Original Research Article

New Protein settings to support *in vivo* antimalarial activity in *Plasmodium berghei* infected mice after Garlic-Arteether Therapy

ABSTRACT

Many malaria endemic nations are pursuing malaria elimination and these technical challenges require the development of integrated approaches, among which safe and effective malaria vaccines could be a crucial tool. Due to non-availability of malaria vaccine, the control efforts relay heavily on treatment with new antimalarial agents preferably acting on newer targets. In this study, the protected serum proteomics after garlic and arteether combination treatment to *P.berghei* infected mice has been analyzed by western blotting. One of the identified host parasites specific proteins, peptidyl-prolyl-*cis-trans* isomerase A (PPIA) is known to catalyze the interconversion of the *cis* and *trans* and mediate certain protein folding events both in *in vitro* and *in vivo* conditions. This study hypothesizes that, overexpressed PPIA might lead to misfold of the parasite protein which are needed for parasite multiplication and in turn lead to the parasite death or in the protection of combination drug treated samples.

Keywords: *Plasmodium berghei*; Malaria; Serum protein; peptidyl-prolyl-*cis-trans* isomerase A; over expression; Misfolding; Protection.

1. INTRODUCTION

Despite of continuing efforts to eradicate malaria, the global number of malaria cases increased from 211 to 219 million between 2015-17, with a concomitant increase in deaths, especially in children below 5 years of age and approximately 97% are from Sub-Sahara Africa and Southeast Asia [1]. The measures to control malaria have become less effective due to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes [2, 3]. Many malaria endemic nations are pursuing malaria elimination [4] and these technical challenges require the development of integrated approaches, among which safe and effective malaria vaccines could be a crucial tool [5]. Due to non-availability of malaria vaccine, the control efforts rely heavily on treatment with new antimalarial agents preferably acting on newer targets.

The use of herbal plants as health promoters is gaining attention in recent years as they are proven to be a valuable source for the discovery of novel antimalarial therapeutic agents since the discovery of the first antimalarial drug in 1800s [6]. Garlic (*Allium sativum*) is one such traditional Ayurvedic medicine, widely used for various risk factors associated with several diseases. It has been proven beneficial treatment for several infection and inflammation [7] with its known anticoagulant, antibiotic, hypocholesterolemic, hypoglycemic and

hypotensive activities [8]. Garlics antimicrobial properties [9,10] have been recorded as early as 1940s [11] and its effects against plant pathogens [12], pathogenic fungi [13] and human pathogens [14] have been well studied. Previous studies from our laboratory have proved, three oral dose of garlic pearl oil (for three constitutive days) in combination with intramuscular single dose of arteether, an artemisinin derivate given to 72 h post-infected mice with *Plasmodium berghei* (*P.berghei*) is completely protected from malaria [15]. This combination therapy also revealed the immunomodulatory activity *via* nitric oxide pathway in *Plasmodium berghei*-infected mice [16]. In recent years, proteomic studies have thrown substantial contribution in understanding the host-pathogen relationship [17,18] and reflecting potential changes occurring due to infection and infection-induced proteins [19], which will serve either as an early indicator for diagnosis or therapeutic targets. There are reports which show specific proteins are overexpressed due to *P.berghei* infection [20] which mimic *P.falciparum*–induced cerebral malaria in human [21]. In this study, we have compared *P.berghei*-infected and protected mice serum after garlic-arteether treatment, initially by western blot analysis and the unique protein expressed only in protected sample was subjected to MALDI-TOF/TOF mass spectroscopy and confirmed by semiquantitative RT-PCR and further analyzed its interacting proteins using STRING v.91 (search tool for the retrieval of interacting genes/proteins). One of the identified parasite specific proteins in this study was turned out to be peptidyl-prolyl-*cis*-*trans* isomerase A (PPIA). The peptidyl *cis*-*trans* prolyl isomerase was first isolated by Fischer in 1984 [22] and is found in both prokaryotes and eukaryotes [23]. PPIA is known to catalyze the interconversion of the *cis* and *trans* isomers at the N-terminus site of Xaa-proline residues of the peptidyl-prolyl bonds in peptide and protein substrates and mediate certain protein folding events both in *in vitro* and *in vivo* conditions [24, 25]. Thisconformational change of the peptide bond is necessary during protein folding [26, 27] and it accelerates the rate-limiting steps in the refolding of several proteins in *in vivo* and hence called as peptidyl-prolyl *cis*-*trans* isomerase or cyclophilin or rotamase. Cyclophilins have been isolated from a variety of animal species, as well as from fungi, bacteria, plants, recently in the protozoan *Toxoplasma gondii* [28] and *P. falciparum* [29]. This study will conclude that the malaria protection in garlic and arteether combination therapy is *via* PPIA and lead to possible drug target for malaria control.

