

Brief Original Article

Investigation of antimalarial activity and cytotoxicity profiling of a Bangladeshi plant *Syzygium cymosum*

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Abstract

Introduction: The persistent increase of resistance to existing antimalarials underscores the needs for new drugs. Historically, most of the successful antimalarial are derived from plants. The leaves of the *S. cymosum* is one of the plant materials used by traditional healers in malaria-endemic areas in Bangladesh for treatment of malaria. Here, we investigated the crude extract and its fractions against chloroquine (CQ)-sensitive 3D7, CQ-resistant Dd2, and artemisinin (ART)-resistant IPC 4912 Mondulkiri strains of *Plasmodium falciparum*.

Methodology: The antimalarial activities were tested using HRP II based *in-vitro* antimalarial drug sensitivity ELISA described by WWARN and half inhibitory concentrations (IC₅₀) were calculated by non-linear regression analysis using GraphPad Prism. The cytotoxicity of the crude methanolic extract was assessed using the MTT assay on Vero cell line.

Results: The methanolic crude extract revealed promising activity against 3D7 (IC₅₀ 6.28 µg/mL), Dd2 (IC₅₀ 13.42 µg/mL), and moderate activity against IPC 4912 Mondulkiri (IC₅₀ 17.47 µg/mL). Among the fractionated portions, the chloroform fraction revealed highest activity against IPC 4912 Mondulkiri (IC₅₀ 1.65 µg/mL) followed by Dd2 (1.73 µg/mL) and 3D7 (2.39 µg/mL). The crude methanolic extract also demonstrated good selectivity with the selectivity indices of > 15.92, > 7.45, and > 6.91 against 3D7, Dd2, and IPC 4912, respectively when tested against Vero cell line.

Conclusions: This is the first report on *S. cymosum* for its putative antimalarial activity, and is imperative to go for further phytochemical analyses in order to investigate possible novel antimalarial drug compound(s).

Key words: antimalarial activity; *Syzygium cymosum*; cytotoxicity; medicinal plants.

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Introduction

Malaria is a deadly disease with an estimated 228 million cases and 405,000 deaths globally in 2018 [1]. Bangladesh is one of the ten malaria-endemic countries of the WHO Southeast Asian region and it is regarded as hypo-endemic in malaria transmission. Although the country is yet to document any clinical or molecular resistance to current treatments by ACT (artemisinin based combination Therapy), this has been found ineffective in neighboring countries in the east [2]. At the same time, consistent high prevalence of CQ resistance in Chittagong Hill Tracts of Bangladesh [3], accentuate the needs for new antimalarial in the near future as an alternative to ACTs failure. Scientists all over the world are trying to find out new antimalarial

from natural sources after being inspired by the discovery of quinine [4] and artemisinin [5] from plant sources [6,7].

Bangladesh has a long history of using medicinal plants by traditional health practitioners [8]. It was reported that tribal, indigenous, and native people use 27 species of plants in the Madhupur forest area of Tangail district [9], 24 plant species in the Rangamati district [10] and 7 species in Thanchi of the Bandarban district [11] for treating fever (including malaria and dengue fever). However, to date none of those plants were tested for *in-vitro* antimalarial activity. In the present study, we attempted to investigate the antimalarial properties of a Bangladeshi plant, *Syzygium cymosum*, locally known as Khudijam (small

black pulm), often prescribed to treat febrile patients by the traditional healers in Sylhet districts, a malaria-endemic area of Bangladesh. It grows up to 15-20 m in height with simple and broad leaf that is mainly used for wood and dye-stuff locally.

Methodology

Plant Materials

Plant material (leaves) was collected from the Sylhet district of Bangladesh. Plant collection was based on its traditional uses suggested by the local community and traditional healers. The plant was collected in November 2016, taxonomically identified in Bangladesh National Herbarium, and voucher specimen deposited with ID- DACB accession number 42860.

Preparation of crude extracts

Leaves were sun dried for 5 days, and ground into a coarse powder using a grinding mill. The powder (400g) was soaked, using a flat-bottom round flask, in methanol and stirred regularly for three weeks. Plant bioactive constituents were obtained through extraction using the maceration process [12]. The filtrate was collected through several filtrations with markin cloth and cotton plug as well as decantation. Finally, the filtrate was evaporated to dryness through rotary evaporation and lyophilization.

Preparation of fractions

Fractionation by solvent-solvent partitioning was performed using the Kupchan method [13] of partitioning with slight modification. In brief, 5g of crude extract was triturated with 90% methanol, and extracted successively with n-hexane and chloroform. Later these three fractions of n-hexane, chloroform, and the left-over mother liquid were evaporated to dryness using a rotary evaporator and freeze dryer.

