Characterization of *P. falciparum* and *P. vivax* L-Lactate Dehydrogenase: Genetic polymorphism and screening of ethnopharmacological antimalarial herbs as potential inhibitors

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DECLARATION

Statement under O. Ph.D.8/ (iii) of The M. S. University of Baroda, Vadodara, India

The work presented in the thesis has been carried out by me under the guidance of Dr. Sanjay Ingle, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

Place: Vadodara — Priyadarshan Keluskar

Date: — Candidate

This is to certify that the above declaration is true.

Place: Vadodara — Dr. Sanjay Ingle

Date: — Research Guide

Dedicated to my family

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of variance
$APAD^+$	-	Acetylpyridine adenine dinucleotide
APADH	-	Acetylpyridine adenine dinucleotide (reduced form)
BSA	-	Bovine serum albumin
EDTA	-	Ethylenediamine tetra acetic acid
HRP2	-	Histidine rich protein
IC_{50}	-	half maximal inhibitory concentration
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
LDH	-	L-Lactate dehydrogenase
MEGA	-	Molecular Evolutionary Genetics Analysis
NAD^+	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
NIMR	-	National Institute of Malaria Research
Ni-NTA	-	Nickel-nitrilo triacetic acid
O.D.	-	Optical density
ORF	-	Open reading frame
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PDB	-	Protein Data Bank
PfLDH	-	*Plasmodium falciparum* specific L-Lactate dehydrogenase
pLDH	-	*Plasmodium* specific L-Lactate dehydrogenase

PMSF	-	Phenylmethylsulfonyl fluoride
ppm	-	Parts per million
PvLDH	-	*Plasmodium vivax* specific L-Lactate dehydrogenase
QMEAN	-	Qualitative Model Energy ANalysis
RDT	-	Rapid diagnostic tests
RFLP	-	Restriction fragment length polymorphism
RMSD	-	Root mean square deviation
rPfLDH	-	Recombinant *Plasmodium falciparum* specific lactate dehydrogenase
rPvLDH	-	Recombinant *Plasmodium vivax* specific lactate dehydrogenase
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssc	-	Single strand conformers
SSCP	-	Single strand conformation polymorphism

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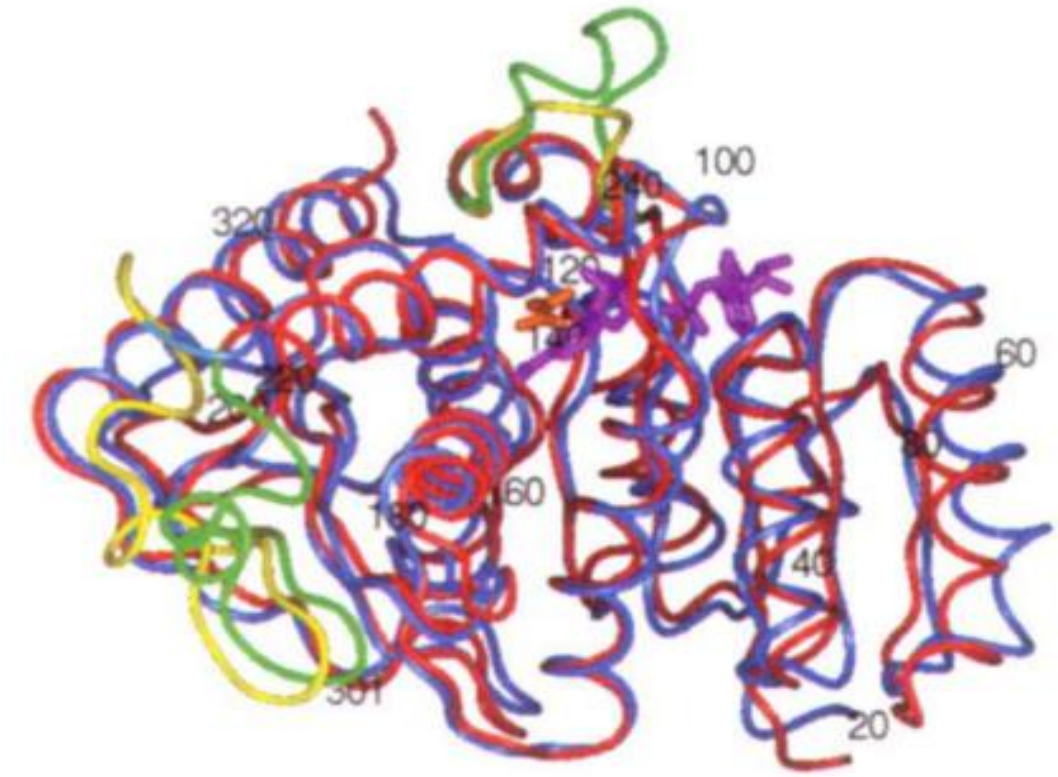
Chapter 1

Introduction



(Dunn et al. 1996)

CHAPTER 1

Introduction

1.1 History

Malaria is a mosquito-borne protozoan disease, older than recorded history and influenced settlement patterns of prehistoric man (Carter & Mendis 2002). The most ancient records for the prevalence of the disease date back to 484-425 BC (Nerlich et al. 2008). The name malaria (mal-aria) has been derived from Italian words: *"mal"-"bad"* and *"aria"-air"*. Charles Louis Alphonse Laveran, a French army surgeon, first noticed and recorded the parasites in microscopic examination of the blood of a patient suffering from malaria (Figure 1.1) (Laveran 1880). Ronald Ross, a British medical officer in Hyderabad, India, discovered that malaria is transmitted by mosquitoes and it was subsequently confirmed by the Italian professor Giovanni Battista Grassi, who further showed that human malaria could only be transmitted by *Anopheles* mosquitoes (Tuteja 2007). For thousands of years, traditional herbal remedies have been used to treat malaria but the first record of treatment utilizing quinine-rich bark of the Cinchona tree was in the 16th century, in Peru (Butler et al. 2010).

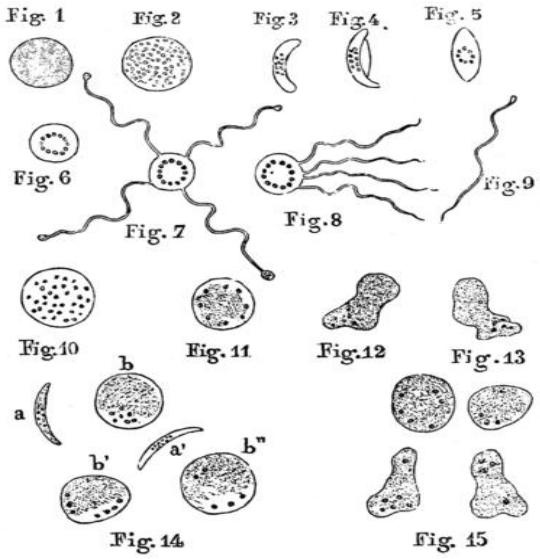


Figure 1.1: Pigmented parasites observed by Lavern (1880).

1.2 Global status of malaria

The global distribution of malaria is highly complex and is determined by both geographic and anthropogenic factors, hence malaria-afflicted and malaria-free areas are often dynamic (Greenwood & Mutabingwa 2002). Over two billion people, representing more than 40% of the world's population, are at risk of contracting malaria. In the year 2010, 216 million cases of malaria with 0.6 million deaths were estimated globally (WHO 2011). Malaria affects several tropical and subtropical countries and many areas of the tropics are endemic for the disease (Figure 1.2). The European and North American countries are nearly free from malaria burden and are observing a consistent decrease in the malaria cases. The South American, Middle East and Western Pacific countries also have achieved substantial control over the disease. The disease is most rampant in the sub Saharan Africa and south-east Asian countries (Hay et al. 2004). More than 90% of malaria cases and deaths occur in tropical Africa. Along with being the cause of death for around 25% children under the age of five, malaria is also a major financial hurdle for Africa with estimated cost of more than $12 billion annually (Snow et al. 2004). The remaining burden of disease is mostly clustered in India, Brazil, Afghanistan, Sri Lanka, Thailand, Indonesia, Vietnam, Cambodia, and China (Breman et al. 2001).

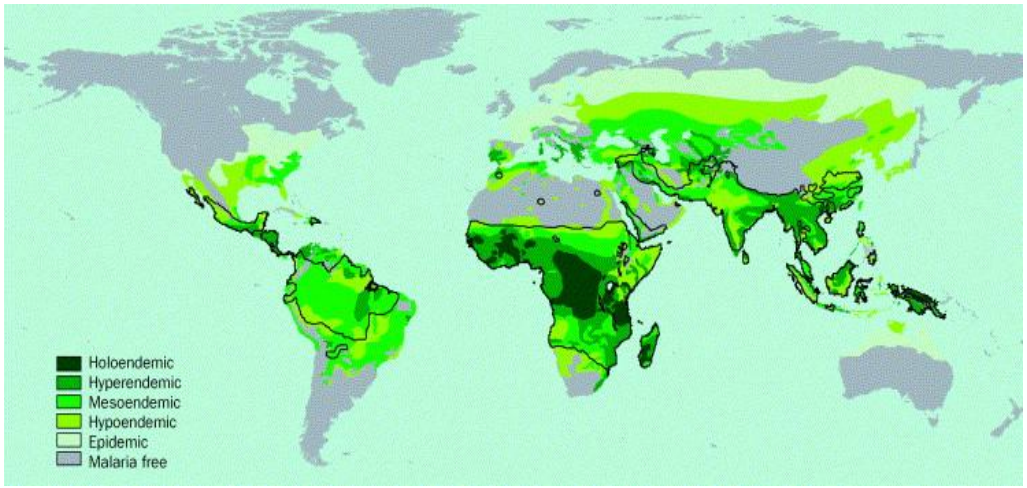


Figure 1.2: Global distribution of malaria (Hay et al. 2004).

1.3 Status of malaria in India.

India is one of the major contributor to the global malaria incidence. Malaria is endemic in entire India except regions above 1800 meters altitude and About 60% of malaria cases in Southeast Asia is contributed by India (WHO 2011). The factors that have established India as a hot spot for malaria infection are – a plethora of different species of malaria vectors; a hospitable environment for growth and proliferation of the parasites and vectors and finally a malaria-susceptible human lineage (Singh et al. 2009). In the colonial times (early 20th century) there was no aspect of life in the country that was not affected by malaria. The economic loss due to the loss of man-days caused by malaria was estimated to be at Rs. 10,000 million per year in 1935 (Panda & Mohapatra 2004). In 1947, around 75 million malaria cases and 0.8 million deaths were estimated in India. National Malaria Control Program (NMCP) was launched in 1953 to control malaria cases in India. A dramatic reduction in the cases of malaria by 1958, had encouraged launching of National Malaria Eradication Program (NMEP) and its implementation brought down malaria cases to 0.1 million without any deaths by 1965. However, there was reappearance of malaria by 1976 that led to a sharp rise in the number of malaria cases to 6.47 million (Table 1) (Narasimhan & Attaran 2003; Panda & Mohapatra 2004). In the recent past (1994-1996), outbreaks of malaria were observed at few places in the country, raising the death toll above 1000. The primary reason of resurgence included:

a) Resistance to insecticide like DDT became widespread amongst *Anopheles* mosquitoes (Sharma 2003).

b) Parasites acquired resistance to common anti-malarials such as chloroquine (A. Kumar et al. 2007).

c) Inadequate management in disease control at grass roots level had a setback in the malaria control program (Panda & Mohapatra 2004).

Table 1.1: Incidences of malaria in India. Source: National vector borne disease control program (http://nvbdcp.gov.in/malaria-new.html)

Year	Total cases	*P. falciparum*	Deaths
1947	75 million	?	800,000
1961	49151	?	--
1965	99667	?	--
1976	6.47 million	0.75 million	59
1984	2.18 million	0.65 million	247
1985	1.86 million	0.54 million	213
1986	1.79 million	0.64 million	323
1987	1.66 million	0.62 million	188
1988	1.85 million	0.68 million	209
1989	2.05 million	0.76 million	268
1990	2.02 million	0.75 million	353
1991	2.12 million	0.92 million	421
1992	2.13 million	0.88 million	422
1993	2.21 million	0.85 million	354
1994	2.51 million	0.99 million	1122
1995	2.93 million	1.14 million	1151
1996	3.04 million	1.18 million	1010
1997	2.57 million	0.99 million	874
1998	2.09 million	0.91 million	648
2002	1.84 million	0.87 million	973
2003	1.86 million	0.85 million	1006
2004	1.91 million	0.89 million	949
2005*	1.81 million	0.80 million	963

? no data; -- not detected

* 2005 onwards updates, in detail, are presented in appendix I.

Currently, 80.5% population of India lives in malaria risk areas. Of this, 4.2%, 32.5% and 43.8% live in areas of high, moderate and low risk to malaria respectively. The states of Rajasthan, Gujarat, Karnataka, Goa, Southern Madhya Pradesh, Chhattisgarh, Jharkhand, Odisha and North-eastern states have the highest incidences of malaria (Figure 1.3; further details in appendix 1). The highest burden of malaria is experienced by Odisha state. Although the state has a population of 36.7 million (3.5% of India's total population), it contributes 25% of a total of 1.5 – 2 million reported annual malaria cases and 30% of deaths caused by malaria in India (A. Kumar et al. 2007).

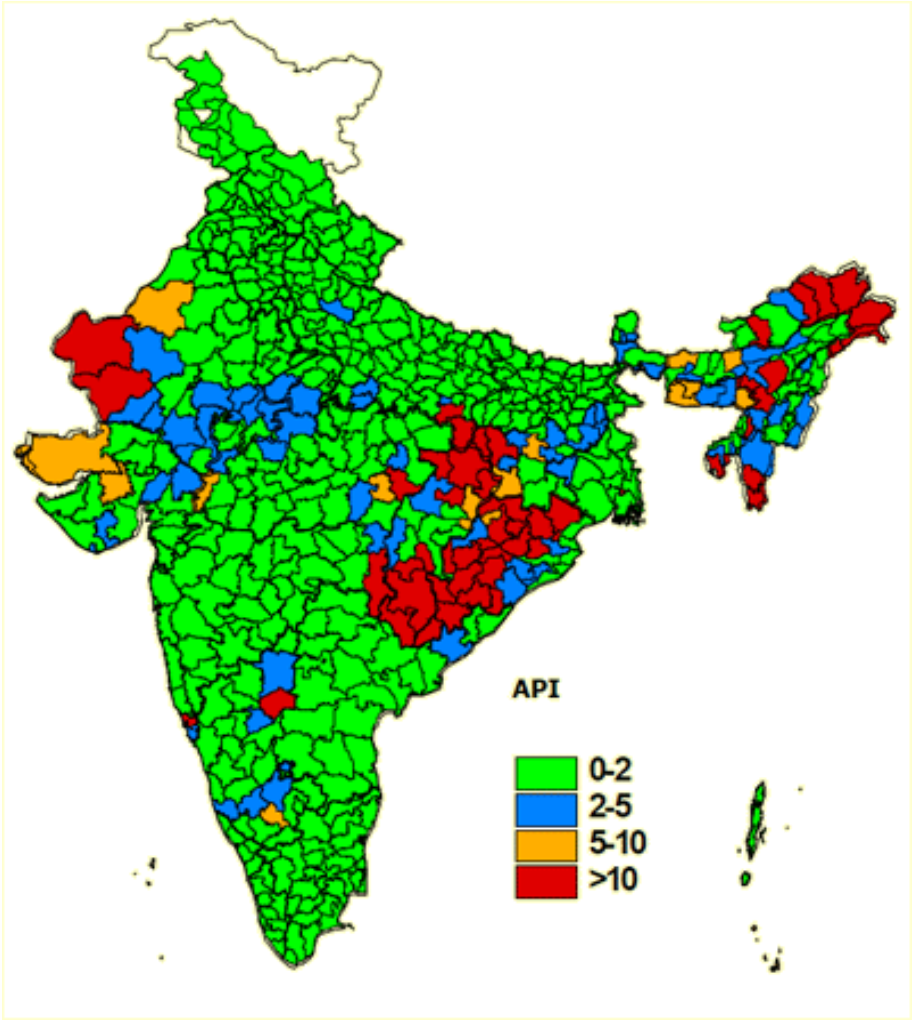


Figure 1.3: Prevalence of malaria cases in India. Annual Parasite Incidence (API) is a malariometric index to express malaria cases per thousand population (A. Kumar et al. 2007).

1.4 *Plasmodium*: The malaria parasite

Malaria is caused by eukaryotic single-celled microorganisms belonging to the genus *Plasmodium*. More than 100 species of *Plasmodium* can infect numerous animal species such as reptiles, birds and various mammals. These are generally host specific and vector specific in that each species will only infect a limited range of hosts and vectors. Four distinct species that infected humans under natural conditions are: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Tuteja 2007). The species differ in regards to their morphology, details of their life cycles, clinical manifestations, geographical distribution and in their drug responses. ***P. falciparum*** is the agent of severe, potentially fatal malaria. Almost every malarial death is caused by *P. falciparum.* It is much more prevalent in sub-Saharan Africa than in many other regions of the world (Snow et al. 2004). Moreover, erythrocytes infected by *P. falciparum* develop cyto-adherence properties that lead to binding of RBC to each other (rosettes) and to endothelial walls of capillaries which obstruct small blood vessels and if this occurs in the brain, it results in cerebral malaria, a complication that is often fatal, particularly in African infants (Tuteja 2007). ***P. vivax*** is the most frequent and widely distributed cause of recurring malaria. Infections caused by this species are rarely fatal but the recent report of severe vivax malaria has necessitated the reassessment of the benign nature of *P. vivax* malaria (Rogerson & Carter 2008). ***P. ovale*** is the least common malaria parasite, which is restricted to West Africa, New Guinea, and the Philippines (Carter & Mendis 2002). ***P. malariae*** is found worldwide, but with relatively low frequency and is the most benign among other malaria species. *P. ovale* and *P. vivax* may develop hypnozoite, which is a liver stage that may remain dormant, from few weeks to many years, before the onset of a new round of pre-erythrocytic schizogony, resulting in relapses of malaria infection. In some cases, *P. malariae* can produce long-lasting blood-stage infections, which can persist asymptomatically in the

human host for periods extending to several decades. Such cases, if left untreated, can act as a reservoir of the parasite (Tuteja 2007).

P. knowlesi is a primate malaria species generally infecting long tailed macaques. Humans become accidental hosts when they enter areas inhabited with macaques. *P. knowlesi* may cause re-infection and can be severe in areas where it is endemic (Lau et al. 2011). An emergence of *P. knowlesi* into the human population as a new species, causing malaria, could be devastating and the prevention of this situation deserves serious consideration (Cox-Singh & Singh 2008).

1.5 Anopheles Mosquito-The Vector

As a part of its complex life cycle, *Plasmodium* infects *Anopheles* mosquitoes, in order to be transmitted from human to human. *Plasmodium* species are finely evolved, to efficiently exploit the obligation of female *Anopheles* mosquitoes to feed on vertebrate blood, to complete its life cycle. Normally mosquitoes feed on nectar but female Anopheles mosquitoes need human blood for progeny production via a process called anterior station transfer (Singh et al. 2009). The female mosquito has a specialized apparatus called proboscis to penetrate the skin of its victim (Figure 1.4). At the end of the slender proboscis, there are two pairs of cutting stylets that slide against one another to slice through the skin. Once through the skin, the mosquito's proboscis begins probing for a tiny blood vessel. If it does not strike one on the first try, the mosquito pulls back proboscis slightly and tries again at another angle through the same hole in the skin. Inside the proboscis, there are two hollow tubes, one that injects saliva into the microscopic wound and one that withdraws blood. The mosquito's saliva includes a combination of antihemostatic and anti- inflammatory enzymes that disrupt the clotting process and inhibit the pain reaction (Budiansky 2002).

Various species of *Anopheles* have been found to be vectors in the different parts of the world. *Anopheles gambiae* is the chief vector in Africa and *A. freeborni* in North America. Nearly 45 species of the

mosquitoes have been found in India of which 9 species are involved in the active transmission of malaria. *A. culicifacies* is the major vector of rural malaria and is widely distributed while *A. stephensi* is responsible for disease transmission in urban areas. *A. fluviatilis* is a vector in the foothills of the Himalayas and A. *minimus*, *A. nivipes* and *A. dirus* are found mainly in the northeastern states of India. *A. sundaicus* is restricted to the Andaman and Car Nicobar islands. Besides these, *A. annularis* and *A. varuna* are considered secondary vectors with wide distribution (Kumar et al. 2012).

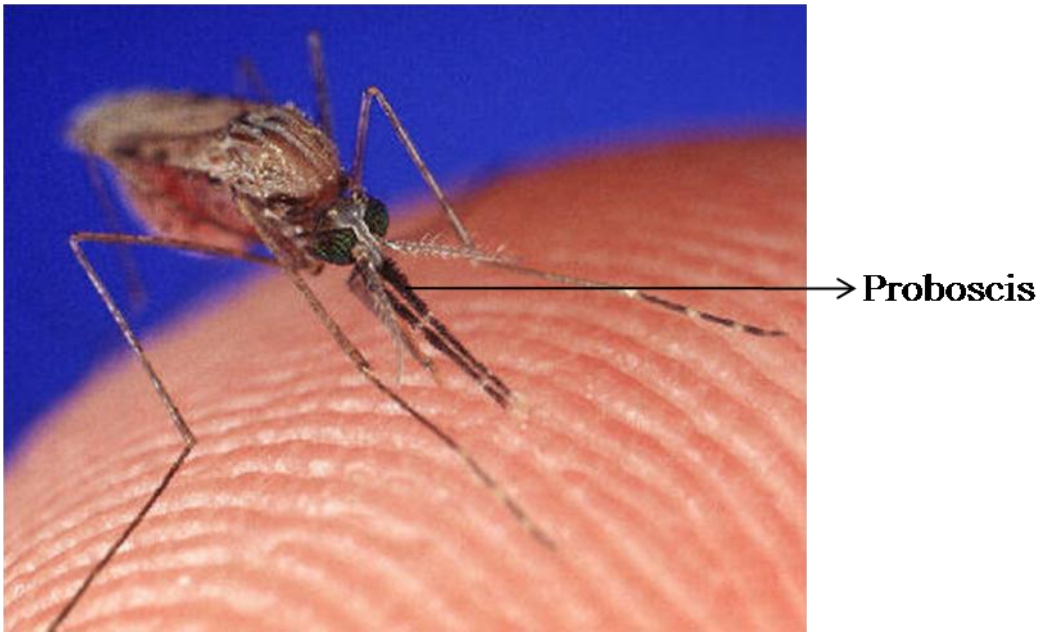


Figure 1.4: A female *Anopheles* mosquito having blood meal (Image from http://science.howstuffworks.com/life/genetic/gm-mosquito.htm).

1.6 Life cycle of *Plasmodium* species

Plasmodium species belong to the phylum apicomplexa and exhibit a heteroxenous life cycle involving vertebrate, a secondary host, in which the parasite completes its asexual stages of the life cycle and an arthropod vector which is a definitive host in which the parasite completes its sexual stages of the life cycle (Figure 1.5). Vertebrate hosts for *Plasmodium* species include: reptiles, birds, rodents, monkeys and humans, while *Anopheles* is the only arthropod vector for all *Plasmodium*

species. The disease is transmitted by the bite of an infected female *Anopheles* mosquito. Once injected into the bloodstream via saliva of the mosquito, infective sporozoites reach the liver and penetrate the liver cells (hepatocytes) where they remain for 9–16 days and undergo asexual replication, known as exorythrocytic schizogony. The circumsporozoite protein and thrombospondin-related adhesive protein (TRAP) on the surface of the sporozoites are responsible for the hepatocyte invasion (Frevert et al. 1993). Each sporozoite, through exo-erythrocytic schizogony, generates tens of thousands of merozoites inside the hepatocyte and once released from the liver, each merozoite can invade a red blood cell (RBC). Liver-stage parasites manipulate their host cells to guarantee the safe delivery of merozoites directly into the bloodstream (Tuteja 2007).

Merozoite invasion of erythrocytes is a highly complex process. It is initiated by recognition and reversible attachment of the merozoite to the erythrocyte membrane, followed by reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of substances from the rhoptry and microneme organelles, leading to the formation of the parasitophorous vacuole (Miller et al. 2002). The movement of the junction towards the posterior end of the parasite leads to the invagination of the erythrocyte membrane around the merozoite. This process is accompanied by the removal of the merozoite's surface coat protein. Finally, erythrocyte membrane is resealed to form parasitophorous vacuole that completes the of merozoite invasion (Miller et al. 2002). Soon after the invasion, parasites start asexual division inside the erythrocyte. The early trophozoites have a characteristic morphology called 'ring form'. Metabolically trophozoites are highly active, which is reflected by glycolysis of large amounts of imported glucose, the ingestion of host cytoplasm and the proteolysis of haemoglobin into constituent amino acids. Malaria parasites cannot degrade heme, which is a byproduct of hemoglobin degradation and free heme is potentially toxic to the parasite. Therefore, during hemoglobin

degradation, most of the liberated heme is polymerized into hemozoin (malaria pigment), a crystalline substance that is stored within the food vacuoles. The end of this trophic stage is marked by multiple rounds of nuclear division without cytokinesis resulting in the formation of schizonts. Each mature schizont contains around 20 merozoites and these are released after lysis of the RBC to invade further uninfected RBCs. The process occurs quite synchronously at approximately the same time of day and release of infected RBC contents stimulates the production of tumor necrosis factor and other cytokines, which are responsible for the episodic chills and fever (Tuteja 2007).

A small proportion of the merozoites in the red blood cells eventually differentiate to produce micro- and macrogametocytes. These forms of the parasite are no longer infective to humans but are essential for transmitting infection to mosquitoes, which ingest these gametocytes into their midgut. After ingestion, change in the environment leads to gametogenesis, where macrogametocytes form macrogametes and exflagellation of microgametocytes produces microga- metes. These gametes fuse, undergo fertilization and form a zygote,which is further transformed into an ookinete, which penetrates the midgut cell lining and develops into an oocyst. Sporogony within the oocyst produces many sporozoites and these sporozoits, after the oocyst ruptures, migrate to and invade the salivary glands. Once the sporozoits infect the salivary glands, the mosquito remains infective for 1–2 months. When an infected mosquito bites a susceptible host, the *Plasmodium* life cycle begins again (Miller et al. 2002; Tuteja 2007).

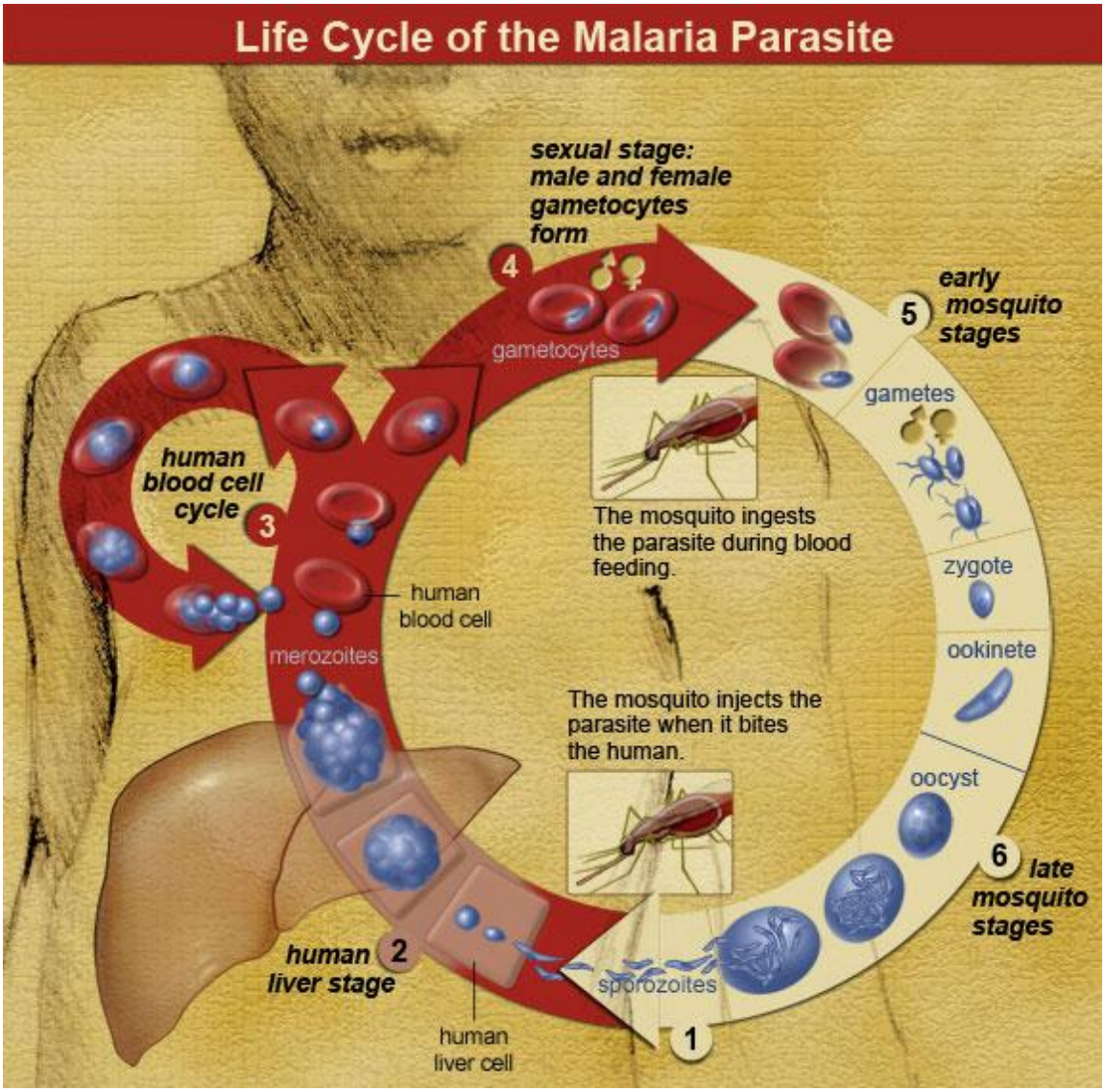


Figure 1.5: Life cycle of *Plasmodium* species (Image from http://www.niaid.nih.gov/topics/malaria/pages/lifecycle.aspx).

1.7 Clinical symptoms of malaria (Hoffman et al. 2011)

The pathology and clinical symptoms associated with malaria are mostly due to the asexual erythrocytic stage parasites. *Plasmodium* infection causes an acute febrile illness which is most notable for its periodic fever paroxysms occurring at either 48 or 72 hour intervals. The severity of the attack depends on the *Plasmodium* species as well as on the state of immunity, general health and nutritional status of the infected individual.

The symptoms of malaria usually start to appear 10-15 days after the bite of an infected mosquito. The typical prepatent (the time between sporozoite inoculation and the appearance of parasites in the blood) and incubation periods (the time between sporozoite inoculation and the onset of symptoms) following sporozoite inoculation vary according to species. All four species can exhibit non-specific prodromal symptoms a few days before the first febrile attack. These prodromal symptoms include: headache, slight fever, muscle pain, anorexia, nausea and lassitude. The symptoms tend to correlate with increasing numbers of parasites.

The prodromal symptoms are followed by febrile attacks, also known as the malarial paroxysms with periodicities of 48 hrs for *P. vivax, P. ovale*, and *P. falciparum*, and a 72-hr periodicity for *P. malariae.* The malarial paroxysm usually lasts for 4-8 hrs and begins with a sudden onset of chills in which the patient experiences an intense feeling of cold despite having an elevated temperature. This is often referred to as the cold stage and is characterized by a vigorous shivering. Immediately following this cold stage is the hot stage. The patient feels an intense heat accompanied by severe headache, fatigue, dizziness, anorexia, myalgia, and nausea. It is followed by a period of profuse sweating and decline of the fever. The patient feels exhausted and weak and usually falls asleep. Upon awakening the patient usually feels good, other than being tired, and does not exhibit symptoms until the onset of the next paroxysm.

1.8 Malaria disease control

Control on the incidence and spread of malaria in endemic countries like India requires strategic planning which includes framing technical guidelines and policies, and monitoring implementation through regular reports on malaria control. In India, The National Vector Borne Disease Control Program (NVBDCP), the agency responsible for the malaria control (along with other vector borne diseases) have strategized the following objectives for the malaria control (Das et al. 2012):

1. Provide a rapid and long lasting clinical cure to individual malaria patient.
2. Prevent progression of uncomplicated malaria to severe disease and death.
3. Reduce the occurrence of malaria-associated anaemia in populations residing in areas of high malaria transmission
4. Reduce consequences of placental malaria infection and maternal malaria-associated anaemia through chemoprophylaxis or preventive intermittent treatment during pregnancy.
5. Delay development and spread of resistance to antimalarial drugs.

One of the key deciding factors to achieve these goals is early diagnosis and prompt treatment of malaria. In the last century, malaria was treated with fast-acting and inexpensive drugs. Since the parasites causing the disease, have a comparatively short life cycle (and thus high evolutionary rate) as compared to the vectors and host, they are able to adapt and grow quickly to changing conditions. Hence, *Plasmodium* species have rapidly acquired resistance to most of the present line of antimalarial drugs, thereby hindering the control and eradication of malaria (Singh et al. 2009). Our present arsenal of drugs to fight against malaria (Table 1.2) is getting inefficient due to rapid emergence and spread of resistance to several drugs around the world (Fidock 2010).

Table 1.2: Existing antimalarial drugs and their use (Fidock 2010)

Common name	Chemical class	Clinical use	Resistance
Artemisinins (artemether, artesunate, dihydroartemisinin)	Sesquiterpene lactone endoperoxide	In artemisinin-based combination therapies (ACTs)	Possibly emerging
Lumefantrine	Arylamino alcohol	Most common first-line antimalarial therapy in Africa, in combination with artemether	No evidence of high-level resistance
Amodiaquine	4-Aminoquinoline	In combination with artesunate in parts of Africa	Limited cross-resistance with chloroquine
Piperaquine	Bisquinoline	In combination with dihydroartemisinin in parts of southeast Asia	Observed in China following single-drug therapy
Mefloquine	4-Methanolquinoline	In combination with artesunate in parts of southeast Asia	Prevalent in southeast Asia
Pyronaridine	Acridine-type Mannich base	Being registered for combined use with artesunate	No cross-resistance with other drugs reported
Quinine/quinidine	4-Methanolquinoline	Mainly for treating severe malaria, often with antibiotics	Exists at a low level
Atovaquone	Naphthoquinone	In combination with proguanil (a biguanide) for treatment or prevention	Has been observed clinically
Chloroquine	4-Aminoquinoline	Former first-line treatment for uncomplicated malaria	Widespread
Pyrimethamine	Diaminopyrimidine	For intermittent preventive treatment, combined with sulphadoxine (a sulphonamide)	Widespread
Primaquine	8-Aminoquinoline	For eliminating liver-stage parasites, including dormant forms of *Plasmodium vivax*	Unknown

In India, chloroquine was considered as a first line of drug for the treatment of malaria (Sharma 2007). Increasing chloroquine resistance in *P. falciparum* has led to changes in the species dynamics in India and by 2007, the proportion of *P falciparum* cases increased to 49% of the total burden, from 13% in 1978 (Figure 1.6). This is an issue of concern because *P falciparum* is associated with high mortality (Shah et al. 2011). Currently, the only fully effective class of antimalarial drug is the artemisinins and recent reports indicating emergence and spread of resistance to artemisinin, have triggered an alarm over the situation (Phyo et al. 2012). Drug resistance was largely acquired by mutations in the parasite genes coding for key targets or transporter proteins, under the selection pressure of the existing drugs (Hyde 2007). Therefore, one of the major agenda to contain scourge of the disease is the insistent search for novel drugs with more efficient targets (Hall and Fauci, 2009).

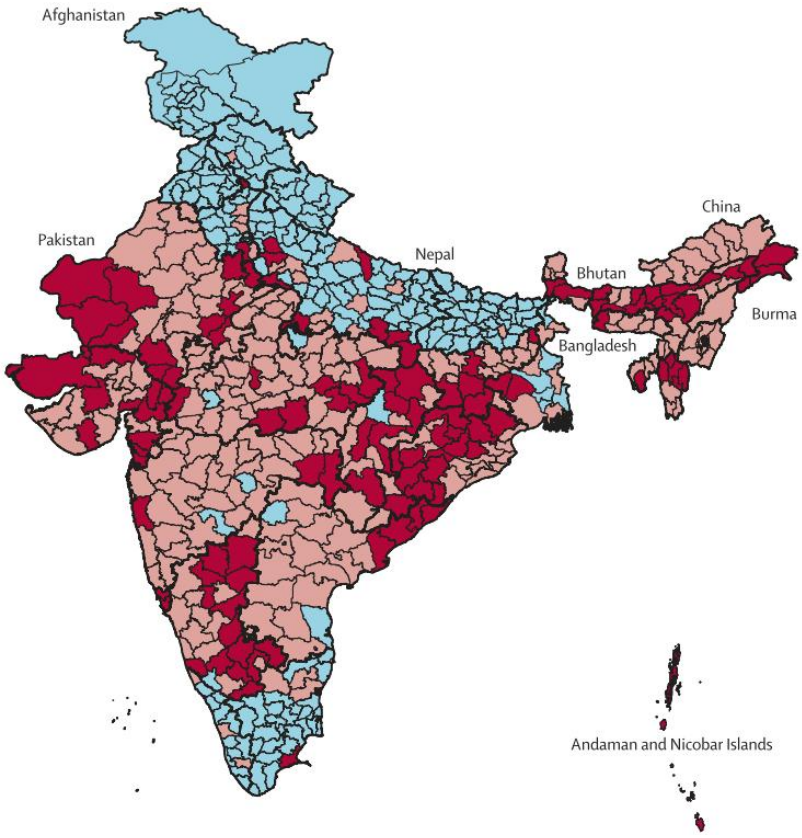


Figure 1.6: Distribution of chloroquine resistance in India. Red – districts with 10% or greater chloroquine-treatment failure; pink – *P. falciparum* endemic districts; blue – districts without reported *P. falciparum* transmission (blue) (Shah et al. 2011).

1.8.1 Malaria diagnostic tools

Global estimates of the number of malaria cases show a wide range, reflecting a lack of precision in the current malaria statistics. The most important cause of such a conjecture is inaccurate malaria diagnosis and insufficient public health data reporting system and healthcare access (Wongsrichanalai et al. 2007). Clinical diagnosis is the least expensive and commonly used method in endemic areas. The overlapping of malaria symptoms with other tropical diseases impairs the specificity of clinical diagnosis and hence encourages the indiscriminate use of antimalarials for managing febrile conditions (Wongsrichanalai et al. 2007). This practice was adequate in the past when inexpensive and well-tolerated antimalarials were still effective.

However, the changing situation of resistance to relatively cost effective therapy is making it unacceptable to diagnose and treat malaria only on the basis of clinical symptoms. In such endemic areas, parasitological confirmation of suspected malaria cases has become essential (Murray et al. 2003; Mariette et al. 2008). Thick and thin peripheral blood smears, stained with Giemsa stain (or, alternatively, Wright's or Field's stains), is the 'gold standard' for routine parasitological confirmation. Malaria smears permit both, species identification and quantification of parasites (Moody & Chiodini 2000). However, processing and interpretation of malaria smears requires appropriate equipment as well as considerable training and expertise. These factors limit their use in the remote areas of endemic regions (Murray et al. 2003).

In early 1990s, rapid malaria diagnostic tests (RDT) were introduced to overcome the limitations of the 'gold standard' of malaria diagnosis (Murray et al. 2003). RDTs are intended to allow simple, swift, precise diagnosis of malaria in areas where standard laboratory diagnosis is not available. The most practical of these are the rapid antigen detection tests, which detect parasite proteins in finger-prick blood samples by immunochromatography.

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Immunochromatographic tests are based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody coated on a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line (Figure 1.7). Incorporation of a goat antimouse antibody band, on a strip, ensures that the system is controlled for migration (Piper et al. 1999). The most common antigens targeted by these assays are:

histidine-rich protein-2 (HRP-2), aldolase and *Plasmodium* specific lactate dehydrogenase (pLDH) (Murray et al. 2003). HRP-2 is a protein produced by asexual stages and young gametocytes of *P. falciparum*. It is expressed on the RBC membrane surface, and because of its abundance in *P. falciparum*, it was the first antigen to be used to develop an RDT for its detection (Moody 2002). Parasite specific enzymes were used as other diagnostic targets. Aldolase, a key enzyme in the glycolysis pathway in malaria parasites, is well conserved across all human-specific species of *Plasmodium* and is used as a panmalarial antigen target (Murray et al. 2008). pLDH, the terminal enzyme in the malaria parasite's glycolytic pathway, is also an antigen target for detection of sexual and asexual malaria parasites. Monoclonal antibodies have now been developed that can target a conserved domain of pLDH on all human malaria species (panmalarial) or specific regions unique to *P. falciparum* or *P. vivax*. Although the number of RDTs detecting HRP-2 surpass those detecting pLDH (WHO 2009), the latter has advantages over the HRP-2 based RDTs, such as absence of the prozone effect (Gillet et al. 2009) and direct correlation between the level of parasitaemia and the titer of pLDH antigen in the patient's blood (Piper et al. 1999).

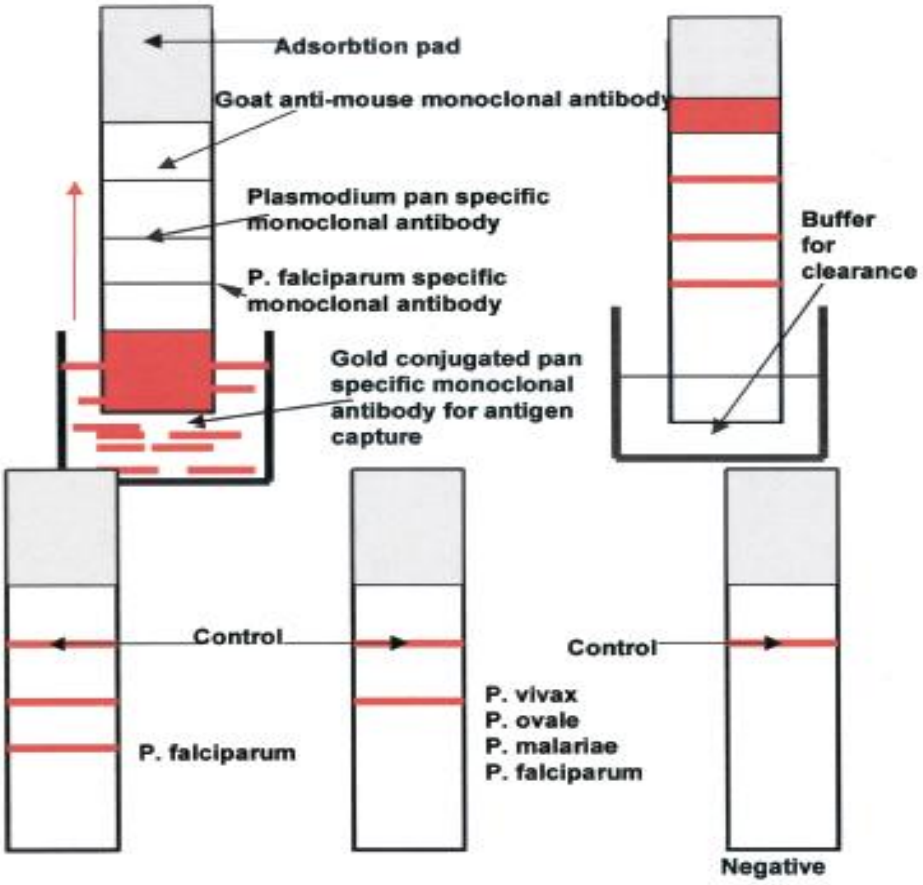


Figure 1.7: Schematic diagram showing principle of immunochromatography based RDT for malaria (Moody 2002).