2. MATERIALS AND METHODS

2.1. Drugs and chemicals

Commercially available antimalarial drug α-β arteether (E MAL™, Themis Medicare Ltd., Uttarakhand, India) and ayurvedic medicine garlic pearl oil (Sun Pharmaceutical Ind. Ltd., Mumbai, India) was procured locally.

2.2. Animals

Experimental animals used in this study were Swiss albino mice of 4-5 weeks old, obtained from the Central Animal Facility, Indian Institute of Science, Bangalore and were maintained on a 12 h light-dark cycle with food and water *ad libitium*. All the precautions were undertaken to minimize suffering throughout the experiments and followed the guidelines of the "Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 48/1999/CPCSEA) and as approved by the Institutional Animal Ethics Committee (IAEC) (CAF/ Ethics/282/2012 and 585/2018).

2.3. In vivo infection and drug treatment protocols

The infections were initiated with 60-70% parasitized erythrocytes obtained from a passaged donor animal and injected intraperitoneally on the beginning of experiment (Day 0)with appropriate dilution. All the control mice died between Day 5 and Day 6 post infection. Drug treatment started after 72 h of post infection (Day 3), when the parasitemia was about 2-4%, with either single dose of intramuscular arteether (500 µg) and/or three oral doses of garlic pearl oil (100 µl/mouse) on Day 3, 4 and 5 to non-anesthetized mice both in mono and combination as shown in the treatment regimens (Table 1). Blood collected from tail vein and smears with Giemsa stain were checked for parasitemia progression or inhibition at regular time intervals and mortality of animals were monitored. *In vivo* antimalarial activity was examinedin groups of ten male mice in three independent experiments.

Table 1: Treatment regimens for mice

No.	Animal Groups	*P. berghei* Infection	Treatment Type	Treatment Day	Purpose
1	Uninfected	No	-	-	Negative control
2	Infected	Yes	-	-	Positive control
3	Infected + AE	Yes	AE	D3	Arteether monotherapy
4	Infected + G	Yes	G	D3, D4, D5	Garlic monotherapy
5	Infected + AE + G	Yes	AE+G	D3, D4, D5	Garlic and arteether combination therapy

AE - arteether; G - garlic

2.4. Serum sample preparation

Blood was collected from different experimental groups by cardiac puncture before the death of the infected control mice. It was allowed to clot for 2 h at room temperature then centrifuged at 5000 rpm for 20 min to separate the clotted material. Serum from normal mice without infection and with infection severs as our control. The pooled sera from two animals of each group were obtained and 100 µl aliquots were stored at -80°C until further analysis.

2.5. Parasite protein lysate preparation

Soluble parasite proteins were prepared as described by Ang et al [30]. In brief, the parasites were collected from infected blood after saponin lysis and suspended in an appropriate volume of phosphate buffer saline followed by sonication (5 min at 4ºC) and centrifugation at 10,000 rpm for 10 min. The supernatant was stored at -80ºC until further analysis.