Malaria parasites and culture

CQ-sensitive *P. falciparum* (3D7), CQ-resistant *P. falciparum* (Dd2), and ART-resistant *P. falciparum* (IPC-4912 Mondulkiri Cambodia 2011) strains were used. All parasites were obtained from BEI-resources (MR4/ATCC®, Manassas, VA, USA). The continuous culture of asexual blood stage was maintained by the Trager and Jensen *in-vitro* culture technique [14] with little modifications [15]. In brief, parasites were cultivated in human erythrocytes (obtained from o-positive blood group donor) and RPMI-1640 (Gibco by Life Technologies, Grand Island, NY, USA) culture medium enriched with 10% heat-inactivated non-

immune human AB+ serum plus 0.5% Albumax II (Gibco by life technologies, Grand Island, NY, USA) serum supplement powder (complete medium), 11 mM glucose, 25 mM HEPES, 23.81 mM NaHCO₃, 200 μM hypoxanthine, and 2 mL gentamicin of 10 mg/mL solution keeping at 37 °C under Candle Jar condition. Red blood cells were washed three times with RPMI 1640 (incomplete medium) before use in culture, and regular haematocrit was 2% in 5 mL culture condition. Parasite growth was monitored in every 24 hours with the daily change of fresh culture medium (complete medium), and parasitaemia was kept < 5%. The daily parasitaemia was calculated by observing blood smears stained with Giemsa (Merck, Darmstadt, Germany) and analyzed using light microscopy.

Assessment of in-vitro antimalarial activity

The *P. falciparum* histidine-rich protein II (HRPII) based ELISA (enzyme-linked immunosorbent assay)-sandwich technique is one of the methods for quantifications of cell growth [16] which was applied in experiments of screening many plant extracts and fractions. The crude methanolic extract and each of the fractions were evaluated against 3D7, Dd2, and IPC-4912 Mondulkiri strains to determine the concentrations that inhibit 50% of parasite growth (IC₅₀) using histidine-rich protein II antibody (anti-HRPII Monoclonal Mouse IgG and IgM, Immunology Consultants Laboratory, Portland, Oregon, USA) ELISA [16] with the help of WWARN (the worldwide antimalarial resistance network) protocol [17]. Parasites were synchronized with 5% D-sorbitol according to Lambros and Vanderberg [18] and adjusted to 0.1% parasitaemia with 2% haematocrit, and placed into 96-well plates (Nunc, Thermo Scientific, Roskilde, Denmark) as 180 μL culture and 20 μL test and control drug (chloroquine diphosphate, SIGMA-ALDRICH Co, St. Louis, MO, USA), applied in triplicate. The plates were incubated for 72 hours, subsequently frozen at -80°C and thawed until the erythrocytes were fully lysed.

Parasite survival was determined by HRPII ELISA using primary antibody MPFM-55A (Monoclonal Mouse IgM anti-*Plasmodium falciparum* HRP II, Immunology Consultants Laboratory, Inc. Portland, OR, USA) and the second antibody MPFG-55P (HRP Conjugated Monoclonal Mouse IgG anti-*Plasmodium falciparum*, Immunology Consultants Laboratory, Inc. Portland, OR, USA), absorbance was read at 450 nm by a microplate reader (BioTek, ELx808, Winooski, VT, USA). The IC₅₀ values were calculated using a non-

linear dose–response curve fitting analysis via Graph Pad Prism v.7.0 software.

Cytotoxicity assessment

Cytotoxic property of methanolic crude extract was assessed by functional assay, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] using Vero cells with little modifications from the methods described by Bagavan *et al.* [19] where OptiPRO SFM (Gibco by life technologies, Grand Island, NY, USA) containing 2% HI-FBS (Gibco by life technologies, Grand Island, NY, USA), and 2% sodium bicarbonate (Gibco by life technologies, Grand Island, NY, USA) were used as cell culture medium. Formazan formation was read on a microplate reader (BioTek, ELx808, Winooski, VT, USA) at 570 nm and percentage cell viability calculated manually using the following formula [20].

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

Results

The initial methanolic crude extract showed IC₅₀ of 6.28 µg/mL against 3D7 strain, 13.42 µg/mL against Dd2, and 17.47 µg/mL against IPC 4912 Mondulkiri, then the crude extract was undertaken for further solvent-solvent fractionation which yielded three different fractions of n-hexane, chloroform, and the left over aqueous methanol. These fractions were then applied to 3D7 strain and gave the IC₅₀s of 3.47, 2.39, and 7.60 respectively at the µg/mL level (Table 1). Their antimalarial activity was categorized as: high (IC₅₀ < 5 µg/mL), promising (5 < IC₅₀ < 15 µg/mL), moderate (15 < IC₅₀ < 50 µg/mL) and inactive (IC₅₀ > 50 µg/mL) based on previous reports [21-23]. Since the chloroform fractions showed the highest activity, it was then applied to CQ-resistant Dd2 and ART-resistant IPC 4912 Mondulkiri which gave IC₅₀s of 1.73 µg/mL and 1.65 µg/mL, respectively indicating its higher

activity on ART-resistant IPC 4912 Mondulkiri and CQ-resistant Dd2 compared to CQ-sensitive 3D7. The cytotoxicity of methanolic crude extract was investigated on Vero cells which gave an IC₅₀ of > 100 µg/mL leading to the selectivity indices of > 15.92, > 7.45 and > 6.91 against 3D7, Dd2, and IPC 4912 Mondulkiri, respectively as shown in Table 1. In summary, the chloroform fraction possesses highest antimalarial activity following n-hexane and methanol fraction. We considered the IC₅₀s of crude extract against 3D7 (6.28 µg/mL) and CQ-resistant Dd2 (13.42 µg/mL) as promising and ART-resistant IPC 4912 Mondulkiri (17.47 µg/mL) moderately active [21-23] with no cytotoxicity on Vero cells.