1.8.2 Malaria vaccine – a distant reality

A safe, effective and affordable malaria vaccine is expected to provide a long-lasting protection from infection, reduce disease severity, prevent death and interrupt transmission. Various asexual and sexual stages of the *Plasmodium* provide numerous targets for mounting an immunological attack by vaccines. Malaria vaccines have long been the subject of intense research, but unfortunately, any clinically approved vaccine is not yet available. One of the several challenges faced by malaria vaccine developers is dealing with the question of parasite genetic polymorphism reflected in continuous antigenic variation within the parasite surface antigens resulting in evasion of the immune response by the parasite (N. Kumar 2007). It is hoped that at least a partially efficacious malaria vaccine will be available in the next 5-10

years and it will form the basis for the development of other more effective malaria vaccines (Chauhan 2007).

1.8.3 Discovery of novel drug/drug target – a need of the time.

The major concern about malaria and its global health burden is evolving drug resistance in the *Plasmodium* parasite. In *P. falciparum* resistance has arisen against all classes of the first line antimalarial drugs and several of these drugs had been withdrawn from use in many countries. Chloroquine, for instance, is no longer recommended for the treatment of *P. falciparum* in India, and has been replaced with more expensive artemisinin-based combination therapy (ACT). It is now accepted as inevitable by the World Health Organization that all drugs will eventually fail in the face of resistance evolution. The only solution to tackle this failure is constant development of new therapies (Das et al. 2012).

Antimalarial drug development can follow several strategies, ranging from minor modifications of existing agents, to the design of novel agents that act against new targets (Rosenthal 2003). One approach is to optimize the dose and combination of existing antimalarial drugs. The combination of amodiaquine and sulfadoxine/pyrimethamine has demonstrated excellent antimalarial efficacy in regions of East Africa, with fairly high levels of resistance to each individual agent (Dorsey et al. 2002).

Another approach is to improve the existing antimalarial drugs by chemical modifications of these compounds. The chemical alterations need not be necessarily guided by the knowledge of the mode of action or the biological target of the parent compound. This approach has been used for the development of many existing antimalarials. For example, chloroquine, primaquine and mefloquine were discovered through chemical strategies to improve upon quinine (Rosenthal 2003).

Third and time tested approach is to screen plant derived compounds for antimalarial activity. Medicinal plants, traditionally used to treat malaria, possess a repertoire of candidate antimalarial drugs and have been exploited globally for many centuries, with excellent examples being quinines and artemisinin (Klayman 1985). Ethnopharmacological analysis of such traditional plants can lead to novel, affordable and accessible antimalarial drugs (Karunamoorthi and Tsehaye, 2012). Several studies have screened herbs, traditionally used to treat febrile illness in malaria endemic regions, for parasiticidal activity (Tona et al. 1999; Andrade-Neto et al. 2003; Chenniappan and Kadarkarai 2010; Muthaura et al. 2011; Ruiz et al., 2011;). Moreover, Kantamreddi and Wright (2012) have recently confirmed efficacy of this ethnopharmacology guided approach over random screening of plants in the process of drug discovery.

The most innovative approach in the antimalarial drug discovery process is *de novo* identification of novel drug targets followed by identification and/or synthesis of the compounds which can act upon them. Recent developments in genome technology and the availability of parasite genome data have facilitated this approach of drug discovery. The genome of *P. falciparum* clone 3D7 was the first to be sequenced and annotated in the *Plasmodium* species (Gardner et al. 2002). Later, genome sequence data of *P. vivax* (Salvador I) was released (Carlton, Adams, et al. 2008). The genome sequence analyses of the *Plasmodium* species have revealed a large number of possible new drug targets that are coded by genes important for parasite biology and pathogenesis. Genome information for *Plasmodium* species is available at http:// www.plasmodb.org. The available information has opened new prospects of reverse pharmacology based drug discovery (Guantai and Chibale, 2011). In the process of reverse pharmacology, existing drug targets are verified or new targets are identified, which are crucial for the survival of the parasite and are absent or are distinctly different in the human host, by using available databases and bioinformatics tools. This is followed

by the screening of compounds that can inhibit the activity of the target (Takenaka 2001).

1.9 *Plasmodium* L-Lactate dehydrogenase (pLDH)

Plasmodium species specific L-Lactate dehydrogenase **(pLDH; L-Lactate : NAD^+-oxidoreductase, EC 1.1.1.27)** is the last enzyme of the glycolytic pathway. It is expressed in the endoerythrocytic stage of the parasite and plays an indispensable role in the survival of the parasites (Royer et al., 1986). Hence, the enzyme is a good target for screening and development of novel antimalarial drugs by reverse pharmacology approach, as described in the previous section (Section 1.8.3).

Exoerythrocytic schizogony is the fastest growing and the metabolically most active stage in the life cycle of the parasite. The clinical manifestations of the disease are the consequences of the parasite's successful completion of this stage in its host, as discussed in the section 1.6. To support this high metabolic rate, the parasite consumes large quantities of glucose – some 30 to 50 folds higher than the host cell (Vander Jagt et al. 1990). Though the genomic data of *P. falciparum* has confirmed the presence of the necessary enzymes needed for a functional tricarboxylic acid (TCA) cycle, it remains unclear as to whether this pathway is used for energy production or for the production of biochemical intermediates for other pathways (Gardner et al. 2002). Thus, anaerobic glycolysis is the sole source of energy production for the parasite, which is evident by the high levels of expression of many of the glycolytic pathway enzymes in the parasite, compared to the erythrocyte. Due to the almost complete dependence of *P. falciparum* on glucose metabolism for ATP production, all the enzymes of glycolysis represent potential drug targets (Vander Jagt et al. 1990).

The last enzyme of glycolysis, pLDH, produces L-lactate from pyruvate while regenerating NAD^+ for continued use in glycolysis and hence is essential for energy production in the parasite. It was one of the first enzymes purified from *P. falciparum* and since then it has been strongly

suggested to be an attractive drug target on the basis of a number of unique kinetic properties that pLDH exhibits (Vander Jagt et al. 1981). The three strikingly different kinetic properties of *Plasmodium* enzyme, compared with most LDHs studied to date are a) It is not inhibited at high pyruvate concentrations; b) It is very active with a synthetic coenzyme, acetyl pyridine adenine dinucleotide ($APAD^+$) and c) It is much more sensitive to inhibition by gossypol and its derivatives than the human equivalent (Sessions *et al.* 1997).

The molecular basis for these distinct kinetic properties was deciphered by comparing the amino acid sequence of *P. falciparum* specific LDH (PfLDH) with that of other LDHs including human LDH (Figure 1.8), which had presented some significant differences in the primary structure of the enzyme (Sessions et al. 1997). Sequence comparison has confirmed that *Plasmodium* LDHs are more distantly related to their mammalian counterparts, although the catalytic residues (Arg 109, Arg 171, His 195, and Asp 168) are strictly conserved (Chaikuad et al. 2005). Asp 168 and His 195 act as hydrogen donors, while the side chain of Arg 171 interacts with the carboxylate of pyruvate. The side chain of Arg 109 interacts with the ketone oxygen of pyruvate leading to polarization of the ketone carbonyl and hydride attack from NADH (Choi, Pradhan, et al. 2007).

The substrate inhibition observed in mammalian LDH is caused by the interaction of pyruvate with enzyme-NAD^+ complex, at higher pyruvate concentrations. In most LDHs the enzyme-NAD^+ complex is stabilized by a hydrogen bond formed between carboxy-amide side chain of the nicotinamide ring in NAD^+ and the hydroxyl group of Ser 163. In PfLDH, this interaction is hindered by Ser 163 to Leu conversion and hence enzyme-NAD^+ complex is detached and substrate inhibition is relieved (Sessions et al. 1997).

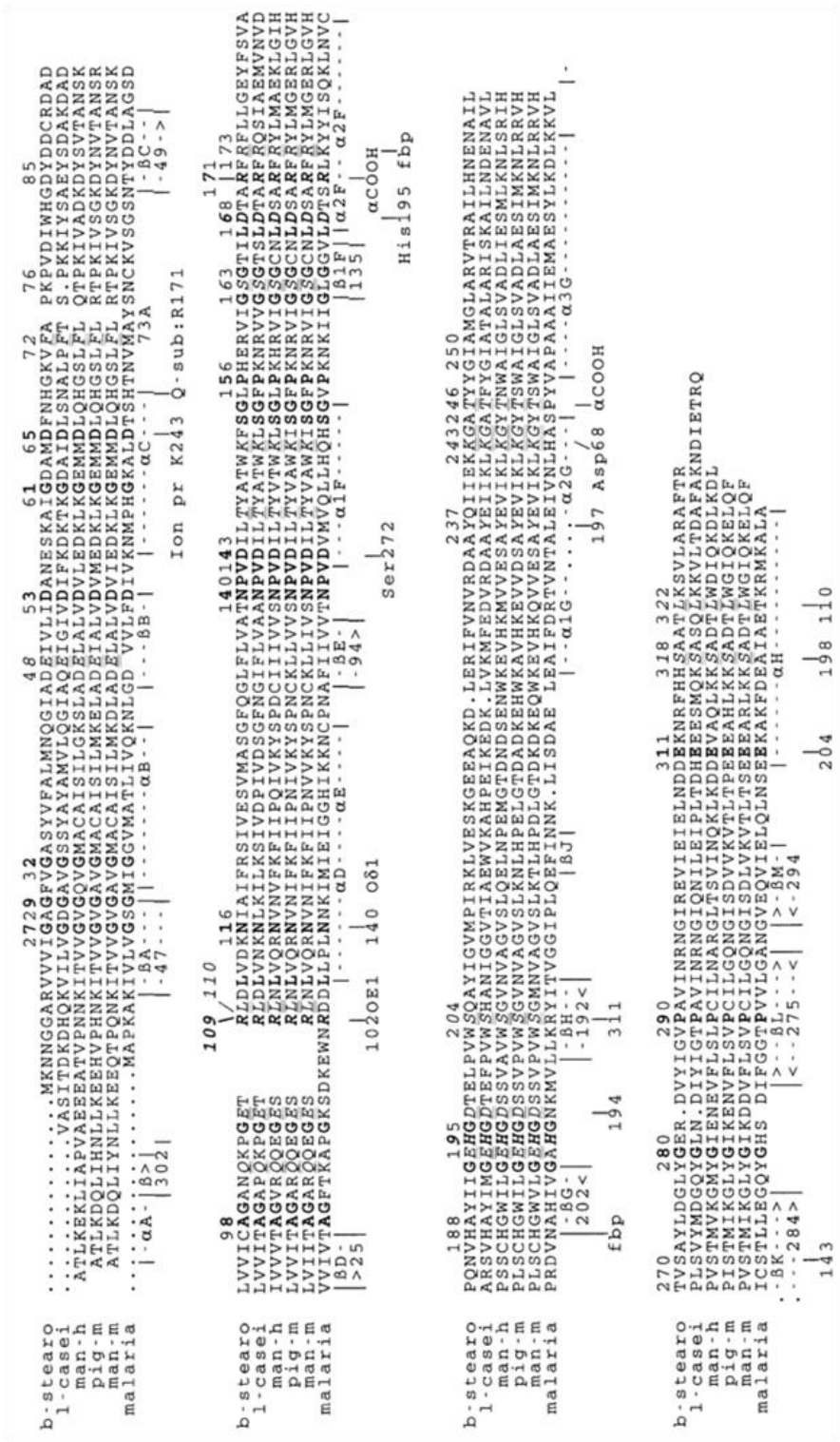


Figure 1.8: Sequence alignment of six LDHs. Residues conserved in nearly all LDHs, including PfLDH, are in bold, key catalytic residues are in bold italics, and residues conserved in nearly all LDHs but not in PfLDH are in underlined italics (Sessions et al. 1997).

The same amino acid variation was found to be liable for variable response of pLDH towards $APAD^+$, compared to other LDHs (Chaikuad et al. 2005). The nicotinamide side chain present in NAD^+ is replaced by a small acetyl side chain in $APAD^+$. This analogue cannot be utilised by the human enzyme because the methyl group in its acetyl side chain is unable to form hydrogen bond with Ser 163. However, in PfLDH the methyl group of the acetyl side chain can pack well against the hydrophobic Leu 163 and hence is efficiently used as a cofactor (Chaikuad et al. 2005).

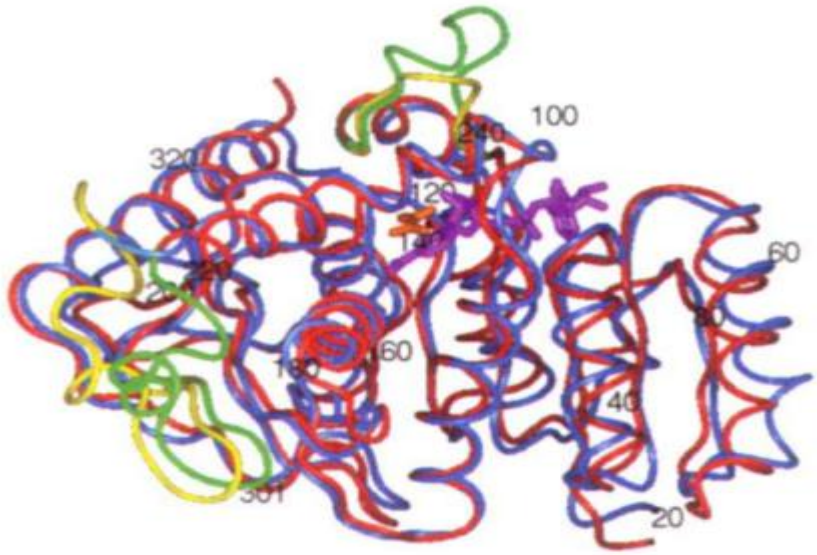


Figure 1.9: Cα trace of superimposed PfLDH (red) on pig LDH (blue). The NADH (pink) and oxamate (orange) are shown bound to PfLDH structure on which every 20th Cα position is labeled. The major structural differences, 101 – 109 loop (top) and 205 – 221 loop (bottom) are highlighted with different color (yellow for pig and green for PfLDH). Note: Amino acid numbering here and throughout the study is according to original dogfish LDH numbering (Dunn et al. 1996).

Homology modeling studies had predicted that the derivative of gossypol, gossylic nitrile diacetate (GNDA) interacts with PfLDH at the catalytic specificity loop in the 101 – 109 sequence position. In mammalian LDH, binding of GNDA to the enzyme is stearically hindered by a comparatively small loop. The extended loop of PfLDH at this position (insertion of 5 amino acid residues) renders binding of bulky GNDA at

this position and hence PfLDH can be selectively inhibited by GNDA (Sessions et al. 1997).

Structural differences between pLDH and mammalian LDH were deduced by deciphering the crystal structures of major human malaria parasites *P. falciparum* (PfLDH) (Figure 1.9) (Dunn et al. 1996) and *P. vivax* (PvLDH) (Chaikuad et al. 2005) and comparing these structures with mammalian LDH. Alignment of Cα residues showed a root mean square deviation of 2.2 Å between PvLDH and hLDH-A and 2.3 Å between PfLDH and hLDH-A. In addition to the characteristic five-residue insertion in the active site loop and Ser 163 to Leu exchange, the *Plasmodium* LDH enzymes also feature a number of substitutions in the cofactor-binding groove that distinguishes *Plasmodium* and human forms of LDH. These changes include exchange of Ile 250 and Thr 246 with Pro. Both are active site residues and define substrate and cofactor binding sites (Dunn et al. 1996). Likewise, the conserved active site residue Asp 197 is also replaced by Asn in PfLDH.

These structural differences have suggested pLDH as a suitable drug target and encouraged by this, several studies have developed and screened selective inhibitors of pLDH and have demonstrated anti-malarial activity *in vitro* and *in vivo.* Choi, Beeler and et al (2007) have developed a library of synthetic derivatives of oxamic acid and screened for potential antimalarial compounds that can selectively inhibit PfLDH over mammalian LDHs. A series of heterocyclic azole-based compounds, showing selective affinity for PfLDH, were also demonstrated to have anti-malarial activity in vitro and in vivo (Vivas et al. 2005). Recently, five different classes of compounds were identified, by computational biology methods as PfLDH specific inhibitors. The virtually predicted compounds were chemically synthesized and were assayed for both enzyme based inhibition and parasite culture based in vitro antimalarial activity (Ambre et al. 2012). These studies have highlighted the significance of pLDH as a potential therapeutic target in the development of novel antimalarial drugs.

Appendix I

State wise distribution of malaria cases in India (2008-2012). Source: National vector borne disease control program (http://nvbdcp.gov.in/malaria-new.html).

MALARIA SITUATION

STATEs/UTs.	2008				2009				2010				2011				2012(P)			
	Blood Slide Examination	Malaria cases	Pf cases	Deaths	Blood Slide Examination	Malaria cases	Pf cases	Deaths	Blood Slide Examination	Malaria cases	Pf cases	Deaths	Blood Slide Examination	Malaria cases	Pf cases	Deaths	Blood Slide Examination	Malaria cases	Pf cases	Deaths
Andhra Pradesh	8964918	26424	15947	0	9189256	25152	14841	3	9120643	33393	23259	20	9368740	34949	24089	5	7578798	21171	13145	2
Arunachal Pradesh	250884	29146	8219	27	213893	22066	6602	15	190063	17944	5412	103	197626	13950	4856	17	107129	5767	1920	5
Assam	2687755	83939	58124	86	3021920	91413	66557	63	4309287	68353	48330	36	4130216	47397	34707	45	3471279	27346	18697	15
Bihar	147279	2541	1712	0	115174	3255	2408	21	133757	1908	933	1	167561	2643	1273	0	139462	2146	780	0
Chhattisgarh	3052934	123495	94803	4	3250904	129397	104055	11	3426558	152209	120080	47	3444641	136899	107472	42	2776772	87810	67764	5
Goa	397349	9822	2727	21	417110	5056	1056	10	459861	2368	275	1	418722	1187	135	3	381789	1435	135	0
Gujarat	9065142	51161	11712	43	10180104	45902	8485	34	10689221	66501	13729	71	10967041	89764	16112	127	8874443	64548	7404	19
Haryana	2571866	35683	1397	0	2083245	30168	781	0	2340573	18921	764	0	2907380	33401	1133	0	2216401	23426	464	1
Himachal Pradesh	384835	146	2	0	397327	192	0	0	393203	210	2	0	367499	247	2	0	328476	200	3	0
Jammu & Kashmir	394922	217	17	1	464748	346	21	0	473268	802	43	0	484704	1091	45	0	417618	777	29	0
Jharkhand	2551489	214299	73521	25	3347069	230683	91194	28	3383496	199842	89357	16	3441614	160653	70302	17	3079214	113792	40185	9
Karnataka	8994881	47344	9864	8	9321098	36859	5723	0	9281666	44319	7936	11	9205620	24237	2648	0	7188700	14120	1051	0
Kerala	1819294	1804	222	4	2054473	2046	249	5	2143497	2299	271	7	2153277	1993	271	2	1455637	1346	143	3
Madhya Pradesh	9286269	105312	42355	53	9609659	87628	24581	26	9230400	87165	31092	31	9900131	91851	31940	109	7492151	63365	17757	36
Maharashtra	13371478	67333	22257	148	14770338	93818	24962	227	16118905	139198	32387	200	16098563	96577	21401	118	14282487	50399	8673	68
Manipur	134755	708	356	2	114720	1069	620	1	117986	947	487	4	120615	714	314	1	102115	232	68	0
Meghalaya	353071	39616	36301	73	501419	76759	74251	192	437167	41642	39374	87	391397	25143	24018	53	305399	18305	17473	41
Mizoram	165441	7361	6172	91	171793	9399	7387	119	334991	15594	14664	31	213149	8861	8373	30	141091	8635	8214	20
Nagaland	135910	5078	835	19	156259	8489	2893	35	182804	4959	1877	14	205520	3363	950	4	166585	2397	654	1
Orissa	5029677	375430	329631	239	5015489	380904	336047	198	5240458	395651	350428	247	4650799	308968	281577	99	3884614	209576	193795	54
Punjab	2979882	2494	38	0	2996929	2955	35	0	3140465	3477	71	0	3120544	2693	64	3	2470476	1598	32	0
Rajasthan	8041283	57482	3954	54	7845840	32709	1767	18	8732582	50963	2331	26	8591970	54294	2973	45	6223343	35723	963	16
Sikkim	6164	38	10	0	6688	42	16	1	6526	49	14	0	6969	51	14	0	5787	74	13	0
Tamil Nadu	6300226	21046	739	2	7801419	14988	448	1	7838638	17086	623	3	7841899	22171	925	0	5741050	13811	427	0
Tripura	341246	25894	23588	51	361848	24430	22952	62	330608	23939	21254	15	288076	14417	13812	12	230383	10313	9748	7
Uttarakhand	226903	1059	47	0	208350	1264	43	0	214763	1672	183	0	246641	1277	123	1	235189	1788	103	0
Uttar Pradesh	4150306	93383	2310	0	3527695	55437	660	0	4066059	64606	1382	0	4110871	56968	1857	0	3343170	42157	581	0
West Bengal	4465619	89443	24453	104	5336895	141211	36982	74	5440313	134795	24693	47	5044278	66368	10858	19	4375468	48352	6769	28
A.N.Islands	165631	4688	3173	0	133504	5760	3056	0	121760	2484	803	0	97946	1918	607	0	81403	1278	554	0
Chandigarh	77716	347	6	0	94301	430	4	0	98930	351	6	0	75368	582	9	0	99271	223	3	0
D & N Haveli	51804	3037	1007	0	62279	3408	1181	0	65104	5703	2243	0	58949	5150	2082	0	95749	4877	2160	1
Daman & Diu	27155	115	19	0	24123	97	19	0	25502	204	60	0	31856	262	55	0	28445	165	25	0
Delhi	593882	253	0	0	509231	169	0	0	503926	251	1	0	377122	413	1	0	320831	358	0	0
Lakshadweep	229	0	0	0	426	8	0	0	440	6	0	0	578	8	0	0	3970	0	0	0
Puducherry	127963	72	5	0	90550	65	1	0	86009	175	0	0	241778	196	6	1	154504	97	1	0
All India Total	97316158	1526210	775523	1055	103396076	1563574	839877	1144	108679429	1599986	834364	1018	108969660	1310656	665004	753	87799199	877607	419733	331

(P)Provisional Upto Oct.2012

AIM AND SCOPE OF THE THESIS

Malaria, a disease caused by infection with a unicellular protozoan parasite *Plasmodium,* is one of the foremost public health problems. Despite our knowledge about the disease; malaria remains a major cause of human morbidity and mortality in tropical regions of the world. *P. falciparum*, is the most lethal species of the parasite causing severe and complicated malaria whereas *P. vivax* is the most widespread and, therefore, is responsible for the greatest burden of the disease in India. *Plasmodium* species have acquired resistance to most of the present antimalarial drugs and have thereby hindered the control and eradication of malaria. One of the major steps to control and eradicate malaria is a persistent search for novel drugs with more efficient targets. Development in the genomic research of major human malaria parasites, has opened new prospects of reverse pharmacology based drug discovery wherein parasite enzymes and receptors, determined as potential drug targets by using available databases and bioinformatics tools, are exploited in the screening of compounds to find their specific inhibitors. *Plasmodium* species specific L-Lactate dehydrogenase (pLDH), the last enzyme of the glycolytic pathway is one amongst such targets that has been used to screen specific inhibitors to develop antimalarial drugs. Apart from being indispensable for the parasite to continue glycolysis in the endoerythrocytic stage, pLDH has significantly different structural and kinetic properties, compared to human LDH isoforms. The structural differences have been utilized in molecular diagnosis of malaria. Thus, the enzyme has a unique significance in malaria research of being both, potential therapeutic and diagnostic target. Considering an ancient origin and extensive polymorphism in the genome of the *Plasmodium* parasite, polymorphism analysis is a prerequisite for any diagnostic or therapeutic target to envisage the performance of any diagnostic test.

The indigenous medical knowledge systems in many countries have used medicinal plants to treat malarial fever since antiquity and such medicinal plants possess a repertoire of potential antimalarial drugs. Ethnopharmacological analysis of such traditional plants can lead to novel, affordable and accessible antimalarial drugs

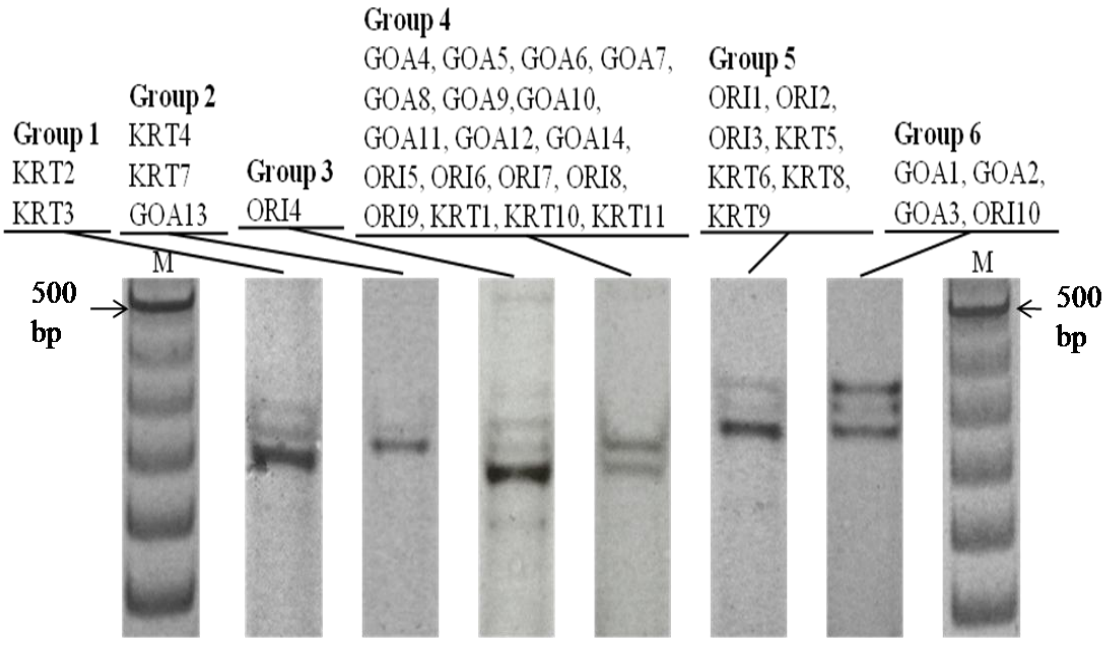
In view of the above, the present investigation was undertaken towards evaluating significance of pLDH as a therapeutic and diagnostic target with regard to the extant polymorphism in the pLDH gene in India followed by the search for novel drug for the treatment of malaria using an innovative approach, wherein ethanopharmacology and reverse pharmacology based drug discovery approaches were concertedly applied. The study was extended towards identifying affinity of the inhibitors towards the target (pLDH) and understanding the kind of molecular interactions between the two.

The major objectives set for the study are listed below:

1. A detailed genetic polymorphism analysis of the genes coding for PfLDH and PvLDH in Indian *P. falciparum* and *P. vivax* population.
2. Cloning, expression and characterization of L-Lactate dehydrogenase (target enzyme used in the study) from *P. falciparum* (PfLDH) and *P. vivax* (PvLDH) in *E. coli.*
3. Screening of selective traditional Indian herbs which can bring about robust pLDH specific inhibition.
4. Analysis of the enzyme inhibition, exhibited by the plant extracts.

Chapter 2

Genetic diversity analysis of L-Lactate Dehydrogenase gene in Indian *Plasmodium falciparum* and *Plasmodium vivax* population



CHAPTER 2

Genetic diversity analysis of L-Lactate Dehydrogenase gene in Indian *Plasmodium falciparum* and *Plasmodium vivax* population

2.1 INTRODUCTION

One of the most important means by which the scourge of malaria can be controlled in endemic countries is implementation of efficient Rapid Diagnostic Test (RDT) in the field as discussed earlier (Section1.8.1). RDTs presently are developed based on several antigenic targets mainly including histidine rich protein-2 (HRP-2), lactate dehydrogenase (LDH) and aldolase (Murray et al. 2003). Amongst these antigenic targets, pLDH has an unique significance in the malaria research as it is considered as both, therapeutic (Padmanaban et al. 2007) and diagnostic target (Piper et al. 1999). Pharmacogenetic studies have provided strong evidence of variable drug response linked to the polymorphism in the genes coding for drug targets (Johnson 2003). Likewise, the sensitivity of HRP-2 based RDT has been reported to be associated with the genetic polymorphism in HRP-2 (Baker et al. 2005; Lee et al. 2006;). Reports of the polymorphism in the pLDH gene are scarce. Talman et al. (2007) had reported both variable and conserved regions in pLDH gene; but the variations were studied at continental level. Considering the unique epidemiology of malaria in India (Singh et al. 2009), it is important to evaluate variations in pLDH in the Indian scenario. Detailed studies on pLDH variation within India have not yet been reported so far. Hence, one of the objectives of the present study was to analyze polymorphism in the genes coding for PfLDH (*P. falciparum* specific LDH) and PvLDH (*P. vivax* specific LDH).

One of the most significant molecular techniques to determine and quantify variability within and between the species is DNA sequencing. Often, analysis of genetic polymorphism distributed in the population

requires sequencing of large number of samples and yet many samples included in the study, may carry the same sequence (Sunnucks et al. 2000). Genetic polymorphism analysis of a population can be effectively carried out by the concerted use of sequencing dependent and independent molecular methods. Sequencing independent methods of genetic polymorphism analysis can determine samples with polymorphic nucleotide sequence from a set of samples without providing actual sequence data. Thus, the amount of sequencing required, is greatly reduced as only an informative subset of samples, predicted to be polymorphic by these methods, are needed to be sequenced (Sunnucks et al. 2000). Some common and sensitive sequencing independent methods include, i) Restriction Fragment Length Polymorphism (RFLP) analysis (Jeffreys et al. 1986), which detects variation in the length of the DNA fragment that is generated by the digestion of DNA using restriction endonucleases. ii) Heteroduplex analysis, which involves detection of variation in the electrophoretic mobility of duplex DNA generated by annealing the wild type and the mutant DNA. Any base substitution in the mutant DNA leads to generation of heteroduplexs which have slower mobility in native gels (White et al. 1992). iii) Denaturing Gradient Gel Electrophoresis (DGGE) that differentiates DNA molecules with sequence variation on the basis of the differences in DNA melting temperature, which leads to change in the final electrophoretic mobility of DNA (Collins & Myers 1987). iv) Single-strand conformational polymorphism (SSCP) analysis is a simple and sensitive technique that differentiates mobility of single-stranded DNA in nondenaturing gels (Orita et al. 1989). The mobility of single-stranded DNA conformers in nondenaturing polyacrylamide gels is very sensitive to primary sequence, because slight sequence changes can have major effects on the structure of single stranded conformers (ssc) of DNA that are generated under nondenaturing conditions. These conformational changes result in the detectable differences in the mobility of the ssc (Nakabayashi 1996). This technique has the potential to discriminate even a single base pair differences between DNA fragments and is used to analyze genetic

polymorphism from different organisms. The sensitivity of SSCP to determine the single nucleotide variation depends on the size of DNA fragment and electrophoresis conditions. Sensitivity of the method to reveal single-base changes decreases significantly for larger fragments (>200 bp). SSCP analysis, being highly sensitive sequencing independent method to genotype samples, has wide application in malaria research and was employed for polymorphism detection in different genes within the parasite population (Kain et al. 1996; Raj et al. 2004; Raj et al. 2005; Mishra et al. 2006).

Hence, the objective of the present study was to analyze polymorphism in PfLDH (*P. falciparum* specific LDH) and PvLDH (*P. vivax* specific LDH) by using PCR-Single Strand Conformation Polymorphism (PCR-SSCP) as a primary screening method followed by sequencing of a subset of DNA samples representing each polymorphic pLDH genotype predicted by PCR-SSCP. Protein sequences and model structures of LDH, resolved from the sequence data of representative samples, were analyzed to understand the phenotypic consequences of genetic polymorphism. pLDH has also been used as one of the markers in evolutionary genetic analysis to determine the genealogy of *Plasmodium* species (Liu et al. 2010). Hence, detailed evolutionary genetic analysis was carried out using pLDH sequences, obtained in this study along with those available in the nucleotide databases, to comprehend the evolutionary aspects of *Plasmodium* species.

2.2 MATERIALS AND METHODS

2.2.1 Collection of samples

Parasite infected blood was used as source of *P. falciparum* and *P. vivax* samples. All the samples were obtained from National Institute of Malaria Research (NIMR), New Delhi. Twenty *P. falciparum* and thirty five *P. vivax* samples were collected from malaria endemic states of India (Odisha; Karnataka and Goa). These samples were collected from March to May and from September to November (peak transmission seasons for

malaria infections in India) in 2007 and 2008. Samples were obtained in the form of finger-prick blood spots on Whatman filter paper (5–7 spots per filter paper) and were kept at 4°C for storage till further processing. The necessary clearance for blood collection in the field trips was obtained from the ethical committee of NIMR, and informed consents were received from each patient. Blood samples collected were confirmed by microscopy for positivity of infection (Moody & Chiodini 2000) and diagnosed for single infection of *P. falciparum* or *P. vivax* by PCR analysis (Gupta et al. 2010), by trained experts of NIMR.

Additionally, a *P. falciparum* standard strain 3D7 and 25 *P. falciparum* isolates from different endemic and mesoendemic states of India (Odisha, Tamil Nadu, Mizoram, Meghalaya, Rajasthan, Chhattisgarh, Uttarpradesh, Gujarat), maintained in the Malaria parasite bank (NIMR, New Delhi), were included in the study. Thus, a total of 81 samples were used in the study (Figure 2.1).

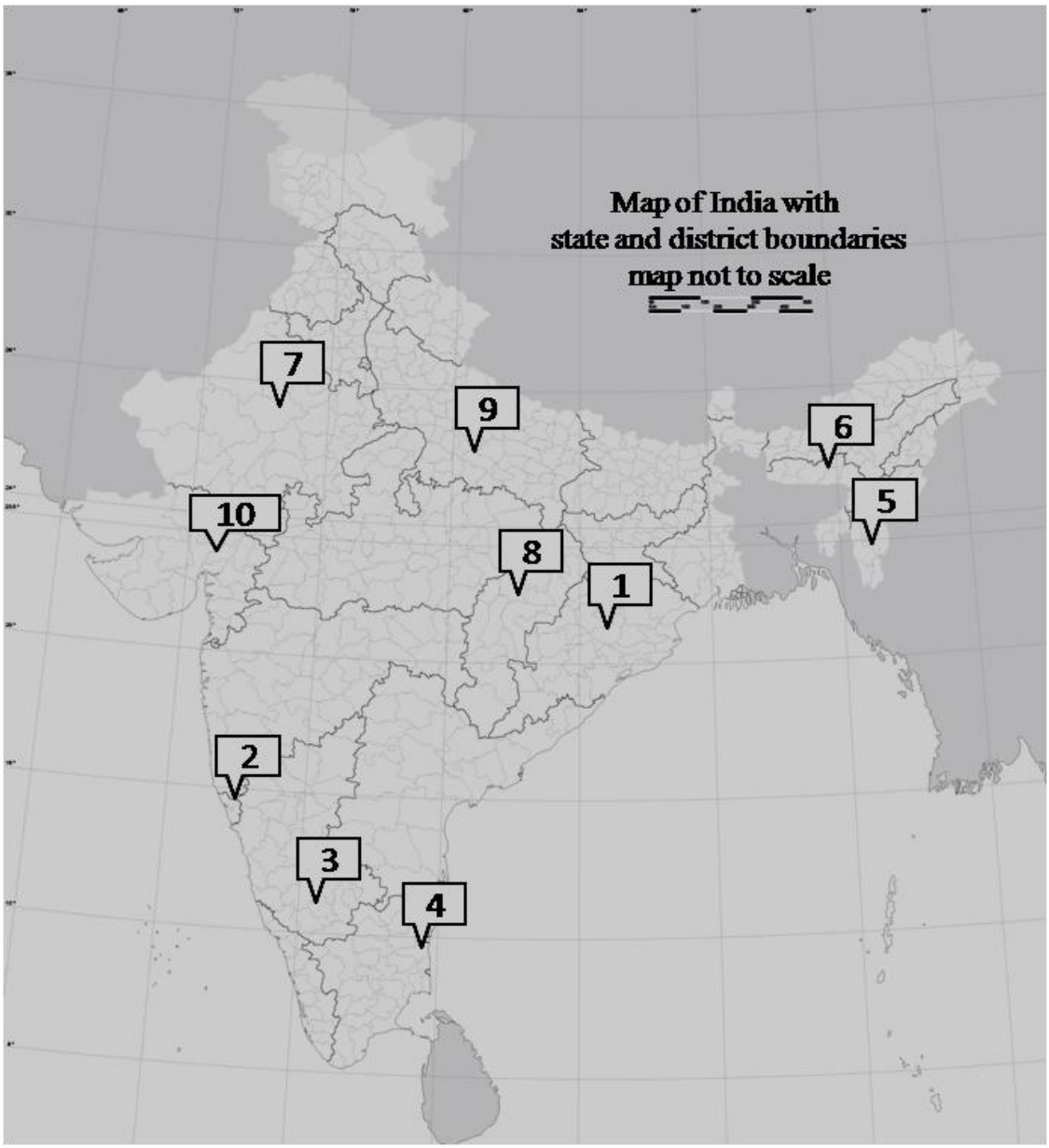


Figure 2.1: Statewise distribution of locations used for sample collection. **1.** Odisha: [8] *Pf* and [10] *Pv*; **2.** Goa: [7] *Pf* and [14] *Pv*; **3.** Karnataka: [5] *Pf* and [11] *Pv*; **4.** Tamilnadu: [2] *Pf*; **5.** Mizoram: [4] *Pf*; **6.** Meghalaya: [2] *Pf*; **7.** Rajasthan: [3] *Pf*; **8.** Chhattisgarh: [4] *Pf*; **9.** Uttar Pradesh: [1] *Pf*; **10.** Gujarat: [9[*Pf*. [*Pf: P. falciparum; Pv: P. vivax*] values in parenthesis indicate number of *P. falciparum* and *P. vivax* samples collected from the respective states.

2.2.2 PCR amplification of PfLDH and PvLDH gene fragments

Genomic DNA was extracted from blood samples, using QIAamp mini DNA extraction kit (Qiagen, Germany) according to manufactures' instructions. Primers were designed using available LDH sequences from *P. falciparum* 3D7 (GenBank ID: XM_001349953) and *P. vivax* Sal1 (GenBank ID: XM_001615570.1) (Table 2.1). DNA fragments to be used for SSCP analysis were amplified by nested PCR approach. In the first round of amplification, DNA fragments of 951bp, coding for complete ORF of PfLDH and PvLDH, were amplified by PfF and PfR primer pair and PvF and PvR primer pair respectively. These PCR products were then used as a template to amplify two fragments each in PfLDH and PvLDH genes using internal primers (Table 2.1). The size and position of internal fragments amplified by these primers are illustrated in figure 2.2.

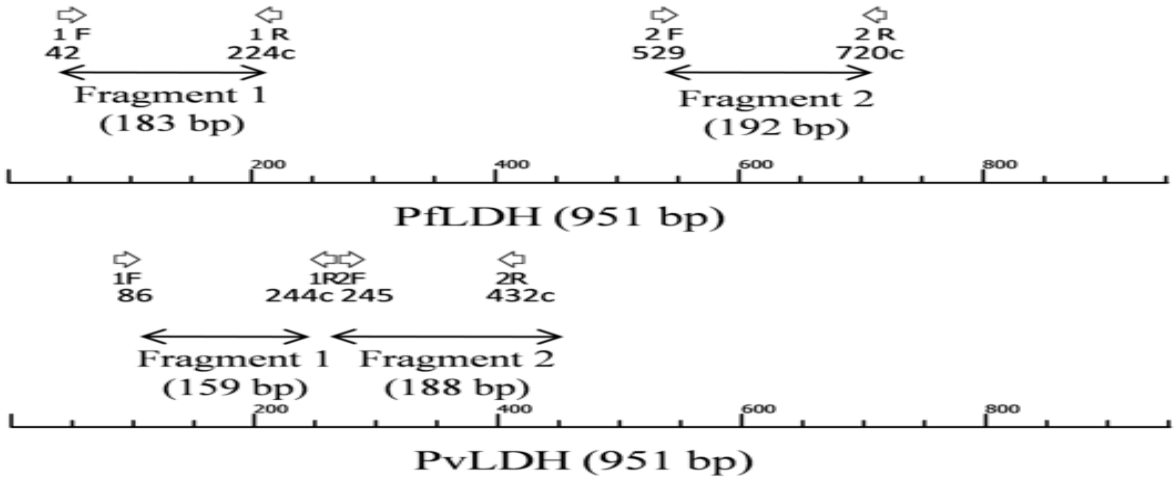


Figure 2.2: Position of PfLDH and PvLDH gene fragments used in the SSCP analysis. For PfLDH gene – 1F: PfSSCP1 forward primer, 1R: PfSSCP1 reverse primer pair, 2F: PfSSCP2 forward primer and 2R: PfSSCP2 reverse primer. For PvLDH gene – 1F: PvSSCP1 forward primer, 1R: PvSSCP1 reverse primer, 2F: PvSSCP2 forward primer and 2R: PvSSCP2 reverse primer. Numbers below primer designate their binding position in the respective gene sequence (c: complementary strand). DNA fragments amplified by the primer pairs are shown as two sided arrows with their respective sizes.

