JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Brief Original Article

Anti-malarial investigation of *Acorus calamus*, *Dichapetalum gelonioides*, and *Leucas aspera* on *Plasmodium falciparum* strains

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Abstract

Introduction: Malaria is a significant global health concern and adversely affects people in developing countries including Bangladesh. The causative agent *Plasmodium falciparum* is resistant to several currently available anti-malarial drugs, such as mefloquine, chloroquine, and artemisinin-based combination therapy (ACT), and this has been a major global challenge towards the control of the disease. There is urgent need for novel anti-malarial chemotherapeutic agents.

Methodology: The present study aimed to evaluate antimalarial activity of methanolic extracts of three Bangladeshi medicinal plants- *Acorus calamus*, *Dichapetalum gelonioides* and *Leucas aspera* - against both chloroquine sensitive (3D7) and resistant (Dd2) strains of *P. falciparum*. Histidine-rich protein 2 (HRP2) based ELISA was used to evaluate the in vitro inhibitory activity of the extracts.

Results: *D. gelonioides* extract showed moderate (IC50 = 19.15 µg/mL) and promising activity (IC50 = 10.43 µg/mL) against 3D7 and Dd2 strains respectively. *A. calamus* remained inactive against both 3D7 (IC50 = 72.29 µg/mL) and Dd2 strain (IC50 = 67.81 µg/mL). *L. aspera* initially remained inactive against 3D7 strain (IC50 = 60.51 µg/mL), but displayed promising activity (IC50 = 7.693) against Dd2 strain.

Conclusions: This is the first time these plant materials have been assessed for their in vitro antimalarial properties. It is pivotal to conduct further phytochemical analysis of *D. gelonioides* and *L. aspera* to evaluate the presence of potential novel antimalarial drug compounds.

Key words: *Plasmodium*; in vitro; anti-malarial resistance; 50% inhibitory concentration (IC_{50}).

J Infect Dev Ctries 2022; 16(11):1768-1772. doi:10.3855/jidc.16741

(Received 25 April 2022 – Accepted 05 September 2022)

Introduction

Malaria is a fatal disease caused by *Plasmodium,* a genus of protozoan parasite [1]. Each year, it affects between 200 and 400 million people, killing nearly 400,000 people and adversely affecting children in Sub-Saharan Africa [2]. The region with the highest malaria burden is Sub-Saharan Africa, where *P. falciparum* is the predominant form [3]. Bangladesh is a densely populated nation with endemic malaria. Most of the cases are found in the thirteen endemic districts near or bordering India and Myanmar [4,5]. The Chittagong Hill Tracts (Bandarban, Rangamati and Khagrachari) have the highest malaria burden accounting for nearly 90% of all malaria cases in Bangladesh [6].

Resistance to antimalarial drugs is a major concern in the global fight against malaria. Chloroquine and sulfadoxin-pyrimethamine resistance were reported in Bangladesh as early as 1970 and 1985, respectively [7,8]. Despite the fact that no clinical or molecular resistance to the current artemisinin-based chemotherapy (ACT) treatments have been documented in the country, it has been discovered that this is ineffective in the countries bordering on the east [9]. This highlights the need to identify new antimalarials in the near term, as a backup in case of ACT failure.

Plants have been investigated as antimalarial agents as a direct outcome of the two potent antimalarial drugs, quinine and artemisinin, both of which are derived from plants [10]. The anti-malarial activity in plants is attributed to a variety of phytoconstituents such as alkaloids, terpenes, steroids, and flavonoids [11]. Traditional healthcare practitioners in Bangladesh have a long history of using medicinal plants [12].

In this study, we investigated three plants that grow locally in Bangladesh, *Acorus calamus* (sweet flag; locally known as bach), *Dichapetalum gelonioides* (gelonium poison-leaf; locally known as moacurra) and

*Leucas aspera (*common leucas; locally known as ghal ghas) for antimalarial properties. All the plant materials were collected from Bandarban, Chattogram. These plants were selected based on their ethnomedicinal values. Leaves and rhizomes of *A. calamus* are used in medicinal preparations to treat various diseases [13]. *D. gelonioides* has traditionally been used to treat amenorrhea and mouth ulcers, and *L. aspera* is used to cure cold, cough, and skin disorders [14,15]. The prime objective of the study is to evaluate antimalarial efficacy of methanolic extracts of these plants.

Methodology

Study site and period

The current study was performed at the Emerging Infections and Parasitology Laboratory, International Centre for Diarrhoeal Disease Research, Bangladesh from November 2020 to June 2021.

Plant materials

Acorus calamus (sweet flag), *Dichapetalum gelonioides* (gelonium poison-leaf) and *Leucas aspera* (common leucas) were collected from Bandarban, Chattogram.