Discussion

Our extensive literature search revealed no scientific information on antimalarial activity of *Syzygium cymosum* either *in-vitro*, *in-vivo* but there is substantial report of its antioxidant properties [24]. The results found in this study indicate that this plant possesses active components capable of inhibiting *P. falciparum in-vitro* having no cytotoxic effects which also meet the answer to its traditional use.

Other than *S. cymosum*, different species of the genus *Syzygium* have been found to have antimalarial activities. The ethyl acetate and methanol extracts of flower bud of *S. aromaticum* showed antimalarial activity at IC₅₀s of 13 µg/mL and 6.25 µg/mL, respectively [25]. The crude leaf extract of *S. guineense* exhibited a considerable parasite suppression and schizontocidal activity on *Plasmodium berghei in-vivo* [26]. Antimalarial silver and gold nanoparticles from leaf and bark extract of *S. jambos* with no significant cytotoxicity have been also reported [27]. Bioassay-guided fractionation of *S. cumini* bark leading to isolation of ellagic acids and its derivatives found to inhibit parasite growth of *P. falciparum in-vitro* [28]. The roots of *Syzygium* sp. was found to have antimalarial activity of an IC₅₀ ≤ 10 µg/mL against

Table 1. *In-vitro* anti-plasmodial activity and cytotoxicity of crude *Syzygium cymosum* extract and its fractions against reference strains.

<i>S. cymosum</i> Extracts	Anti-plasmodial activity IC ₅₀ (µg/mL)			Cytotoxicity on Vero cell (IC ₅₀ , µg/mL)	Selectivity index (SI)		
	3D7	Dd2	IPC4912		3D7	Dd2	IPC4912
Crude	6.28	13.42	17.47	> 100	> 15.92	> 7.45	> 6.91
Fractions	Aqueous	7.60	NP*	NP*	NP*		
	n-Hexane	3.47	NP*	NP*	NP*		
	CHCL3	2.39	1.73	1.65	NP*		
Control drug (CQ)	Anti-plasmodial activity IC ₅₀ (nM)						
	3D7	Dd2	IPC4912				
	10.99	86.26	78.40				

*NP = not performed.

either CQ-sensitive or CQ-resistant clones of *P. falciparum* [29]. Experiment of *S. aromaticum*-derived oleanolic acid on *P. berghei*-infected rats revealed its ability to clear and reduce malaria parasites [30]. These findings support our results (Table 1) since the plant investigated in this study belongs to the same genus *Syzygium*. Further investigations are needed to reveal if the plant *S. cymosum* possesses the same active compounds as other species of its genus. Thus, this plant might be of great importance in terms of its putative compounds active against *P. falciparum*.

The higher IC₅₀ of chloroform fraction on CQ-sensitive 3D7 than the IC₅₀ of n-hexane and methanol fraction of *S. cymosum* (Table 1) suggests that the active compounds were more soluble in chloroform than n-hexane and methanol. The higher activity of chloroform fraction on CQ-resistant Dd2 and ART-resistant IPC-4912 Mondulkiri also confirm this. Since the chloroform fraction gave a consistent activity on all three strains, we assumed that the very same would happen to n-hexane as well. Therefore, n-hexane activities against Dd2 and IPC-4912 Mondulkiri were not investigated. Moreover, we assumed that there is no significant difference in solubility of the active compound(s) in *S. cymosum* leaf between n-hexane and chloroform given that the almost same IC₅₀ values in both fractions. However, the most active chloroform fraction has been selected for further investigation with an aim to isolate and characterize the active compounds solely responsible for *P. falciparum* growth inhibition at any stages of its life cycle.

Conclusion

It is recommended that a plant extract should be investigated both *in-vitro* and *in-vivo* to justify its antimalarial activity; yet, introduction of *in-vitro* *P. falciparum* culture for the first time in our lab, shortage of time, and limited resources only allowed *in-vitro* study of this plant. However, this is the first ever report on potential *in-vitro* antimalarial activity of *S. cymosum*. On the other hand, this report substantiates the traditional use of this plant. Further phytochemical studies such as bioguided assay fractionation, TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography), NMR (Nuclear Magnetic Resonance) etc. of the *S. cymosum* leaf are underway to isolate, purify and identify the active compounds which might be the potential drug candidates with the therapeutic value in combating malaria.

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Authors' contributions

MRHH, MKN, RH, MSR, AAC, MAR and MSA participated in the design of the study. MRHH, SAS, MKN and MTA carried out the laboratory experiments and data analysis. MRHH drafted the manuscript. MSA conceived the concept, designed and coordinated the study, supervised the study, and revised the manuscript. All authors read and approved the final manuscript.

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