Table 2.1: Primers used in the study

Primer	Sequence	T_a (°C)	amplicon size (bp)
PfSSCP1 F	5' GATTGGAGGAGTAATGGCTACC 3'	60	183
PfSSCP1 R	5' ACATCTGCTCCAGCCAAATC 3'		
PfSSCP2 F	5' CACATTGTAGGTGCTCATGG 3'	57	192
PfSSCP2 R	5' AGCTGGTGCAACATATGG 3'		
PvSSCP1 F	5' GGGACGTAGTGATGTTTGAC 3'	58	159
PvSSCP1 R	5' ATCCCGCAGTGACGATCACC 3'		
PvSSCP2 F	5' TTACTAAAGCACCAGGAAAG 3'	54	188
PvSSCP2 R	5' TTTTGGGACTCCGGAATG 3'		
Pf F	5' ATGGCACCAAAAGCAAAAATC 3'	54	951
Pf R	5' TTAAGCTAATGCCTTCATTCTC 3'		
Pv F	5' ATGACGCCGAAACCCAAAATTG 3'	58	951
Pv R	5' TTAAATGAGCGCCTTCATCC 3'		

T_a: Annealing temperature used in the PCR reaction for the respective primer pair

The PCR mixture (50 µl) contained 50 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, approximately100 ng of genomic DNA and 2.5 U of *Pfu* polymerase. PCR program used for the amplification: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 sec, respective annealing temperature for each primer pair (Table 1) for 30 sec, extension at 74°C for 75 sec (amplification of complete ORF) / 30 sec (amplification of internal fragments); followed by a final extension of 72°C for 10 min. Silver staining of PCR products, run on native PAGE gel, was carried out to check their purity (Sambrook & Russel 2001) and the molecular weight of each amplicon, compared to the reference (50 bp O'range ruler, Fermentas Inc), was determined by AlphaEaseFC version 4.0 software.

2.2.3 SSCP analysis of PfLDH and PvLDH gene fragments

SSCP analysis was carried out as described by Sambrook and Russel (2001) with modifications, for optimum resolution of single strand conformers. In brief, 2 µl of PCR products were denatured in 18 µl of gel loading dye (98% formamide, 20 mM EDTA and 0.05% xylene cyanol) at 94°C for 10 min and immediately plunged in ice. The samples were then loaded in 8% native PAGE gels (0.75 mm thick, 15 X 15 cm) without glycerol. Single stranded DNA conformers were resolved by electophoresis using DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA) at constant 70 V, at 25°C for 12 hrs. Silver staining was used in the present study to visualize the band pattern of single strand conformers in the polyacrylamide gels. Relative electrophoretic mobility of each single strand conformer was calculated in the AlphaEaseFC version 4.0 software using a 50 bp O'range ruler (Fermentas Inc.) as a reference. Samples were genotyped on the basis of similarity in the band pattern of the single strand conformers. Abundance of each genotype amongst the samples was calculated as percentage of occurrence and statewise distribution of genotypes was illustrated by plotting a Venn diagram. Genotype diversity amongst the studied population was determined using Danoff-Burg and Chen's Abundance curve calculator (Available at www.columbia.edu/itc/cerc/danoff-burg/Biodiversity Calculator.xls)

2.2.4 Sequence and structure analysis of PvLDH genotypes

DNA fragments of 951 bp, coding for PfLDH and PvLDH open reading frame (ORF), were cloned in *E. coli* DH5α using CloneJET PCR cloning Kit (MBI, Fermentas Inc., USA) and sequenced in both directions (SPA sequencing services, Merk Inc., Bengaluru, India) using pJET1.2 sequencing primers, included in the cloning kit. Sequences were edited using Chromas Pro Software version 1.49 beta (Technelysium Pty Ltd., Australia) to remove terminal vector sequences and were submitted to the GenBank database.

DNA sequences and *in silico* translated protein sequences of PfLDH and PvLDH gene, were aligned with respective reference LDH sequences (GenBank ID: XM_001349953 and GenBank ID: XM_001615570.1) by Clone Manager 7 / Align Plus 5 program (Scientific & Educational Software, USA). Protein structures for PvLDH genotypes with amino acid sequence variation were predicted by homology modeling and the quality of these models was assessed by QMEAN score determination function (Benkert et al. 2011) in SWISS-MODEL workspace (Arnold et al. 2006). Model structures of PvLDH protein from each genotype were compared with the known structure of PvLDH (PDB ID: 2A92) as a reference and were visualized in Accelrys DS Visualizer (v2.0.1.7347).

2.2.5 Phylogenetic analysis of pLDH

The PfLDH and PvLDH sequences obtained in this study and LDH gene sequences of *Plasmodium* species infecting human and other mammalian hosts, available in Genbank Database, were included in the phylogenetic analysis of *Plasmodium* species. LDH sequences used in the present study are listed in table 2.2. Phylogenetic trees showing genetic distance were constructed using the neighbor joining (Saitou & Nei 1987) and minimum evolution (Rzhetsky & Nei 1993) methods in MEGA version 5.0 software. Trees constructed were validated with 1000 pseudo-replicates by the bootstrap method (Felsenstein 1985). The branch lengths representing evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura & Nei 1993).

Table 2.2 LDH sequences used in the present study

Sr. No	Name	Accession No.	Reference
PfLDH gene sequences			
1	3D7	XM_001349953	Standard strain, Harris et al. (Direct submission)
2	FCBR	DQ198262	Balik & Holbrook 2001
3	Jind	EU330208	Berwal et al. 2008
4	K1	DQ198261	Balik & Holbrook 2001
5	FCC1/HN	DQ825436	XU et al. 2007
6	Honduras 1	M93720	Bzik et al. 1993
7	Madagascar 1	EU589948	Mariette et al. 2008
8	Madagascar 2	EU589947	Mariette et al. 2008
9	Ori1	JN547218	This study
10	Mzr1	JN547219	This study
PvLDH gene sequences			
11	Sal1	XM_001615570	Standard strain, Carlton (Direct submission)
12	Anhui	GU078391	Fang et al. (Direct submission)
13	Belem	DQ060151	Turgut-Balik et al. 2004
14	Hainan	FJ527750	Lu and Can (Direct submission)
15	Gwalior	EU262983	Berwal et al. (Direct submission)
16	Madagascar 1	EU589951	Mariette et al. 2008
17	Madagascar 2	EU589952	Mariette et al. 2008
18	Madagascar 3	EU589953	Mariette et al. 2008
19	Madagascar 4	EU589954	Mariette et al. 2008
20	Madagascar 5	EU589955	Mariette et al. 2008
21	Madagascar 6	EU589956	Mariette et al. 2008
22	Madagascar 7	EU589957	Mariette et al. 2008
23	Goa4	JN547220	This Study
24	Ori1	JN547221	This Study
25	Krt2	JN547222	This Study
26	Goa1	JN547223	This Study

27	Krt4	JN547224	This Study
28	Krt1	JN547225	This Study
29	Ori4	JN547226	This Study
LDH sequences from other human malaria parasites			
30	*P. malariae* Uganda 1	AY486059	Brown et al. 2004
31	*P. ovale*	AY486058	Brown et al. 2004
Nonhuman mammalian malaria species			
32	*P. reichenowi* (Chimpanzee)	AB122147	Tanabe et al. 2004
33	*P. berghei* strain ANKA (Murine rodents)	XM_674309	Hall et al. 2005
34	*P. knowlesi* H (primate, long tailed macaques)	JF958130	Vandana Singh et al. 2012
35	*P. chabaudi chabaudi* (murine rodents)	XM_740087	Hall et al. 2005
36	*P. yoelii yoelii* 17XNL (rodents)	XM_719008	Carlton et al. 2002
LDH sequences from recently reported new clades			
• **Chimpanzee clades**			
37	C1 strain GTptt212_SGA2.5	HM235118	Liu et al. 2010
38	C2 strain MTptt157_SGA5.20	HM235131	Liu et al. 2010
39	C3 strain GTptt503_SGA2.1	HM235125	Liu et al. 2010
• **Gorilla clades**			
40	G1 strain GTgor118_SGA2.11	HM235128	Liu et al. 2010
41	G2 strain LBgor1222_SGA2.4	HM235124	Liu et al. 2010
42	G3 strain GTgor119_SGA5.4	HM235140	Liu et al. 2010

2.3 RESULTS

2.3.1 Amplification PfLDH and PvLDH gene fragments

PfLDH and PvLDH gene ORF (951 bp each) were amplified using genomic DNA from 46 *P. falciparum* samples and 35 *P. vivax* samples. Subsequently, using these products as a template, two internal fragments were amplified from both the genes. The fragments obtained by this nested PCR approach were of the expected size of about 183 and 192 bp for PfLDH and 159 and 188 bp for PvLDH respectively. LDH amplicons in all reactions were found to be devoid of any other non-specific amplification (Figure 2.3).

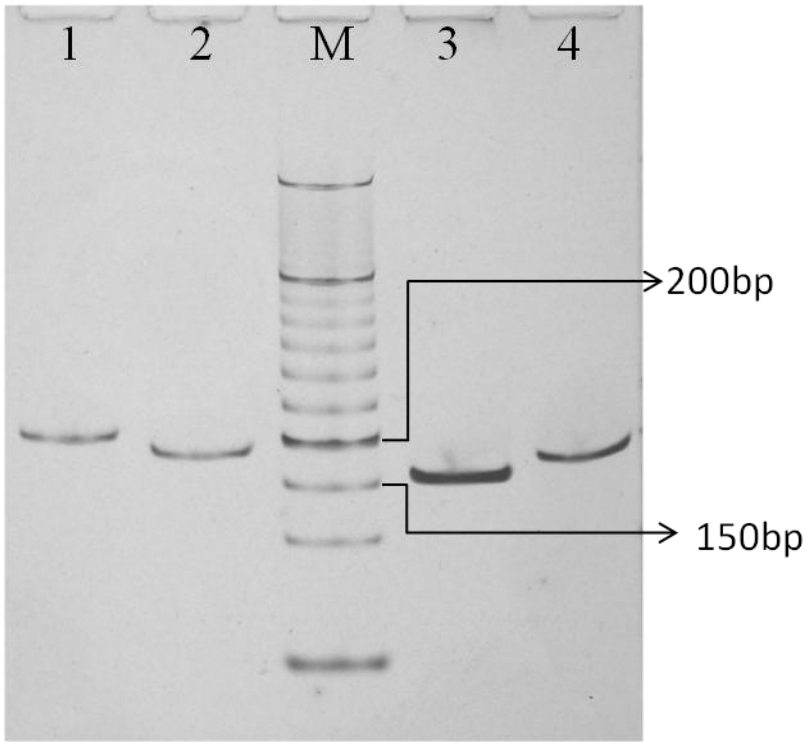
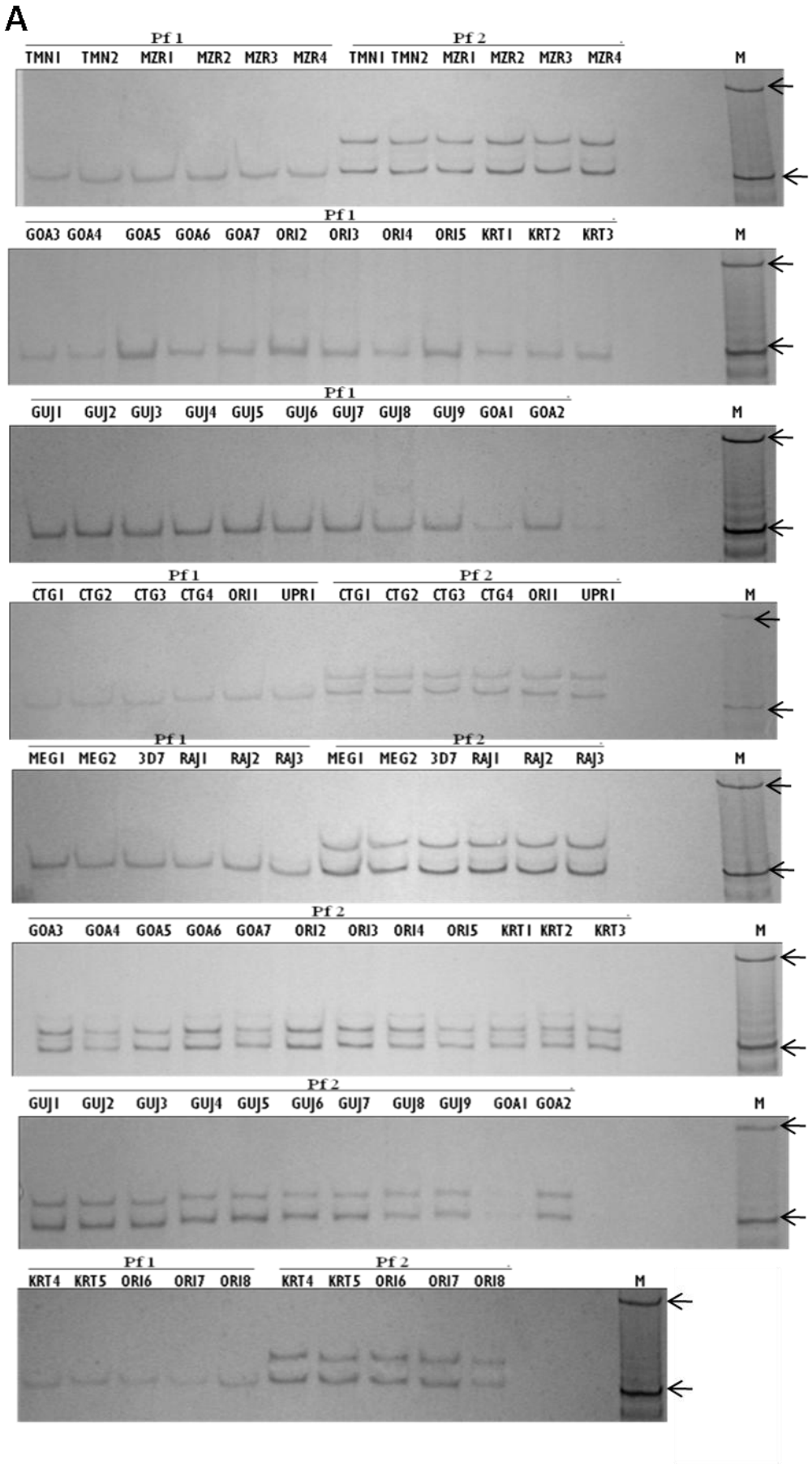


Figure 2.3: Amplification of PfLDH and PvLDH gene fragments. Lane 1: PfLDH fragment 2 (192 bp); lane 2: PfLDH fragment 1 (183 bp); lane 3: PvLDH fragment 1 (159 bp); lane 4: PvLDH fragment 2 (188 bp); M: 50 bp ruler.

2.3.2 SSCP analysis PfLDH and PvLDH gene fragments

PCR-SSCP was used as a tool to analyze genetic variation in PfLDH and PvLDH gene fragments. Relative electrophoretic mobility of single strand conformers for both PfLDH and PvLDH gene fragments are shown in Figure 2.4 A and B. All *P. falciparum* samples examined in the present study, showed identical relative electrophoretic mobility for Single Strand Conformers derived from 183 bp fragments as well as from 192 bp fragments of the gene (Figure 2.4A). Thus, all *P. falciparum* samples were grouped in single PfLDH genotype. Likewise, in all *P. vivax* samples, variation was not observed in the electrophoretic pattern of 159 bp fragments of PvLDH gene. However, Single Strand Conformers of 188 bp fragments of PvLDH gene had different relative electrophoretic mobility in different samples (Figure 2.4B).


A
Pf 1
TMN1 TMN2 MZR1 MZR2 MZR3 MZR4
Pf 2
TMN1 TMN2 MZR1 MZR2 MZR3 MZR4
M
Pf 1
GOA3 GOA4 GOA5 GOA6 GOA7 ORI2 ORI3 ORI4 ORI5 KRT1 KRT2 KRT3
M
Pf 1
GUJ1 GUJ2 GUJ3 GUJ4 GUJ5 GUJ6 GUJ7 GUJ8 GUJ9 GOA1 GOA2
M
Pf 1
CTG1 CTG2 CTG3 CTG4 ORI1 UPR1
Pf 2
CTG1 CTG2 CTG3 CTG4 ORI1 UPR1
M
Pf 1
MEG1 MEG2 3D7 RAJ1 RAJ2 RAJ3
Pf 2
MEG1 MEG2 3D7 RAJ1 RAJ2 RAJ3
M
Pf 2
GOA3 GOA4 GOA5 GOA6 GOA7 ORI2 ORI3 ORI4 ORI5 KRT1 KRT2 KRT3
M
Pf 2
GUJ1 GUJ2 GUJ3 GUJ4 GUJ5 GUJ6 GUJ7 GUJ8 GUJ9 GOA1 GOA2
M
Pf 1
KRT4 KRT5 ORI6 ORI7 ORI8
Pf 2
KRT4 KRT5 ORI6 ORI7 ORI8
M

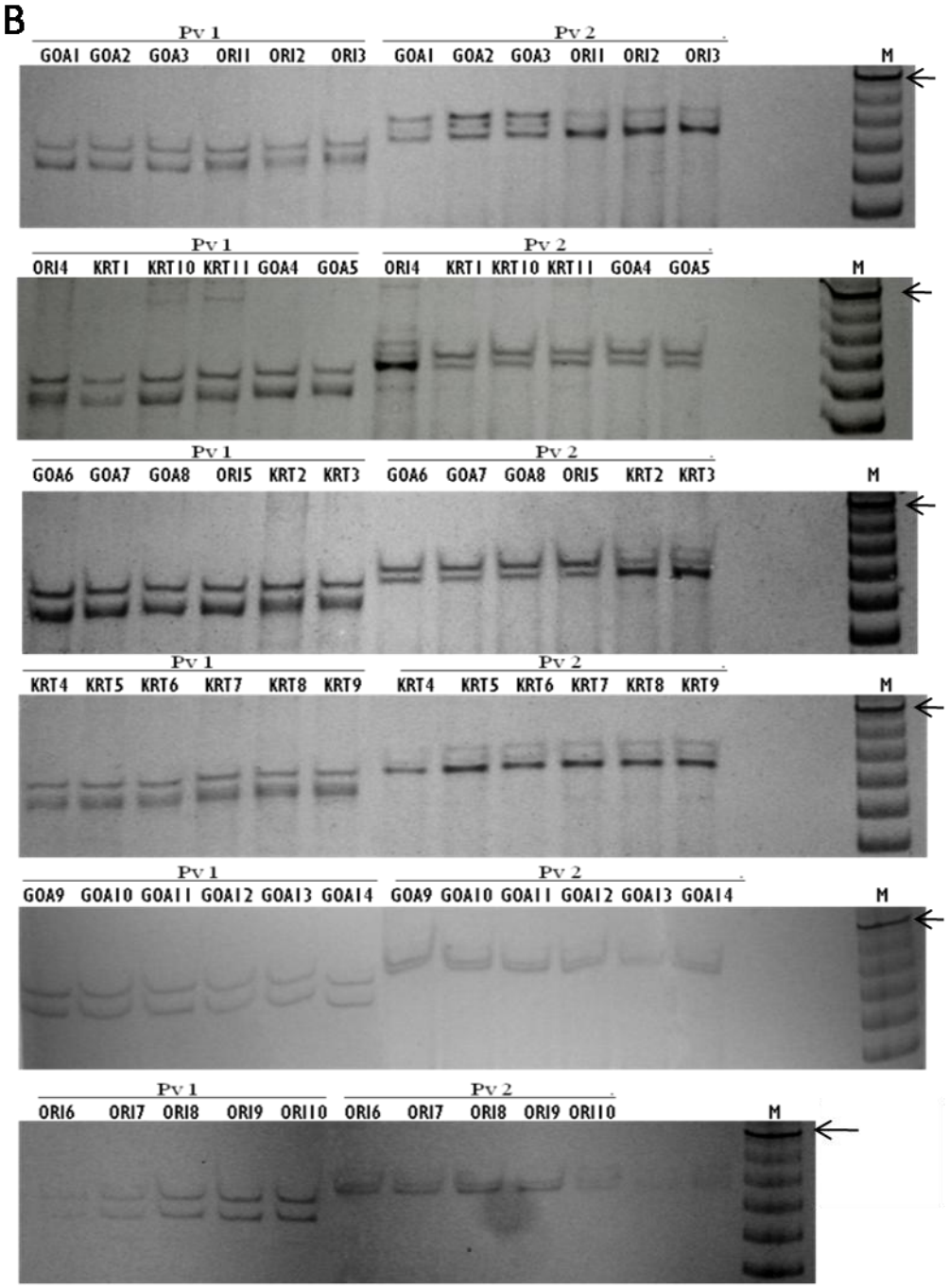


Figure 2.4: Relative electrophoretic mobility of single strand conformers as visualized by silver staining. A) Pf1 and Pf2: 183 bp and 192 bp fragments of PfLDH gene respectively. B) Pv1 and Pv2: 159 bp and 188 bp fragments of PvLDH gene respectively. M: 50 bp ruler marked with 1000 bp (upper arrow) and 500 bp (lower arrow) bands in section A and 500 bp band (arrow) in section B.

Six discreet band patterns of single strand conformers, derived from the 188 bp fragment, were observed amongst the *P. vivax* samples and on account of these variations, *P. vivax* samples were divided in 6 genotypes (Figure 2.5).

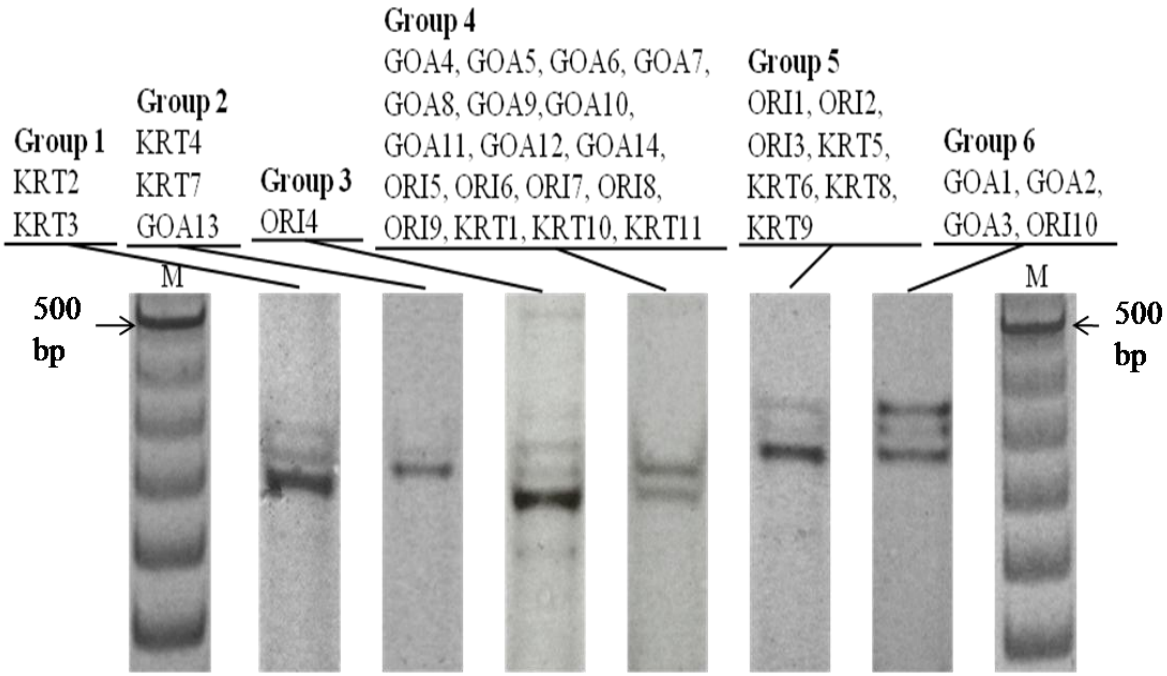


Figure 2.5: Genotyping of *P. vivax* samples based on the SSCP patterns observed in a 188 bp fragment of PvLDH gene. A representative pattern observed for each genotype is displayed. M: 50 bp ruler as a reference for electrophoretic mobility.

Distribution of genotypes was not even amongst the studied states (Odisha, Karnataka and Goa). Genotype 4 was the most abundant with 51.43% occurrence and was present in all the three states, while genotypes 1 and 3 were sporadic with 5.71% and 2.86% occurrence and were geographically restricted to Karnataka and Odisha states respectively (Table 2.2, Figure 2.6). Rest all genotypes were scant with restricted distribution to one or two states, with exception of Karnataka, where genotype 5 was more abundant than genotype 4 (Table 2.2).

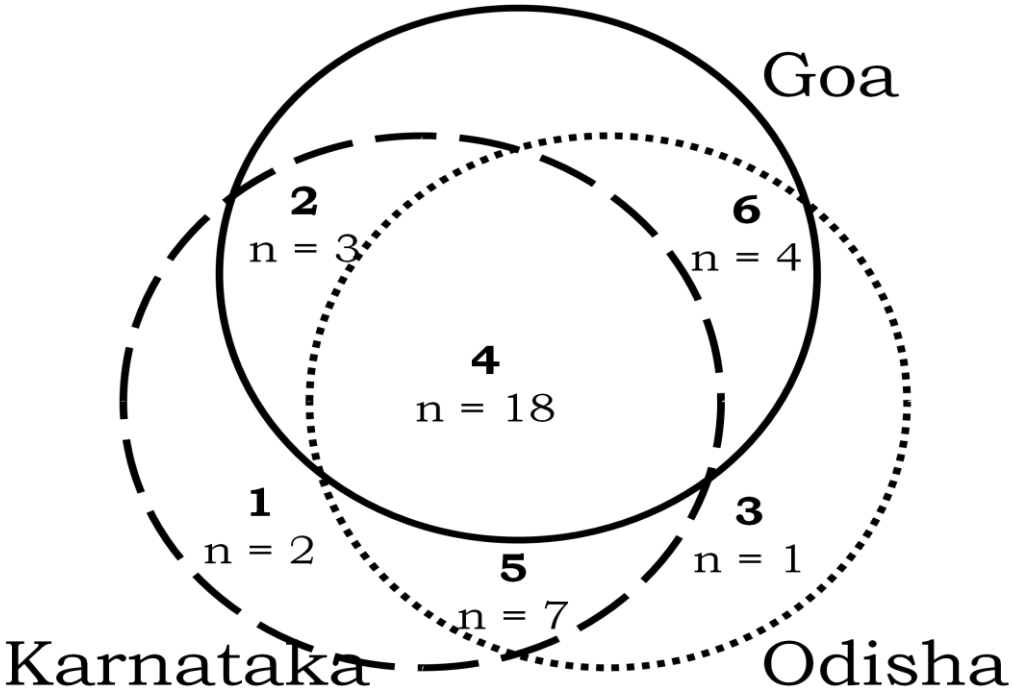


Figure 2.6: Venn diagram representing the statewise distribution of PvLDH genotypes.

PvLDH genetic diversity was nearly equal in Karnataka and Odisha (Simpson's index of diversity *D*: 0.8 and 0.71 respectively) while Goa had comparatively lesser genetic diversity (*D*: 0.47). The overall diversity index (*D*) for samples in all states was 0.69. Genotypes were more evenly distributed in Karnataka and Odisha (Shannon index of evenness *E:* 0.97 and 0.84 respectively) than Goa (*E:* 0.69), as genotype 4 was observed predominantly in Goa. The overall evenness index (*E*), representing distribution of samples in the states studied, was 0.77.

Table 2.3: PvLDH genotypes observed in the study, with their percentage abundance

Genotype	Abundance (%)			
	Karnataka	Odisha	Goa	Total
1	18.18	Absent	Absent	5.71
2	18.18	Absent	7.14	8.57
3	Absent	10	Absent	2.86
4	27.27	50	71.42	51.43
5	36.36	30	Absent	20
6	Absent	10	21.42	11.43

2.3.3 Sequence and structure analysis of PvLDH from samples representing each genotype

Complete ORF (951 bp) coding for PfLDH, from Ori1 and Mzr1 samples and PvLDH, from Goa1, Goa4, Ori1, Ori4, Krt1, Krt2 and Krt4 samples, were cloned in pJET1.2 vector and sequenced from their respective clones. Sequences obtained contain vector sequences at 5' and 3' ends as vector specific primers were used for sequencing and hence each sequence was edited to remove these terminal vector sequences. The sequences of complete ORF coding for PfLDH and PvLDH are available in GenBank under following accession numbers. **PfLDH:** Ori1 – JN547218, Mzr1 – JN547219; **PvLDH:** Goa4 – JN547220, Ori1 – JN547221, Krt2 – JN547222, Goa1 – JN547223, Krt4 – JN547224, Krt1 – JN547225 and Ori4 – JN547226.

PfLDH gene sequences were aligned with *P. falciparum* 3D7 LDH sequence and it was observed that the complete ORF sequences of both the samples were highly conserved with only single synonymous

substitution of A15 to G in Mzr1 (Figure 2.7A); whereas multiple sequence alignment analysis of PvLDH genes with *P. vivax* Sal1 LDH sequence revealed polymorphism at multiple loci in *P. vivax* samples. Within the regions analyzed by SSCP, 159 bp fragment had single nucleotide variation in Krt1, Krt2 and Ori1 samples but at different loci. The 188 bp fragments were observed to be conserved in Goa4 and Krt1, which belong to the same genotype (Figure 2.5) and the same fragment (188 bp) from the samples belonging to different genotypes, was found to be polymorphic, for e.g. single nucleotide variation was observed in Ori4, two nucleotide variations in Krt4 and Ori1, three nucleotide variations in Krt2 and four nucleotide variations in Goa1. Polymorphism was interspersed throughout the gene and was also observed in the regions, which were not included in the SSCP analysis (Figure 2.7B).

A

```
3D7        1 ATGGCACCAAAAGCAAAAATCGTTTTAGTTGGCTCAGGTATGATTGGAGGAGTAATGGCTACCTTAATTGTTCAGAAAAA
Ori1       1 ATGGCACCAAAAGCAAAAATCGTTTTAGTTGGCTCAGGTATGATTGGAGGAGTAATGGCTACCTTAATTGTTCAGAAAAA
Mzr1       1 ATGGCACCAAAAGCGAAAATCGTTTTAGTTGGCTCAGGTATGATTGGAGGAGTAATGGCTACCTTAATTGTTCAGAAAAA

3D7       81 TTTAGGAGATGTAGTTTTGTTCGATATTGTAAAGAACATGCCACATGGAAAAGCTTTAGATACATCTCATACTAATGTTA
Ori1      81 TTTAGGAGATGTAGTTTTGTTCGATATTGTAAAGAACATGCCACATGGAAAAGCTTTAGATACATCTCATACTAATGTTA
Mzr1      81 TTTAGGAGATGTAGTTTTGTTCGATATTGTAAAGAACATGCCACATGGAAAAGCTTTAGATACATCTCATACTAATGTTA

3D7      161 TGGCATATTCAAATTGCAAAGTAAGTGGTTCAAACACTTATGACGATTTGGCTGGAGCAGATGTAGTAATAGTAACAGCT
Ori1     161 TGGCATATTCAAATTGCAAAGTAAGTGGTTCAAACACTTATGACGATTTGGCTGGAGCAGATGTAGTAATAGTAACAGCT
Mzr1     161 TGGCATATTCAAATTGCAAAGTAAGTGGTTCAAACACTTATGACGATTTGGCTGGAGCAGATGTAGTAATAGTAACAGCT

3D7      241 GGATTTACCAAGGCCCCAGGAAAGAGTGACAAAGAATGGAATAGAGATGATTTATTACCATTAAACAACAAGATTATGAT
Ori1     241 GGATTTACCAAGGCCCCAGGAAAGAGTGACAAAGAATGGAATAGAGATGATTTATTACCATTAAACAACAAGATTATGAT
Mzr1     241 GGATTTACCAAGGCCCCAGGAAAGAGTGACAAAGAATGGAATAGAGATGATTTATTACCATTAAACAACAAGATTATGAT

3D7      321 TGAAATTGGTGGTCATATTAAGAAGAATTGTCCAAATGCTTTTATTATTGTTGTAACAAACCCAGTAGATGTTATGGTAC
Ori1     321 TGAAATTGGTGGTCATATTAAGAAGAATTGTCCAAATGCTTTTATTATTGTTGTAACAAACCCAGTAGATGTTATGGTAC
Mzr1     321 TGAAATTGGTGGTCATATTAAGAAGAATTGTCCAAATGCTTTTATTATTGTTGTAACAAACCCAGTAGATGTTATGGTAC

3D7      401 AATTATTACATCAACATTCAGGTGTTCCTAAAAACAAGATTATTGGTTTAGGTGGTGTATTAGATACATCAAGATTGAAG
Ori1     401 AATTATTACATCAACATTCAGGTGTTCCTAAAAACAAGATTATTGGTTTAGGTGGTGTATTAGATACATCAAGATTGAAG
Mzr1     401 AATTATTACATCAACATTCAGGTGTTCCTAAAAACAAGATTATTGGTTTAGGTGGTGTATTAGATACATCAAGATTGAAG

3D7      481 TATTACATATCTCAGAAATTAAATGTATGCCCAAGAGATGTAAATGCACACATTGTAGGTGCTCATGGAAATAAAATGGT
Ori1     481 TATTACATATCTCAGAAATTAAATGTATGCCCAAGAGATGTAAATGCACACATTGTAGGTGCTCATGGAAATAAAATGGT
Mzr1     481 TATTACATATCTCAGAAATTAAATGTATGCCCAAGAGATGTAAATGCACACATTGTAGGTGCTCATGGAAATAAAATGGT

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Mzr1     561 TCTTTTAAAAAGATACATTACTGTAGGTGGTATCCCTTTACAAGAATTTATTAATAACAAGTTAATTTCTGATGCTGAAT

3D7      641 TAGAAGCTATATTTGATAGAACTGTTAATACTGCATTAGAAATTGTAAACTTACATGCATCACCATATGTTGCACCAGCT
Ori1     641 TAGAAGCTATATTTGATAGAACTGTTAATACTGCATTAGAAATTGTAAACTTACATGCATCACCATATGTTGCACCAGCT
Mzr1     641 TAGAAGCTATATTTGATAGAACTGTTAATACTGCATTAGAAATTGTAAACTTACATGCATCACCATATGTTGCACCAGCT

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Mzr1     721 GCTGCTATTATCGAAATGGCTGAATCCTACTTAAAAGATTTGAAAAAAGTATTAATTTGCTCAACCTTGTTAGAAGGACA

3D7      801 ATATGGACACTCCGATATATTCGGTGGTACACCTGTTGTTTTAGGTGCTAATGGTGTTGAACAAGTTATCGAATTACAAT
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3D7      881 TAAATAGTGAGGAAAAAGCTAAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA
Ori1     881 TAAATAGTGAGGAAAAAGCTAAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA
Mzr1     881 TAAATAGTGAGGAAAAAGCTAAATTTGATGAAGCCATAGCTGAAACTAAGAGAATGAAGGCATTAGCTTAA
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B

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Sal1        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCCACGCTGATTGTGCAGAAGAA
Goa4        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCCACGCTGATTGTGCAGAAGAA
Krt1        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCCACGCTGATTGTGCAGAAGAA
Ori4        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCCACGCTGATTGTGCAGAAGAA
Krt2        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCCACGCTGATTGTGCAGAAGAA
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Ori1        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGAGGGCCACGCTGATTGTGCAGAAGAA
Goa1        1 ATGACGCCGAAACCCAAAATTGTGCTCGTCGGGTCGGGCATGATCGGAGGCGTGATGGCCACGCTGATTGTGCAGAAGAA

Sal1       81 CCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATGCCCCAAGGAAAGGCACTAGATACGTCTCACTCGAATGTGA
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Krt2       81 CCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATGCCCCAAGGAAAGGCACTAGATACGTCTCACTCGAATGTGA
Krt4       81 CCTGGGGGACGTAGTGATGTTTGACGTAGTGAAAAACATGCCCCAAGGAAAGGCACTAGATACGTCTCACTCGAATGTGA
Ori1       81 CCTGTGGGACGTAGTGATGTTTGACGTAGTGAAAAACATGCCCCAAGGAAAGGCACTAGATATGTCTCACTCGAATGTGA
Goa1       81 CCTGCGGGACGTAGTGATGTTTGACGTAGTGAAAAACATGCCCCAAGGAAAGGCACTAGATACGTCTCACTCGAATGTGA

Sal1      161 TGGCTTATTCCAATTGCAAGGTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGATCGTCACTGCG
Goa4      161 TGGCTTATTCCAATTGCAAGGTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGATCGTCACTGCG
Krt1      161 TGGCTTATTCCAATTGCAAGGTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGATCGCCACTGCG
Ori4      161 TGGCTTATTCCAATTGCAAGGTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGATCGTCACTGCG
Krt2      161 TGGCTTATTCCAATTGCAAGGTGACTGGCTCGAACTCGTATGATGACTTGAAGGGAGCCGACGTGGTGATCGTCACTGCG
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Krt2      241 GAATTTACTAAAGCACCAGGAAAGAGCGTCAAGGAATGGAACCGAGATGATTTACTCCCCTTGAATAACAAAATTATGAT
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Krt1      321 TGAGATTGGGGGACATATTAAGAACCTTTGCCCCAATGCCTTTATCATTGTGGTGACGAACCCAGTGGACGTGATGGTGC
Ori4      321 TGAGATTGGGGGACATATTAAGAACCTTCGCCCCAATGCCTTTATCATTGTGGTGACGAACCCAGTGGACGTGATGGTGC
Krt2      321 TGAGATTGGGGGACATATTAAGAACCTTTGCCCCAATACCTTTATCATTGTGGTGACGAACCCAGTGGACGTGATGGTGC
Krt4      321 TGAGATTGGGGGACATATTATGAACCTTTGCCCCAATGCCTTTATCATTGTGGTGACGAACCCAGTGGACGTGATGGTGC
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Sal1      401 AGTTACTCTTCGAGCATTCCGGAGTCCCAAAAAATAAAATCATCGGATTAGGTGGTGTGCTAGATACATCTAGACTGAAA
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Krt1      401 AGTTACTCTTCGAGCATTCCGGAGTCCCAAAAAATAAAATCATCGGATTAGGTGGTGTGCTAGATACATCTAGACTGAAA
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Sal1      561 TCTCCTGAAAAGGTACATCACAGTTGGAGGTATCCCATTGCAAGAATTTATTAATAACAAAAAGATTACAGATGAAGAAG
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Krt4     801 ATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTTATCGGGGGCACCGGAGTTGAGCAAGTCATCGAGTTGCAGC
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Goa1     801 ATACGGCCACAGCAACATCTTTGGTGGTACTCCTCTCGTTATCGGGGGCACCGGAGTTGAGCAAGTCATCGAGTTGCAGC

Sal1     881 TGAATGCCGAGGAGAAGACCAAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA
Goa4     881 TGAATGCCGAGGAGAAGACCAAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA
Krt1     881 TGAATGCCGAGGAGAAGACCAAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGATGAAGGCGCTCATTTAA
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Krt2     881 TGAATGCCGAGGAGAAGACCAAGTTCGACGAGGCAGTTGCGGAGACTAAAAGGAAGAAGGCGCTCATTTAA
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Goa1     881 TGAATGCCGAGGAGAAGACTAAGTTCGACGAGGCAGTTGCGGAACTAAAAGGATGAAGGCGCTCATTTAA
```

Figure 2.7: Multiple sequence alignment of PfLDH (A) and PvLDH (B) with respective reference sequences. 3D7: *P. falciparum* 3D7 LDH (GenBank ID: XM_001349953); Sal1: *P. vivax* Sal1 LDH (GenBank ID: XM_001615570.1). Polymorphic nucleotides are presented in red. Regions analysed by SSCP fragment 1 of both PfLDH and PvLDH are shown in bold where fragment 2 of both are shown as bold and underlined.

Variations in the PvLDH gene sequence included both synonymous and nonsynonymous nucleotide substitutions. Resulting amino acid sequence variations were analyzed by multiple sequence alignment. Variations were interspersed throughout the protein sequence, the catalytic residues (Arg 109, Asp 168, Arg 171 and His 195) and the *Plasmodium* specific active site residues which are markedly different from human LDH (Lys 107, Leu 163, and Ser 245 – Tyr 247) were conserved in all genotypes. Variations were observed in the active site loop in Ori1 (Ser 108 to Gly and Asp 109_a to Asn), Goa1 (Asp 109_a to Asn) and in Ori4 and Krt2 (Asp 109_a to Val in both) (Figure 2.8).

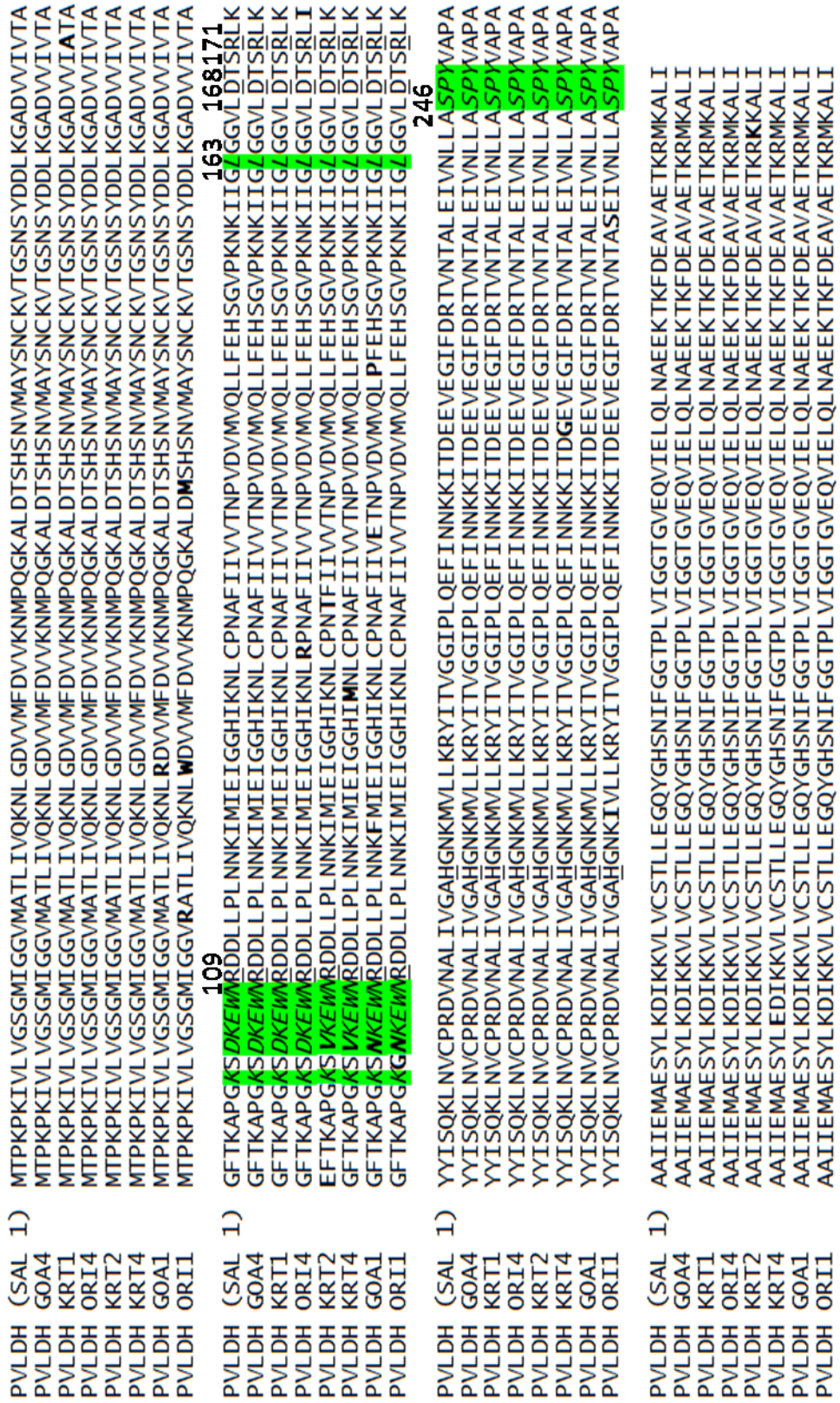


Figure 2.8: Amino acid sequence comparison of PvLDH. Variable residues are in bold and catalytic residues are underlined. pLDH specific active site residues are in italics and highlighted with green background.