2.6. SDS-PAGE and western blot analysis

For Western blot, 100 µg of parasite proteins were electrophoresed on a 10% SDS-PAGE gel under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad). Membranes were cut in vertical strips, blocked with 5 % BSA dissolved in PBS-T (PBS with 0.05% Tween 20) for 2 h at room temperature and probed with individual serum samples diluted to 1:50 in PBS, pH 7.4. Antibody responses were revealed with monoclonal secondary antibodies in dilution of 1:1000 followed by incubation with AP-conjugated anti-mouse antibodies (Dako, Hamburg, Germany). Western blots were performed with SDS-PAGE gels; all loaded with the same protein preparation and run under identical electrophoretic parameters. The strips shown are originated from a single blot. The blot was developed and visualized using a Pierce ECL Western Blotting Kit (Thermo Scientific).

2.7. Mass spectrometry protocol

Unique protein bands appeared only in samples of mice undergone garlic and arteether combination therapy. The corresponding protein bands in the western blot were excised manually from Coomassie stained gel and washed with 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and incubated at room temperature for 15 min. The wash was repeated until the coomassie dye was completely removed. The gel was later dehydrated with 100% ACN for 5 min. The gel was rehydrated with reduction solution containing 10 mM DTT and 100 mM ammonium bicarbonate for 30 min at 56°C. Next, the gel was treated with alkylation solution containing 50 mM iodoacetamide and 100 mM ammonium bicarbonate, incubated for 30 min in dark. The gel was subjected to another round of wash and dehydration. Later, the gel was rehydrated with protease digestion solution (20 µg of trypsin in 1 ml of 50 mM ammonium bicarbonate) and incubated for overnight digestion at 37°C. Peptide fragments were extracted with 60% ACN and 0.1% Trifluroacetic acid (TFA) and resuspended with 50% ACN and 0.1% TFA. 0.5 µl of the processed sample was spotted on MALDI plate followed by 0.5 µl of alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50% ACN and 0.1% TFA). Calibration was done with internal tryptic peaks of 842.5 and 2211.1 Da. The MALDI mass spectra were obtained using an ULTRA FLEXtreme MALDI TOF/TOF (Bruker Daltonics).

2.8. Peptide mass fingerprinting and database search

The collected peptide mass data from MALDI-TOF were processed and were searched against SwissProt database employing Mascot (Matrix Science) database for protein identification and for STRING v9.1 analysis (search tool for the retrieval of interacting genes), which is an application that aggregates available databases of known proteins. The search parameters included: consideration of probable modifications, such as carboxy methylation of cysteine residues and oxidation of methionine residues, and maximum missed cleavage of 1 using trypsin as a cleavage enzyme. Peptide mass tolerance was set at +/-100 ppm. Protein matches with high score ($p < 0.05$) were considered as significant.

2.9. RNA extraction from blood & confirmation by RT-PCR

The identified protein was further confirmed by semi-quatitative RT-PCR. Total RNA from blood of different groups was extracted with Trizol reagent (Invitrogen, USA) and the extracted RNA samples were subjected to DNAse (Promega) treatment to avoid genomic DNA contamination. 2 µg of total RNA was used for cDNA synthesis using random primer. One tenth of the volume (20 µl) was taken and set up PCR for PPIA by using primers sequences - forward 5'-AGGGTGGTGACTTTACACGC3' and reverse5'-CATTCCTGGACCCAAAACGC-3' along with endogenous constitutively expressing GAPDH primer 5′-AGGGATGTATTCG CTTTATTTAATGC-3′ and TCTTGTCCAAACAATTCATCATATC-3′ sequence. PCR conditions: initial denaturation at 95°C for 45 sec. annealing at 55°C (for PPIA) and 50°C (for GAPDH) for 40 sec. and extension at 72°C for 40 sec, with a final extension for 10 min. Control reaction was included under the same conditions using DNAs treated RNA as template along with the different sample groups. Amplified products were loaded on 1% agarose gel electrophoresis and visualized after ethidium bromide staining using Kodak EDAS120 system.

2.10. ELISA protocol

The different serum samples from all the groups were further subjected to PPIA detection by ELISA technique, using commercially available PPIA ELISA kits (Life Span Bio Sciences LS-F13411) and followed the protocol according to the manufacturer's recommendations. The concentrations were calculated from standard curves established according to the protocol. The results observed were supportive towards higher level of PPIA in combination treated group serum as compared rest of the group.

