Synopsis of the thesis on

Studies on the Antiplasmodial Activity of Medicinal Plants by Ethnopharmacological Approach

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Introduction

Malaria, an infectious disease caused by eukaryotic protozoan *Plasmodium* parasites and transmitted through female *Anopheles* mosquito vectors, is one of humanity's greatest health concerns. Female *Anopheles* mosquitos feed on human blood to obtain proteins from the blood plasma, which they need to nourish their eggs. The disease is widespread throughout the tropical and subtropical regions of the earth, spanning large ranges of South America, Africa, the Middle East, and Asia. It is associated with poverty as poor sanitary conditions contribute to its transmission, furthermore, infected populations experience reduced economic production. In 2019, 229 million cases of malaria have been reported worldwide (WHO, 2018). The genus *Plasmodium* has over 200 species that have been formally described to date (Sato, 2021). *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* are the five *Plasmodium* species that naturally infect humans and cause malaria. *P. falciparum* is the most lethal of these parasites, accounting for most of the malaria cases every year. The presence of a staggering number of sequence and gene arrays involved in pathogenesis and immune evasion contributes to the virulence of this species (Crabb and Cowman, 2002).

Chloroquine (CQ) served as the mainstay therapy for malaria for at least four decades (Deshpande and Kuppast, 2016), until CQ-resistant *P. falciparum* and *P. vivax* strains emerged, making it less effective. CQ, sulfadoxine-pyrimethamine and artemisinin are the most common antimalarial drugs. Due to the continuous threat of emergence of resistant *Plasmodium* strains to the available drugs, artemisinin derivatives are used in combination with a 'partner drug' from a different drug class and the resulting drug combinations are known as artemisinin-based combination therapies (ACTs). ACTs remain the most effective method to treat the resistant parasite at present but again with the reports of multidrug resistance, besides artemisinin (partial) resistance and partner drug resistance in four Greater Mekong Subregion (GMS) countries and the countries outside GMS (WHO, 2018). Consequently, in addition to understanding the emergence and selection of drug-resistant strains, which is necessary to stop the constant battle, there is an urgent need to discover new antimalarial drugs, before the massive death history repeats itself.

One of the most vital metabolic processes for the survival of the *Plasmodium* parasite is hemoglobin degradation. Plasmespsin I (PM I) and plasmepsin II (PM II), the food vacuole aspartic acid proteases in *P. falciparum*, are known to initiate the process of hemoglobin degradation by cleaving the native molecule in a highly conserved hinge region (Banerjee et al., 2002). They perform the first cleavage of the molecule between Phe33 and Leu34 of the α-chain that results in protein unfolding and release of the heme moiety (Gupta et al., 2010). They share only about 35% sequence homology with human aspartic protease, cathepsin D (Silva et al., 1996). Inhibitors designed against them have demonstrated inhibition of *P. falciparum* growth *in vitro* (Silva et al., 1996). Additionally, sound X-ray crystallographic data can be found on PMs. Overall, this strongly suggests that PM I and PM II as good targets for new antiplasmodial drugs.

Plants produce a plethora of molecules with various pharmacological properties, and many of these molecules have yet to be discovered. People have relied on plants for medicine since antiquity. Ayurveda, India's traditional medicinal system, and traditional practices involving the use of plants as medicine provide information on the various properties of the indigenous plants. The ethnopharmacology approach can be a successful tool for utilizing the knowledge embedded in traditional medicinal systems to discover the inhibitors of PM I and PM II from plants.

Rationale

In light of the above, the current study aimed at investigating the indigenous plants to find out antiplasmodial agents that are inhibitors of *P. falciparum* PM I and PM II. The selection of plants was done using the ethnopharmacological approach. PM I and PM II were expressed recombinantly to meet the requirement of the studies. To date, recombinant expression of PMs and achieving their active forms have proven difficult. Therefore, efforts were made to establish an expression system to achieve the enzymes in their active forms. Further, using *in silico* approach the interactions between the putative inhibitors of the enzymes PM I and PM II and the enzymes were studied to gain insight into their mechanism of action.

Objectives

To achieve the aforementioned aim, the objectives of the study were identified as the following:

1. Screening of medicinal plant extracts for antiplasmodial activity.
2. Cloning and expression of PM I and PM II from *P. falciparum*.
3. Screening of the antiplasmodial plant extracts to find out putative PM I and PM II inhibitors.
4. *In silico* studies on PM I and PM II and their putative inhibitors from the plant extracts.