PvLDH model structures, generated by homology modeling using protein sequences, were of good quality with QMEAN (Qualitative Model Energy ANalysis) score4 value > 0.75 and low Z - score values (Table 2.3). All the models matched significantly with the physically derived crystal structure of PvLDH [PDB (Protein Data Bank) ID: 2A92] with < 0.1Å RMSD (Table 2.3).

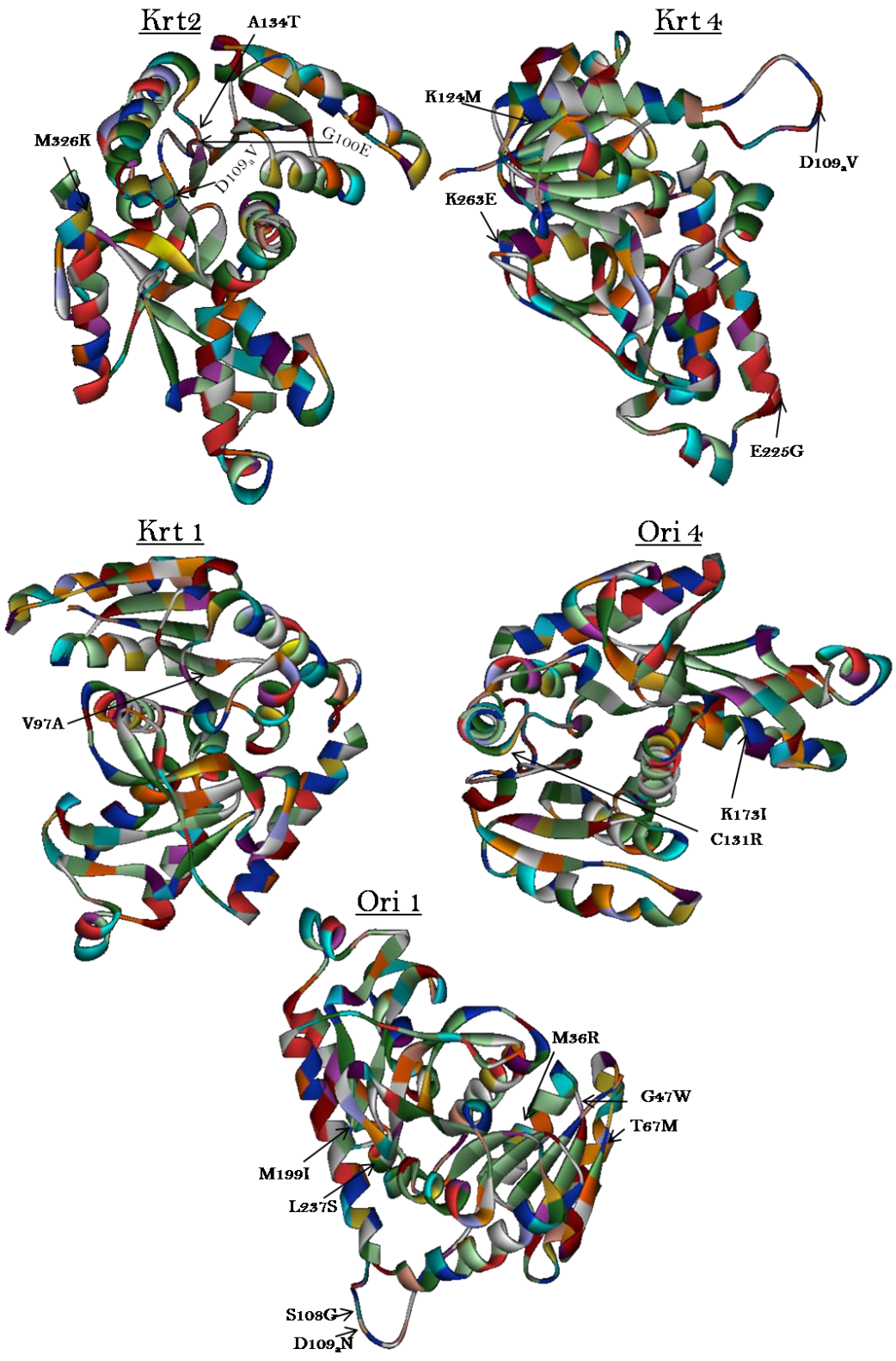
Table 2.4: Quality estimation scores of PvLDH models

Genotype (Representative Sample)	Quality estimation of the predicted protein model		Protein sequence homology [%][a]	RMSD [Å][b]
	QMEAN score4	QMEAN Z-Score		
1 (Krt 2)	0.78	0.26	98.7	0.01
2 (Krt 4)	0.779	0.25	98.7	0.01
3 (Ori 4)	0.799	0.57	99.4	0.01
4 (Krt 1)	0.789	0.40	99.7	0.00
5 (Ori 1)	0.776	0.20	97.8	0.03
6 (Goa 1)	0.768	0.08	98.4	0.03

[a] Protein sequence homology compared to reference PvLDH (GenBank ID: XM_001615570.1)

[b] RMSD (Å) of model PvLDH structures compared to reference (PDB Id: 2A92)

All PvLDH model structures were identical with the physically derived crystal structure of PvLDH (PDB Id: 2A92) from *P. vivax* Sal1; with only exception of Goa1 PvLDH, where Leu 149 to Pro substitution in α1F helix (Helix numbering is in accordance with Sessions et al. (1997) had resulted in helix to loop transition in a small region (spanning 3 amino acid residues) without altering the topology of surrounding domain (Figure 2.9).


Krt2
A134T
M326K
G100E
D109V
Krt 4
K124M
D109V
K263E
E225G
Krt 1
V97A
Ori 4
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M199I
L237S
S108G
D109N

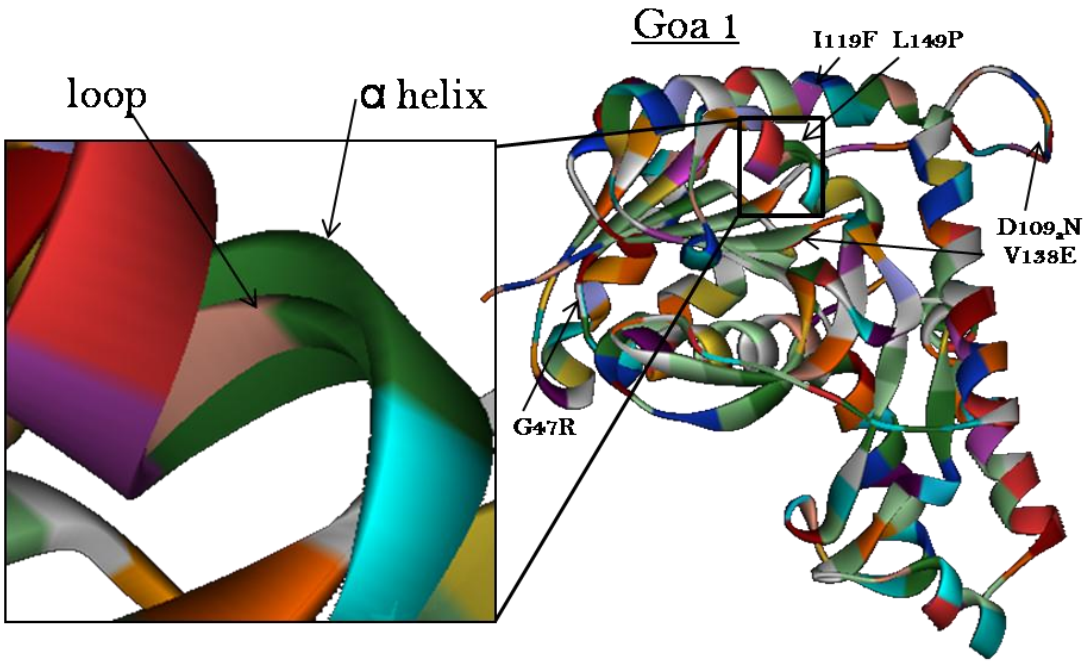


Figure 2.9: Model structure comparisons of PvLDH with reference. The predicted model structure of PvLDH from each genotype was overlapped with the known crystal structure of PvLDH (PDB ID: 2A92). Structures are displayed as cartoons representing secondary structures with each amino acid in different colors (Orientation of molecules was adjusted independently to visibly display sequence and structural variations). Arrows represent variable amino acid residues and structural variations. The only visible structural difference observed in Goa1 PvLDH is magnified in the inset.

2.3.4 Malaria evolutionary genetics analysis with pLDH as a marker

Phylogenetic trees were constructed using 43 nucleotide sequences which involved pLDH sequences, obtained in this study and sequences available in GenBank. Neighbor Joining and Minimum Evolution methods led to identical phylogenies with same tree topology and the tree inferred from Neighbor Joining method is presented in figure 2.10.

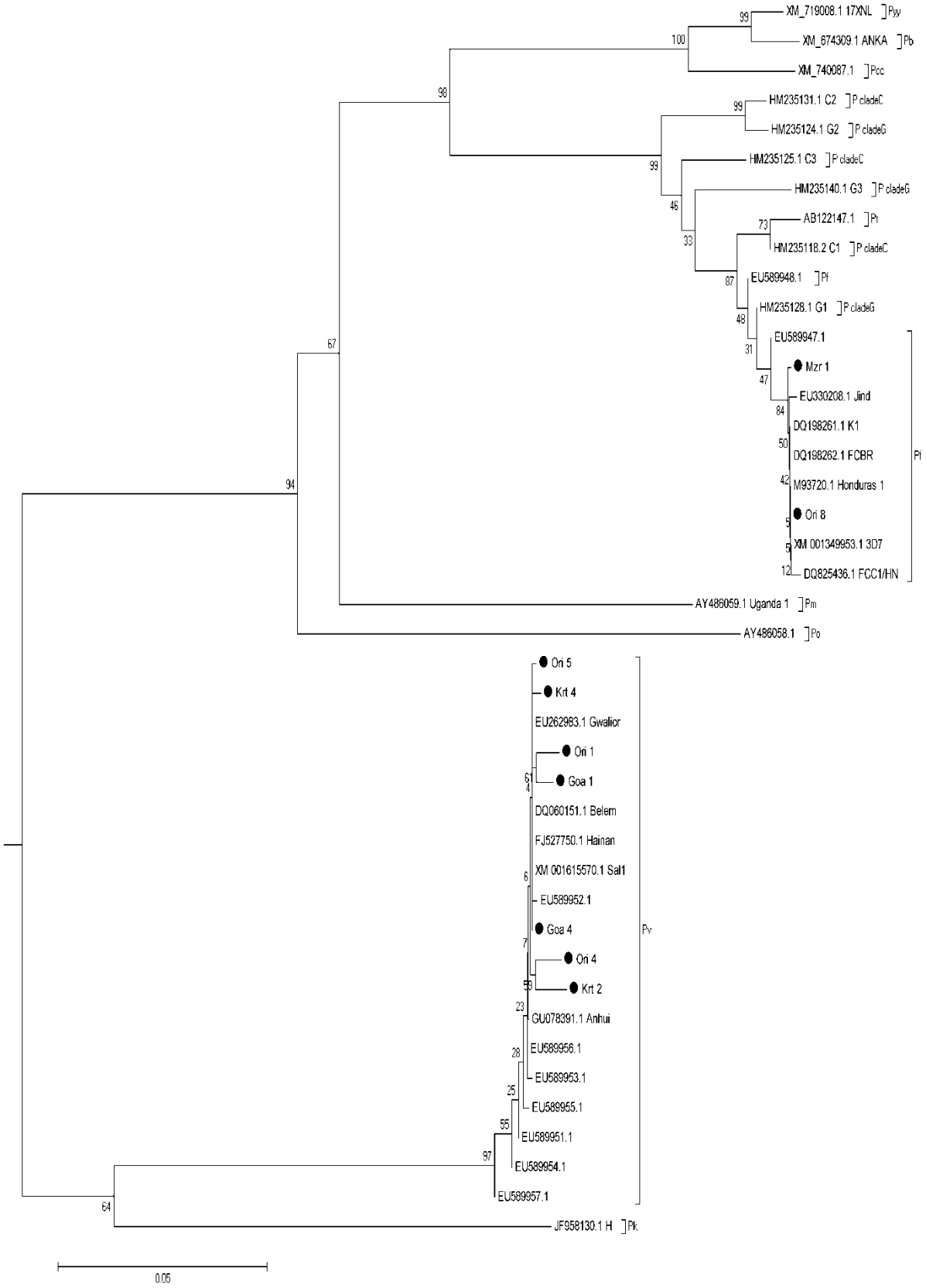


Figure 2.10: The phylogenetic tree inferred from pLDH sequences using the Neighbor Joining method. The percentage of replicate trees in which

the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale and the units represent the number of base substitutions per site. Dots represent sequences reported in this study. Accession numbers followed by strain name (if available) represent sequences from Genbank. Species names are represented by box bracket on right (Pf: *P. falciparum*; Pv: *P. vivax*; Po: *P. ovale*; Pm: *P. malariae*; Pr: *P. reichenowi;* Pb*: P. berghei;* Py*: P. yoelii yoelii,* Pc: *P. chabaudi chabaudi;* Pk*: P. knowlesi* and P clade G or C: *Plasmodium* caldes of Gorilla and Chimpanzee respectively).

Phylogenetic analysis of the studied pLDH sequences showed two main branches separating *P. vivax* LDH and *P. falciparum* LDH sequences in two separate lineages. First lineage consisted mainly of *P. vivax* LDH sequences, showed sequence similarity with *P. knowlesi* LDH, forming its sister branch in the pLDH phylogenetic tree. The second lineage consisted of *P. falciparum* LDH sequences clustered tightly together that showed sequence similarity with LDH belonging to the lineages of Gorrila and Chimpanzee clades G and C with high stability (99% bootstrap value). Rodent malaria species (*P. berghei, P. yoelii yoelii* and *P. chabaudi chabaudi*) stably clustered together with 100% bootstrap value and formed a sister branch to *P. falciparum* LDH lineage. *P. ovale* and *P. malariae* LDH sequences diverged more distantly from *P. falciparum* LDH forming a separate branch each, with 94% and 67% bootstrap values respectively, in the group belonging to *P. falciparum* LDH sequences.

2.4 DISCUSSION

In malaria research, pLDH has a unique significance of being both therapeutic and diagnostic target (Padmanaban 2007; Piper et al., 1999). Hence, the study was designed to analyse pLDH genetic polymorphism in Indian scenario, which is an important aspect in determining its efficiency either in therapeutics or diagnostics, like any other target molecule.

pLDH has been reported to be highly conserved within the species (Talman et al. 2007) and therefore SSCP analysis, owing to its high sensitivity in detection of polymorphism, was applied in the present study to detect any possible variation within pLDH samples from India. Care was taken to avoid erroneous results during SSCP analysis by confirming the purity of the DNA fragments through silver staining.

Initially, *P. falciparum* and *P. vivax* samples were analyzed from Odisha, Karnataka and Goa which are regions with highest annual parasite index of > 5 (A. Kumar et al. 2007). PfLDH gene fragments were found to be conserved in these states and the results instigated further analysis of *P. falciparum* samples from other states (Tamilnadu, Mizoram, Meghalaya, Rajasthan, Chhattisgarh, Uttar Pradesh and Gujarat) to verify the polymorphism status. The extended study confirmed that PfLDH was conserved nationwide. On the contrary, 6 PvLDH genotypes were observed in *P. vivax* samples collected from Odisha, Karnataka and Goa states, confirming presence of genetic variability in PvLDH gene. Based on the observed frequency of occurrence and geographic distribution of genotypes (Table 2.3), it can be stated that more genotypes are likely to be present in other states of India too. Albeit, the extension of the study to other states was restricted due to lack of facility like Malaria Parasite Bank for *P. vivax* isolates owing to difficulty in culturing *P. vivax*.

Recently, comparative genomic analysis had confirmed that *P. vivax* genome has more polymorphism than *P. falciparum* (Neafsey et al. 2012).

Moreover, global pLDH sequence comparison also found variability in PvLDH and not in PfLDH (Talman et al. 2007). In agreement with these findings, the present study displayed high polymorphism (6 genotypes) in PvLDH compared to PfLDH, which was found to be conserved countrywide (Figure 2.4).

Here, for the first time, nonsynonymous substitutions were observed in the PvLDH genotypes (Figure 2.8). Despite having difference in the overall amino acid sequence, the catalytic residues were found to be conserved (Figure 2.8) and hence all PvLDH variants were presumed to be functionally active. This was apparent as pLDH is crucial for the survival of the parasite in endoerythrocytic stage (Gomez et al. 1997). The insertion of 5 residues in the active site loop, as a whole (Asp 109a, Lys 109b, Glu 109c, Trp 109d, and Asn 109e,) is necessary for the distinctive functions of pLDH, first two residues (Asp 109a and Lys 109b) have been demonstrated to be redundant for enzyme activity in the mutation studies (Turgut-Balik et al. 2006). Hence, a substitution at position 109a is inadequate to significantly vary the enzyme activity in the genotypes (Goa1, Ori1, Krt2 and Krt4) (Figure 2.8).

Three dimensional structure of a protein essentially determines its biological function and hence to explore the functional consequence of amino acid variations in PvLDH genotypes, protein structure of the enzymes from each genotype were predicted by homology modelling and these models were compared with the PvLDH crystal structure. The quality of the models was tested using Qualitative Model Energy ANalysis (QMEAN) Z-score, which is an estimate of the absolute quality of a model with reference to experimentally solved crystal structures of similar size (+/− 10%) which are deposited in PDB (Benkert et al. 2009). The score indicates the standard deviations in the values of the quality and the energy of the model from structures solved experimentally. Protein models with values < 0.6 are reasonably trustworthy (Benkert et al. 2011) and all the models of PvLDH had < 0.6 QMEAN Z-score (Table 2.3), which confirmed the reliability of predicted PvLDH structures.

These structures were nearly identical with physically resolved crystal structure of PvLDH (Table 2.4, Figure 2.9). Hence, it was confirmed through the homology modelling that sequence variations in the PvLDH genotypes had not culminated in the conformation change of a protein.

The prevalence of single PfLDH genotype was confirmed by genetic polymorphism analysis, earlier in the study (Section 2.3.3), in Indian *P. falciparum* population. Further confirmation of identical PvLDH structures in all genotypes have suggested that pLDH epitopes, used by immunochromatography based RDTs would be conserved in the parasite population. Thus, variation in the response of RDT due to polymorphic epitopes, will not be observed in India, in spite of the occurrence of different PvLDH genotypes. Field performance assessment of pLDH based RDTs in India (N. Singh et al. 2003) and other countries (Fogg et al. 2008; Valéa et al. 2009) have also shown successful use of these RDT. In congruence with the field evaluation of pLDH based RDTs, this study confirmed the reliability of these tests for diagnosis of malaria in the field conditions. The study has also confirmed pLDH to be ideal for therapeutics in the Indian scenario, on the same grounds of lack of polymorphism.

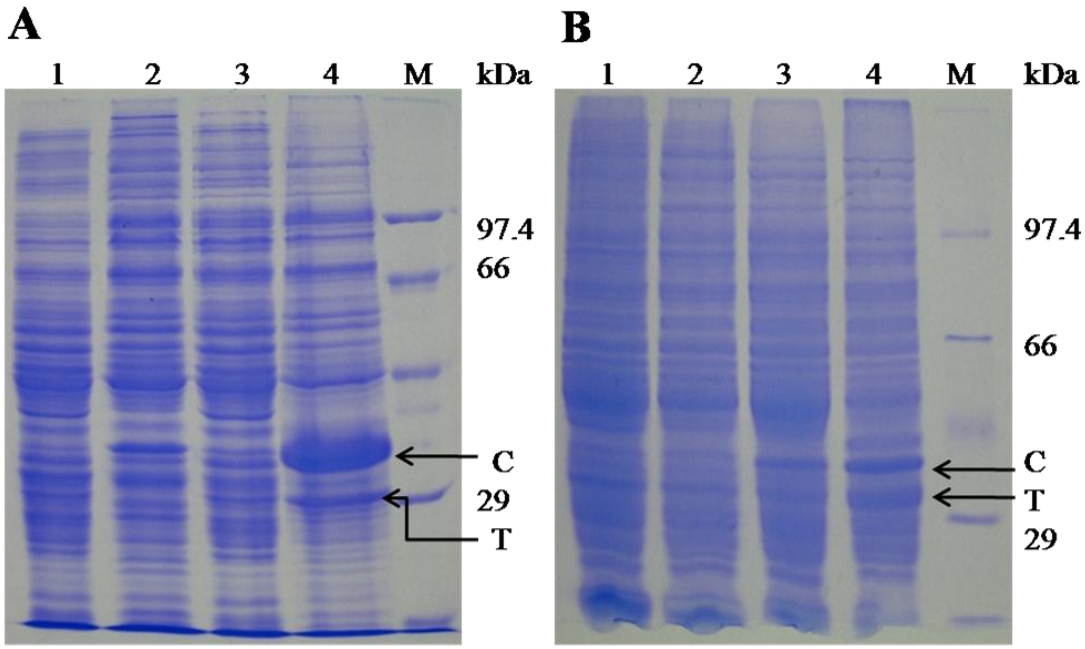
The genealogy of *Plasmodium* species inferred from the phylogenetic tree constructed using pLDH sequences was resembling with the trees inferred from the parasite mitochondrial genome sequences (Carlton, Escalante, et al. 2008; Duval et al. 2010) with the exception of the 2 rare human malaria species *P. malariae* and *P. ovale* which were not monophyletic with *P. vivax* in pLDH based tree. Branch lengths representing evolutionary distances were lowest within *P. falciparum* samples indicating paucity in the genetic polymorphism. This is in congruence with the hypothesis given by Rich et al (1998) stating the origin of extant global populations of *P. falciparum* have recently evolved from a single ancestral strain. Moreover, clustering of Gorilla clade G1 within *P. falciparum* confirmed close evolutionary proximity of *P. falciparum* with gorilla clade. These findings are analogous with the

recent findings which indicate that *P. falciparum* is of gorilla origin and not from chimpanzee, bonobo or ancient human origin (Liu et al. 2010).

In conclusion PfLDH was observed to be conserved throughout the country whereas PvLDH was observed to be polymorphic. This study reports nonsynonymous mutation in the PvLDH gene for the first time apart from synonymous mutations. These changes did not lead to variation in the structure and activity of the enzyme and thus pLDH has been proffered as a molecule with unique significance in Malaria research. Along with being a therapeutic and diagnostic target, pLDH was also confirmed as a target molecule, to infer evolutionary history of *Plasmodium* spp.

Chapter 3

Cloning, expression and characterization of *Plasmodium falciparum* and *Plasmodium vivax* specific L-Lactate dehydrogenase



CHAPTER 3

Cloning, expression and characterization of *Plasmodium falciparum* and *Plasmodium vivax* specific L-Lactate dehydrogenase

3.1 INTRODUCTION

A molecule that plays a vital role in the life of a parasite and is absent or has significantly different structural and functional properties compared to its host equivalent molecule is a good target in the search for novel drugs. As described in earlier chapters, *Plasmodium* specific L-Lactate dehydrogenase is a good target for novel antimalarial drug discovery and development as its inhibition results in death of the parasite within the cultured red blood cells (Royer et al. 1986). Presence of genetic polymorphism in pLDH in the parasite population has been illustrated to be insignificant for functional variance of the enzyme (chapter 2). This has further supported the candidature of the enzyme as a drug target.

pLDH was one of the first malaria parasite enzymes that was electrophoretically and kinetically confirmed to be distinct from its corresponding host enzyme (Sherman 1979). Detailed structural and functional analyses of the parasite enzymes eventually helps in the development of antimalarial drugs that can selectively inhibit it (Bzik et al. 1993). The prerequisite for this target based drug discovery approach is a constant source of the target protein. Initial approaches to obtain adequate quantities of the enzyme involved, purification of the enzyme from parasite culture (Vander Jagt et al. 1981) but it was not possible to yield high quantities of the purified enzyme from the parasite culture. To overcome yield limitation of this approach, parasite gene coding for *P. falciparum* LDH (PfLDH) was identified, cloned and expressed in *Escherichia coli* (Bzik et al. 1993). Later, several groups have reported enhanced soluble expression of LDH from all the four human malaria

parasites from different geographical locations (Turgut-Balik et al. 2001; Brown et al. 2004; Turgut-Balik et al. 2004; XU et al. 2007).

The prime objective of the study was to use pLDH as a therapeutic target to screen plants traditionally used in the treatment of malaria. This required over-production of the enzyme which was achieved by cloning and expressing LDH from indigenous strains of the two most common malaria species in India, *P. falciparum* and *P. vivax* followed by purification of the recombinant enzymes to homogeneity and further, the purified protein was characterized to confirm its functional identity.

3.2 MATERIALS AND METHODS

3.2.1 Cloning of *P. falciparum* LDH (PfLDH) and *P. vivax* (PvLDH) ORF from Indian strains

Genomic DNA obtained from patient's blood samples (Ori1 and Krt1) (Refer section 2.2.1), were used for cloning of genes coding for PfLDH and PvLDH respectively. Species specific primers (Table 3.1) were designed based on *P. falciparum* 3D7 (GenBank ID: XM_001349953) and *P. vivax* Sal1 (GenBank ID: XM_001615570.1) *ldh* sequence to amplify complete open reading frame (ORF) coding for PfLDH and PvLDH respectively.

Table 3.1: Primer sequences used in the study

Primers	Gene	Sequence[a]
Pf F	PfLDH	5' CCATGGCACCAAAAGCAAAAATC 3'
Pf R	PfLDH	5' ATCGTCCTCGAGAGCTAATGCCTTCATTC 3'
Pv F	PvLDH	5' CCATGGCTATGACGCCGAAACCCAAAATTG 3'
Pv R	PvLDH	5' CTCGAGAATGAGCGCCTTCATCC 3'

[a] Restriction sites in primer sequence are underlined.

Restriction sites for *Nco*I (CCATGG) and *Xho*I (CTCGAG) were incorporated in forward and reverse primers respectively. The PCR mixture (50 µl) contained 50 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, approximately 100 ng of genomic DNA and 2.5 U of *Pfu* polymerase. PCR program was as follows: initial denaturation of 5 min at 94°C, 40 cycles of 30 sec at 94°C, 45 sec at 54°C for PfLDH and 58°C for PvLDH, 2 min at 72°C and a final extension for 10 min at 72°C. Amplified PCR products were purified by QIAquick Gel Extraction Kit (Qiagen) and cloned in *E. coli* DH5α using CloneJET PCR cloning Kit (Fermentas) as per the manufacturer's instructions. Clones harboring constructs with PfLDH and PvLDH inserts were confirmed by PCR using Pf F, Pf R and Pv F, Pv R primer pairs respectively and by double restriction digestion using *Nco*I and *Xho*I. Putative clones were further confirmed by sequencing using Single Pass Analysis sequencing services (Bangalore Genei, India) using pJET1.2 sequencing primers. Sequences were edited in Chromas Pro Software version 1.49 beta (Technelysium Pty Ltd., Australia) to remove terminal vector sequences and the identities of thus processed sequences were confirmed by BLASTN analysis.

3.2.2. Protein expression and characterization

PfLDH and PvLDH genes were subcloned in the expression vector pET-28a(+) (Merck Inc., India). Inserts from pJET constructs were released by *Nco*I and *Xho*I double restriction digestion and were ligated into vector pET-28a (linearised by double digestion using the same set of restriction enzymes). Competent *E. coli* DH5α cells were transformed using the ligated products by $CaCl_2$ heat shock method (Sambrook & Russel 2001). Putative clones were confirmed for the presence of the desired insert by PCR and restriction digestion as described in section 3.2.1. Confirmed clones, with constructs having PfLDH and PvLDH genes, were termed as pETPFL and pETPVL respectively. These were used to transform competent *E. coli* BL21 (DE3) lysogen by $CaCl_2$ heat shock method (Sambrook & Russel 2001). Transformant clones were grown overnight

in Luria Bertani broth supplemented with 30 µg/ml kanamycin and then inoculated (1%) in 50 ml of same medium in 250 ml baffled flask and incubated at 37°C with agitation till 1.0 $O.D._{600nm}$ was achieved. Induction was given at this stage by 1 mM IPTG and cultures were further incubated at 20°C for 12 hr with agitation for protein expression. Cells were harvested by centrifuging at 6,000 x g at 4°C for 10 min and lysed by French Press at 1000 psi. Cell lysates were centrifuged at 19,200 x g at 4°C for 10 min to pellet down cell debris and the clarified supernatants were tested for protein expression on 12% SDS-PAGE gels and visualized by coommassie staining. Identity of rPfLDH and rPvLDH in lysate supernatants were confirmed by immunochromatography based species specific *Plasmodium* LDH detection kit 'Vector screen' (IND Diagnostics Inc., Canada). LDH activity was determined as described by Bernt (1981). The assay mix contained 3 ml of 5 mM pyruvate in 50 mM phosphate buffer (pH 7.5) and 50 µl of 11.3 mM β-NADH. As an enzyme source, appropriately diluted cell free lysates (30 µl) were added in the assay mix, such that enzyme activity was maintained < 250 U/L. $O.D._{340nm}$ was measured for 4 min at 1 min interval and change in absorbance (ΔA)/min was calculated. Enzyme units were determined by formula

'Enzyme activity = 5064 × mean ΔA/min (i)

1 unit was defined as 1 µmol of NADH oxidized to NAD^+ per minute.

The protein concentration was determined by Bradford's method (1976).

3.2.3 Purification of rPfLDH and rPvLDH

The recombinant enzymes were purified by nickel affinity chromatography using Ni-NTA (nitrilo triacetic acid) resin based kit (Invitrogen, USA) by following manufacturer's protocol. Excess of imidazole from purified protein was removed by size exclusion chromatography using protein desalting column (Merck, India). $O.D._{280nm}$ and enzyme activity of the fractions collected was measured to

determine the fractions containing sufficient quantities of the enzyme and subsequently such fractions were pooled. Purification efficiency of the process for both the enzymes was determined by calculating protein concentration in the pooled fractions and by calculating change in the enzyme activity measured by enzyme assay. Furthermore, molecular weight estimation and densitometry analysis of protein bands developed by silver staining of SDS-PAGE gels was also carried out using AlphaEase FC software version 6.0 (Alpha innotech corp., USA).

3.2.4 Steady state enzyme kinetics studies of rPfLDH and rPvLDH

Recombinant parasite LDH were diluted to 18 kU/L concentration in phosphate buffered saline (pH 7.4) containing 1 mg/ml BSA and 1 mM PMSF and from this, 30 µl enzyme was used in the assay. Kinetic constants for both rPfLDH and rPvLDH with respect to substrate (pyruvate) and cofactor (NADH) were determined by linear regression analysis of double reciprocal plots (Lineweaver-Burk plot). Affinity constant K_m was calculated by formula (ii)

$$K_M = m/c \qquad \text{(ii)}$$

Where m = slope and c = y intercept of line in double reciprocal plot.

The enzyme activity was determined, as described in the section 3.2.2, at varying concentrations of pyruvate (0.02 mM – 5 mM) with fixed concentration of NADH (11.3 mM) to calculate kinetic constants for substrate. Similarly, kinetic constants for cofactor were calculated based on the enzyme activity determined at varying concentrations of NADH (0.01 - 0.1 mM) and at 5 mM pyruvate. Further, catalytic rate constants (k_{cat}) and catalytic efficiency values (k_{cat}/K_M) were also calculated as follows.

$$k_{cat} = V_{max}/E_t \qquad \text{(iii)}$$

$$V_{max} = 1/c \qquad \text{(iv)}$$

E_t is the total concentration of enzyme in the reaction.

Kinetic constants of both the enzymes for $APAD^+$ were calculated from the enzyme activity that was determined in lactate to pyruvate direction in the presence of cofactor analogue $APAD^+$. The assay mix contained 3 ml of 100 mM lactate, 50 mM KCl in 100 mM Tris buffer (pH 8) and varying concentrations of $APAD^+$ (0.01 - 0.2 mM). Absorbance was measured at 363nm for 4 min at one min interval and change in absorbance (ΔA)/min was calculated. Enzyme units were determined from the molar extinction coefficient of APADH of 8900 M^{-1} cm^{-1} and enzyme unit was defined as 1 µmol of APADH produced from $APAD^+$ per minute. Substrate inhibition of enzyme was determined by calculating enzyme activity at higher pyruvate concentration (0.63-50 mM). The dissociation constant (K_i) of the pLDH specific inhibitors, gossypol and chloroquine, were determined from double reciprocal plots by linear regression analysis (Brown et al. 2004). Enzyme activity in the presence of chloroquine was determined by measuring change in absorbance at 370nm to avoid strong absorbance of chloroquine at 340nm. Hence, enzyme units were defined as mean ΔA_{370nm}/min/L.

3.3 RESULTS

3.3.1 Cloning of PfLDH and PvLDH ORF from Indian strains

Specificity of the primers designed in the study (Table 3.1) and of the PCR program used, was validated by analyzing PCR amplification obtained in the presence of template genomic DNA from *P. falciparum* (Ori1) and *P. vivax* (Krt1) from malaria patient's blood and from genomic DNA from a healthy human blood, using both sets of primes. When PfLDH specific primers were used, amplification of the desired size (962 bp) was obtained only in *P. falciparum* sample, while in *P. vivax* and healthy human sample multiple bands of low molecular weight were observed (Figure 3.1A). Similarly, with PvLDH specific primers amplification of the desired size (962 bp) was obtained only in *P. vivax* sample whereas nonspecific amplifications were observed in *P. falciparum* and healthy human sample (Figure 3.1B). Before commencing

for cloning, DNA fragments of the desired size were gel purified from both PfLDH and PvLDH PCR amplified products, to remove nonspecific amplicons obtained during PCR.

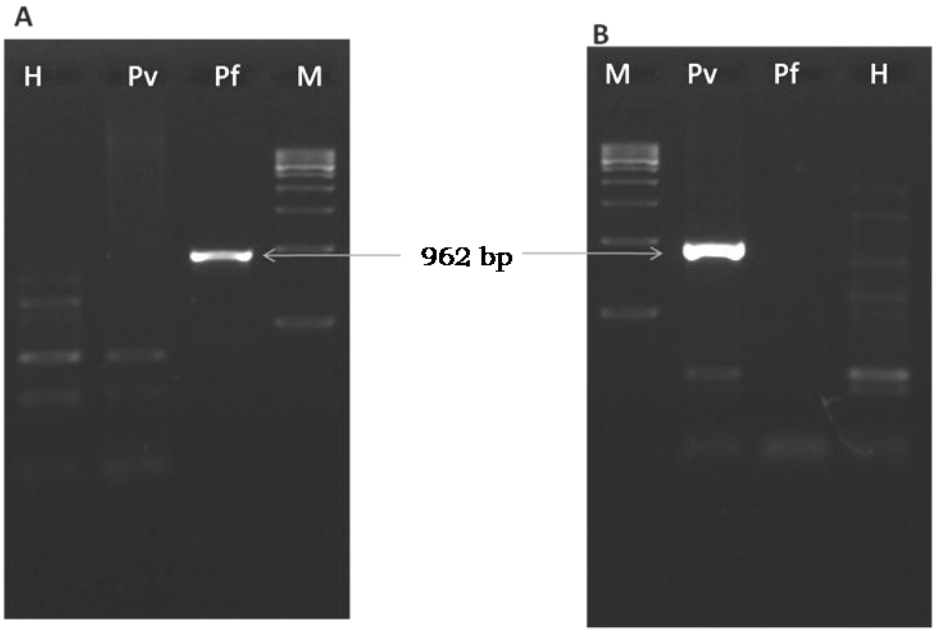


Figure 3.1: Primers and PCR method validation. A) PfLDH specific primers; B) PvLDH specific primers. Lanes – M: 500 bp ladder; Pf: PCR with Ori1 template DNA; Pv: PCR with Krt1 template DNA; H: PCR with healthy human template DNA (Control).

Complete ORF coding for PfLDH and PvLDH was cloned in *E. coli* using the CloneJet cloning kit yielding 38 and 115 clones respectively. The clones of these libraries were labeled pJETPFL$_{1\text{-}38}$ and pJPVL$_{1\text{-}115}$ respectively. Screening of randomly selected 20 clones from each library confirmed presence of the desired insert in 15 pJETPFL and 12 pJETPVL clones, by double restriction digestion and PCR. The clones, from which a 962 bp fragment was released from the vector on *Nco*I and *Xho*I double restriction digestion and which have yielded amplification of the same size on PCR with Pf F – Pf R and Pv F – Pv R primer pairs, were putatively considered to harbor the desired construct. Results of the representative putative clone from both libraries (pJETPFL$_2$ and pJETPVL$_4$) are displayed in figure 3.2.

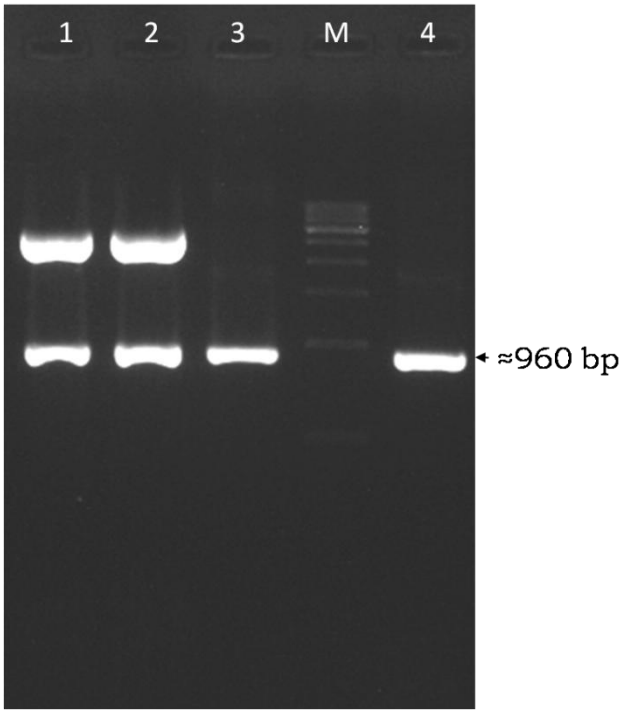


Figure 3.2: Clone confirmation by restriction digestion and amplification of PfLDH and PvLDH genes. Lanes – 1, 2: Double restriction digestion of pJETPFL$_2$ and pJETPFL$_4$ with *Nco*I and *Xho*I. 3: PCR amplification with pJETPFL$_2$ using Pf F and Pf R primers. 4: PCR amplification with pJETPVL$_4$ using Pv F and Pv R primers. M: 500 bp ladder.

Sequencing and BLAST analysis of insert sequences from pJETPFL$_2$ and pJETPVL$_4$ confirmed the presence of complete ORF of 948 bp coding for PfLDH and PvLDH in these clones respectively (Table 3.2).

Table 3.2: BLASTN Results of the clones confirming their identity

Clone	Maximum identity of insert with GenBank sequence (ID)	Score	Identity	E value
pJETPFL$_2$	*Plasmodium falciparum* 3D7 L-lactate dehydrogenase mRNA, complete cds (XM_001349953.1)	1757	100%	0
pJETPVL4	Plasmodium vivax SaI-1 lactate dehydrogenase partial mRNA (XM_001349953.1)	1751	99%	0

3.3.2 Heterologous expression of recombinant pLDH in *E. coli*

DNA fragments containing PfLDH and PvLDH ORF were released from pJETPFL$_2$ and pJETPVL$_4$ constructs respectively by double restriction digestion with *Nco*I and *Xho*I and were ligated with pET28a vector. Transformation of competent *E. coli* DH5α with these ligated products have yielded library of 36 transformant clones for PfLDH and more than 100 transformant clones for PvLDH. Screening of randomly selected 20 clones from each library by double restriction digestion and PCR, confirmed the presence of the desired insert in 18 PfLDH and 19 PvLDH clones (Figure 3.3). These confirmed clones were labeled pETPFL$_{1-18}$ and pETPVL$_{1-19}$ respectively.

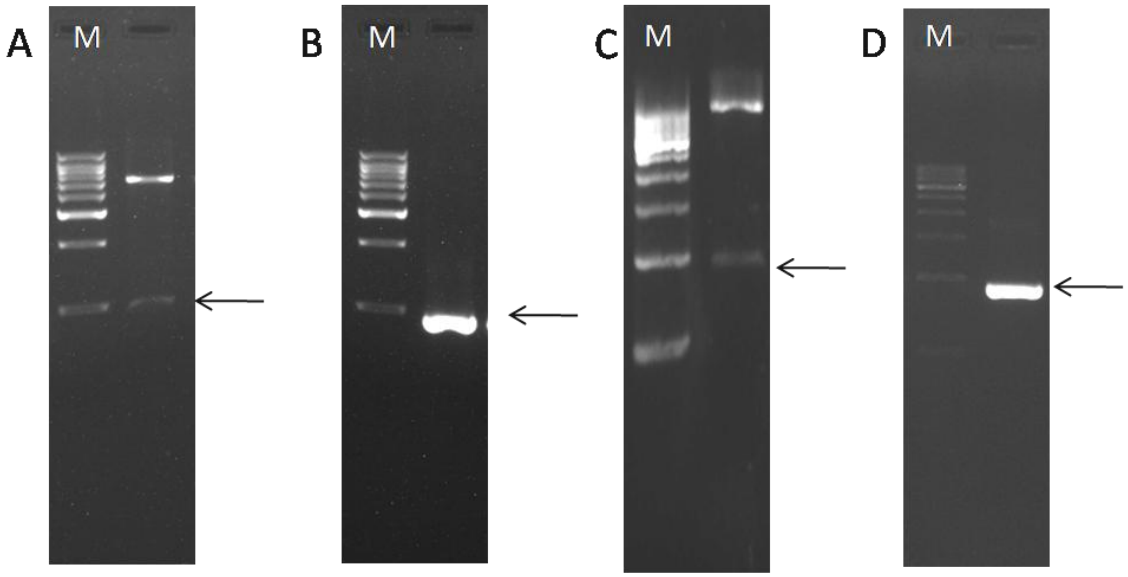


Figure 3.3: Clone confirmations by restriction digestion and amplification of PfLDH and PvLDH genes from representative samples. A and C: Double restriction digestion of pETPFL$_1$ and pETPVL$_1$ with *Nco*I and *Xho*I. B: PCR amplification with pETPFL$_1$ using Pf F and Pf R primers. D: PCR amplification with pETPVL$_1$ using Pv F and Pv R primers. M: A and B 1 Kb ladder, C and D 500 bp ladder. Arrows indicate approximate positions of a band of 970 bp DNA.

