Table 2: Primary antitrypanosomal properties of fractions and crude extracts of *Piliostigma thonningii*

Samples	Concentration (µg/mL)	% Inhibition
PT	20	10 ± 0.93
PTA	20	8 ± 2.39
PTB	20	0
PTC	20	0
PTE	20	0
PTF	20	100 ± 0.00
PTG	20	99 ± 0.00
Amphotericin B	2	99
Alpha-difluoromethylornithine	20	99
Pentamidine	10	100

PT: *Piliostigma thonningii* crude methanol extract; PTA: DCM fraction; PTB: EtOAc fraction; PTC: BuOH fraction; PTE: DCM 8: EtOAc 15: MeOH 4: H2O 1 subfraction; PTF: DCM 6: EtOAc 10: MeOH 4: H2O 1 subfraction; PTG: DCM 4: EtOAc 6: MeOH 4: H2O 1 subfraction.

Table 3: Secondary and tertiary antitrypanosomal properties of fractions and crude extracts of *Piliostigma thonningii*

Samples	Concentration (µg/mL)	(IC50)	(IC90)
PT	20 – 0.8	>20	>20
PTA	20 – 0.8	>20	>20
PTB	20 – 0.8	>20	>20
PTC	20 – 0.8	>20	>20
PTE	20 – 0.8	>20	>20
PTF	20 – 0.8	13.35	17.60
PTG	20 – 0.8	13.36	18.12
Pentamidine	10 – 0.008	0.002	0.003
Amphotericin B	2 – 0.08	XX	XX
Alpha-difluoromethylornithine	20 – 0.08	3.593	9.897

PT: *Piliostigma thonningii* crude methanol extract; PTA: DCM fraction; PTB: EtOAc fraction; PTC: BuOH fraction; PTE: DCM 8: EtOAc 15: MeOH 4: H2O 1 subfraction; PTF: DCM 6: EtOAc 10: MeOH 4: H2O 1 subfraction; PTG: DCM 4: EtOAc 6: MeOH 4: H2O 1 subfraction.

Table 4: Primary antibacterial properties of *Piliostigma thonningii* fractions & crude extract

Samples	Concentration (µg/mL)	MRSA	*Eschericha coli*	VRE	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
PT	200	0	14 ± 1.27	12 ± 1.33	9 ± 1.02	3 ± 1.94
PTA	200	1 ± 0.00	9 ± 0.19	10 ± 3.07	4 ± 0.08	5 ± 0.06
PTB	200	3 ± 0.01	9 ± 0.42	15 ± 1.61	0	7 ± 1.03
PTC	200	4 ± 0.64	12 ± 1.26	7 ± 0.34	0	8 ± 2.32
PTE	200	1 ± 0.15	3 ± 0.34	12 ± 2.11	0	4 ± 0.23
PTF	200	5 ± 0.25	6 ± 1.18	3 ± 0.96	0	5 ± 1.05
PTG	200	0	9 ± 0.71	19 ± 0.85	0	7 ± 2.13
Methicillin	100	99	11	69	5	21
Vancomycin	100	99	60	27	26	18
Meropenem	100	75	99	79	98	22
Cefotaxime	100	98	100	84	3	22
Ciprofloxacin	100	0	100	100	5	15

PT: *Piliostigma thonningii* crude methanol extract; PTA: DCM fraction; PTB: EtOAc fraction; PTC: BuOH fraction; PTE: DCM 8: EtOAc 15: MeOH 4: H2O 1 subfraction; PTF: DCM 6: EtOAc 10: MeOH 4: H2O 1 subfraction; PTG: DCM 4: EtOAc 6: MeOH 4: H2O 1 subfraction.

Discussion

Extraction and fractionation results for leaves of *P. thonningii* showed that *P. thonningii* methanol crude extract had fractionation achieved by vacuum liquid chromatography with water / methanol/ dichloromethane / ethyl acetate solvent mixtures.

Methanolic crude extract of P. thonninngii examined in this research work revealed that extracts differ in their properties according to different methods and solvents used in partitioning / fractionation of the crude extracts. Biological activity of extracts from various plants is typically determined by nature and type of the solvents used. The varying solvent polarities used employed in extraction equally plays a vital function in enhancing the solubility of phytochemicals hence giving different activities (Naima et al., 2015).

Previous studies on *P. thonningii* leaves crude extract showed the presence of notable phytochemicals as shown in the work by Bello et al., (2013) where crude methanolic extract from the leaves of *P. thonningii* was tested positive for tannins, saponins, alkaloids, cardiac glycosides and flavonoids. These phytochemicals when present in an extract are found to be responsible for a variety of bioactivities. The presence of alkaloids, tannins, saponins, terpenoids, flavonoids, steroids as well as phenols, in any extract is an indication that it would be of biological or pharmacological relevance (Ekhaise et al., 2010).

The crude extracts' antimalarial assay revealed very little antimalarial activity; however, fractionation greatly enhanced their antimalarial activity. Since terpenoids and flavonoids are phytochemicals that have been differently linked to the antiplasmodial properties of numerous plants, this may be explained by their existence in these fractions (Mbah et al., 2012).

The crude extract of *P. thonningii* did not show observable antitrypanosomal activity but the EtOAc and BuOH fractions showed activity with IC50 of 13.35 and 13.36 µg/mL respectively. This could be attributed to further purification of the extract hence enhancing better results as equally observed in the antimalarial assay results shown earlier (Afolayan et al., 2023). The antitrypanosomal activity of the EtOAc and BuOH fractions are still quite lower compared to that of the standard drugs used which offers a suggestion that isolating pure compounds from the respective fractions may afford isolates that will exhibit antitrypanosomal activity which may be comparable if not better than the standard drugs currently in use for the treatment of trypanasomiasis.

The fractions and also the crude extract did not show any noticeable antimicrobial property which is in tandem with earlier results by Ighodaro et al., 2012. However, Ibewuike et al., (1997) in an earlier study confirmed antibacterial properties of some C-methyl flavonols isolated from the same plant which also lays credence to the fact that further purification of the extracts may be able to increase antimicrobial activity.

Conclusion

The in vitro biological assays conducted on the methanolic crude extract and fractions of *P. thonningii* revealed significant antitrypanosomal activity in two of the fractions, indicating the plant's potential as a source of antiprotozoan agents. These findings provide scientific support for its traditional medicinal use and highlight the potential for developing new, natural compounds from this plant. Such compounds could offer safe and effective treatments for malaria and trypanosomiasis—protozoan diseases that pose significant economic and health challenges, particularly in tropical regions like Africa.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

None.

Consent for publications

All authors have read and approved the manuscript for publication.

Funding/support

None.

Authors' contributions

AM initiated the research concept and made the framework. AM, AS, and SB conducted all experiments and made the manuscript initial draft, AS and SB did literature survey. AM supervised all the research work. The final manuscript was read and approved by AM, AS, and SB for publication.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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