3. STATISTICAL ANALYSIS

The data are presented as the mean ± SD or mean ± SEM (n=3). Differences between groups were calculated by Student's t-test using SPSS software (Version 13.0, SPSS, Chicago) and $p<0.05$ was considered as statistically significant.

4. RESULTS

4.1. In vivo efficacy of garlic, arteether mono and combination therapy

It was interesting to examine the mechanism of action of garlic-arteether combination treated mice infected with *P. berghei* using a rodent model of malaria. Swiss mice infected with *P. berghei* were treated with single injection of arteether along with three oral dose of garlic, not only cleared parasite in the blood but also protected mice from malaria. This is confirming the previous results from the laboratory.

4.2. *Western blot and MALDI Analysis*

The sera collected from different groups were tested against *P. berghei* total protein by Western blotting. The results showed that the unprotected sera (un-treated and mono therapy group) did not recognize

any parasite-specific proteins, whereas sera from the protected (combination therapy) mice interestingly showed two parasite-specific proteins with molecular weight of 90kDa and 110kDa(marked by arrow) (Fig.1). The corresponding protein bands of western blot were identified in Coomassie stained SDS-PAGE gels, excised and subjected MALDI-TOF/TOF analysis and one of the bands identified was a very interesting protein, Peptidyl-prolyl cis-trans isomerase A (Band 2-PPIA) and the other one turned out to be C-X-C motif chemokine 11 (Band 1-CXCL11). We have done further analysis only with PPIA since this protein showed significant higher scorein Mascot (Matrix Science) analysis (Table 2).

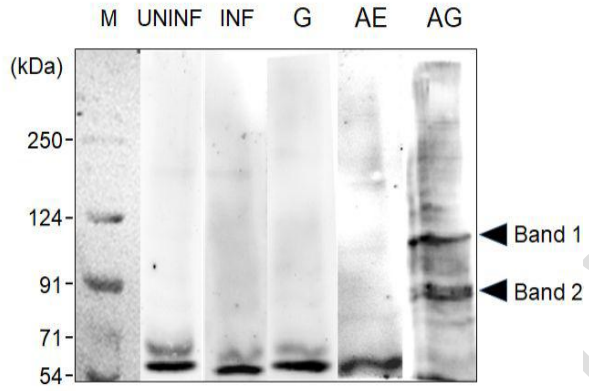

Fig 1: Western blot analysis of parasite proteins reacted with different sera samples (pooled sera from two animals of each group), UNINF-Uninfected; INF-Infected; G-Garlic treated; AE-Arteether treated; AG-Arteether and garlic treated; M-Molecular weight marker; Band 1 & 2 – parasite specific proteins detected only in combination treated protected mice sera.

Table 2

MALDI-TOF analysis of Band 1 and 2 and proteins identified by employing Mascot (Matrix Science) with the respective protein sequence

Sample	Identified Protein	Protein sequence coverage (Matched peptides shown in bold)
Band 1	C-X-C motif chemokine 11 (CXCL11) (Score:27)	MNRK**VTAIALAANWATAAQGFLMFKQGR**C LCIGPGMKAV **KMAEIEKASV IYPSNGCDKV EVIVTMK**AHK RQRCLDPRSK QAR**LIMQAIE** **K**KNFLRRQNM

Band 2	Peptidyl-prolyl cis-trans IsomeraseA (PPIA) (Score: 45)	M**VNPTVFFDI TADDEPLGRV SFELFADK**VP KTAENFRALSTGEKGFGYKGSSFHRIIPGFMCQGGDFTRHNG TGGRSIYGEKFEDENFILK**HTG PGILSMANAGPNTNGSQFFICTAKTEWLDGKHVVFGK**VK**E GMNIVEAMER**FGSRNGKTS KKITISD CGQL

4.3. RT-PCR Results

Semi-quantitative RT-PCR carried out for constitutively expressed endogenous control,GAPDH with 390 bp and specific PPIA with 550 bp PCR product and the results confirmed the overexpression of PPIA in combination treated group along with the basal values in the other group samples. Relative mRNA expression levels were normalized with GAPDH levels. This result gave us an important validation that PPIA is significantly upregulated in combination treated samples as compared to other groups (Fig.2).