Results and Discussion

Objective 1: Screening of medicinal plant extracts for antiplasmodial activity.

The plants selected for the study are as follows: *Aristolochia bracteolata* Lam. (Leaves, Roots), *Andrographis paniculata* (Burm.f.) Nees (Aerial parts), *Cassia angustifolia* M.Vahl (Leaves, Flowers, Seeds), *Cassia tora* L. (Leaves, Seeds), *Centella asiatica* (L.) Urb. (Leaves, Roots), *Commiphora wightii* (Arn.) Bhandari (Stem), *Curcuma zedoaria* (Christm.) Roscoe (Leaves, Rhizome), *Cyperus rotundus* L. (Leaves, Roots), *Glycyrrhiza glabra* L. (Roots), *Ficus religiosa* L. (Leaves, Roots), *Ocimum sanctum* L. (Leaves), *Phyllanthus amarus* Schumach. & Thonn. (Aerial Parts), *Terminalia chebula* Retz. (Leaves, Fruits), *Terminalia bellirica* (Gaertn.) Roxb. (Leaves, Fruits), *Tinospora cordifolia* (Willd.) Miers (Leaves), and *Vitex negundo* L. (Leaves).

Plant extracts were sequentially extracted using n-hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH) and water (AQ). The screening of the plant extracts for antiplasmodial activity was performed *in vitro* using CQ-sensitive *P. falciparum* 3D7 strain. Among all the 129 extracts tested, the following 22 extracts were found to exhibit > 70% antiplasmodial activity against the parasite. *Aristolochia bracteolata* (Leaves) AQ, (Roots) EtOH ; *Andrographis paniculata (*Aerial parts) EtOH, AQ; *Cassia tora* (Leaves) AQ, (Seeds) EtOH; *Commiphora wightii* (Stem) EtOH, AQ; *Curcuma zedoaria* (Leaves, Rhizome) DCM, EtOH, AQ; *Glycyrrhiza glabra* (Roots) AQ; *Ficus religiosa* L. (Leaves) Hex, (Roots) EtOAc, EtOH, AQ; *Ocimum sanctum* (Leaves) DCM; *Phyllanthus amarus* (Aerial Parts) DCM, EtOAc; *Terminalia chebula* (Fruits) AQ; *Tinospora cordifolia* (Aerial parts) EtOH; *Vitex negundo* L. (Leaves) AQ. Phytochemicals belong to different classes,

which can be selectively extracted using different organic solvents. Thus, the *in vitro* screening results indicated the potential as well as the class of an antiplasmodial agent in a plant.

The putative antiplasmodial plant extracts demonstrating *Plasmodium* parasite inhibition > 70% were evaluated for their ability to inhibit the mPM I and mPM II, the recombinantly expressed PM I and PM II respectively. Of all the extracts tested, the *A. paniculata* EtOH, *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM extracts significantly inhibited both the enzymes with % inhibition more than 50%. These extracts were selected for further studies. The Pearson correlation coefficient r, between the % inhibition of mPM I and that of mPM II was found to be 0.996, which suggested a strong positive correlation between them.

The antiplasmodial IC_{50} value for the *A. paniculata* EtOH, *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM extracts was found to be of 4.12 µg/ml, 5.34 µg/ml, 4.38 µg/ml and 5.82 µg/ml. When tested for cytotoxicity against HEK-293 cells *in vitro*, the *A. paniculata* EtOH and *C. wightii* AQ extracts were found to be non-toxic to HEK-293 cells, while the *C. zedoaria* DCM and *P. amarus* DCM extracts were found exhibit cytotoxic activity against the cells. During i*n vitro* hemolytic activity analysis all the four extracts were found to be non-hemolytic to human erythrocytes. Altogether, the *A. paniculata* EtOH and *C. wightii* AQ extracts were concluded to be safe and were studied further.

Objective 2. Cloning and expression of PM I and PM II from *P. falciparum*.

The fragments carrying the mature regions of the PM I and PM II were cloned into pET-28a(+) vector to express mPM I and mPM II. The molecular weight of the His-tag fusion enzymes, mPM I and mPM II was 39.51 kDa and 39.37 kDa respectively. Following the purification pipeline, the pure enzymes mPM I and mPM II without His-tag of 37.63 kDa and 37.49 kDa respectively were achieved. Further, using thermal-assisted refolding technique the enzymes were successfully converted to their active forms which could degrade their natural substrate hemoglobin. The yields of the final purified active forms of mPM I and mPM II enzymes were found to be 110 µg/l culture and 120 µg/l culture respectively.