Transformation of *E. coli* BL21 (DE3) by pETPFL$_1$ and pETPVL$_1$ yielded more than 100 clones in both the cases. Induction and protein expression studies were performed by using one clone from each set of transformants. Under the given expression conditions (IPTG concentration, aeration and post-induction incubation temperature / time) recombinant enzymes (rPfLDH and rPvLDH) were expressed specifically on induction with IPTG (Figure 3.4). Two new protein bands were observed in the cell lysate of both the induced clones (Figure 3.4). The size of the higher molecular weight bands was close to 35 kDa while the size of lower molecular weight bands was close to 30 kDa. Band intensity was higher in induced lysate of pETPFL clone compared to pETPVL suggesting variation in the expression level of rPfLDH and rPvLDH (Figure 3.4).

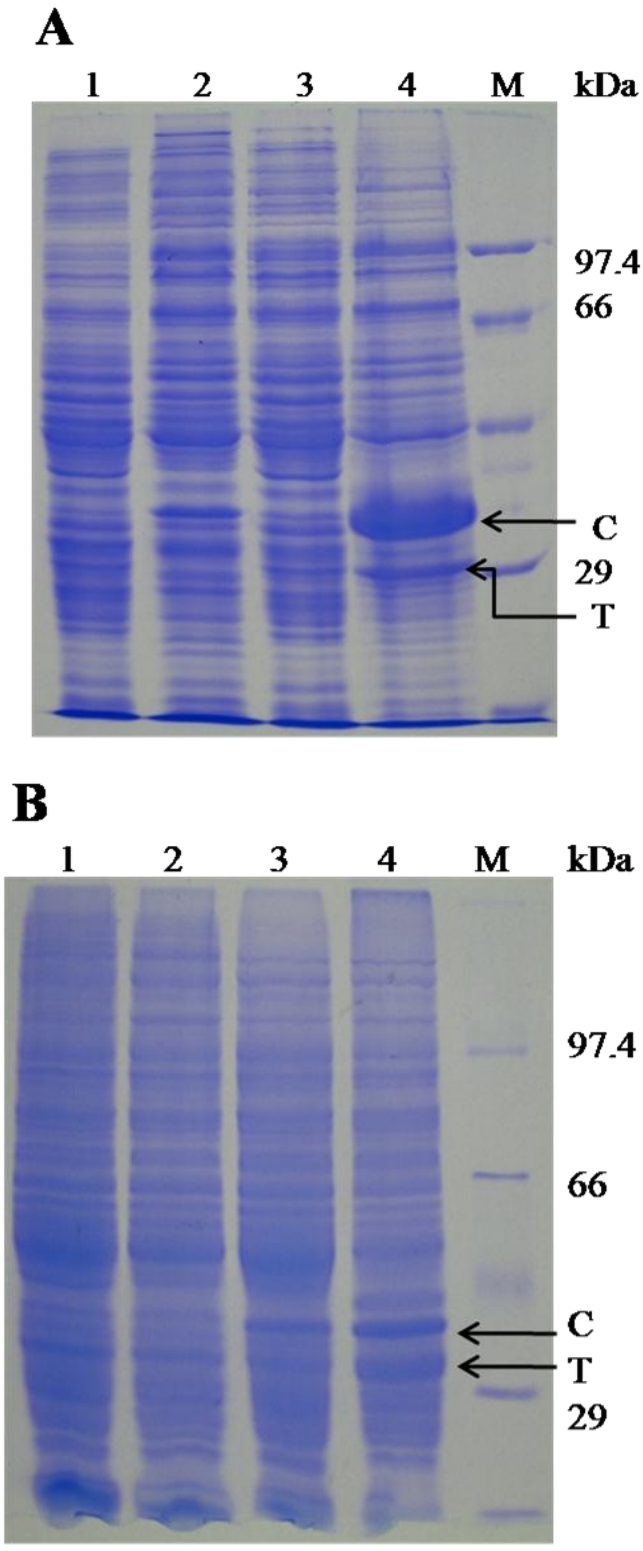


Figure 3.4: SDS-PAGE analysis of *E. coli* cell lysate, with pET28a (only vector) under IPTG uninduced (Lane 1 of A and B) and induced conditions (Lane 2 of A and B), with pETPFL and pETPVL under uninduced conditions (Lane 3 of A and B respectively) and with pETPFL

and pETPVL under induced conditions (Lane 4 of A and B respectively). Arrows indicate band positions of complete (C) and truncated (T) proteins, expressed in lysate. Protein bands in the gel were visualized by Coommassie Blue staining. Molecular weights of marker bands (M) are shown on the right hand side of the gel.

Presence of rPfLDH in the induced lysate of pETPFL clones was also confirmed by the development of bands corresponding to PfLDH specific and pan specific (Human *Plasmodium* species LDH other than PfLDH) monoclonal antibodies in immunochromatography based dip stick test (Figure 3.5). Similarly, development of pan specific band in the induced lysate of pETPVL clones confirmed the presence of pan specific LDH, specifically rPvLDH in the present study (Figure 3.5).

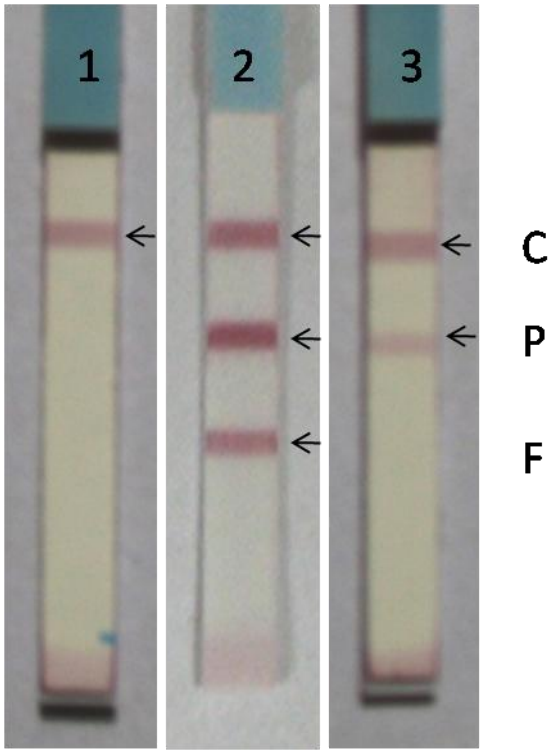


Figure 3.5: Immunochromatography based dip stick assay of induced *E. coli* lysate with 1. pET28a, 2. pETPFL construct and 3 pETPVL construct. Band development at *P. falciparum* (F) and pan specific (P) position indicate presence of rPfLDH; at only pan specific (P) position indicate presence of rPvLDH. Assay control bands (C) are developed at top.

A substantial increase in the LDH activity was observed in the induced lyastes of pETPFL and pETPVL clones (22312 U/L and 18939 U/L respectively) as compared to their respective uninduced clones (374 U/L and 897 U/L respectively) (Figure 3.6) indicating presence of functional rPfLDH and rPvLDH. The LDH assay used in the study was not *Plasmodium* LDH specific and hence enzyme activity was also observed in the controls and it was attributed to *E. coli* LDH (Figure 3.6).

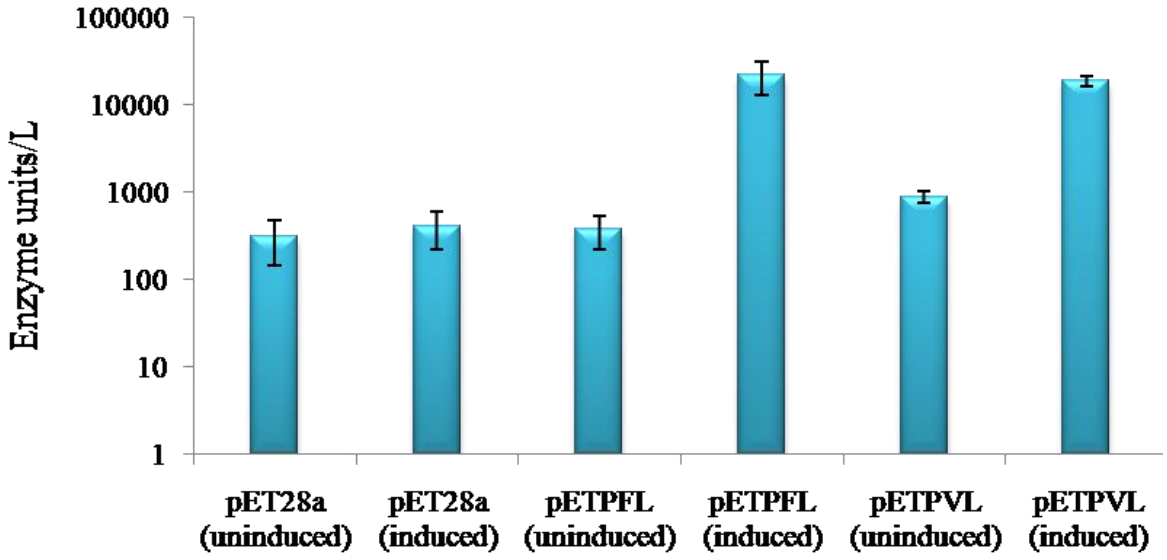


Figure 3.6: LDH activity in lysate supernatants of uninduced and induced clones.

3.3.3 Purification of recombinant proteins

Hexahistidine tag at the C' terminus of the recombinant proteins had facilitated single step protein purification by metal chelation chromatography. Recombinant proteins were selectively adsorbed on Ni-nitrilotriacetic acid agarose and were eluted using high concentrations of imidazole, which was subsequently removed by gel filtration chromatography. The specific activity of rPfLDH and rPvLDH was increased by 10.6 and 9.7 fold in the purification process. Silver stained SDS-PAGE gel analysis confirmed ≥ 90% purity of both the recombinant proteins. Their molecular weights (rPfLDH 34.5 kDa; rPvLDH 35 kDa)

were close to expected molecular weights calculated using amino acid sequence (rPfLDH 34.9 kDa; rPvLDH 35.1 kDa) (Figure 3.7)

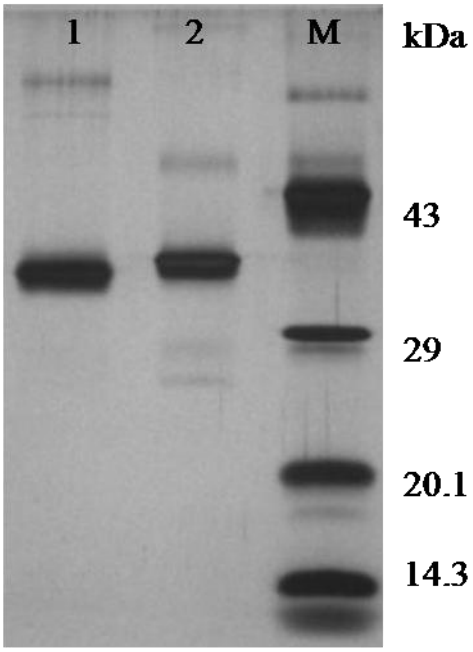
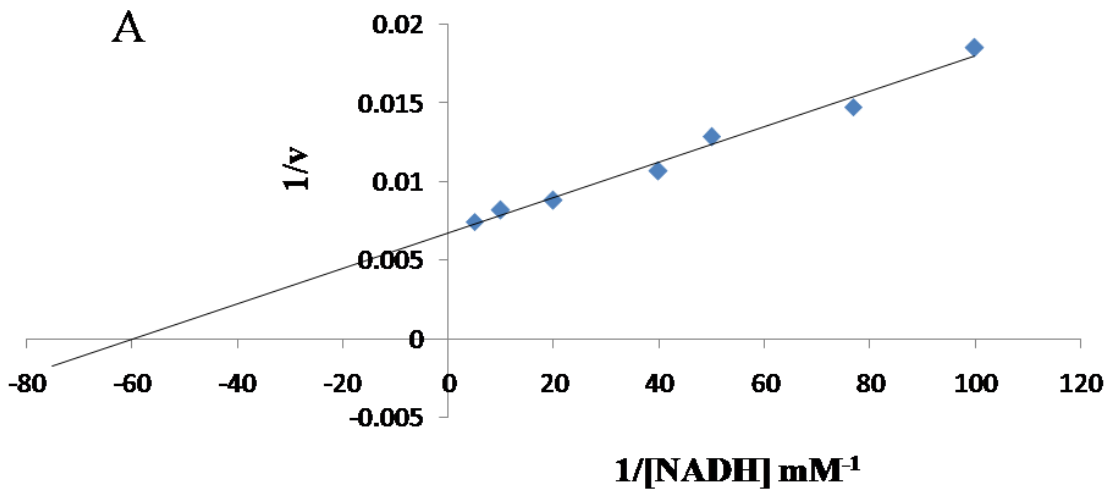


Figure 3.7: Purified recombinant enzymes as visualized by silver staining on SDS-PAGE gel. Lane 1. rPvLDH, Lane 2. rPfLDH. Molecular weights of marker bands (M) are shown on the right hand side of the gel.

3.3.4 Enzyme kinetic studies of rPfLDH and rPvLDH

Double reciprocal plots of the enzyme activity of rPfLDH and rPvLDH at varying concentrations of substrate and cofactor are presented in figure 3.8.


A
0.02
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0.01
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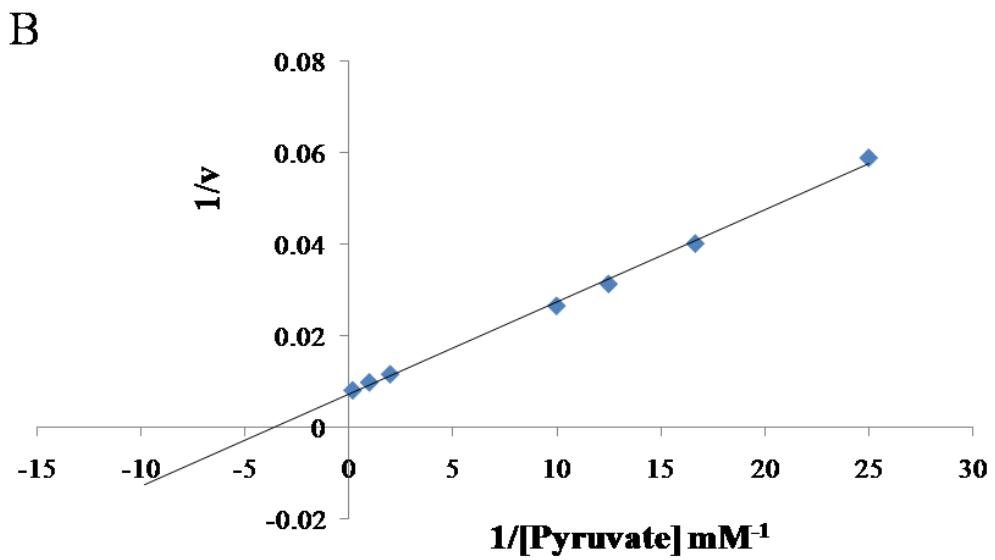
80
100
120
1/[NADH] mM-1


B
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0.06
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1/[Pyruvate] mM-1

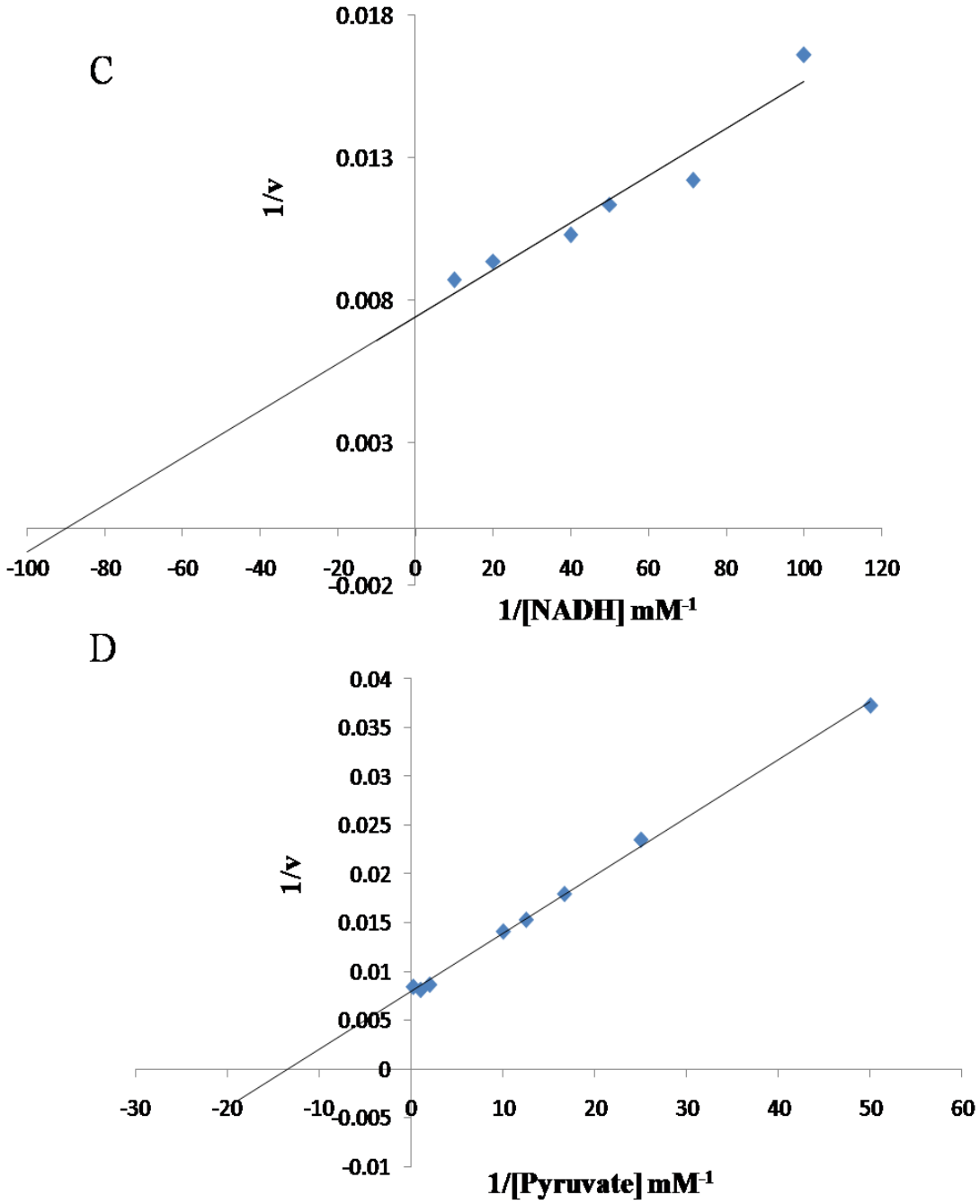


Figure 3.8: Double reciprocal plots of enzyme activity. A) rPfLDH at varying NADH concentration. B) rPfLDH at varying pyruvate concentration. C) rPvLDH at varying NADH concentration. D) rPvLDH at varying pyruvate concentration.

The steady state kinetics constants (K_M, k_{cat} and k_{cat}/K_M) for both rPfLDH and rPvLDH were calculated based on the plots and are tabulated in table 3.3.

Table 3.3: Kinetic constants of rPfLDH and rPvLDH for substrate and cofactor

Enzyme	**Substrate**	**K_M (µM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_M (M^{-1} min^{-1})**
PfLDH	NADH	14.9 ± 0.7	(4.63 ± 2) x 10^4	3.1 x 10^9
	Pyruvate	278 ± 2		1.66 x 10^8
PvLDH	NADH	10.8 ± 1.5	(9.65 ± 1.4) x 10^3	8.94 x 10^8
	Pyruvate	75 ± 0.6		1.29 x 10^8

The double reciprocal plots of enzyme activity of rPfLDH and rPvLDH with $APAD^+$ are presented in figure 3.9.

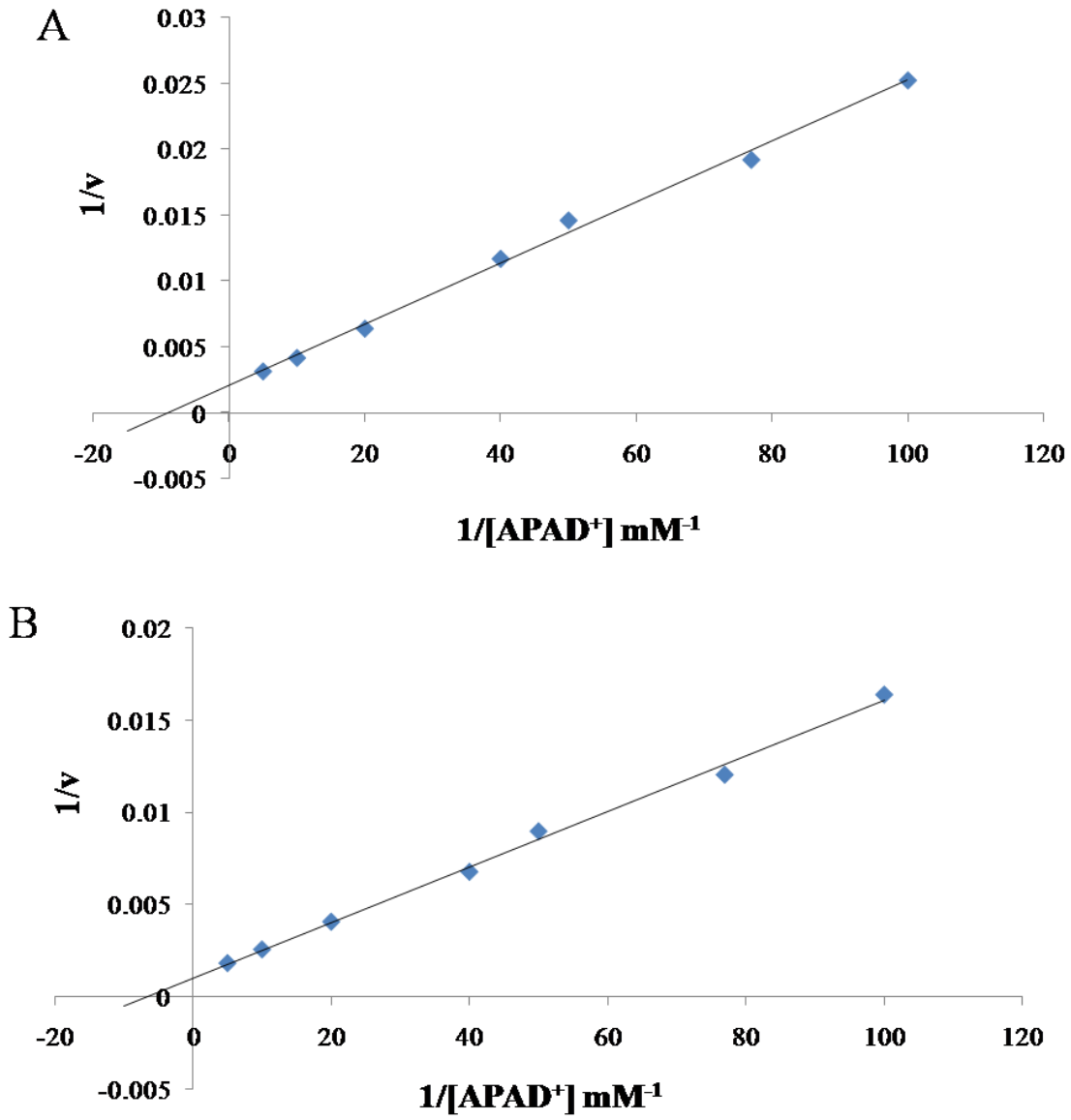


Figure 3.9: Double reciprocal plots of enzyme activity at varying concentrations of $APAD^+$ A) rPfLDH B) rPvLDH.

The steady state kinetics constants for rPfLDH and rPvLDH, calculated based on the plots are tabulated in table 3.4.

Table 3.4: Kinetic constants of rPfLDH and rPvLDH for $APAD^+$

Enzyme	K_M (µM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} M^{-1})
rPfLDH	95.24 ± 4	(1.59 ± 0.8) x 10^5	1.67 x 10^9
rPvLDH	200 ± 6	(3.3 ± 0.2) x 10^5	1.65 x 10^9

Substrate inhibition of both parasite LDHs was assessed by calculating enzyme activity upto 50 mM pyruvate concentration. More than 80% activity was retained by both the enzymes upto 30 mM pyruvate concentration and even at 50 mM concentrations of pyruvate, activity of rPfLDH and rPvLDH were not reduced to 50% suggesting weak substrate inhibition of the parasite LDH (Figure 3.10).

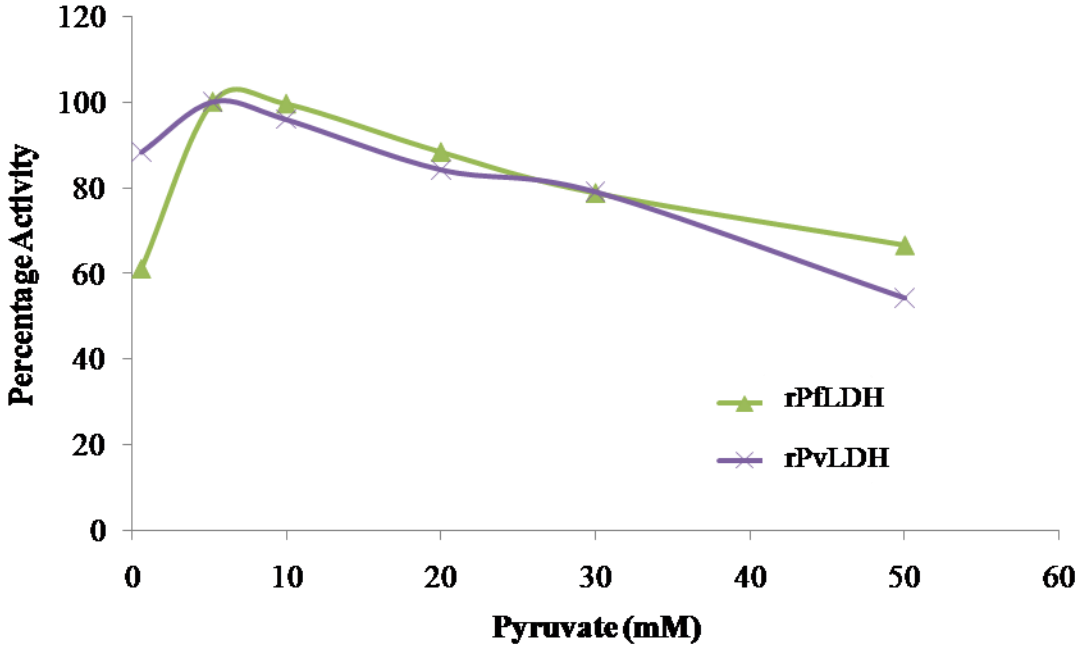


Figure 3.10: Substrate (pyruvate) inhibition of *P. falciparum* and *P. vivax* LDH.

Dissociation constant (K_i) of gossypol for rPfLDH and rPvLDH was 1 µM and 5.31 µM respectively. Based on the linear regression analysis of double reciprocal plot, it was inferred that gossypol exhibited competitive inhibition, with respect to NADH, on both parasite enzymes (Figure 3.11).

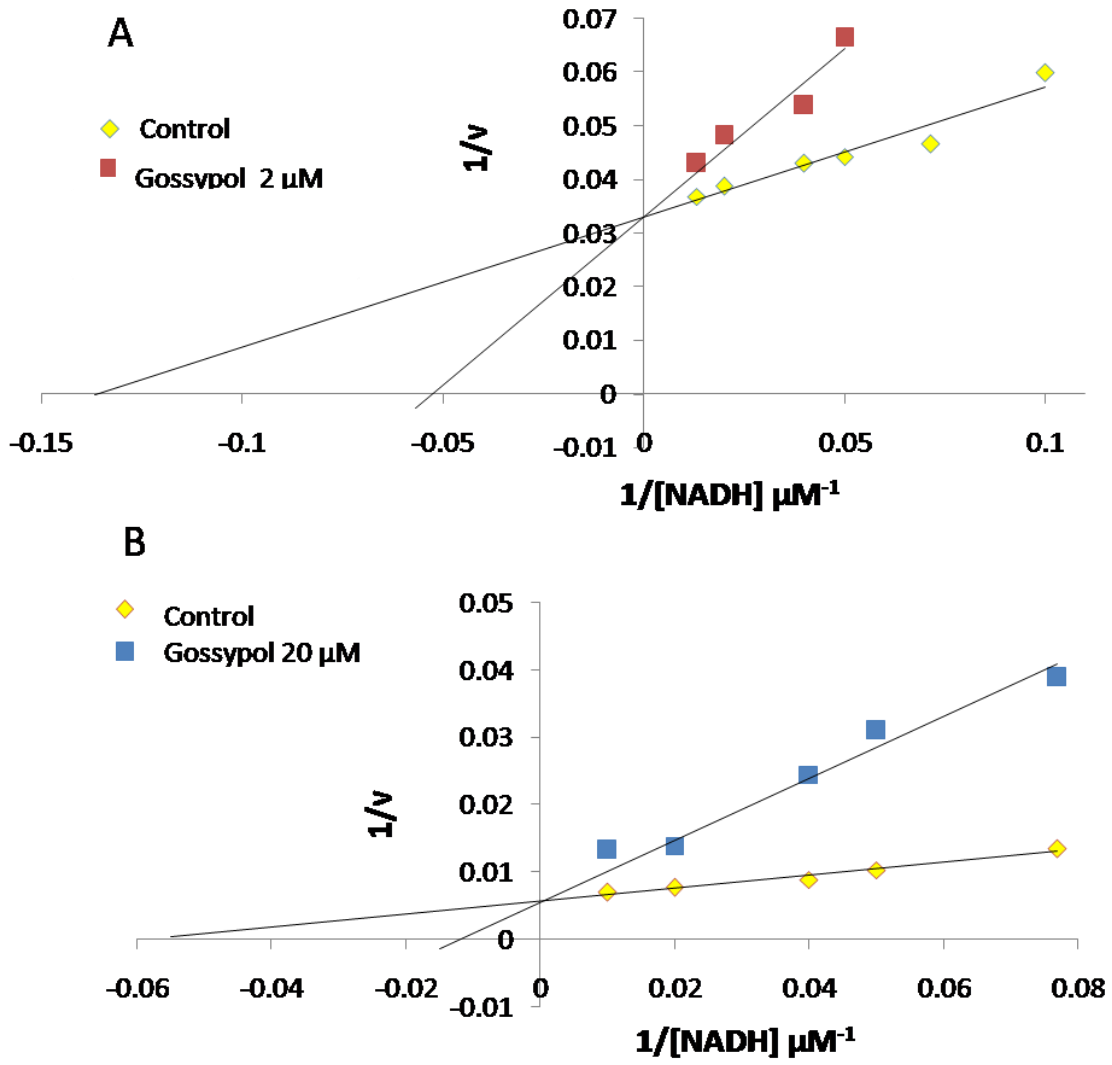


Figure 3.11: Double reciprocal plots of enzyme activity in the presence of gossypol A) rPfLDH and B) rPvLDH.

Dissociation constant (K_i) of chloroquine for rPfLDH and rPvLDH was 5.03 mM and 14.2 mM respectively and the interaction of chloroquine with rPfLDH was observed to be competitive with NADH while with rPvLDH the interaction was non-competitive with NADH (Figure 3.12).

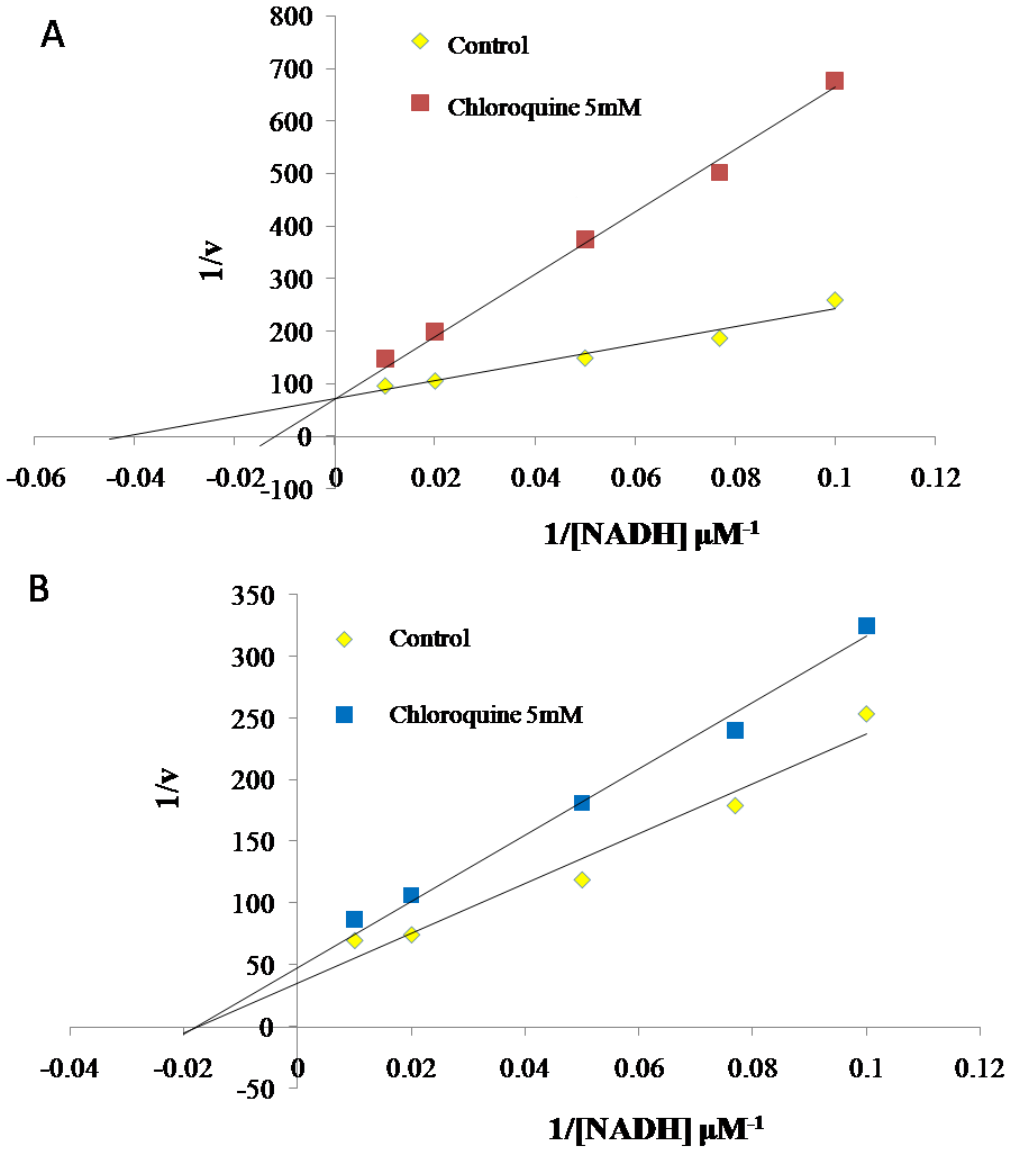


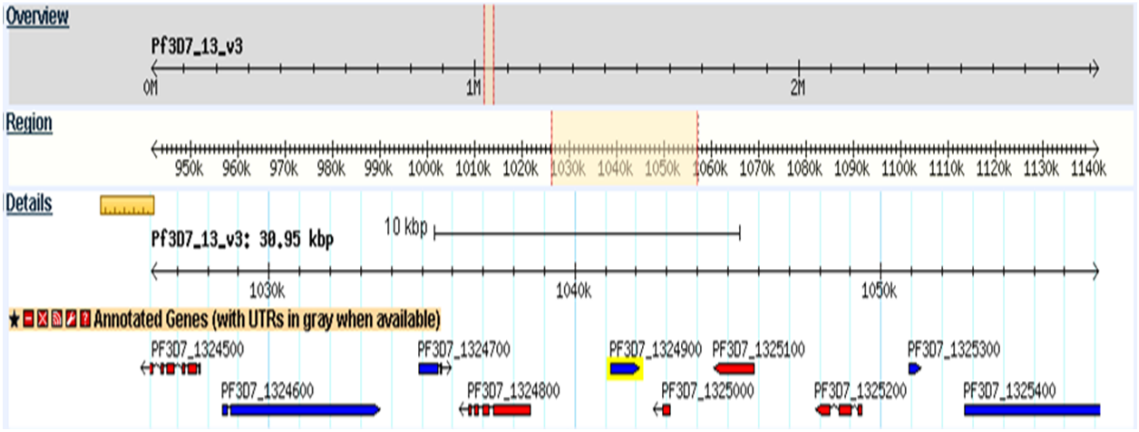
Figure 3.12: Double reciprocal plots of enzyme activity in the presence of chloroquine A) rPfLDH and B) rPvLDH.

3.4 DISCUSSION

Overproduction of the therapeutic target protein plays an important role by providing abundant enzyme for the drug screening and in this view, cloning of LDH from major Indian malaria species *P. falciparum* and *P. vivax* was the first step in the search for antimalarials. rPfLDH was cloned using blood sample from Odisha state which has the highest share of total malaria cases in India (25%) with a high proportion of *P. falciparum* malaria cases, whereas rPvLDH was cloned using blood sample from Karnataka state which also has substantial share of total malaria cases in India (7%) but with more proportion of *P. vivax* malaria cases (Kumar et al., 2007).

Plasmodium LDH is a protein with 316 amino acids, coded by a single copy gene on chromosomes 13 in *P. falciparum* and on chromosome 12 in *P. vivax* (Figure 3.13). It is expressed in all asexual blood stages of the parasite as a single 1.6 kb mRNA without any intron (Bzik et al. 1993). Thus, reverse transcription step to generate cDNA was not required. Cloning strategy included amplification of the complete ORF coding for PfLDH and PvLDH by PCR and inserting these amplicons in pJET1.2/blunt vector followed by subcloning using pET28a vector, to generate constructs (pETPFL and pETPVL) for protein expression (Figure 3.14).

P. falciparum Chromosome 13



P. vivax Chromosome 12

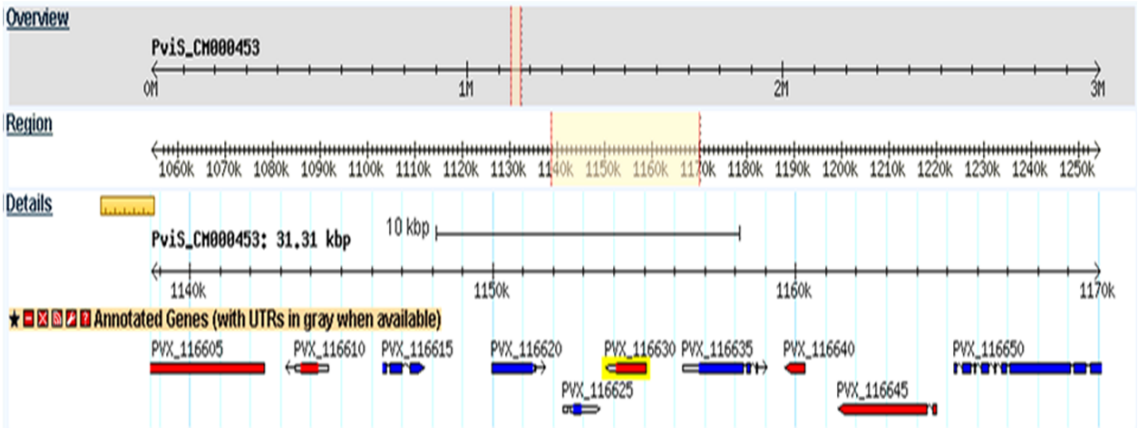


Figure 3.13: Position of PfLDH and PvLDH genes in *P. falciparum* and *P. vivax* genome respectively. Both the genes are highlighted with a yellow background [Map generated in Plasmo DB GBrowse V2.48 for location of PfLDH (Plasmo DB Id. PF3D7_1324900) and PvLDH (Plasmo DB Id. PVX_116630) genes].

As *Taq* DNA polymerase has high error rate of base incorporation, *Pfu* DNA polymerase, an enzyme with proofreading activity, was used in PCR amplification of PfLDH and PvLDH gene to ensure correct amplification of the gene. Suitable restriction sites were not available in the flanking regions of both ORF and hence, sites for *Nco*I and *Xho*I were incorporated in the primer sequence and were subsequently used for cloning. Optimum restriction digestion of PCR amplicons with these enzymes was difficult to achieve as the sites were at the ends of amplicons. The amplicons were therefore cloned in pJET1.2 vector (a

high copy number, blunt end cloning vector) using cloneJet PCR cloning kit to internalize the restriction sites.

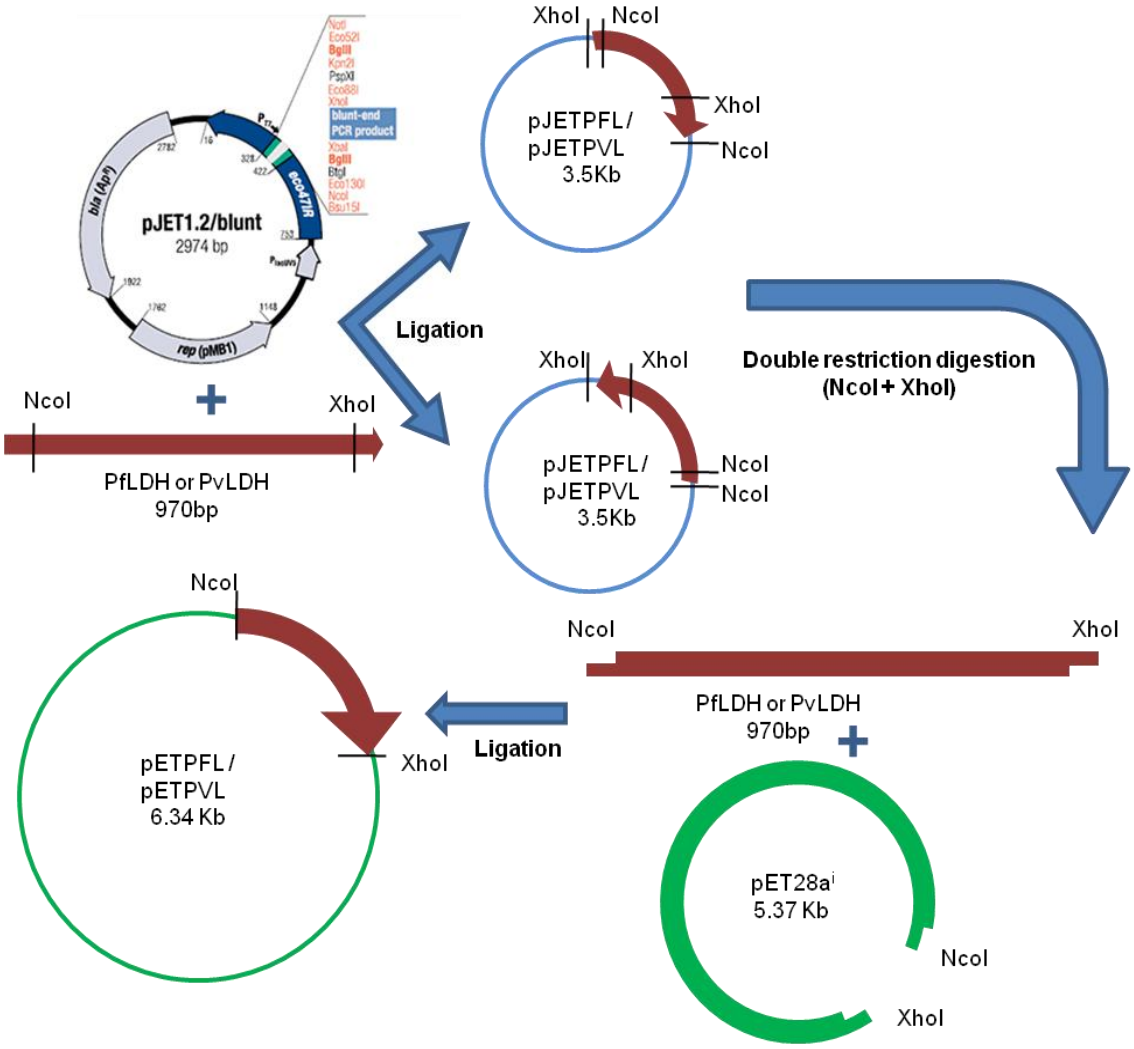


Figure 3.14: Schematic representation of the cloning strategy.