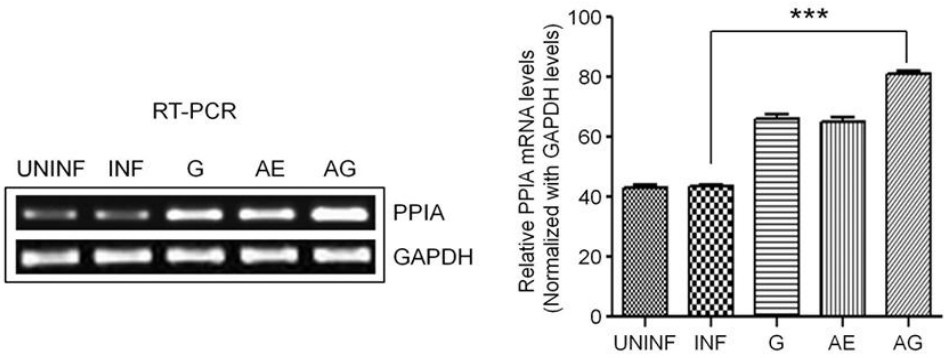

Fig 2: PPIA mRNA expression confirmed by semi-quantitative RT-PCR analysis of different groups. Relative mRNA expression levels of PPIA from blood of *P. berghei*-infected and treated animals. UNINF, uninfected; INF - infected; G - garlic treated; AE - arteether treated; AG - arteether + garlic treated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) (one-way ANOVA).

4.4. The protein-protein interaction networks of PPIA

The PPIA protein identified by MALDI analysis was further subjected to functional association networks (Fig.3) by STRING database. Results of protein-protein interaction analysis of PPIA revealed several predicted protein interactions, which are known from curated data bases and some of them are experimentally determined. The predicted interactions depend on gene neighbourhood, co-occurrence or fusions and other interactions like co-expression of proteins and also by studying protein homology. Most of these results proves that the involvement of PPIA in any biological and molecular processes is by binding to other proteins or by its catalytic activity. Table 3 shows the identified protein and their functional interaction depending on the interaction network analysis.