Objective 3: Screening of the antiplasmodial plant extracts to find out putative PM I and PM II inhibitors.

The *A. paniculata* EtOH and *C. wightii* AQ extracts with PM inhibition property were subjected to phytochemical analysis. After qualitative and quantitative analysis, the major class of phytochemicals found in the case of *A. paniculata* EtOH extract were alkaloids, carbohydrates, phenolics, proteins, steroids and terpenoids, while in the case of *C. wightii* AQ extract they were alkaloids, amino acids, carbohydrates, phenolics and saponins. The principle components identified from *A. paniculata* EtOH extract by LC-MS-QTOF analysis included 14-deoxy-11,12-didehydroandrographolide and andrographolide which belong to the class of terpenoids, that constituted almost 50% of the extract as was indicated by the quantitative phytochemical analysis. The extract was found to have unexpected over-abundance of phospholipids. Explaining this condition of phospholipids profusion presents a new research question. The LC-MS-QTOF analysis of *C. wightii* AQ identified seven principle compounds of the extract along with a compound similar to Scroside D. The presence of large amounts of C_{35}-C_{39} fragments that are suggested to be derivatives of compounds belonging to the class saponins were also found. The absence hemolytic activity found in the case of *C. wightii* AQ extract despite the abundance of saponins corroborates the statement that various structural aspects, such as the nature of the aglycone backbone, the complexity of sugar moieties and the number, length and position of sugar side chains, may affect hemolytic activity (Vo et al., 2017).

Objective 4: *In silico* studies on PM I and PM II and their putative inhibitors from the plant extracts.

The interactions between the compounds identified from *A. paniculata* EtOH and *C. wightii* AQ extracts and the enzymes mPM I and mPM II were studied *in silico*. The 3D structures for the enzymes were predicted by the Phyre2 Protein Fold Recognition Server using 2BJU as a template. Total 13 compounds from the two extracts were docked against both the enzymes using the docking software tool AutoDock 4.2.6. Four compounds viz., 2-amino-2-ethyl-4-(methylsulfonimidoyl)butanoic acid (Ligand 1) and andrographolide (Ligand 3) from the *A. paniculata* EtOH extract, and safrole (Ligand 11) and sorbitol hexaacetate (Ligand 12) from the *C. wightii* AQ extract were found to have high binding affinity for the active site of both the enzymes. The two canonical catalytic aspartic acid residues, Asp34 and Asp214 of the enzymes PM I and PM II were represented by Asp40 and Asp220 in

mPM I and mPM II. Ligand 1 and Ligand 3 formed H-bond with the amino acid residues in the active site of the enzymes including the catalytic aspartic acid residue Asp220. These compounds also formed hydrophobic contacts with the other aspartic acid residue from the catalytic dyad Asp40 and the residues in the vicinity of the catalytic dyad of both the enzymes. Ligand 11 and Ligand 12 were found to make hydrophobic contacts with the catalytic dyad aspartic acid residues and the residues in the vicinity of the catalytic dyad of both the enzymes. The blocking of the active site residues is suggested as the possible mechanism of mPM I and mPM II inhibition by the four compounds which was found during the *in vitro* studies with the extracts. The fact that H-bonds form stable protein-ligand complex proposes 2-amino-2-ethyl-4-(methylsulfonimidoyl)butanoic acid and andrographolide as more promising inhibitors of mPM I and mPM II as compared to safrole and sorbitol hexaacetate.

Conclusion

The plant extracts namely, *A. paniculata* EtOH, *C. wightii* AQ, *C. zedoaria* DCM and *P. amarus* DCM were found to inhibit both the recombinantly expressed PMs, mPM I and mPM II. The recombinant expression of PM I and PM II in their mature forms was done and they were converted successfully into their active forms using thermal-assisted refolding technique. The work introduces an approach to achieve PM I and PM II in their active forms, which is simpler than those reported earlier. The compounds 2-amino-2-ethyl-4-(methylsulfonimidoyl)butanoic acid and andrographolide which form more stable complexes with the enzymes as compared to the other compounds can be taken as lead structures to develop new antiplasmodial drugs that are inhibitors of PM I and PM II.

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