Preparation of methanolic extract

Fresh leaves of *A. calamus* and entire plants of *D. gelonioides* and *L. aspera* were collected and sun dried. The leaves of *A. calamus* were cut into small pieces and sun dried for 7 days, while the *D. gelonioide*s and *L. aspera* plants took 10 and 12 days respectively to dry. The dried plant parts were converted into powder using a laboratory grinder. 250 g of dried and powdered plant material was soaked in 1000 mL methanol at 25 ± 2°C for 7 days in the case of leaves and 14 days in the case of whole plant in airtight bottles and the mixture was stirred every 18 hours using a sterile glass rod. Thereafter, the solution was vacuum filtered using Whatman Grade 1 filter paper. All the extracts were concentrated and dried in a rotary evaporator, followed by a water bath. The extracts were then stored in airtight containers and kept in a refrigerator at 4ºC to protect against light and humidity until used.

Malaria parasites and culture

P. falciparum strain 3D7 is sensitive to all anti-malarial drugs available in the market; and *P. falciparum* strain Dd2 is resistant to chloroquine. These two strains were used in the study. They were collected from the Malaria Research and Reference Reagent Resource Center (MR4), which includes the American Type Culture Collection (ATCC). The Trager and Jensen in vitro culture technique was used with some modification to maintain the continuous culture of the asexual blood stage [16]. Briefly, the parasites were cultivated in O +ve erythrocyte and maintained in RPMI-1640 media (Gibco by Life Technologies, Grand Island, NY, USA). In addition, 0.5% Albumax II (Gibco by Life Technologies, Grand Island, NY, USA) serum supplement powder, 25 mM HEPES, 11 mM glucose, 23.81 mM $NaHCO_3$, 200 μM hypoxanthine, and 20 mg/L gentamicin solution were also added to the media. The cultivated parasites were kept in a 25 cm^2 Corning® culture flask (Corning Inc., NY, USA) with 2% hematocrit at 37 °C inside a candle jar to maintain anaerobic condition. Routine microscopy was performed to monitor and ensure parasite growth at < 5% every 24 hours with the daily change to fresh culture medium.

Evaluation of in-vitro antimalarial activity

Worldwide Antimalarial Resistance Network (WWARN) protocols were used for the experiment. Assay plate preparation were done by the WWARN protocol INV03 and Histidine-rich protein 2 (HRP2) Enzyme-linked immunosorbent assay (ELISA) was done by WWARN protocol no: INV09 (WWARN, Oxford, United Kingdom) [17]. A batch of drug plates were prepared by adding 40 μL of stock solution and 160 μL of RPMI 1640 (Roswell Park Memorial Institute) media. Serial dilutions of each set of plant extracts were made in triplicates in 96 well microtiter plates with concentration ranging from 0.003-1.67E-08 gm/mL.). In each well, 8 μL of diluted plant extract and 192 μL of parasitized culture was added in concentrations ranging from 200 μg/mL–0.0976 μg/mL. Parasitized red blood cell cultures with Chloroquine (CQ) were used as a positive control; the last well was drug free and was used as the negative control. The assay plates were incubated for 72 hours at 37°C in a candle jar. After the 72 hour incubation period, the plates were removed from the incubator and stored at –20°C until all the wells was completely frozen. Then the plates were thawed for hemolysis. HRP2 ELISA technique measures the quantity of HRP2 produced by *P. falciparum* during the 72 hour incubation and its inhibition by anti-malarial drugs.

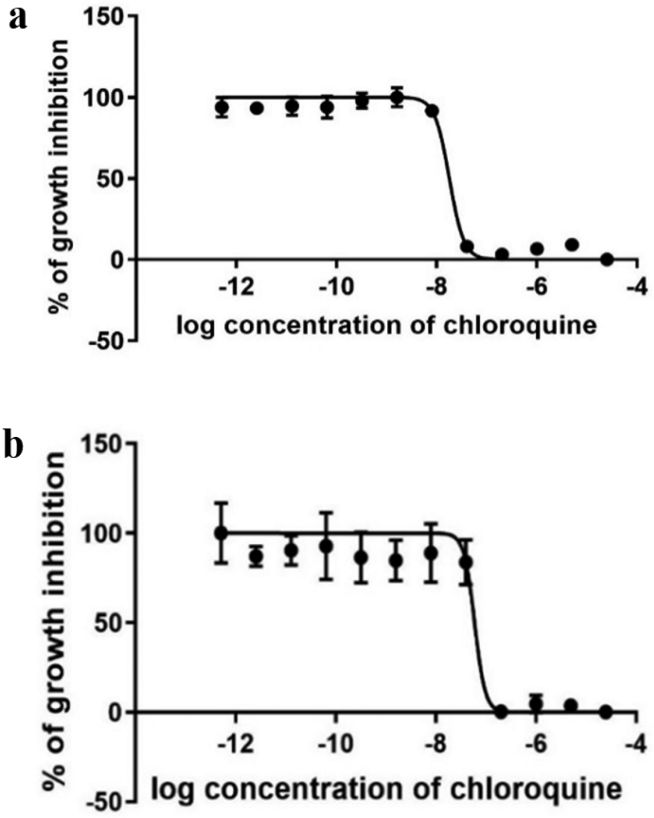
The percentage inhibition values were calculated from normalized activities (activity expressed as percentage of solvent control) for assessing the anti-malarial activity. The concentration of extracts that caused 50% inhibition of *P. falciparum* (IC50 values) was also calculated using the GraphPad Prism Software Version 8.4.3 (La Jolla, CA 92037 USA).