Insertion of PfLDH and PvLDH ORF in pET28a vector in the proper orientation (Directional cloning) was achieved by releasing inserts from pJET constructs with two restriction enzymes (*Nco*I and *Xho*I). Furthermore, the use of *Nco*I at 5' end had ensured that the inserts were ligated with the vector in the proper reading frame for the expression of the recombinant protein as the start codon of the expression cassette lies within the *Nco*I restriction site (Figure 3.15).

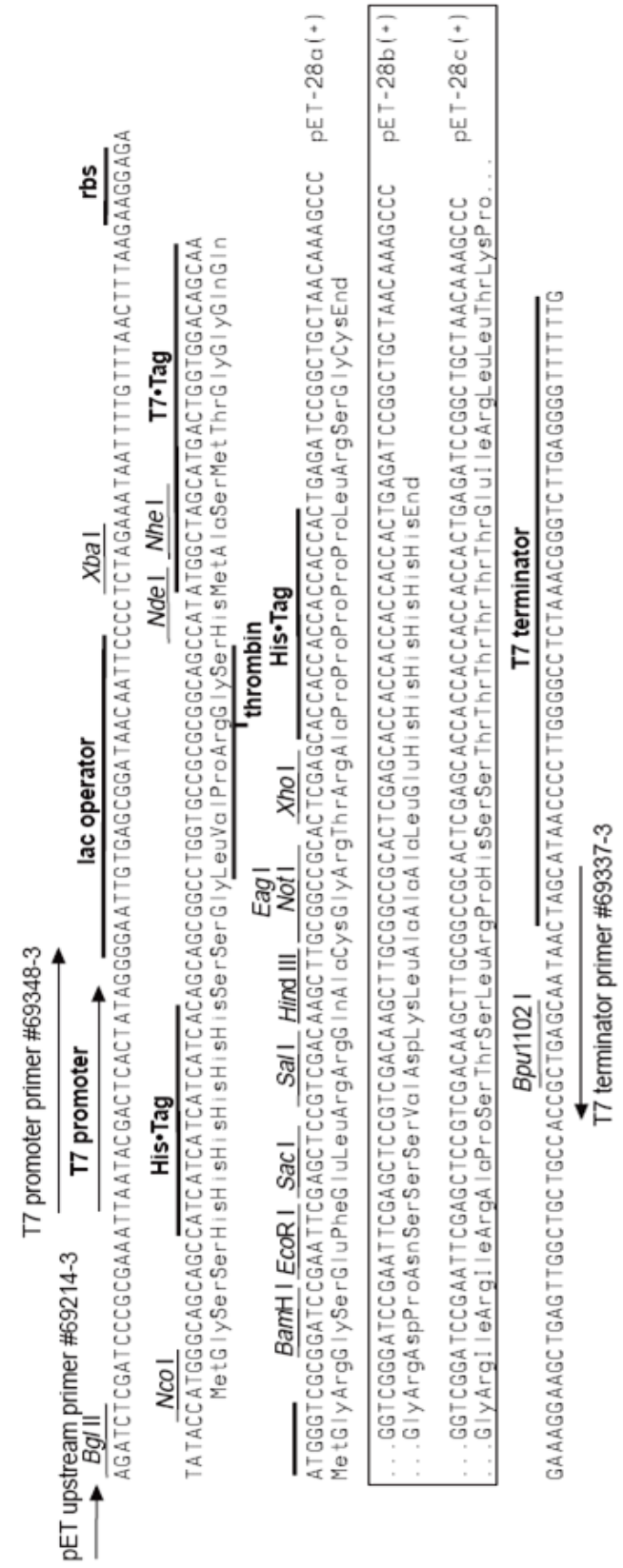


Figure 3.15: Cloning/expression region of the pET28 vector (pET 28a-c instruction manual).

pET system was used for the heterologous expression of the parasite enzymes in *E. coli* as it is one of the most powerful systems yet developed for cloning and expression of recombinant proteins in *E. coli*. Expression of the genes cloned in pET28a vector is controlled by strong transcription and translation signals of bacteriophage T7 (Fig 3.15). This expression system, comprising of a pET vector and an *E. coli* host having an inducible source of T7 RNA polymerase under *lacUV5* promoter control, like *E. coli* BL21 (DE3) lysogen (Figure 3.16), is so selective that within few hours of induction more than 50% of the total cell protein can comprise the desired product (Blaber 1998).

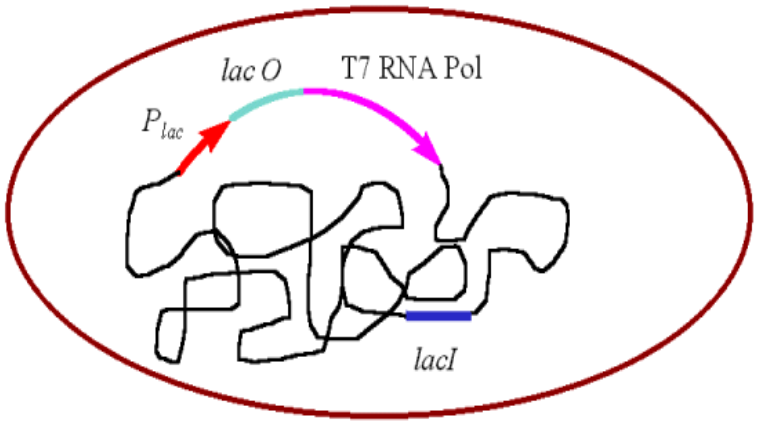


Figure 3.16: Schematic representation of *E. coli* BL21 (DE3) host chromosome characteristics (Blaber 1998).

The expression of the recombinant proteins was attained in soluble form by incubating cultures at lower temperatures after induction to maintain slow growth rate (Section 3.2.2). Incubation at suboptimum temperatures has dual advantages of reduced transcription and translation rates and of reduced hydrophobic interactions strength which are responsible for inclusion body formation due to protein misfolding (Baneyx & Mujacic 2004). A substantial increase in the LDH activity observed in the clarified lysate confirmed soluble expression of rPfLDH and rPvLDH proteins (Section 3.3.2).

Co-expression of truncated and complete ORF of rPfLDH in *E. coli* (Figure 3.4 A) was attributed to the presence of internal Shine Dalgarno

sequence in the rPfLDH ORF (Figure 3.17A) as described by Turgut-Balik et al. (2001). Interestingly, co-expression of truncated protein was also observed in rPvLDH (Figure 3.4B). Sequence analysis of rPvLDH showed presence of a similar internal Shine Dalgarno sequence (Figure 3.17B), which may have led to the co-expression of a truncated protein in rPvLDH (Figure 3.4). Amino acid sequence of PfLDH and PvLDH have 90% homology and the rest 10% variation can lead to differences in stability and solubility of the two heterologously expressed proteins in *E. coli,* justifying the different quantities of rPfLDH and rPvLDH expression under the same conditions (Figure 3.4).

A) PfLDH (Ori1)

```
   1  atggcaccaa aagcaaaaat cgttttagtt ggctcaggta tgattGGAGG agtaatggct accttaattg
  71  ttcagaaaaa tttaggagat gtagttttgt tcgatattgt aaagaacatg ccacatggaa aagctttaga
 141  tacatctcat actaatgtta tggcatattc aaattgcaaa gtaagtggtt caaacactta tgacgatttg
 211  gctggagcag atgtagtaat agtaacagct ggatttacca aggccccagg aaagagtgac aaagaatgga
 281  atagagatga tttattacca ttaaacaaca agattatgat tgaaattggt ggtcatatta agaagaattg
 351  tccaaatgct tttattattg ttgtaacaaa cccagtagat gttatggtac aattattaca tcaacattca
 421  ggtgttccta aaaacaagat tattggttta ggtggtgtat tagatacatc aagattgaag tattacatat
 491  ctcagaaatt aaatgtatgc ccaagagatg taaatgcaca cattgtaggt gctcatggaa ataaaatggt
 561  tcttttaaaa agatacatta ctgtaggtgg tatcccttta caagaattta ttaataacaa gttaatttct
 631  gatgctgaat tagaagctat atttgataga actgttaata ctgcattaga aattgtaaac ttacatgcat
 701  caccatatgt tgcaccagct gctgctatta tcgaaatggc tgaatcctac ttaaaagatt tgaaaaaagt
 771  attaatttgc tcaaccttgt tagaaggaca atatggacac tccgatatat tcggtggtac acctgttgtt
 841  ttaggtgcta atggtgttga acaagttatc gaattacaat taaatagtga ggaaaaagct aaatttgatg
 911  aagccatagc tgaaactaag agaatgaagg cattagctta a
```

B) PvLDH (Krt1)

```
   1  atgacgccga aacccaaaat tgtgctcgtc gggtcgggca tgatcGGAGG cgtgatggcc acgctgattg
  71  tgcagaagaa cctgggggac gtagtgatgt ttgacgtagt gaaaaacatg ccccaaggaa aggcactaga
 141  tacgtctcac tcgaatgtga tggcttattc caattgcaag gtgactggct cgaactcgta tgatgacttg
 211  aagggagccg acgtggtgat cgccactgcg ggatttacta aagcaccagg aaagagcgac aaggaatgga
 281  accgagatga tttactcccc ttgaataaca aaattatgat tgagattggg ggacatatta agaacctttg
 351  ccccaatgcc tttatcattg tggtgacgaa cccagtggac gtgatggtgc agttactctt cgagcattcc
 421  ggagtcccaa aaaataaaat catcggatta ggtggtgtgc tagatacatc tagactgaaa tattacatat
 491  cgcagaagtt gaacgtctgc ccgagagatg ttaatgcact cattgtcggt gcacatggga acaagatggt
 561  tctcctgaaa aggtacatca cagttggagg tatcccattg caagaattta ttaataacaa aaagattaca
 631  gatgaagaag tggaaggcat atttgatcgc actgtgaaca ctgctttgga gattgtgaac ctccttgcct
 701  ctccttatgt tgccccagct gctgccatca tcgaaatggc cgaatcttat ttgaaggata taaagaaagt
 771  gcttgtttgt tccactctac tagagggaca atacggccac agcaacatct ttggtggtac tcctctcgtt
 841  atcgggggca ccggagttga gcaagtcatc gagttgcagc tgaatgccga ggagaagacc aagttcgacg
 911  aggcagttgc ggagactaaa aggatgaagg cgctcattta a
```

Figure 3.17: ORF sequence of A) PfLDH (JN547218) and B) PvLDH (JN547225). Start codons are underlined and internal Shine Dulgarno sequences are presented in bold and upper case.

Region coding for hexahistidine tag towards N' terminal end was removed from pET28a vector due to the use of *Nco*I site in cloning and hence only C' terminal end hexahistidine tag was present in the recombinant proteins (Figure 3.10). This tag was sufficient for the purification of the desired proteins using nitrilotriacetic acid (Ni-NTA) resin based affinity chromatography. The resin (Ni-NTA) contains, a tetradentate chelating ligand, in a highly cross-linked 6% agarose matrix. NTA binds Ni^{2+} ions by four coordination sites. The resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins, thus, both the enzymes were purified to >90% in a single step of purification (Figure 3.7). Interestingly, truncated proteins, co-expressed with the desired proteins (Figure 3.4), were not purified efficiently, which can be attributed to reduced stability of the truncated proteins.

Unlike the thrombin site at N' terminal end, any endopeptidase acting site was not available between coding region and C' terminal histidine tag in both pETPFL and pETPVL constructs. Hence, hexahistidine tag was not removed from the purified recombinant enzymes.

The affinity constants of both the enzymes for cofactor were close to the earlier reports. Affinity of the substrate was comparatively reduced but the catalytic rate constants (k_{cat}) and catalytic efficiency values (k_{cat} /K_M) of rPfLDH and rPvLDH (Table 3.3) were comparable to the earlier reported values (Brown et al. 2004). This comparative analysis of steady state kinetic constants had confirmed the identity and enzymatic activity of the recombinant enzymes.

Based on the kinetic data, it was apparent that both rPfLDH and rPvLDH catalyze the reaction at nearly same rates and have similar affinities for cofactor NADH. Though the affinity of PvLDH for pyruvate was higher than that of PfLDH, the catalytic efficiencies of both the enzyme were similar. These results are in congruence with the structural similarity between both the enzymes. PfLDH and PvLDH have 89% protein

sequence identity and the majority of the changes were reported to be present on the surface of the protein. These changes had not altered the overall conformations of the two proteins significantly (Chaikuad et al. 2005)

Both parasite enzymes (rPfLDH and rPvLDH) utilized $APAD^+$ efficiently as a cofactor and the observed kinetic constants for $APAD^+$ (Table 3.4) were comparable to reported data (Brown et al. 2004). Similarly, weak substrate inhibition was observed in rPfLDH and rPvLDH. These characteristics are exclusively present in apicomplexan parasite LDHs and were attributed to unique structural differences (Brown et al. 2004) between human and malaria parasite LDH, which are described in section 1.9. These properties had confirmed that the purified recombinant enzymes had retained their unique structural and functional properties.

Both gossypol and chloroquine had inhibited rPfLDH and rPvLDH but the dissociation constant of chloroquine was 10^3 fold higher than gossypol suggesting that gossypol is a stronger inhibitor of PLDH than chloroquine. Gossypol was found to be a competitive inhibitor of pLDH, competing with NADH to bind with both the enzymes (Figure 3.11A and B) with dissociation constants close to those reported earlier i.e. 0.7 and 1.4 μM for PfLDH and PvLDH respectively (Brown et al. 2004).

Chloroquine, with respect to NADH, was found to be a competitive inhibitor of rPfLDH and a non-competitive inhibitor of rPvLDH (Figure 3.12A and B). The interaction of chloroquine with rPfLDH, reported here, was in congruence with Read et al. (1999) who has also reported chloroquine to interact with PfLDH at the cofactor binding site with K_i of 1.3 ± 0.2 mM; whereas interaction of chloroquine with rPvLDH leading to non-competitive inhibition is reported here for first time. Variation in the inhibition pattern of chloroquine for rPvLDH in the present study could be attributed to the conformational changes presented by Chaikuad et al. (2005) in the chloroquine binding region (amino acid residues at 53-

62) of the enzyme. This region contains residues which interact with adenyl group of NADH. As chloroquine had differential binding pattern for this region in PfLDH and PvLDH, the variation in this region could be exploited to develop species specific selective inhibitors of pLDH.

In conclusion, soluble expression of the recombinant enzymes (rPfLDH and rPvLDH) was achieved by using cloning strategy designed in the study. Further, both the recombinant enzymes were purified to > 90% purity and their functional identity was confirmed. Both the enzymes, inspite of having C' hexahistidine tag, exhibited kinetic parameters comparable to earlier reports and hence could be used in structure based drug discovery to screen pLDH specific inhibitors.

Chapter 4a

Screening of traditional Indian herbs for selective inhibition of *Plasmodium falciparum* and *Plasmodium vivax* specific L-Lactate dehydrogenase

CHAPTER 4a

Screening of traditional Indian herbs for selective inhibition of *Plasmodium falciparum* and *Plasmodium vivax* specific L-Lactate dehydrogenase

4a.1 INTRODUCTION

Medicinal plants, traditionally used to treat malaria, possess a repertoire of prospective antimalarial drugs and have been exploited globally since antiquity, some excellent examples include quinines and artemisinin (Klayman 1985). The indigenous medical knowledge systems in many countries have found local solutions to treat malarial fever using such medicinal plants (Unnikrishnan et al. 2004). Pharmacological analysis of these traditional plants can lead to novel, affordable and accessible antimalarial drugs (Karunamoorthi and Tsehaye, 2012).

Based on their traditional use in the treatment of recurrent fever or symptomatically diagnosed malaria in India, 8 plants were selected for the present study(Nadkarni & Nadkarani 1954). Following is the brief description of each plant used in the study.

- *Eclipta alba* is a herb and it generally grows in moist places as a weed in warm temperate to tropical areas worldwide (Figure 4a.1A). It is used in the treatment of hepatomegaly, splenomegaly, and skin disorders. Its antimalarial activity was evaluated and confirmed in mice by Bapna et al. (2007).
- *Azadirachta indica* is a fast growing, evergreen tree mainly observed in the Indian subcontinent (Figure 4a.1B). This plant has been used in India for centuries for their antifungal, antibacterial, antimalarial and several other medicinal properties (Biswas et al.

2002). Its leaf extract has been reported to be effective against both sexual and asexual stages of malaria parasites (Udeinya et al. 2008).

- *Andrographis paniculata* is an annual herb widely cultivated in Southern and Southeast Asia (Figure 4a.1C). It is extremely bitter in taste and is reported to have several medicinal properties including antimalarial activity (Mishra et al. 2009).
- *Murraya koenigii* is a small tropical tree and is traditionally used for a range of ailments and has been reported to have antipyretic activity (Patel et al. 2009) (Figure 4a.1D).

A

B

C

D

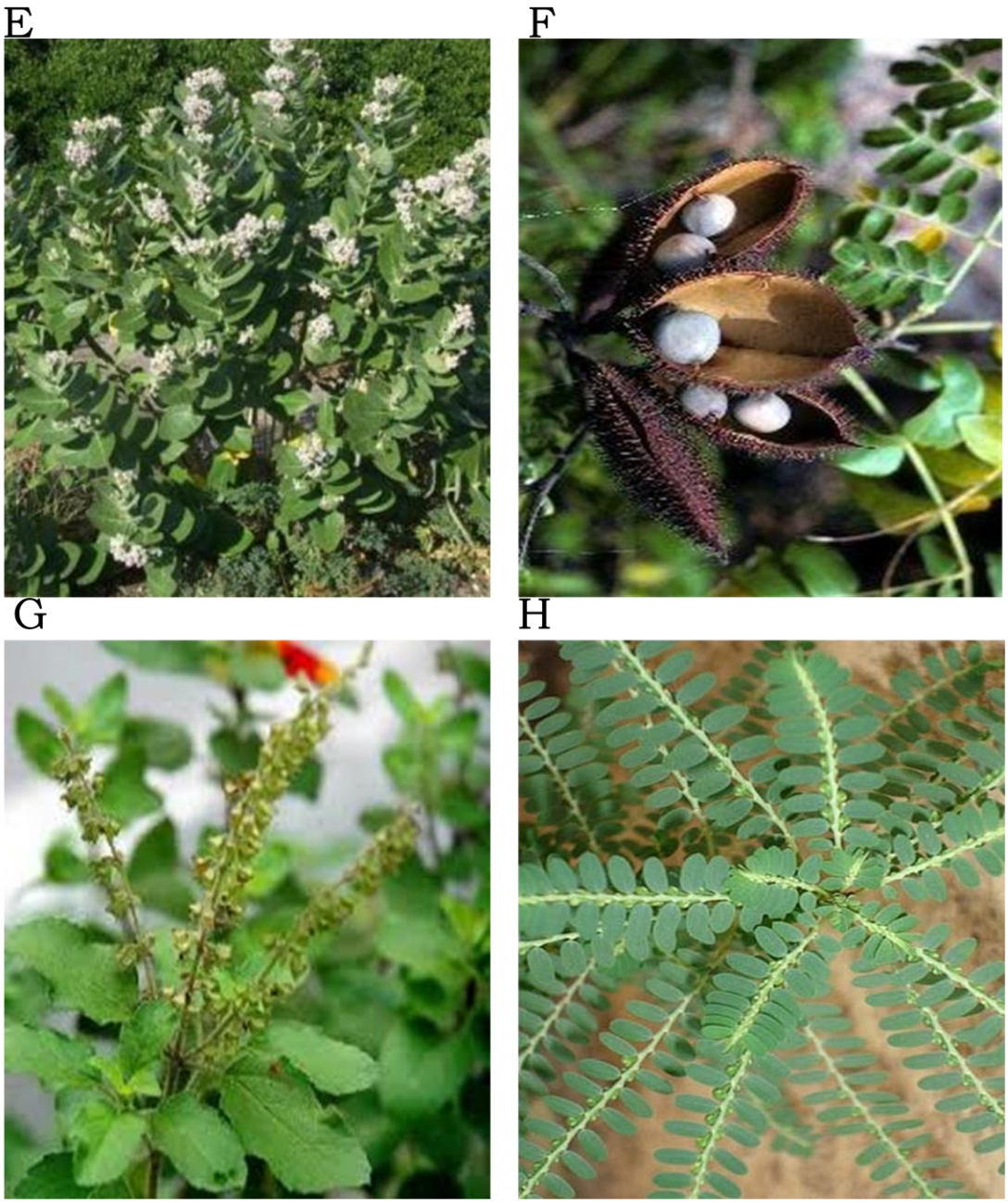


Figure 4a.1: Medicinal plants used in the study. A: *Eclipta alba*, B: *Azadirachta indica*, C: *Andrographis paniculata*, D: *Murayya koenigii*, E: *Calotropis procera*, F: *Caesalpinia crista*, G: *Ocimum sanctum*, H: *Phyllanthus amarus*.

- *Calotropis procera* is a weed commonly found throughout the tropics of Asia and Africa and is used in many traditional systems of medicine (Figure 4a.1E). Evaluation of antimalarial activity of *C.*

procera had confirmed its schizonticidal activity on chloroquine sensitive *P. falciparum* strain (Sharma & Sharma 1999).

- *Caesalpinia crista* is deciduous tree of medium size, commonly found throughout the deciduous forest of India (Figure 4a.1F). Its parts are used to treat colic, convulsions, leprosy, and palsy (Suryawanshi & Patel 2011) and in vivo studies in mice have confirmed the antimalarial activity of *C. crista* seed kernels (Linn et al. 2005).
- *Ocimum sanctum* is an erect, much branched subshrub (Figure 4a.1G). It is native throughout the Eastern World tropics and widespread both as a cultivated plant and an escaped weed. It is an aromatic plant with strongly scented leaves. For many centuries, the plant has been used in Indian and other Southeast Asian medicinal systems for its diverse healing properties and its antiplasmodial activity has been recently confirmed in *in vitro* studies (Venkatesalu et al. 2012).
- *Phyllanthus amarus* is an annual herb having ascending herbaceous branches (Figure 4a.1H). The antimalarial activity of *P. amarus* was confirmed by *in vivo* studies in mice model (Devi et al. 2001).

This ethnopharmacology based selection of plants was exploited in target based drug discovery wherein these plants were screened for inhibition of *P. falciparum* and *P. vivax* specific LDH to identify quality leads towards discovering novel anti-malarial drugs with known mode of action.

4a.2 MATERIALS AND METHODS

4a.2.1 Sample Collection

Materials or parts of the 8 plants used in the present study are listed in table 4a.1. Plant materials were collected in the post monsoon season (October – December) from Vadodara district, Gujarat, India. Leaves were collected in a healthy state (green and without abnormal morphology or colour spots) from *O. sanctum, M. koenigii* and *A. indica*. Similarly, healthy aerial parts (leaves, stem, flowering twigs and fruits) were collected from *P. amarus*, *A. paniculata* and *E. alba*. All collected plant materials were washed with distilled water to remove dirt particles and blotted with paper towels. Plant materials were further desiccated by lyophilization and stored in airtight containers at -20°C. Seeds of *C. crista* were obtained from naturally dried pods. Seed kernels were collected by carefully cracking shells and were stored in airtight containers at -20°C. For collecting latex of *C. procera* incisions were made on the stem at nodes and internodes with a clean blade. Oozing latex was collected in glass vials, desiccated by lyophilization and stored in airtight containers at -20°C.

Prior to biomass collection each plant was identified by botanical experts and voucher specimen of the plants were deposited at Maharaja Sayajirao University of Baroda Herbarium (Biodiversity Collection Index Code: BARO) for further reference (Table 4a.1).

Table 4a.1: Medicinal plants used in the study

Plants	**Common name**	**Local name**	**Herbarium code**
Eclipta alba (L.) Hassk.	False daisy	Maha-Bhringaraj	PKSI 01
Azadirachta indica A. Juss.	Indian lilac	Neem	PKSI 05
Andrographis Paniculata (Burm. F.) Wall. ex Nees	Chirata	Kirayat	PKSI 10
Murraya koenigii (L.) Spreng.	Curry leaf	Currypatta	PKSI 14
Calotropis procera (Aiton) Dryand. ex W. T. Aiton.	Milk weed	Akra	PKSI 18
Caesalpinia crista L.	Bonduc nut	Latakaranj	PKSI 21
Ocimum sanctum L.	Holy basil	Tulsi	PKSI 25
Phyllanthus amarus Schum. and Thonn.	Stone breaker	Bhui Amla	PKSI 30

4a.2.2 Preparation of plant extracts

Petroleum ether (polarity index 0.1), chloroform (polarity index 4.1) and ethanol (polarity index 5.2) extracts were prepared with 0.5 gm dried biomass of each plant in 200 ml of respective solvent. Extraction was carried out in the Soxhlet apparatus for 12 hrs at 50°C for petroleum ether, 55°C for chloroform and 70°C for ethanol. Lyophilized plant material was added in the thimble and kept in the extraction chamber and solvent was taken in a round bottom flask. The assembly of an apparatus was completed by attaching a condenser and the extraction process was carried out by heating the solvent in the round bottom flask. A schematic diagram of the Soxhlet apparatus used in the study is shown in figure 4a.2.

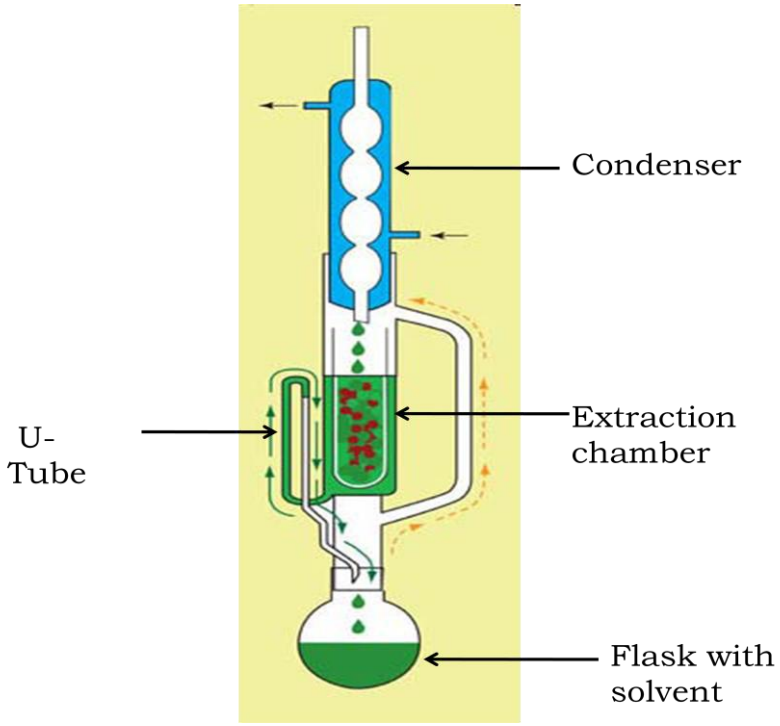


Figure 4a.2: Design of the Soxhlet apparatus used in the study.

Aqueous extracts were prepared by heating 0.5 g of biomass in 50 ml of water at 80°C for 1 hr followed by filtration through Whattman number 3 filter paper. Using rotary vacuum evaporator, extracts were evaporated completely to form dry flakes or powder. These were analyzed gravimetrically and re-dissolved in suitable solvents (petroleum ether and chloroform extracts were re-dissolved in isopropanol, ethanol extracts in ethanol and aqueous extracts in water) to attain 1% w/v solutions. Organic extracts were stored at ambient temperature while aqueous extracts were stored at 4°C.

4a.2.3 Screening of herbal extracts for pLDH inhibitory activity

Recombinant parasite LDH and bovine LDH were diluted to 18 kU/L concentration in phosphate buffered saline (pH 7.4) containing 1 mg/ml BSA and 1 mM PMSF and from this, 30 µl enzyme was used in the assay described in section 2.2. The effect of plant extracts on rPfLDH and rPvLDH was determined by examining variation in the enzyme activity at 50 µg/ml extract concentration in the assay mix and solvents, used for redissolving plant extracts (Section 2.4), were used as control. One way

ANOVA with Holm-Sidak method was performed to determine the extract with significant inhibitory effect, compared to control. Extracts reducing pLDH activity to ≤ 50% were further tested for their specificity by examining their effect on bovine heart and muscle LDH (Sigma Aldrich, India). The effects of the extracts on parasite and mammalian enzymes were compared by plotting correlation graphs and by calculating Pearson's correlation coefficient *r*.

The extracts reducing either or both rPfLDH and rPvLDH activity to ≤ 50% and were further tested for their 50% inhibitory concentration (IC_{50}). Gossypol (Sigma Aldrich, India) was used as a positive control in the study. The enzyme assay was carried out with different inhibitor concentrations and IC_{50} values were determined as described by Xu et al. (2007) using equation (v).

$$IC_{50} = 10^{exp}\,[B + (\{50 - < 50\%\ inhibition\} / \{> 50\%\ inhibition - < 50\%\ inhibition\}) \text{ X } C] \quad \text{(v)}$$

Where $C = A - B$; A = log (*Concentration of the extract at which > 50% inhibition was observed*) and B = log (*Concentration of the extract at which < 50% inhibition was observed*)

Aqueous extract of *P. amarus* was tested for *in vitro* anti-malarial activity at National Institute of Malaria Research, New Delhi, using the method described by Bagavan *et al* (2011).

All the enzyme and *P. falciparum* culture inhibition assays were performed in triplicates and statistical calculations were carried out using SigmaStat version 3.5 software (Systat Software Inc., USA).

4a.3 RESULTS

4a.3.1 Preparation of plant extracts

Biomass yield obtained in the extraction of 8 plants using 4 different solvents was summarized in Table 4a.2. More yields were obtained in the aqueous extracts compared to organic solvent extracts.

Table 4a.2: Yield of plant extracts in different solvents

Plants	% Yield (w/w) in extract of solvent			
	Petroleum ether	Chloroform	Ethanol	Water
E. alba	4.2	6.1	6.8	9.7
A. indica	2.4	5.8	6.2	8.5
A. paniculata	4.3	5.5	6.1	8.1
M. koenigii	3.1	6.5	6.7	8.6
C. procera	7.6	14.5	16.1	18.3
C. crista	9.3	12.3	14.2	20.6
O. sanctum	3.8	7.4	8.3	9.8
P. amarus	4.6	6.9	7.6	10.2

4a.3.2 Inhibition studies of rPfLDH and rPvLDH

pLDH inhibitory activity of the plant extracts was tested by incorporating extracts at 50 µg/ml concentration in the reaction mix. Petroleum ether extract of *C. crista* was precipitated in the aqueous reaction system at this concentration and hence was not tested further. Ethanol extract of *E. alba, A. paniculata* and *P. amarus* reduced rPfLDH activity to < 50%, similarly, chloroform extracts of *A. paniculata* reduced rPvLDH activity to < 50% whereas petroleum ether extract of *C. procera,* chloroform extracts of *M. koenigii* and aqueous extracts of *P. amarus* reduced activity of both pLDH to ≤ 50%. Effects of these 7 extracts were statistically significant at 0.001 levels (Table 4a.3). Though few other extracts also changed pLDH

activity to statistically significant level, the activity was not reduced to ≤ 50% by any of these extracts, suggesting their IC_{50} values would be more than 50 µg/ml and hence were not considered in the further studies.

Table 4a.3: Effect of plant extracts on rPfLDH and rPvLDH activity

Plants	rPfLDH Activity [mean U/l[a] ± SD] (Activity, % of solvent control)				rPvLDH Activity [mean U/l[a] ± SD] (Activity, % of solvent control)			
	Petroleum ether extract	Chloroform extract	Ethanol extract	Aqueous extract	Petroleum ether extract	Chloroform extract	Ethanol extract	Aqueous extract
Solvent Control	134.4 ± 2.6 (100.0)	134.4 ± 4.4 (100.0)	149.1 ± 7.3 (100.0)	152.4 ± 3.3 (100.0)	133.3 ± 10.2 (100.0)	133.3 ± 10.2 (100.0)	143.0 ± 6.0 (100.0)	149.9 ± 4.3 (100.0)
E. alba	115.4 ± 2.6 (85.9)	87.5 ± 7.4[b] (65.1)	29.8 ± 1.1[b] (20.0)	116.2 ± 5.0[b] (76.3)	145.8 ± 1.9 (≥100)	85.0 ± 18.5[b] (63.8)	134.6 ± 0.8 (94.1)	130.4 ± 3.9 (87.1)
A. indica	119.7 ± 2.2 (89.1)	92.0 ± 8.3[b] (68.4)	118.4 ± 1.9[b] (79.4)	161.0 ± 9.6 (≥100)	149.8 ± 0.6 (≥100)	153.7 ± 4.5 (≥100)	139.5 ± 2.8 (97.6)	148.7 ± 3.3 (99.3)
A. paniculata	124.8 ± 4.7 (92.9)	85.8 ± 6.0[b] (63.9)	62.3 ± 2.5[b] (41.8)	149.3 ± 8.8 (98.0)	132.3 ± 6.4 (99.3)	60.2 ± 29.4[b] (45.2)	134.1 ± 2.8 (93.8)	144.9 ± 2.0 (96.8)
M. koenigii	119.7 ± 1.0 (89.1)	41.5 ± 3.6[b] (30.8)	146.4 ± 1.1 (98.2)	150.3 ± 11.3 (98.7)	121.5 ± 2.3 (91.1)	5.2 ± 10.0[b] (3.9)	131.0 ± 3.5[b] (91.6)	137.9 ± 12.7 (92.0)
C. procera	62.6 ± 27.2[b] (46.6)	135.9 ± 3.5 (≥100)	150.3 ± 0.8 (≥100)	134.0 ± 8.9 (88.0)	66.8 ± 8.8[b] (50.1)	108.5 ± 13.7 (81.4)	162.8 ± 2.4[b] (≥100)	148.7 ± 1.3 (99.2)
C. crista	N/D[c]	114.3 ± 2.3[b] (85.0)	150.0 ± 0.7 (≥100)	158.9 ± 4.1 (≥100)	N/D[c]	168.3 ± 3.2 (≥100)	149.0 ± 1.5 (≥100)	164.0 ± 1.1 (≥100)
O. sanctum	129.9 ± 3.1 (96.6)	114.4 ± 12.2[b] (85.1)	119.4 ± 0.6[b] (80.1)	103.8 ± 10.4[b] (68.1)	143.4 ± 3.9 (≥100)	139.7 ± 7.6 (≥100)	137.3 ± 7.3 (96.0)	146.6 ± 3.0 (97.9)
P. amarus	123.7 ± 0.5 (92.0)	138.8 ± 1.7 (≥100)	13.6 ± 3.5[b] (9.1)	3.1 ± 5.4[b] (2.1)	128.0 ± 2.7 (96.0)	141.9 ± 5.2 (≥100)	135.2 ± 3.9 (94.5)	45.9 ± 19.4[b] (30.6)

[a] Enzyme unit, U = 1 µmol of $NADH^+$ utilized per minute.
[b] Significant variation compared to control using one way ANOVA by Holm Sidak method ($P \leq 0.001$).
[c] Not determined.

The extracts that effectively inhibited parasite LDH, were tested for their effect on Bovine Heart and Muscle LDH at the same concentration. Both the enzymes retained > 95% activity in the presence of these extracts, compared to the control (Table 4a.4).

Table 4a.4: Effect of plant extracts on Bovine Heart and Muscle LDH activity

Plant extracts	**Enzyme activity [mean U/L[a] ± SD] (Activity, % of solvent control)**	
	Bovine Heart LDH	Bovine Muscle LDH
Solvent control	149.6 ± 2.6 (100)	130.4 ± 1.8 (100)
Petroleum ether extract of *C. procera*	150.8 ± 0.1 (≥ 100)	130.2 ± 2.2 (100)
Chloroform extract of *M. koenigii*	143.9 ± 2.1 (96.2)	125.2 ± 2.7 (96)
Chloroform extract of *A. paniculata*	148.5 ± 1.3 (99.3)	127.2 ± 1.5 (97.6)
Solvent control	216.2 ± 3.1 (100)	201.6 ± 3.2 (100)
Ethanol extract of *A. paniculata*	227 ± 2.9[b] (≥100)	200.2 ± 0.3 (99.3)
Ethanol extract of *E. alba*	227.8 ± 3.8[b] (≥100)	211.7 ± 2.9[b] (≥100)
Ethanol extract of *P. amarus*	227.5 ± 4.8[b] (≥100)	200.6 ± 2.5 (98.4)
Gossypol[c]	218 ± 0.5 (≥100)	198.3 ± 2.5 (98.4)
Solvent control	225.8 ± 4.8 (100)	210.7 ± 12.2 (100)
Aqueous extract of *P. amarus*	216.4 ± 1.3 (95.8)	203.7 ± 4.7 (96.7)

[a] Enzyme unit, U = 1 µmol of NADH utilized per minute.

[b] Significant variation compared to control using one way ANOVA by Holm Sidak method ($P \leq 0.001$)

[c] Gossypol (Positive control) is strong and selective inhibitor of parasite LDH

The correlation was not observed between the effect of the 7 plant extracts on the rPfLDH and rPvLDH activity (Pearson's coefficient, r = 0.2, P = 0.6), in contrast the effect of extracts on Bovine Heart and Muscle LDH activity were strongly correlated (r = 0.8, $P \leq 0.05$).

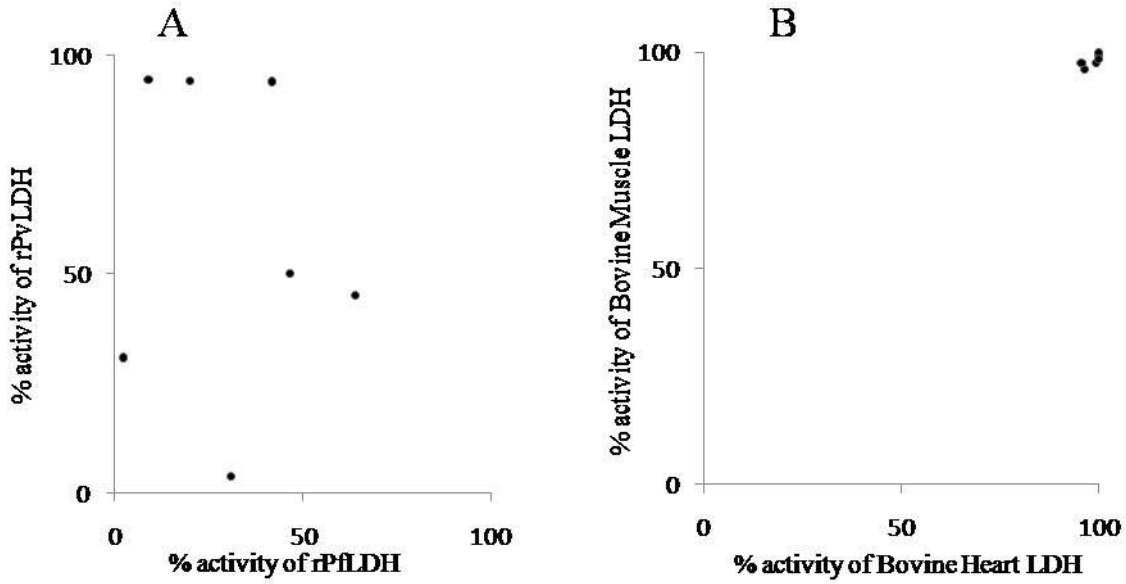


Figure 4a.3: Correlation plots of the percentage enzyme activity with respect to the effect of the 7 plant extracts. A) Percentage activity of PfLDH plotted against percentage activity of PvLDH in the presence of each extract. B) Percentage activity of Bovine Heart LDH plotted against percentage activity of Bovine Muscle LDH in the presence of each extract.

IC_{50} of the selected 7 plant extracts were < 50 µg/ml (Table 4a.5) and the *P. amarus* aqueous extract exerted strongest effect on rPfLDH (IC_{50} = 11.2 µg/ml ± 0.4) and the *M. koenigii* chloroform extract had strongest effect on rPvLDH (IC_{50} = 6.0 µg/ml ± 0.6). Gossypol effectively inhibited both rPfLDH and rPvLDH (Table 4a.5) but was ineffective on mammalian LDH (Table 4a.4). *In vitro* test of the *P. amarus* aqueous extract confirmed its parasiticidal activity on *P. falciparum* NE (chloroquine sensitive) and *P. falciparum* MRC-2 (chloroquine resistant) strains with IC_{50} = 7.1 µg/ml ± 0.5 and 6.9 µg/ml ± 0.7 respectively for the two strains.

Table 4a.5: IC_{50} values of the selected 7 plant extracts

Plant extracts	IC_{50} [mean µg/ml ± SD]	
	rPfLDH	rPvLDH
Petroleum ether extract of *C. procera*	42.8 ± 5.7	48.4 ± 1.9
Chloroform extract of *M. koenigii*	21.3 ± 1.9	6.0 ± 0.6
Chloroform extract of *A. paniculata*	N/D[b]	39.3 ± 0.8
Ethanol extract of *A. paniculata*	45.7 ± 1.2	N/D[b]
Ethanol extract of *E. alba*	35.0 ± 0.4	N/D[b]
Ethanol extract of *P. amarus*	14.6 ± 2.5	N/D[b]
Aqueous extract of *P. amarus*	11.2 ± 0.4	34.9 ± 4.7
Gossypol[a]	2.6 ± 0.8 (5.1 µM)	10.4 ± 0.2 (20.1 µM)

[a] Gossypol (Positive control) is strong and selective inhibitor of parasite LDH.

[b] Not determined.