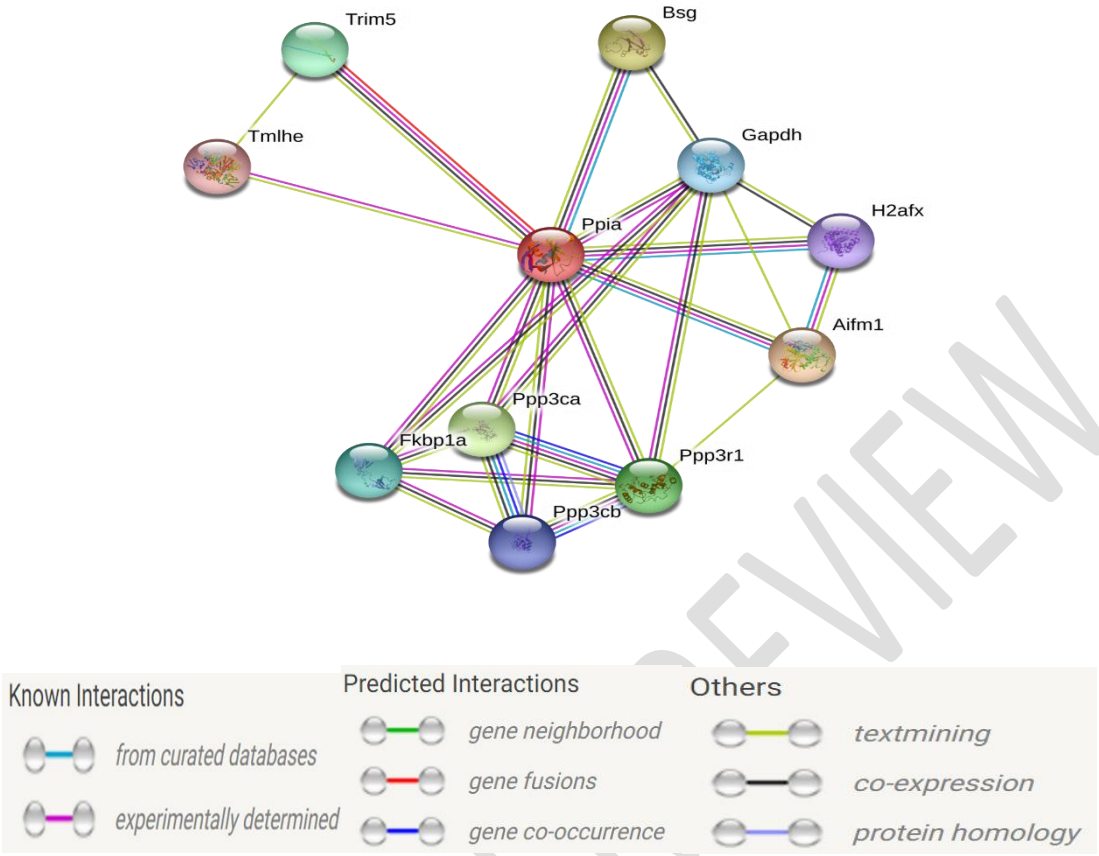

Fig 3: The protein-protein interaction networks of PPIA constructed by STRING Analysis.

Your Input:

		Neighborhood	Gene Fusion	Cooccurence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
Ppia	Peptidyl-prolyl cis-trans isomerase A; PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides; Belongs to the cyclophilin-type PPIase family. PPIase A subfamily (164 aa)									
Predicted Functional Partners:										
Aifm1	Apoptosis-inducing factor 1, mitochondrial; Functions both as NADH oxidoreductase and as regulator of apoptosis. In respon...					●	●	●		0.967
Bsg	Basigin; Plays an important role in targeting the monocarboxylate transporters SLC16A1, SLC16A3, SLC16A8 and SLC16A11 t...						●	●		0.962
Ppp3ca	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform; Calcium-dependent, calmodulin-stimulated protein ...					●				0.814
Ppp3r1	Calcineurin subunit B type 1; Regulatory subunit of calcineurin, a calcium-dependent, calmodulin stimulated protein phosphat...					●				0.770
Trim5	Tripartite motif-containing 5 (497 aa)							●		0.760
Fkbp1a	Peptidyl-prolyl cis-trans isomerase FKBP1A; Keeps in an inactive conformation TGFBR1, the TGF-beta type I serine/threonine k...							●		0.742
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase; Has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities, ...							●		0.736
Ppp3cb	Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform; Calcium-dependent, calmodulin-stimulated protein p...					●				0.730
H2afx	Histone H2AX; Variant histone H2A which replaces conventional H2A in a subset of nucleosomes. Nucleosomes wrap and co...						●			0.708
Tmlhe	Trimethyllysine dioxygenase, mitochondrial; Converts trimethyllysine (TML) into hydroxytrimethyllysine (HTML); Belongs to th...							●		0.705

Table3: Predicted Functional partners of PPIA

4.5. PPI A detection by ELISA

The different serum samples from all the groups were further subjected to PPIA detection by ELISA technique, using commercially available PPIA ELISA kits (LifeSpanBioSciences LS-F13411) and followed the protocol

according to the manufacturer's recommendations. The concentrations were calculated from standard curves established according to the protocol (**Fig 4**). The results observed were supportive towards higher level of PPIA in combination treated group serum as compared rest of the group.

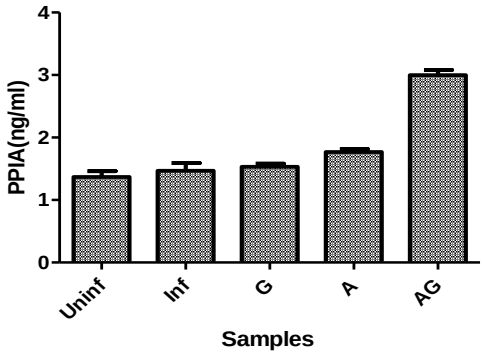

Fig. 4: PPIA detection in different sera samples. Mean (SD) PPIA concentration (ng/ml) was highest in arteether and garlic combination treated sera compared to the normal range in rest of the groups. Data is the mean value of the three independent experiments.