Results

Anti-malarial activity of extracted biota was calculated using results from three independent anti-malarial assays, each carried out in triplicate. The Graphpad prism 8.4.3.686 software was used to construct a graph of non-linear regression of the optical density values of the chloroquine and plant extracts. Dose versus response curves (Figures 1 and 2) were obtained, where concentrations of chloroquine and plant extracts were expressed as logarithmic numbers in the x axis and O.D. values were normalized and expressed as percentage inhibition values. The concentration at which parasite growth was inhibited by 50% (IC50) was calculated from the graph representing the percentage growth inhibition data.

IC50 values for chloroquine drug (positive control) were 17.79 nM and 59.64 nM respectively for 3D7 and Dd2 strain. Based on previous studies, anti-malarial activity can be characterized as high (IC50 < 5 µg/mL), promising (5 < IC50 < 15 µg/mL), moderate (15 < IC50 < 50 µg/mL) and inactive (IC50 > 50 µg/mL) [18-20]. IC50 values of *Dichapetalum gelonioides* were 19.15 µg/mL against 3D7 (CQ- sensitive strain) and 10.43 µg/mL against Dd2 (CQ-resistant strain) exhibiting moderate and promising antimalarial activity respectively. IC50 values of *Leucas aspera* against Dd2 was 7.693, showing promising activity. However, *L. aspera* against 3D7 and *A. calamus* against both the strains remained inactive (Table 1).

Figure 1. Dose vs response curve of positive control (chloroquine) on (**a**) 3D7 and (**b**) Dd2 strains.



Discussion

Research is needed to develop plant-based complementary medicine for malaria since malarial parasites have developed resistance to the synthetic drugs like chloroquine, and ACT [21,22]. We found no studies on antimalarial activity, in vitro or in vivo, of *A. calamus*, *D. gelonioides* and *L. aspera*; however, there are reports on the antioxidant and antihepatotoxic activities of *A. calamus* [23], nematicidal and antifungal activities of compounds extracted from *D. gelonioides* [24], and antioxidant activity of *L. aspera* [25].

Figure 2. Dose vs response curve of *Acorus Calamus* on (**a**) 3D7 and (b) Dd2, *Dichapetalum gelonioides* on (**c**) 3D7 and (**d**) Dd2, and *Leucas aspera* on (**e**) 3D7 and (**f**) Dd2 strains.