4a.4 DISCUSSION

In codified (e.g. *Ayurveda, Siddha* etc.) and noncodified (Verbal folk remedies) traditional medicinal systems of India, plants are often used to treat recurrent fever or symptomatically diagnosed malaria (Unnikrishnan et al. 2004). Eight such plants which are well documented for their antiplasmodial or antipyretic activity (Table 4a.1) and are readily available at *Ayruvedic* shops in India were tested in the present study. Four solvents of different polarity were used to extract maximum compounds from the 8 plants and hence all 32 extracts were expected to contain different concoction of compounds on the basis of their polarity. Seven of these extracts significantly reduced pLDH activity without having any inhibitory effect on bovine heart and bovine muscle LDH (Table 4a.4). These results were analogous to the effect of gossypol and have therefore confirmed the specificity of the extracts towards *Plasmodium* LDH which can be accredited to structural variations between *Plasmodium* and mammalian LDH (Dunn et al. 1996). Unlike mammalian LDH, discrete effects of the extracts on rPfLDH and rPvLDH, observed in the correlation plots (Figure 4a.3), suggested variable interactions of the compounds within the extracts with both the parasite enzymes. Actual molecular interactions of these inhibitors with pLDH can be deciphered using X-ray crystallography

Amongst the tested extracts, *P. amarus* aqueous extract and *M. koenigii* chloroform extract had inhibitory effects close to gossypol, a known selective inhibitor of pLDH (Sessions et al. 1997) (Table 4a.3). Rationally, effective compound/s (responsible for pLDH inhibition) are merely a fraction of the total composition of the crude extract. Hence, if crude extract can inhibit the enzyme comparable to the pure inhibitor (gossypol), its component compound/s responsible for enzyme inhibition, in pure form; will have more effective inhibitory concentrations (IC_{50}).

The aqueous extracts of *P. amarus* had remarkable parasiticidal activity on chloroquine sensitive and resistant strains of *P. falciparum*. These results are analogous to reported PfLDH inhibitors which in turn kill parasite (Royer et al. 1986; Vivas et al. 2005; Choi et al. 2007). Interestingly, *P. amarus* extract had stronger parasiticidal activity (IC_{50} 7.1 µg/ml) than pfLDH inhibitory activity (IC_{50} 11.2 µg/ml) (Table 4a.3 and Table 4a.5). This is possibly caused by the presence of compounds with different mode\s of action on parasite, along with PfLDH inhibition, which act synergistically and kill parasites more efficiently. *P. amarus* is used in several ethnomedical systems to treat malaria and other diseases (Bagalkotkar et al. 2006; Patel et al. 2011). Antimalarial activity of *P. amarus* extract has also been reported earlier *in vivo* in a mouse model wherein the parasitemia was reduced by 73% at 200 mg/kg of oral dose without any toxicological effects (Tona et al. 2001). The parasiticidal activity of the *P. amarus* aqueous extract was comparable to the effect of individual compounds purified from *P. amarus* (IC_{50} values ranging from 1.4 to 32) as reported by Subeki et al. (2005). Our results, together with these reports, strongly indicate the presence of potential antimalarial drug/s in *P. amarus* with PfLDH inhibition as mode of action. Further, detailed investigation can lead to the inclusion of the effective drug in our present inadequate arsenal of antimalarial compounds.

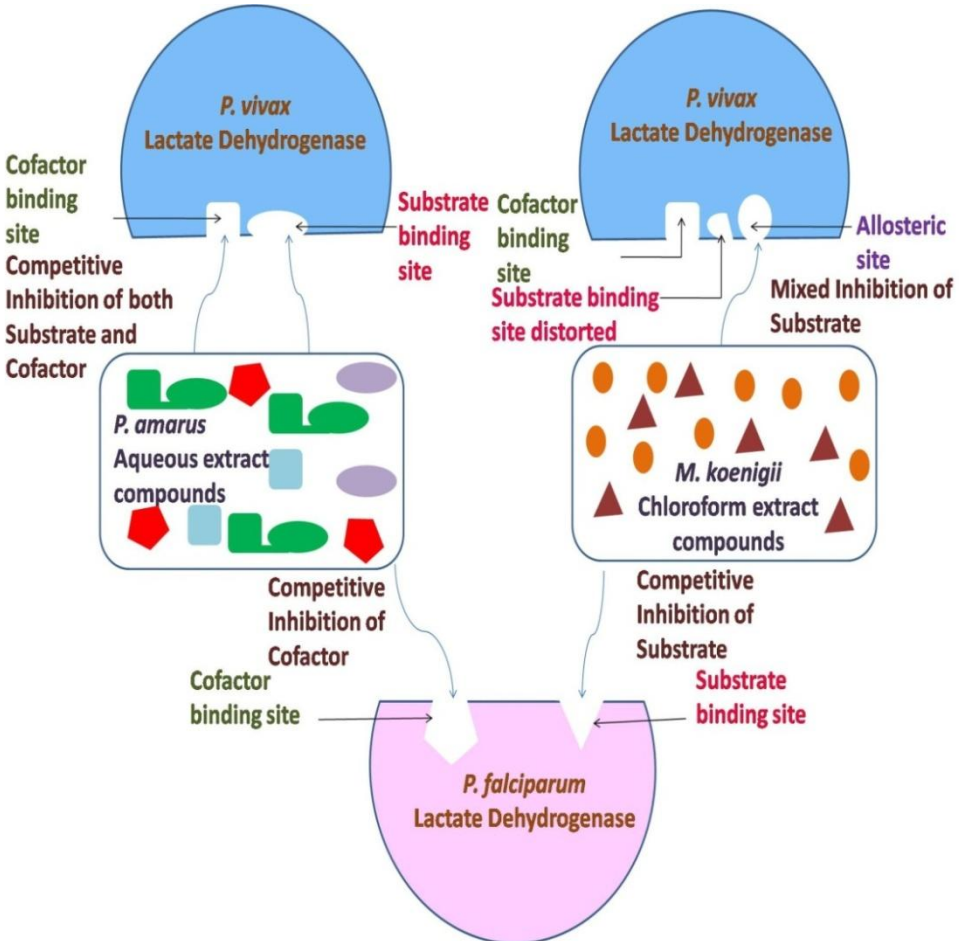
M. koenigii chloroform extract had an inhibitory effect on rPvLDH, more effective than gossypol (Table 4a.3). These results have suggested the presence of extremely efficient and selective rPvLDH inhibitor in the extract. Though *in vitro* activity of *M. koenigii* extract on *P. vivax* culture was not determined, owing to the importance of pLDH for the survival of the parasite (Vivas et al. 2005), strong rPvLDH inhibition can presumably culminate in parasiticidal activity. *M. koenigii* holds medicinal properties such as antipyretic (Patel et al. 2009) antimicrobial (Rahman & Gray 2005) and nephroprotective activity (Yankuzo et al.

2011). Besides it is a common ingredient of Southeast Asian and Indian cuisine, hence is safe for human applications.

In conclusion, *P. amarus* and *M. koenigii* extracts were found to have selective and significant inhibitory effect on *P. falciparum* and *P. vivax* LDH respectively. Using a combined approach of ethnopharmacology and reverse pharmacology, these extracts were obtained as the hits by screening of as low as eight plants. Further, identification and analysis of the effective molecules from these extracts may lead to a new therapeutic molecule with known mode of action.

Chapter 4b

Molecular characterization of the enzyme inhibition exhibited by the compounds from *Phyllanthus amarus* and *Murraya koenigii* extracts



CHAPTER 4b

Molecular characterization of the enzyme inhibition exhibited by the compounds from *Phyllanthus amarus* and *Murraya koenigii* extracts

4b.1 INTRODUCTION

Plasmodium specific L-Lactate dehydrogenase is a bi-substrate enzyme and like other LDHs, pLDH follows an ordered bi-bi kinetic reaction mechanism where cofactor (NADH) binds first to the enzyme followed by substrate (Pyruvate) (Shoemark et al. 2007). Molecules with the binding affinity for either cofactor or substrate binding site on the enzyme can compete with them and consequently may inhibit the enzyme reaction. pLDH inhibitory compounds can be classified on the basis of their different modes of binding to the active site of the enzyme. These inhibitors either bind to the substrate binding site, the cofactor binding site or bridging site between the cofactor and the substrate (Choi, Beeler, et al. 2007). Gossypol, a natural product and its derivatives, in pLDH, bind to the cofactor binding site and the site bridging the nicotinamide chain of the cofactor and the substrate (pyruvate) (Conners et al. 2005) while azole-based inhibitors interact at the substrate binding site (Cameron et al. 2004).

Aqueous extract of *Phyllanthus amarus* and chloroform extract of *Murayya koenigii* had selectively inhibited rPfLDH and rPvLDH respectively, implying that these extracts contain one or more compounds which specifically interact with the parasite enzymes (pLDH). Enzyme inhibition kinetics studies of these extracts can shed light on the binding affinities of the constituent compounds in the extracts, similar to the previous studies of gossypol and their derivatives

which had shown them to be competitive with NADH confirming their affinity for the cofactor binding site (Brown et al. 2004).

Finding a molecule that can potentially bind to a target protein is essential in the drug discovery process and using only experimentation based techniques, makes it an expensive and time-consuming task. This is one of the reasons why computational methods were introduced. Development in the field of computational methodologies and considerable increase in the number of elucidated structures of proteins have made structure based virtual screening techniques as a crucial component of many drug discovery programmes, from hit identification to lead optimization (Kitchen et al. 2004). One of these methodologies is docking of small molecules to the target protein at key binding sites like catalytic centre (Gohlke & Klebe 2002). The algorithms, used in docking predictions, usually calculate molecular interactions like steric, electrostatic and hydrogen bonds. Desolvation energy, rotational entropy and translational entropy are also considered for docking predictions in the algorithm (Kitchen et al. 2004). Docking studies involve prediction of the structure of enzyme inhibitor complex [EI] under equilibrium conditions and prediction of the orientation (posing) of the inhibitor within the binding site of the enzyme. The binding affinity of the inhibitor, which is responsible for the biological activity, is also predicted in the docking studies by calculating the free energy of binding (ΔG).

In the present study, enzyme inhibition kinetic studies were carried out to determine the type of inhibition obtained by the aqueous extracts of *P. amarus* and chloroform extract of *M. koenigii* and based on the type of inhibition, a potential site of interaction of the inhibitor with pLDH was predicted. Further, using molecular docking studies, the molecule predicted to have the best interaction with LDH at the NADH binding site was elucidated.

4b.2 MATERIALS AND METHODS

4b.2.1 Inhibition kinetic studies using plant extracts

The dissociation constant (Ki) of the aqueous extract of *P. amarus* and chloroform extracts of *M. koenigii* for rPfLDH and rPvLDH were determined by linear regression analysis of double reciprocal plots as described in section 3.2.4. The types of inhibition were determined based on variations in the kinetic constants of the enzyme (PfLDH and PvLDH) for the substrate (pyruvate) and the cofactor (NADH) compared to control, as described in table 4b.1 On the basis of the type of inhibition obtained with respect to (w.r.t.) the substrate and cofactor, the site of enzyme inhibitor interactions were determined.

Table 4b.1: Type of inhibition represented by variation in the kinetic constants

Variation in $_{app}K_m$	**Variation V_{max}**	**Inhibition type**
Increase	No	Competitive
No	Decrease	Noncompetitive
Decrease	Decrease	Uncompetitive
Increase	Decrease	Linear mixed

4b.2.2 Compounds of *P. amarus* and *M. koenigii* used in the present study

Compounds reported to be present and distributed throughout the aerial parts of *P. amarus* (Ross 2005) were included in the study as aqueous extracts of aerial parts of *P. amarus* had exhibited strong and selective pLDH inhibitory activity. While chloroform extract of *M. koenigii* leaves exhibited pLDH inhibitory effect and hence compounds present in essential oils extracted from *M. koenigii* leaves (Chowdhury et al. 2008) were discreetly selected for the docking studies (Table 4b.2).

Table 4b.2: Phytocompounds used in the study

Plant	Phytochemical group	Phytocompound	Pubchem compound ID
P. amarus	Tannins	Amariin	5482103
	Alkaloids	Norsecurinine	160648
		Phyllochrysine	267769
	Flavonoids	Kaempferol	5280863
	Proanthocyanidins	Geranin A	44566352
	Phenolic compounds	Beta-glucogallin	124375
		Gallic acid	370
		Astragalin	5282102
		Corilagin	73568
		Ellagic acid	5281855
	Terpenoids	Limonene	440917
		Alpha-phellandrene	7460
M. koenigii	Terpenoids	Beta-myrcene	31253
		Beta-elemene	6918391
		Terpinyl acetate	111037
		Alpha-caryophyllene	5281520
		Camphene	6616
		Thujene	637518
		Cubenol	519857
		Spathulenol	522266
		Carene	26049
		Germacrene D	5373727

4b.2.3 Phytochemical analysis of *P. amarus* and *M. koenigii* extracts

Aqueous extract of *P. amarus* and chloroform extract of *M. koenigii* were tested for the presence of phytochemical constituents as described in table 4b.3. Water and isopropanol (Solvents used for storage of extract, as described in section 4a.2.2) were used as controls in the tests.

Table 4b.3: Phytochemical tests

Plant extract	**Test for the phytocompounds**	**Reference**
Aqueous extract of aerial parts of *P. amarus*	Tannins	(Edeoga et al. 2005)
	Alkaloids (Mayer's test, Dragendorff's test, Wagner's test)	(Mahajan & Badgujar 2008)
	Flavonoids	(Edeoga et al. 2005)
	Proanthrocyanidins	(Mahajan & Badgujar 2008)
	Phenolic compounds	(Mahajan & Badgujar 2008)
	Terpenoids (Salkowski's test)	(Edeoga et al. 2005)
Chloroform extracts of leaves of *M. koenigii*	Terpenoids (Salkowski's test)	(Edeoga et al. 2005)

4b.2.4 Preparation of the protein and the ligand for the docking studies.

Molecular docking studies were performed in collaboration with The Department of Pharmaceutical Chemistry, Bombay College of Pharmacy, Mumbai. The X-ray crystal structures (PDB codes:1T2C, 2A92) of parasite LDH bound with NADH were obtained from the Protein Data Bank (http://www.rcsb.org/pdb). The structures were prepared for the docking studies with the Protein Preparation Wizard in the Schrödinger Suite of programs (Schrödinger 2011 Suite). Water molecules were removed from the crystal, hydrogen atoms were added, atom types and partial charges were assigned based on the OPLS2005 forcefield. The formal charges for amino acids, both acidic and basic were set corresponding to the physiological pH 7.4. The N' and the C' termini were capped with the acetyl (ACE) and the N' methyl amino (NMA) groups, respectively. Subsequently, the system was relaxed using energy minimization until an energy gradient of 0.01kcal/mol/Å was reached with the OPLS2005 forcefield.

Structures of the compounds present in the plant extracts (Table 4b.1) were obtained from The PubChem Project (http://pubchem.ncbi.nlm.nih.gov/) and were prepared using the LigPrep v2.4 in Maestro (Schrödinger 2011 Suite). The atom types and partial charges were assigned based on the OPLS2005 forcefield, corresponding to the physiological pH 7.4. A set of diverse conformations, and tautomeric states were generated for the ligands using the LigPrep in Schrödinger Suite.

4b.2.5 Docking of the compounds from the plant extracts in rPfLDH and rPvLDH.

In the initial phase of docking, a grid was generated in the active site of the selected X-ray crystal structures of both pLDH enzymes with the bound NADH defining the active site and the grid center. The grid box was defined to 20 Å around grid center, to cover both substrate and

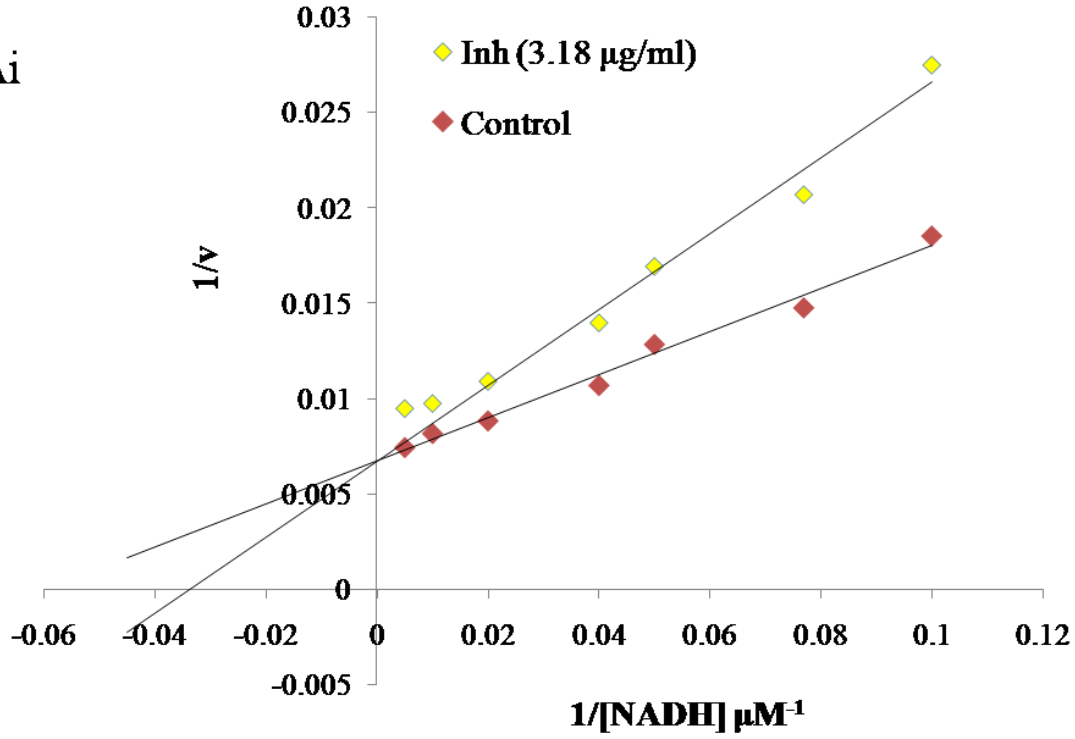
cofactor binding sites and to allow ample space for the interaction of ligand in the binding pocket during the execution of the search and score algorithm. The Van der Waals radius was scaled to 1 Å to soften the potential over the non-polar areas of the enzyme that lie within the grid extents and partial atomic charges were set to the value of 0.25. The receptor atoms beyond the extents of the grid were unscaled. To maximize the probability of forming hydrogen bonds between the residues in the enzyme active site and the ligand, the side chain hydroxyl hydrogen atoms of the amino acids serine, threonine and tyrosine were allowed to rotate. The docking settings were validated based on the efficiency of NADH to bind at its natural binding site (cofactor binding site). On optimization of the protocol, the compounds were docked to find the best poses. In addition to NADH, gossypol was also docked as a positive control as it is a selective inhibitor of parasite LDH with known binding site in the enzyme (Conners et al. 2005). Interaction energies *viz.* electrostatic, Van der Waals and hydrogen bond were computed between every amino acid in the active site and the ligand and on the basis of these, efficiency of the interactions were scored using the GlideScore SP.

4b.3 RESULTS

4b.3.1 Inhibition kinetic studies of plant extracts with rPfLDH and rPvLDH

Comparative double reciprocal plots of the enzyme activity in presence and absence of plant extracts at varying concentrations of substrate (pyruvate) and cofactor (NADH) are presented in figure 4b.1.

Ai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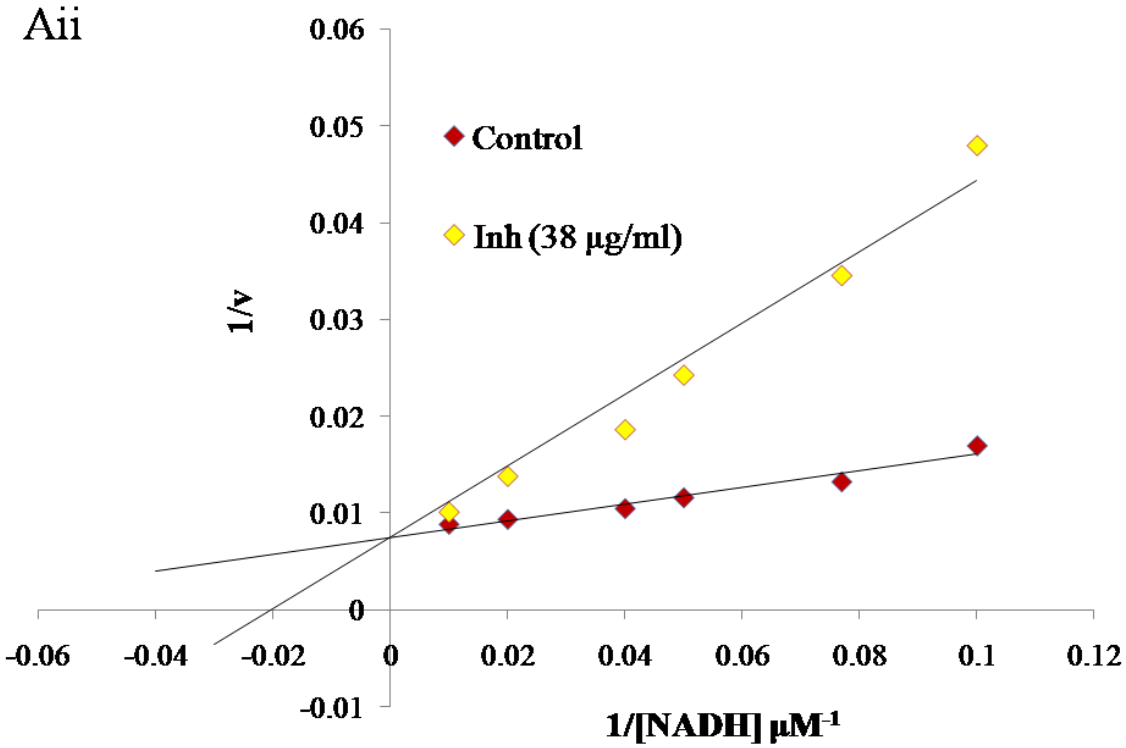
Control
1/v
1/[NADH] µM-1
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.06
-0.04
-0.02
0
0.02
0.04
0.06
0.08
0.1
0.12

Aii


Control
Inh (38 µg/ml)
1/v
1/[NADH] µM-1
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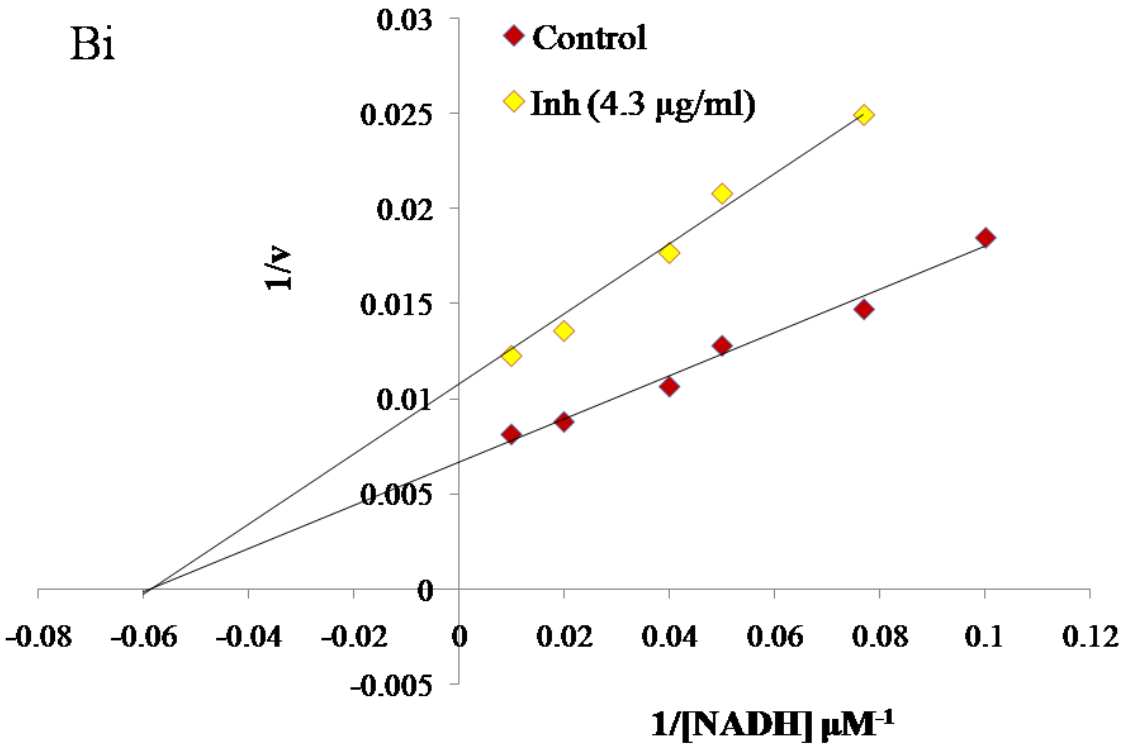
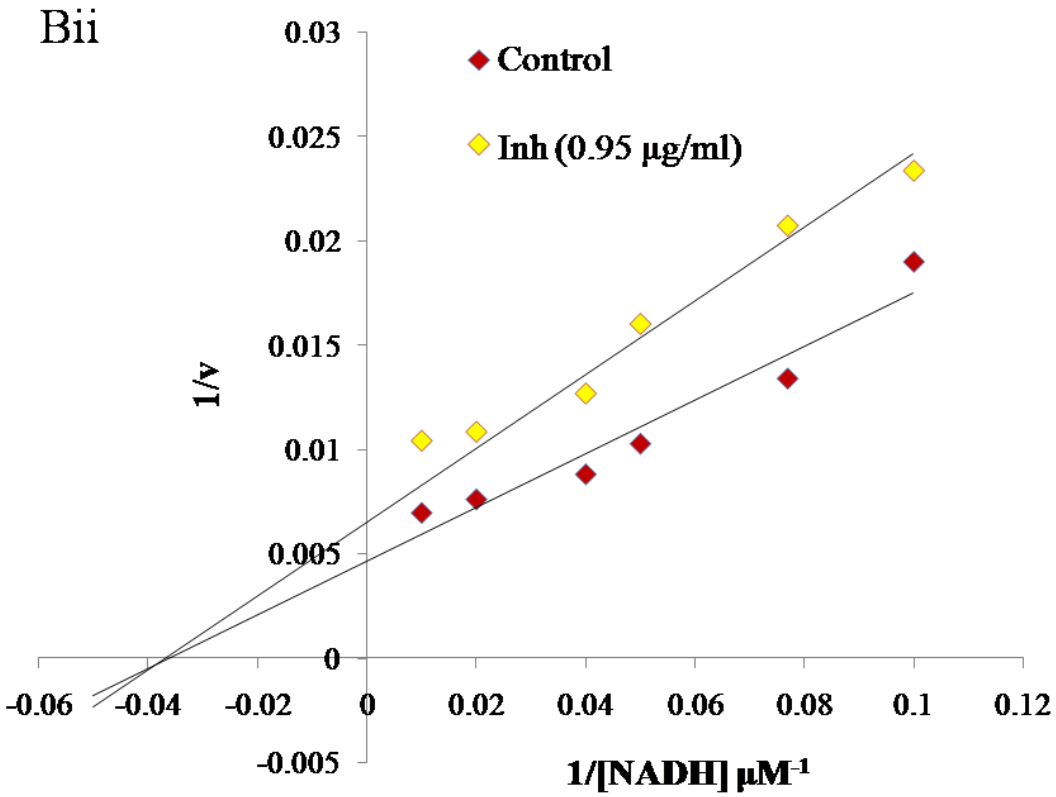
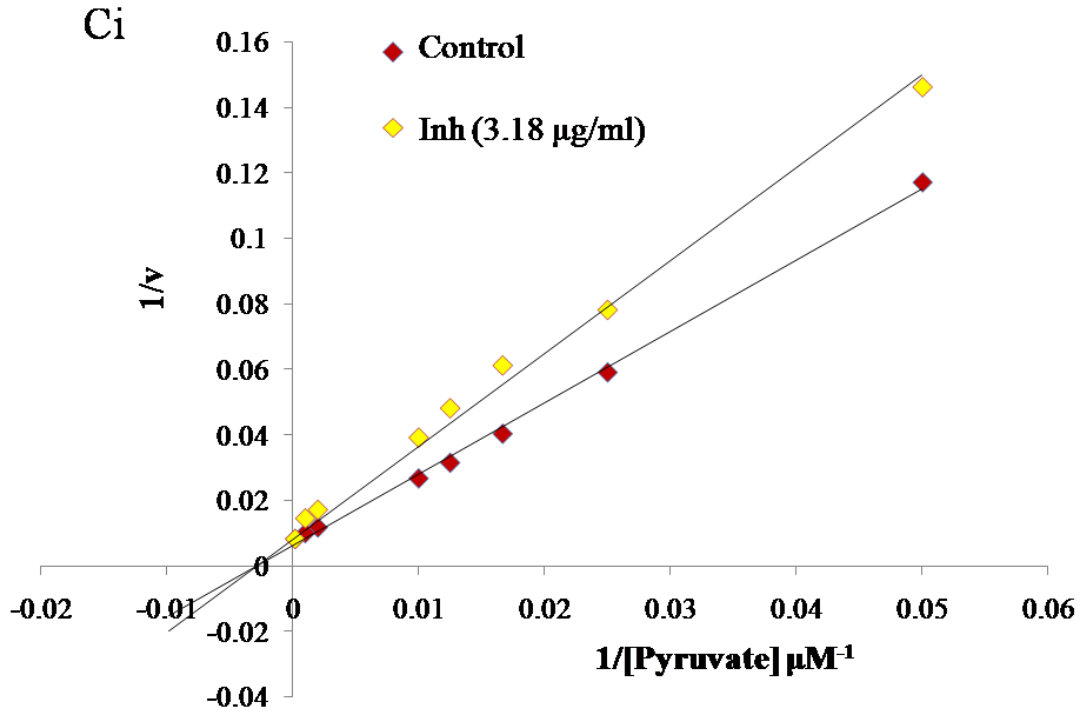
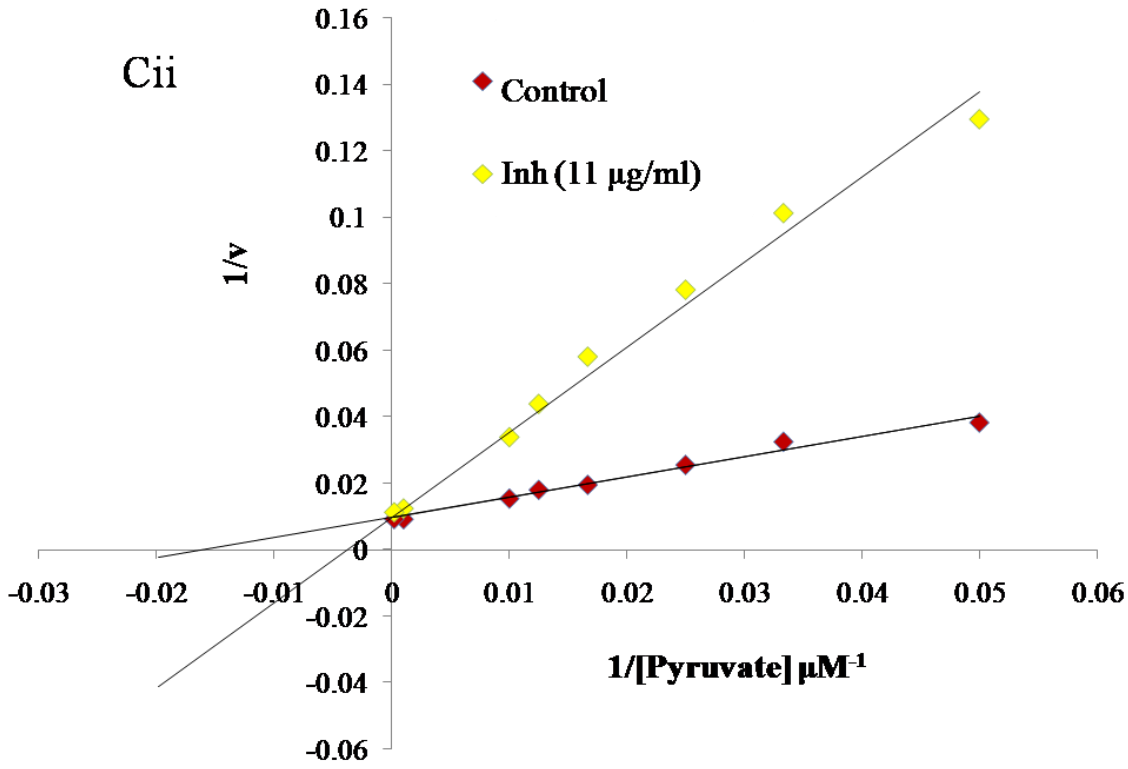
0.05
0.04
0.03
0.02
0.01
0
-0.01
-0.06
-0.04
-0.02
0
0.02
0.04
0.06
0.08
0.1
0.12


Bi
Control
Inh (4.3 µg/ml)
1/v
1/[NADH] µM-1
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.08
-0.06
-0.04
-0.02
0.02
0.04
0.06
0.08
0.1
0.12


Bii
Control
Inh (0.95 µg/ml)
1/v
1/[NADH] µM-1
0.03
0.025
0.02
0.015
0.01
0.005
0
-0.005
-0.06
-0.04
-0.02
0.02
0.04
0.06
0.08
0.1
0.12


Ci
Control
Inh (3.18 µg/ml)
1/v
1/[Pyruvate] µM-1
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.02
-0.01
0
0.01
0.02
0.03
0.04
0.05
0.06


Cii
Control
Inh (11 µg/ml)
1/v
1/[Pyruvate] µM-1
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.03
-0.02
-0.01
0
0.01
0.02
0.03
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0.05
0.06

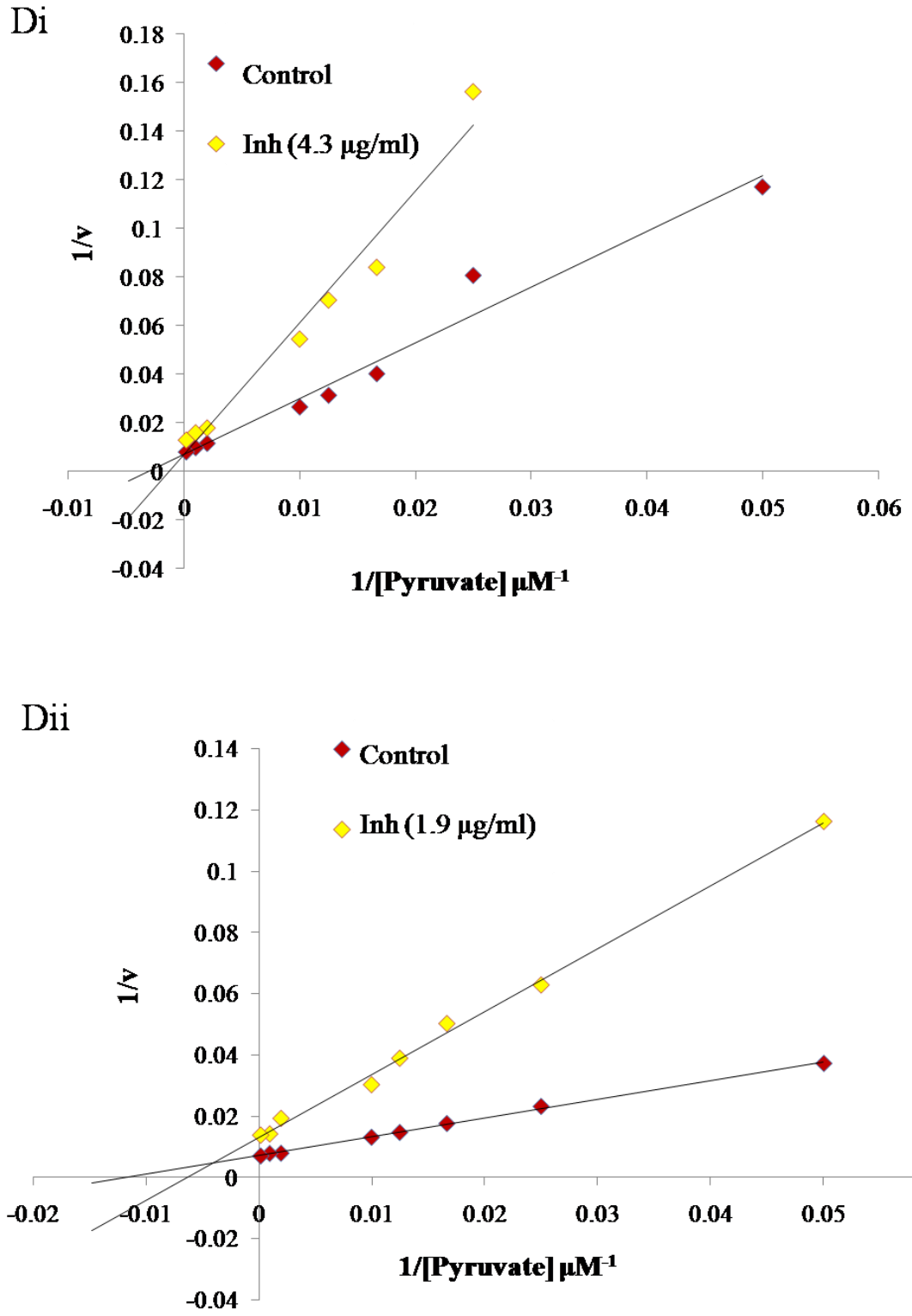


Figure 4b.1: Double reciprocal plots of enzyme activity in the presence of plant extracts. A) Inhibition by *P. amarus* aqueous extract w.r.t NADH as a cofactor i) PfLDH ii) PvLDH B) Inhibition by *M. koenigii* chloroform extract w.r.t. NADH as a cofactor i) PfLDH ii) PvLDH C) Inhibition by *P. amarus* aqueous extract w.r.t. pyruvate as a substrate i) PfLDH ii)

PvLDH D) Inhibition by *M. koenigii* chloroform extract w.r.t. pyruvate as a substrate i) PfLDH ii) PvLDH.

P. amarus aqueous extracts showed competitive inhibition w.r.t NADH in both PfLDH and PvLDH and also w.r.t. pyruvate in PvLDH, whereas it was noncompetitive with pyruvate in PfLDH (Fig 4b.1Ai, ii, Ci, ii). *M. koenigii* chloroform extract showed noncompetitive inhibition with NADH in both PfLDH and PvLDH whereas it showed competitive inhibition with pyruvate in PfLDH and linear mixed with pyruvate in PvLDH (Fig 4b.1 Bi, ii, Di, ii).

Dissociation constant (K_i) of both the extracts and the type of inhibition observed with each, cofactor and substrate were summarised in table 4b.4

Table 4b.4: Enzyme inhibition kinetics of *P. amarus* aqueous extracts and *M. koenigii* chloroform extract

		NADH (Cofactor)		Pyruvate (Substrate)	
Plant Extracts	**Enzymes**	**Type of Inhibition**	**Inhibition constant K_i (µg/ml)**	**Type of Inhibition**	**Inhibition constant K_i (µg/ml)**
P. amarus aqueous extract	PfLDH	Competitive	4.1± 0.7	Noncompetitive	9.6±1.8
	PvLDH	Competitive	11.4±0.2	Competitive	20.8±0.2
M. koenigii chloroform extract	PfLDH	Noncompetitive	7.8±0.7	Competitive	6.6±0.3
	PvLDH	Noncompetitive	2.3±0.4	Linear Mixed	0.8±0.2

4b.3.2 Phytochemical group test

Qualitative tests were performed to determine phytochemicals present in *P. amarus* aqueous extract and *M. koenigii* chloroform extract. These included tests for tannins, alkaloids, flavonoids, proanthocyanidins, phenolic compounds, and terpenoids expected to be present in the aqueous extract of *P. amarus* and presence of terpenoids was tested in the chloroform extract of *M. koenigii*. Their results are displayed in figure 4b.2 and summarised in table 4b.5.

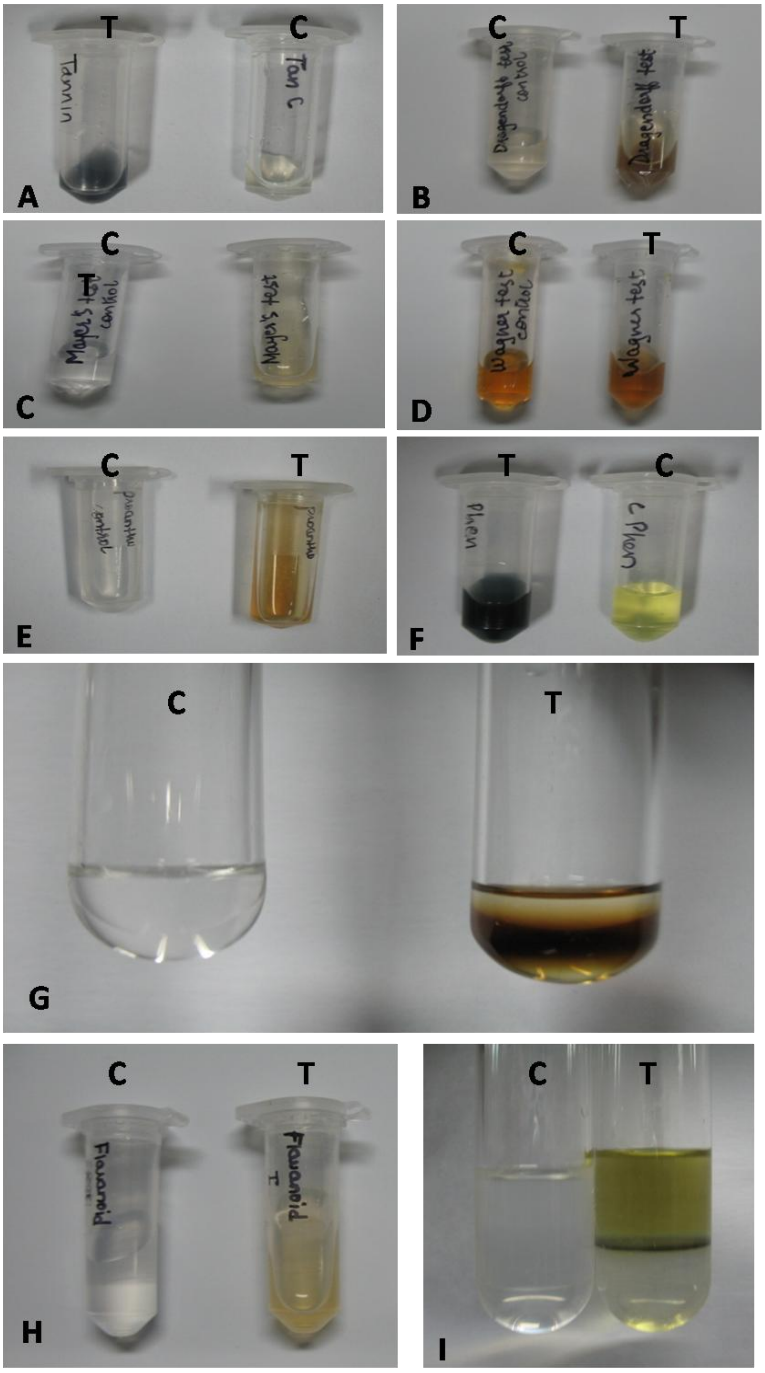


Figure 4b.2: Qualitative tests to determine phytochemicals present in *P. amarus* aqueous extract and *M. koenigii* chloroform extract A) Tannins B) Dragendorff's test for alkaloids C) Mayer's test for alkaloids D) Wagner's test for alkaloids E) Proanthocyanidins F) Phenolic compounds G and I) Salkowski's test for Terpenoids H) Flavonoids. A to H are tests for phytochemicals from *P. amarus* aqueous extract and I is for phytochemicals from *M. koenigii* chloroform extract. C-Control and T-Test.