5. DISCUSSION:

Many pathogens will use the host machinery for its propagation or inhibition of immune system for their survival. In malaria parasites, because of the high replication rate and less machinery of its own, it depends on host for its need of protein. One such host protein identified in this study, PPIA is known to play a critical role in cell process such as protein secretion, RNA processing and cell cycle regulation to pathogenicity (31). It interacts with several proteins as shown in Table 2 to reach the target action. Many PPIA interacting proteins are involved in accelerating the protein folding (FKBP1A) or helping the host to modulate the cytoskeleton structure for in/out transport of proteins via microtubule and membrane associations (GAPDH) or the proteins (H2AX) involved in DNA replication, DNA repair, chromosomal stability, or a complex set of posttranslational modifications. In viruses and some other obligate parasites, it is known that the use of host PPIA for their own protein modification as an essential step for the pathogen multiplication. In this case these modified host PPIA might suppress the pathogenesis and turn out to be a potential target for gene therapy (32). General activity of PPIA and their phenotypic impact is experimentally difficult to define but several characteristics of PPIA can be linked to their *in vivo* pivotal roles. The contribution of PPIA to cellular physiology is thought to be very specific and dependent on different conditions and the niche in which a cell resides. PPIA are highly conserved abundant protein that originate in eukaryotic and prokaryotic cells based on the drug specificity and primary sequence homology (33, 34). This type of PPIA functional role was identified as the major cellular target for CsA (35) and

same kind of upregulation of PPIA protein was also observed by many other conditions like stress factors (36,37) or infection (38, 39) or as a part of repair process (40) which are the conditions might lead to misfold proteins. In malaria parasites, protein quality control is potentially important because of the high replication rate and the rapid transformations of the parasite during life cycle progression. This study hypothesizes that this over expressed PPIA might lead to misfold of the parasite specific protein and quality check in the host, which are needed for parasite multiplication and in turn lead to the parasite death or the protection in combination drug treated samples. To understand the development of novel strategies against malaria parasites, it is important to investigate the therapeutic targets, and molecular mechanisms to control the infections caused by plasmodium parasite. This study, for the first time provides new insights into the role of PPIA in malaria prevention and control of Plasmodium pathogenicity and guides to understanding of the regulation in pathogenic parasite. This identification of PPIA in malaria animal model might lead to our understanding the roles of this protein in cellular biochemistry, host-parasite interaction and the antiparasitic mechanism of the drugs that bind to them. Regarding protection by the host PPIA in this combination drug treatment has opened new perspectives and support the need for a thorough understanding of the involvement of PPIA in infectious diseases. Understanding the exact mode of action during infection still requires extensive research to know the association with malarial pathogen and this renders the new group of virulence factor as an attractive drug target.

6. CONCLUSION:

To understand the development of novel strategies against malaria parasites, it is important to investigate the therapeutic targets, and molecular mechanisms to control the infections caused by plasmodium parasite. All, these results may provide valuable information for designing the controlling infections of malaria. Our study, for the first time provides new insights into the role of PPIA in malaria based on prevention and control of *Plasmodium* pathogenicity and guides to understand the regulation in another pathogenic parasite. This study also provides a novel approach for new promising control strategies with the new combination drugs target for designing the antimalarial drugs. This finding will allow further characterization of PPIA cyclophilins leading to new insights into the malaria parasite biology and a novel target for malaria control. PPIA shows general enzymatic activity and its impact in *in vivo* role, is difficult to define. This identification of PPIA in parasites and the animal model might lead to our understanding of the functional roles of this protein in cellular biochemistry, host-parasite interaction and the antiparasitic mechanism of the drugs that bind to them.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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