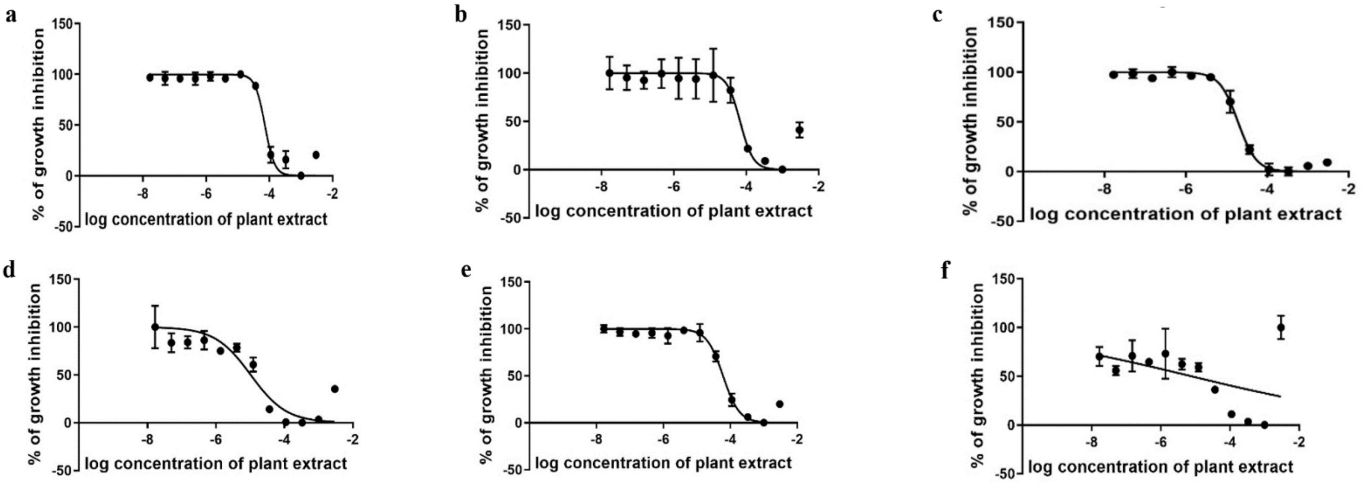


Table 1. Inhibitory concentration (IC50) and antimalarial activity of methanolic extracts of *Acorus calamus, Dichapetalum gelonioides* and *Leucas aspera* against 3D7 and Dd2 strains.

Strain	Plant extract	IC50 (µg/ml)	Activity
3D7	A.C[1] Methanol	72.29	Inactive
	D.G[2] Methanol	19.15	Moderate activity
	L.A[3] Methanol	60.51	Inactive
Dd2	A.C[1] Methanol	67.81	Inactive
	D.G[2] Methanol	10.43	Promising activity
	L.A[3] Methanol	7.693	Promising activity

[1] *Acorus calamus;* [2] *Dichapetalum gelonioides;* [3] *Leucas aspera.*

Our study was designed to evaluate anti-plasmodial activity on two *P. falciparum* strains in the selected plant extracts by HRP2 ELISA technique. The effectiveness of all the extracts against *P. falciparum* parasites was dose-dependent; 0.003 gm/mL was the most effective dose. The initial IC50 value of the plant materials suggested that *D. gelonioides* has moderate and promising activities against 3D7 and Dd2 strains, respectively. *L. aspera* showed promising activity against the Dd2 strain, whereas *A. calamu*s remained inactive against both the strains. The results indicate that two of the studied species of plants, *D. gelonioides* and *L. aspera,* possess active components capable of inhibiting *P. falciparum* in vitro, which is in agreement with its traditional use.

Plant materials may contain phenolics that may be simple (e.g., phenolic acids, anthocyanins) or highly polymerized substances (e.g., tannins). The type of solvent used in the extraction procedure has a big impact on the success of extracting bioactive compounds from plants [26]. Methanol has proven to be a good solvent for extracting the bioactive compounds from the plant materials. Previous studies have shown that the growth of *P. falciparum* in the schizont stage was inhibited by a methanolic leaf extract of the chikadoma plant [27]. Another study reported that the methanolic crude extract of *Syzygium cymosum* had promising effect against 3D7 (IC50 = 6.28 g/mL), Dd2 (IC50 = 13.42 g/mL) [28].

Certain plant extracts can prove to be a good resource for antimalarial properties. *Vitex negundo* leaf extract showed effective anti-malarial interaction against the 3D7 and K1 strains, with IC50 values of 7.21 g/mL and 7.43 g/mL, respectively [29]. Similarly, *Acacia nilotica* plant extracts had antimalarial properties with initial IC50 values of leaves, pods and bark extracts of 1.29, 4.16 and 4.28 µg/mL respectively [30]. The activity of *D. gelenoides* and *L. aspera* against *P. falciparum* strains indicate that these plants can be vital sources of antimalarial agents. The results of the phytochemical investigation of these plants warrants further investigation to determine the active ingredient responsible for their antimalarial activity.

Conclusions

Antimalarial efficacy of plant extracts should be justified in both in vitro and in vivo settings. We used in vitro experiments only due to lack of resources and laboratory settings. However, this is the first ever report of antimalarial activity of *Dichapetalum gelonioides* against both CQ-sensitive and resistant strains, and *Leucas aspera* against CQ-resistant strain. These plants may have some valuable bio-active compounds and further phytochemical analysis of *Dichapetalum gelonioides* and *Leucas aspera* to isolate, purify, and identify the active compounds is recommended for using them as a source of potential drug candidates in the fight against malaria.

Acknowledgements

MFZ was supported by NST fellowship from the Ministry of Science and Technology, Government of the People's Republic of Bangladesh.

Authors' Contributions

MSA, MRHH, HK and PB participated in the design of the study. AB and SAS collected and extracted the plants. SAS and MFZ carried out the laboratory experiments and data analysis. MFZ drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of interests: No conflict of interests is declared.