Amongst the phytochemicals tested tannins, flavonoids, proanthocyanidins, phenolic compounds and terpenoids were found to be present in the aqueous extract of *P. amarus* and terpenoids were found to be present in the chloroform extract of *M. koenigii* (Figure 4b.2 and Table 4b.5).

Table 4b.5: Phytochemical test results

Test	**Observation**	**Result**
Aqueous extract of *P. amarus*		
Test for tannins	Blue black coloration	Present
Mayer's test for alkaloids	No formation of white floccules	Absent
Dragnedorf's test for alkaloids	No red/orange precipitate	Absent
Wagner's test for alkaloids	No formation of brown floccules	Absent
Test for flavonoids	Development of brownish yellow coloration	Present
Test for proanthocyanidins	Development of red coloration	Present
Test for phenolic compounds	Development of black gray coloration	Present
Test for terpenoids	Formation of brown ring	Present
Chloroform extract of *M. koenigii*		
Test for terpenoids	Formation of brown ring	Present

4b.3.3 Docking of compounds from plant extracts in rPfLDH and rPvLDH

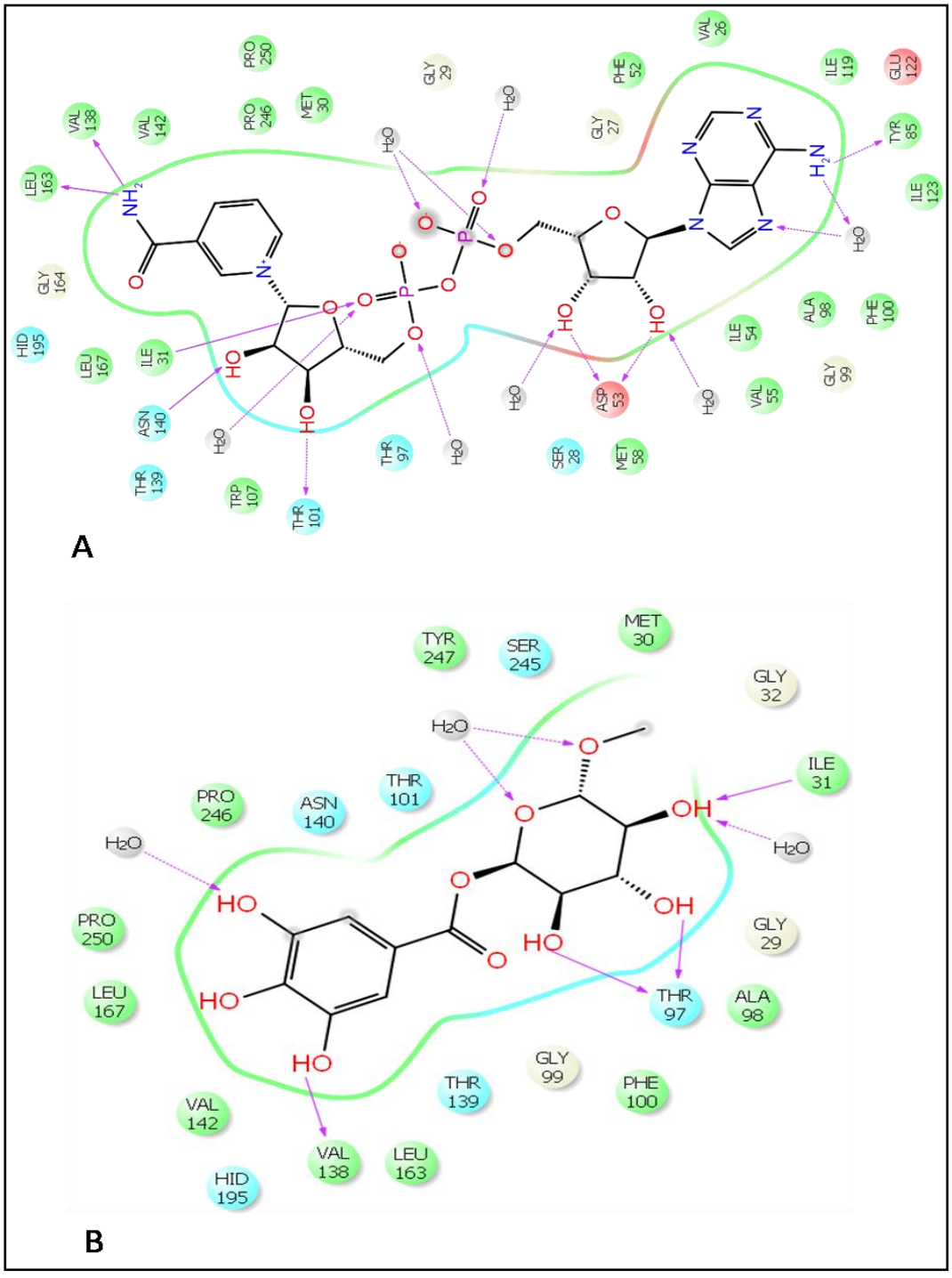
The present study aimed to find an effective molecule with strong affinity for pLDH close to or higher than gossypol, a known selective pLDH inhibitor. Hence, compounds, from phytochemical groups that were confirmed to be present in aqueous extract of *P. amarus* and chloroform extract of *M. koenigii* (Section 4b.3.2), were used in the molecular docking analysis and their efficiency of interaction with rPfLDH and rPvLDH, represented by GlideScore are presented in table 4b.6. To validate the docking score obtained for the compounds, interaction of NADH with PfLDH and PvLDH was taken into consideration. NADH had a similar affinity for PfLDH and PvLDH (Km: 14.9±0.7 µM and 10.8±1.5 µM respectively) observed through enzyme kinetics studies (Table 3.3) and therefore GlideScore was validated by determining the efficiency of NADH interaction with both the enzymes. The GlideScore obtained for NADH interaction with PfLDH and PvLDH (-12.2 and -12.0) were observed to be near identical, confirming the reliability of the scoring function of glide. Amongst all the compounds tested, NADH had strongest affinity for both the enzymes, while amongst the compounds from plant extracts, beta glucogallin from *P. amarus* was found to interact with both enzymes with highest affinity (GlideScore: -9.88 and -8.74 for PfLDH and PvLDH respectively). Interestingly, the affinity of beta glucogallin was even higher than the affinity of gossypol with GlideScore: -8.47 and -7.74 for PfLDH and PvLDH respectively (Table 4b.6). The interaction of both, gossypol and beta glucogallin were stronger with PfLDH compared to PvLDH (Table 4b.6)

Table 4b.6: GlideScore representing strength of molecular interaction

Interaction with PfLDH (Receptor)		Interaction with PvLDH (Receptor)	
Phytocompounds (Ligands)*	**GlideScore**	**Phytocompounds (Ligands)***	**GlideScore**
NADH	-12.2	NADH	-12.0
Beta glucogallin	-9.88	Beta glucogallin	-8.74
Gossypol	-8.47	Gossypol	-7.74
Astragalin	-5.36	Kaempferol	-5.11
Ellagic acid	-5.24	Ellagic acid	-4.93
Kaempferol	-5.14	Geranin A	-4.89
Geranin A	-4.97	Astragalin	-4.8
Cubenol	-4.31	Spathulenol	-4.61
Beta elemene	-4.3	Beta elemene	-4.33
Gallic acid	-4.30	Cubenol	-3.96
Thujene	-3.92	Gallic acid	-3.82
Spathulenol	-3.89	Camphene	-3.79
Camphene	-3.84	Germacrene D	-3.66
Beta myrcene	-3.68	Beta myrcene	-3.65
Carene	-3.68	Thujene	-3.31
Germacrene D	-3.59	Corilagin	-3.29
Corilagin	-3.43	Carene	-3.15
Alpha phellandrene	-3.4	Alpha phellandrene	-3.1
Limonene	-3.38	Limonene	-3.09
Amariin	ND	Terpinyl acetate	-3.01
Terpinyl acetate	ND	Alpha caryophyllene	ND
Alpha caryophyllene	ND	Amariin	ND

* Compounds in blue are from *P. amarus* aqueous extract while compounds in red are from *M. koenigii* chloroform extract. NADH and gossypol were used as control. ND – Not docked efficiently. Compounds are arranged in descending order of their GlideScore.

Amongst the compounds studied for their interactions with both the enzymes, beta glucogallin showed highest affinity for both pLDH and therefore the molecular interactions between beta glucogallin and both the enzymes were analysed further and were compared with the interaction of NADH with pLDH. It was observed that binding site of beta glucogallin was lying within the cofactor binding site of PfLDH and PvLDH (Figure 4b.3).



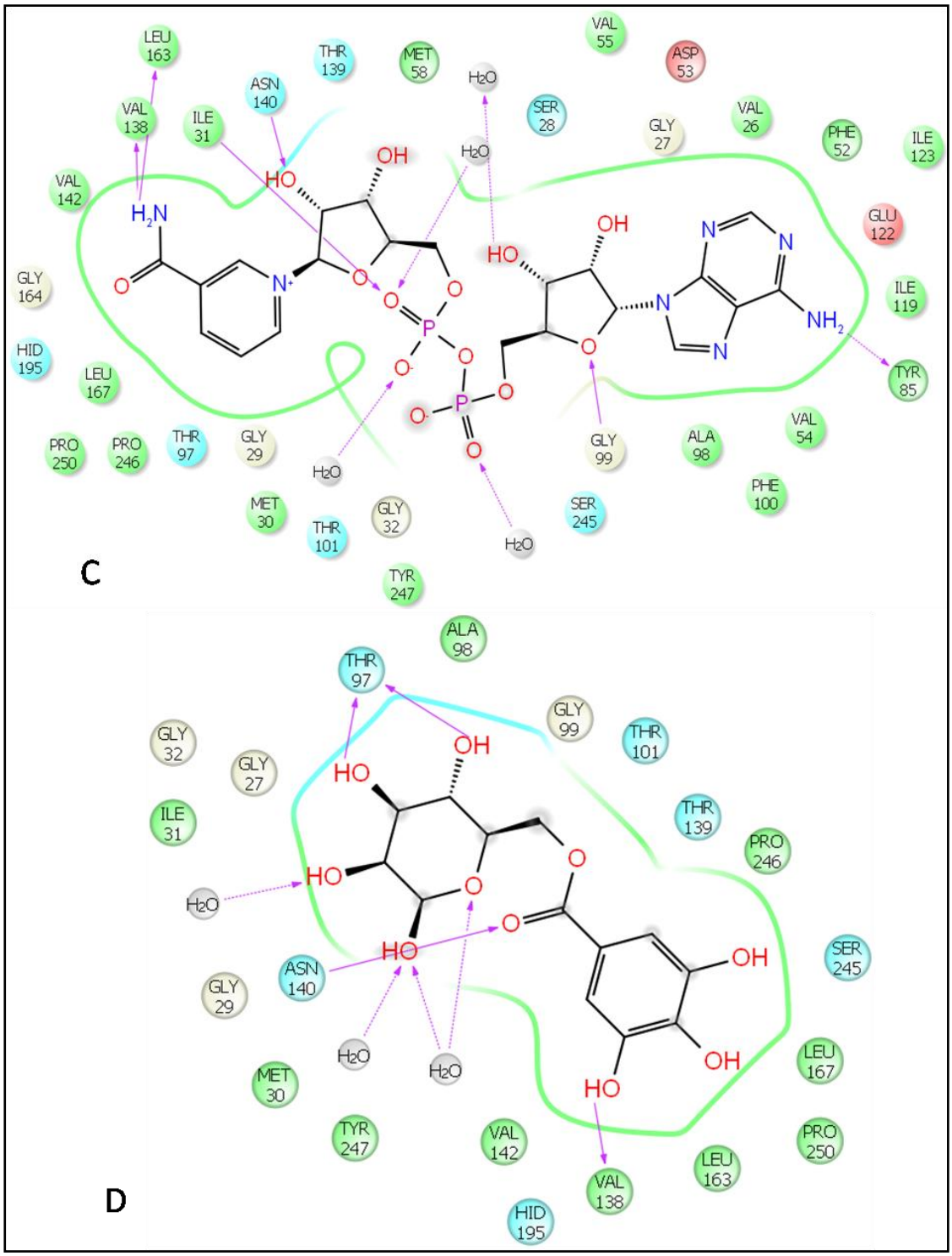


Figure 4b.3: Schematic representation of molecular interactions A) NADH with PfLDH B) Beta gluogallin with PfLDH C) NADH with PvLDH D) Beta gluogallin with PvLDH. Green - hydrophobic interactions, blue – polar interactions, red – negatively charged amino acids, grey – water molecules, arrows – hydrogen bonds, dotted arrows – weak interactions.

The binding site of beta glucogallin in PfLDH covered 20 amino acids of which 15 amino acids comprise the cofactor binding site. The interaction was stabilised by 4 hydrogen bonds formed between hydroxyl groups of beta glucogallin and the amino acid residues in the enzyme binding site (one bond each with Ile 31 and Val 138; two bonds with Thr 97). Out of these 4 bonds, 2 bonds were formed with the same residues which form hydrogen bond with NADH (Ile 31 and Val 138).

Similarly, the binding site of beta glucogallin in PvLDH also covered 20 amino acids and all comprise the cofactor binding site. The interaction was stabilised by 4 hydrogen bonds formed between hydroxyl and carbonyl groups of beta glucogallin and the amino acid residues in the enzyme binding site (one bond each with Asn 140 and Val 138; two bonds with Thr 97). Out of these 4 bonds, 2 bonds were formed with the same residues which form hydrogen bond with NADH (Asn 140 and Val 138).

Phytocompounds from chloroform extracts of *M. koenigii*, considered in the present study, did not show strong affinity towards either of the enzymes and hence were not considered further.

4b.4 DISCUSSION

Mode of inhibition of a compound provides information about the site of interaction of that compound with the enzyme. For example a competitive inhibitor shares the binding site with the substrate and hence competes with it for binding with the enzyme. A mixed inhibitor binds at a site distinct from the substrate binding site and, though binding of inhibitor does not directly block binding of substrate, this interaction partially affects the binding of substrate to its active site. A non-competitive inhibition is a special case of mixed inhibition. Here, binding of inhibitor with an enzyme is a disconnected event and does not affect the binding of substrate to the enzyme. In the fourth case, an uncompetitive inhibitor also binds at a site distinct from the active site

only if the substrate is already bound to its binding site (Nelson & Cox 2000).

The type of enzyme inhibitor interaction can be easily determined by double-reciprocal plot. Based on the enzyme inhibition kinetics results of the aqueous extract of *P. amarus* (Table 4b.4), it was deduced that the extract contained the compound/s which interact with rPfLDH at the cofactor binding site and compete with NADH. On the contrary these compound/s interact with rPvLDH such that, a single compound overlap both, the substrate and the cofactor binding sites or the extract contain more than one compound which individually interact at the substrate and cofactor binding sites. Interestingly, the enzyme inhibition kinetics results of the chloroform extract of *M. koenigii* (Table 4b.4) proposed different mode of interaction. The compounds in this extract interact with rPfLDH at the substrate binding site and compete with pyruvate while they interact with rPvLDH at a site other than both, substrate and cofactor binding site and exhibit mixed inhibition by reducing the affinity of the enzyme for pyruvate. This observation suggests that the binding of the inhibitors with rPvLDH had allosterically altered the substrate binding site to partially reduce the affinity of the enzyme towards substrate (Pyruvate). The predicted model of the enzyme inhibition is schematically represented in figure 4b.4.

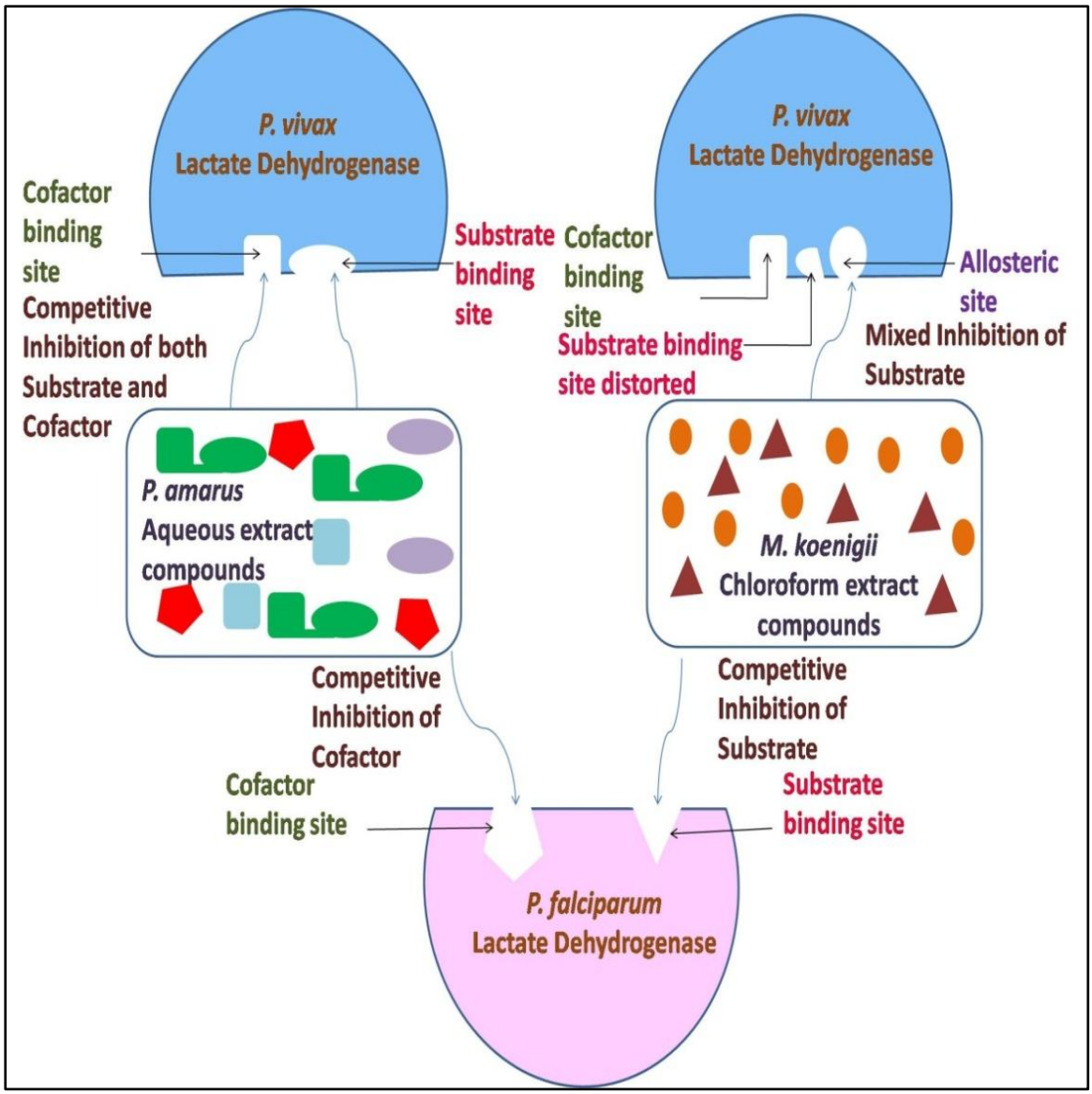


Figure 4b.4: Predicted model of molecular interactions between enzymes and the compounds in the plant extracts.

Though the predicted model is the straight forward interpretation of inhibition kinetics results, other interactions are also possible. For example, an inhibitor may exhibit competitive inhibition without binding to the substrate binding site. This can occur if binding of the inhibitor at a distant site on the enzyme stearically or allosterically blocks substrate binding site. Thus, further verification of the predicted model was necessary which was carried out by docking compounds from both plant extracts individually in available crystal structures of both the enzymes.

Amongst all the compounds tested from both plant extracts, beta glucogallin from aqueous extract of *P. amarus* exhibited strongest interaction with PfLDH and PvLDH (Table 4b.6). Gomez et al. (1997) have reported gossypol to be a selective inhibitor of PfLDH, but interestingly the interaction of beta glucogallin was found to be stronger than gossypol for both PfLDH and PvLDH. The binding site of beta glucogallin was overlapping with the cofactor binding site in both the enzymes. This observation supported the enzyme kinetics data, where *P. amarus* aqueous extract was shown to be a competitive inhibitor of NADH (Table 4b.4).

pLDH has high structural similarities with malate dehydrogenase (Zhu & Keithly 2002). Thus, malate dehydrogenase potentially complements the activity of pLDH if pLDH is selectively inhibited (Tripathi et al. 2004). The key structural similarity between these enzymes is in the cofactor binding site. This has been evident by the observation that inhibitors like gossypol, which interact at the cofactor binding site of PfLDH can effectively inhibit both the enzymes; whereas PfLDH inhibitors that interact at substrate binding site e.g. oxamate, do not inhibit malate dehydrogenase (Choi, Pradhan, et al. 2007) and hence due to complementation of LDH activity by MDH, such inhibitors do not exhibit parasiticidal activity. Thus, antimalarial drugs targeting pLDH should ideally interact with the enzyme at the cofactor binding site. As beta glucogallin inhibits the enzyme by interacting at cofactor binding site, this compound has a strong potential to be developed as novel antimalarial drug. Moreover, purified beta glucogallin has been shown to have potent anti-plasmodial activity with IC_{50} value of 4.6 µg/ml (Subeki et al. 2005).

The Cofactor binding site is not generally considered as a target site in drug development but the report of selective protein kinase inhibitors that bind to ATP binding site, by Parasrampuria et al. (2003), has justified the possibility of a compound, binding at cofactor binding site in highly selective manner and inhibit the enzyme, to be developed as a

novel drug. In congruence with this report, beta glucogallin having strong affinity for the NADH (cofactor) binding site is a promising candidate to be developed as a novel antimalarial drug.

Chloroform extract of *M. koenigii* leaves had strong and selective inhibitory effect on both pLDH (Section 4a.3.2) and this was also confirmed by K_i values of the extract observed in the enzyme kinetic studies (Table 4b.4). Despite these evidences asserting presence of the pLDH inhibitory compounds in the extract, the molecular docking studies did not display any significant interaction of the compounds tested with either of these enzymes. Chloroform, being hydrophobic in nature (polarity index 4.1), could extract non-polar and hydrophobic compounds more efficiently from the biomass. Hence, the rationale behind selectively considering terpenoids, reported to be present in the essential oils extracted from *M. koenigii* leaves (Chowdhury et al. 2008), were to select only hydrophobic compounds, which, consequently restricted broader selection of compounds for the docking studies. The possible reason for not getting compound with significant interaction with pLDH was that the molecules causing inhibition were not included in the studies. Further, detailed phytochemical characterization of chloroform extract could provide more information about chemical composition of the extract. Identification and characterization of such compounds may provide lead compound with potential antimalarial activity.

In conclusion, pLDH inhibitory effect of *P. amarus* aqueous extract was found to be because of competitive inhibition with cofactor (NADH) by the constituents of the extract. This was also confirmed by molecular docking studies and beta glucogallin from *P. amarus* aqueous extract was found as a molecule responsible for enzyme inhibition. Thus, the study had conferred beta glucogallin as a potential antimalarial drug. Study has also highlighted the potential of *M. koenigii* chloroform extract in antimalarial drug discovery which requires further investigation.

Summary

SUMMARY

Chapter 2: Genetic diversity analysis of L-Lactate Dehydrogenase gene in Indian *Plasmodium falciparum* and *Plasmodium vivax* population

- Total 81 samples containing 46 *P. falciparum* samples and 35 *P. vivax* samples were used in the present study for PfLDH and PvLDH genetic polymorphism analysis.

- 951 bp amplicons of PfLDH and PvLDH genes from *P. falciparum* and *P. vivax* samples respectively, coding for the complete ORF, were amplified using primers designed specifically for *P. falciparum* and *P. vivax.*

- Nested PCR was carried out using the 951 bp PfLDH and PvLDH gene fragments as template to get fragment 1 (183 bp) and fragment 2 (192 bp) from *P. falciparum* and fragment 1 (159 bp) and fragment 2 (188 bp) from *P. vivax* samples using specific primers.

- The single strand conformers, derived from 183 bp and 192 bp fragments of PfLDH gene, had the same electrophoretic mobility in all the samples; indicating presence of single PfLDH genotype amongst the studied *P. falciparum* samples. Similarly, differences were not observed in the electrophoretic mobility of 159 bp PvLDH gene fragment, whereas different electrophoretic mobility of SSCs, leading to 6 discreet band patterns were observed in the 188 bp fragment of PvLDH; indicating presence of at least 6 PvLDH genotypes amongst the studied *P. vivax* samples.

- Distribution of the genotypes was examined amongst the states (Odisha, Karnataka and Goa) in consideration in the present study. Genotype 4 was found to be the most abundantly (51.43%)

present in all states followed by genotype 2 and genotype 3 that were present in Odisha and Karnataka states, whereas all other genotypes were scanty and distributed in Goa and Odisha states. Thus, high diversity and eveness was observed amongst the genotypes with Simpson's Index of diversity 0.69 and Eveness index 0.77.

- Sequence analysis conferred with the SSCP analysis of the PfLDH and PvLDH gene fragments.
- PfLDH gene sequences were aligned with *P. falciparum* 3D7 LDH sequence and only one synonymous mutation (A15 to G) was observed in Mzr1 LDH.
- PvLDH gene sequence were found to be polymorphic. This is the first report presenting presence of nonsynonymous mutation in PvLDH genotypes.
- Protein sequence variation was not observed in the active site and key catalytic residues of the enzyme.
- Three dimensional structures of LDH protein derived through homology modeling were found to be reasonably trustworthy with QMEAN Z-score < 0.6.
- These structures were aligned with the crystal structure of PvLDH (PDB ID: 2A92) and were found to be structurally similar with RMSD not more than 0.03 Å.
- Leu 149 to Pro substitution in α1F helix of Goa 1 PvLDH had resulted in helix to loop transition in a small region (spanning 3 amino acid residues) without altering the topology of surrounding domain.
- Sequence variations in the PvLDH genotypes had not culminated in conformation change of the protein. Thus, pLDH epitopes used

in immunochromatography based RDTs would be conserved and hence variation in the response of RDT due to polymorphic epitopes would be at low level in India despite of the occurrence of different PvLDH genotype.

- Phylogenetic analysis of the pLDH gene showed that PvLDH and PfLDH genes formed two different clades.

- Genealogy of *Plasmodium* species inferred by phylogenetic analysis had suggested *P. falciparum* as a recently transferred parasite from African Gorilla host to human and *P. vivax* had comparatively ancient origin.

Chapter 3: Cloning, expression and characterization of *Plasmodium falciparum* and *Plasmodium vivax* specific L-Lactate dehydrogenase

- Amplification of 962 bp PfLDH and PvLDH genes was obtained from genomic DNA of *P. falciparum* (Ori1) and *P. vivax* (Krt1) by designing primers specific for *P. falciparum* and *P. vivax* LDH. *Nco*I and *Xho*I sites were incorporated in the forward and reverse primers respectively to aid in cloning the genes in pET28a vector.

- Cloning of PfLDH and PvLDH genes in *E. coli* DH5α using CloneJet cloning kit yielded 38 and 115 clones of PfLDH and PvLDH genes respectively.

- Inserts from the confirmed clones $pJETPFL_2$ and $pJETPVL_4$ of PfLDH and PvLDH respectively were sequenced which showed presence of the complete ORF of 948 bp coding for PfLDH and PvLDH.

- DNA fragments containing PfLDH and PvLDH ORF were subcloned in *E. coli* DH5α using pET28a vector.

- *E.coli* BL21 (DE3) was transformed with the constructs pETPFL1 and pETPVL$_1$ for protein expression.

- Recombinant proteins (rPfLDH and rPvLDH) were expressed in the soluble form after IPTG induction and their expression was observed on the SDS-PAGE gels as two new protein bands in both the clones.

- The size of the higher molecular weight band was close to 35 kDa while that of the lower molecular weight band was close to 30 kDa suggesting co-expression of truncated protein along with the major protein.

- Co-expression of the truncated rPfLDH along with the complete ORF was attributed the presence of internal Shine Dalgarno sequence in the rPfLDH ORF.

- A similar co-expression of truncated rPvLDH was reported for the first time in the present study. Sequence analysis of rPvLDH also showed presence of similar internal Shine Dalgarno sequence which would be responsible for the co-expression of the truncated protein.

- Presence of rPfLDH and rPvLDH in the induced lysate of pETPFL and pETPVL clones was also confirmed through immunochromagraphy based dip stick test.

- A substantial increase in the LDH activity was observed in the induced lysates of pETPFL and pETPVL clones (22312 U/L and 18939 U/L) compared to uninduced clones (374 U/L and 897 U/L).

- Recombinant proteins were purified using Ni-NTA agarose with the help of hexahistidine tag at the C' terminal end of the recombinant proteins and were eluted using high concentration of imidazole.

- Specific activity of rPfLDH and rPvLDH increased by 10.6 and 9.7 fold respectively with more than 90% purity.

- Molecular weights of rPfLDH and rPvLDH were 34.5 kDa and 35 kDa respectively (values were close to the expected molecular weights of the proteins).

- Enzyme activity of rPfLDH and rPvLDH at varying concentration of substrate (pyruvate) and cofactor (NADH) were plotted as Lineweaver - Burk plot through which it was observed that both the recombinant enzymes had nearly the same rates and have similar affinities for cofactor NADH.

- Feeble substrate (Pyruvate) inhibition was observed in both the enzymes.

- rPfLDH and rPvLDH both could utilize $APAD^+$ efficiently as a cofactor and the observed kinetic constants for $APAD^+$ were comparable to the reported data which confirmed that the purified recombinant enzymes had retained their unique structural and functional properties.

- rPfLDH and rPvLDH could be inhibited by gossypol and chloroquine but the latter had dissociation constant 10^3 fold higher than gossypol indicating that gossypol is a stronger inhibitor compared to chloroquine.

- Gossypol was found to be a competitive inhibitor of both rPfLDH and rPvLDH competing with NADH to bind with both the enzymes.

- Chloroquine was found to be a competitive inhibition of rPfLDH (competitive with respect to NADH) whereas it was a non-competitive inhibitor of rPvLDH. This variation could be attributed to the conformational changes in the chloroquine binding region of the enzyme (amino acid residues 53-62). Further, variations in

this region could be exploited to develop species specific selective inhibitors of pLDH.

Chapter 4a: Screening of traditional Indian herbs for selective inhibition of *Plasmodium falciparum* and *Plasmodium vivax* specific L-Lactate dehydrogenase

- Eight plants traditionally used in the treatment of recurrent fever or symptomatically diagnosed malaria in India were selected in the present study. These included *Eclipta alba, Azadirachta indica, Andrographis paniculata, Murayya koenigii, Calotropis procera, Caesalpinia crista, Ocimum sanctum* and *Phyllanthus amarus*.

- Plant extracts were prepared in four solvents (petroleum ether, chloroform, ethanol and water with different polarity index) in a Soxhlet apparatus. Thus, 32 extracts were to be tested wherein each contained different concoction of compounds on the basis of their polarity.

- Seven of these extracts significantly reduced rPfLDH and rPvLDH activity to < 50% (ethanol extract of *E. alba, A. paniculata* and *P. amarus* reduced rPfLDH activity, similarly chloroform extracts of *A. paniculata* reduced rPvLDH activity whereas petroleum ether extract of *C. procera*, chloroform extracts of *M. koenigii* and aqueous extracts of *P. amarus* reduced activity of both pLDH).

- The seven extracts that effectively inhibited parasite LDH, were also checked for their effect on Bovine Heart and Muscle LDH at the same concentration and they were observed to be ineffective on both Bovine Heart and Muscle LDH.

- These extracts had discrete effects on rPfLDH and rPvLDH (Pearson's correlation coefficient r = 0.2) while their effect was highly correlated on Bovine LDHs (Pearson's correlation coefficient r = 0.8).

- Amongst the tested extracts, *P. amarus* aqueous extract exhibited inhibitory effect on rPfLDH (IC_{50} = 11.2 ± 0.4 µg/ml) and *M. koenigii* chloroform extract exhibited inhibitory effect on rPvLDH (IC_{50} = 6.0 ± 0.6 µg/ml), close to gossypol (IC_{50} = 2.6 ± 0.8 and 10.4 ± 0.2 µg/ml respectively on rPfLDH and rPvLDH) which is a known pLDH inhibitor.

- IC_{50} values of *P. amarus* aqueous extracts for *P. falciparum* NE (chloroquine sensitive strain) and *P. falciparum* MRC-2 (chloroquine resistant strain) were 7.1 µg/ml ± 0.5 and 6.9 µg/ml ± 0.7 respectively which were lower than the IC_{50} values obtained for rPfLDH activity, implying possibility of the presence of compounds with different target in the parasite, which act synergistically and kill parasites more efficiently.

Chapter 4b: Molecular characterization of the enzyme inhibition exhibited by the compounds from *Phyllanthus amarus* and *Murraya koenigii* extracts

- Enzyme inhibition kinetics studies were carried out to determine the type of the inhibition exhibited by aqueous extract of *P. amarus* and chloroform extract of *M. koenigii* by double reciprocal plots of enzyme activity in the presence and absence of plant extracts at varying concentrations of substrate (pyruvate) and cofactor (NADH).

- *P. amarus* aqueous extracts showed competitive inhibition w.r.t NADH in both PfLDH and PvLDH and also w.r.t. pyruvate in PvLDH, whereas it was noncompetitive with pyruvate in PfLDH

- *M. koenigii* chloroform extract showed noncompetitive inhibition with NADH in both PfLDH and PvLDH whereas it showed competitive inhibition with pyruvate in PfLDH and linear mixed with pyruvate in PvLDH.

- Qualitative tests carried out to determine the phytochemical compounds present in the extracts showed presence of tannins, flavanoids, proanthrocyanidins, phenolic compounds and terpenoids in the aqueous extract of *P. amarus* and terpenoids were found to be present in the chloroform extract of *M. koenigii.*

- Compounds from phytochemical groups that were confirmed to be present in aqueous extract of *P. amarus* and chloroform extract of *M. koenigii* were used in the molecular docking analysis and their efficiency of interaction with rPfLDH and rPvLDH were represented by GlideScore.

- Docking score was validated by taking NADH, a natural cofactor of PfLDH and PvLDH, into consideration which expectedly showed similar GlideScore for both PfLDH and PvLDH.

- Amongst all the compounds tested NADH showed maximum affinity towards both the enzyme as it is a natural cofactor of both the enzymes.

- Beta glucogallin obtained from *P. amarus* aqueous extract showed maximum affinity for the enzymes amongst the compounds present in both the extracts. Its affinity was even higher than gossypol.

- Analysis of the molecular interactions of beta glucogallin with both the enzymes showed that its binding site was overlapping with the cofactor binding site of PfLDH and PvLDH.

- In PfLDH the binding site of beta glucogallin covered 20 amino acids of which 15 amino acids comprise the cofactor binding site whereas in PvLDH it covered 20 amino acids and all comprise the cofactor binding site of the enzyme.

- In PfLDH the interaction was stabilised by 4 hydrogen bonds formed between hydroxyl groups of beta glucogallin and the amino

acid residues in the enzyme binding site (one bond each with I31 and V138; two bonds with T97) and out of these, 2 bonds were formed with the same residues which form hydrogen bond with NADH (I31 and V138).

- In PvLDH the interaction was stabilised by 4 hydrogen bonds formed between hydroxyl and carbonyl groups of beta glucogallin and the amino acid residues in the enzyme binding site (one bond each with N140 and V138; two bonds with T97) and out of these, 2 bonds were formed with the same residues which form hydrogen bond with NADH (N140 and V138).

- Phytocompounds from chloroform extracts of *M. koenigii* taken into consideration in the present study did not show strong affinity towards the enzyme.

Conclusion

CONCLUSION

Malaria is one of the most vicious diseases and a major cause of mortality and morbidity worldwide. Resistance acquired by the parasites to the present line of antimalarials has necessitated a persistent search for novel drugs with more efficient targets. *Plasmodium* species specific L-lactate dehydrogenase (pLDH), the last enzyme of glycolytic pathway is one amongst such targets, which has the potential to be used as both therapeutic and diagnostic target. The prerequisite for a target to be effectively used in diagnostics or therapeutics is to be conserved in the population. The present study was therefore initiated with the polymorphism analysis of PfLDH (*P. falciparum* specific LDH) and PvLDH (*P. vivax* specific LDH) genes.

Considering the epidemiology of malaria in India, total 81 samples (46 *P. falciparum* including *P. falciparum* 3D7 and 35 *P. vivax*) from different states of India, were selected in the present study. Using PCR-Single Strand Conformation Polymorphism (PCR-SSCP) as a primary screening method, only a single PfLDH genotype was observed to be prevalent throughout India while 6 PvLDH genotypes were prevalent in Karnataka, Goa and Odisha states. Sequence analysis of two representative PfLDH samples confirmed lack of genetic polymorphism. Similarly, sequence analysis of representative samples of each PvLDH genotype validated the PCR-SSCP based genotyping. Moreover, nonsynonymous substitutions along with synonymous substitutions were reported to be present in the PvLDH genotypes for the first time. Though the protein sequence was found to be variable, the catalytic residues and active site residues were conserved in all PvLDH genotypes. Model structures of PvLDH, derived from protein sequences of all the genotypes, were nearly identical to the reference PvLDH structure. Hence, we propose that PvLDH from different genotypes will have the same enzyme activity and drugs targeting pLDH might not experience response variation. The study therefore confirmed PfLDH and PvLDH as suitable drug target. Moreover, the structural

analysis of PvLDH from all genotypes also confirmed that pLDH epitopes used in immunochromatography based RDTs would be conserved and hence LDH based rapid diagnostic tests would be highly efficient in detecting malaria cases. Phylogenetic analysis of pLDH gene sequences had shed light on the genealogy of *Plasmodium* species. As compared to *P. vivax*, *P. falciparum* was found to have recent origin through a host transfer from African gorilla species.

Reverse pharmacology based drug discovery approach requires ample source of target protein for screening of the inhibitors. pLDH was considered as a drug target in this study and hence to acquire persistent supply of the enzyme, gene coding for LDH was cloned from *P. falciparum* and *P. vivax* in *E. coli* using pET28a expression vector. Recombinant proteins (rPfLDH and rPvLDH) were expressed in *E. coli* and subsequently purified to homogeneity and their identities were confirmed. This is the first report of successful cloning and expression of PvLDH from *P. vivax* from India, which is a major contributor of vivax malaria cases. Functional characteristics of the recombinant enzymes were found to be comparable to the earlier reported values for pLDH, through enzyme kinetics studies.

Medicinal plants, traditionally used to treat malaria, possess a repertoire of prospective antimalarial drugs and have been exploited globally in indigenous medical practices since antiquity, some excellent examples are quinines and artemisinin. In the present study ethnopharmacology guided drug screening was carried out in conjunction with target based drug discovery approach, wherein 8 herbs that are traditionally used to treat symptomatic malaria in India were rationally selected and screened for specific inhibition of pLDH. Amongst 8 plants studied, aqueous extract of *P. amarus* and chloroform extract of *M. koenigii* were found to have selective and significant inhibitory effect on *P. falciparum* and *P. vivax* LDH respectively. Moreover, strong parasiticidal activity was also observed in the aqueous extract of *P. amarus*.

Molecular interactions of active compounds in the plant extract with pLDH were predicted using enzyme inhibition kinetics analyses. The study had suggested that compounds present in *P. amarus* aqueous extracts interact at cofactor binding site of PfLDH and at both cofactor and substrate binding sites of PvLDH, whereas compounds present in *M. koenigii* chloroform extracts interact at substrate binding site of PfLDH and allosterically reduce the affinity of the substrate for PvLDH. Amongst the compounds from *P. amarus* and *M. koenigii*, that were used in molecular docking analysis, beta glucogallin from *P. amarus* was found to be strongly interacting at the cofactor binding sites of both PfLDH and PvLDH by forming hydrogen bonds using molecular docking studies. Interestingly, the interaction observed was stronger than gossypol, which is a known pLDH inhibitor.

Using the combined approach of ethnopharmacology and reverse pharmacology, *P. amarus* and *M. koenigii* extracts were obtained as hits by screening of as low as eight plants. Further, molecular analysis of these interactions have proffered beta glucogallin from *P. amarus* to be potentially developed as novel antimalarial drug with pLDH inhibitory activity. In the light of the urgent need for novel drugs against malaria and other drug resistant pathogens, this kind of approach can effectively translate the rich ethnic medicinal repertoire throughout the globe into effective drugs. Nevertheless, this study is more economical over high throughput screening and excludes need of post drug development studies on determination of target and mode of action. Thus, in the context of tropical infectious disease like malaria, where limitation of funds is a major concern, this study introduces an efficient and economical approach for drug discovery.

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Publications and Presentations

LIST OF PUBLICATIONS AND PRESENTATIONS

- **Publications**

1. Keluskar, P. and Ingle, S. (2012). Ethnopharmacology guided screening of traditional Indian herbs for selective inhibition of Plasmodium specific lactate dehydrogenase. Journal of ethnopharmacology. *Journal of ethnopharmacology*. **144** (1), 201-207. doi:10.1016/j.jep.2012.09.006

2. Keluskar, P., Singh, V., Gupta, P. and Ingle, S. Genetic polymorphism analysis of Plasmodium Lactate Dehydrogenase from Indian isolates: a therapeutic and diagnostic target. **Manuscript under review**.

3. Evaluation of Novel *Plasmodium falciparum* and *Plasmodium vivax* specific Lactate Dehydrogenase Inhibitors from *Phyllanthus amarus* and *Murayya koenigii* by enzyme kinetics and virtual docking studies. **Manuscript in preparation.**

4. Population genetic analyses of *Plasmodium* species specific lactate dehydrogenase gene to ascertain the evolutionary link. **Manuscript in preparation**.

- **Presentations**

1. Poster Presentation: "Inhibition kinetics of *Plasmodium* Lactate Dehydrogenase with herbal extracts suggest possible enzyme inhibitor molecular interaction" at international conference on'Challenges in Malaria Research: Progress towards elimination' in Basel, Switzerland (10^{th} -12^{th} Oct. 2012).

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2. Oral Presentation: "Screening of Traditional Indian Herbs for Selective Inhibition of Plasmodium specific Lactate Dehydrogenase as Drug Target" (2012). at Regional Science Congress on 'Science for shaping the future of India' jointly organized by The M. S. University of Baroda and Indian Science Congress Association (Baroda Chapter), Vadodara, Gujarat (India). (15^{th}-16^{th} Oct, 2012)

Awarded: **Best oral presentation award**

3. Poster presentation: "In silico identification of novel lead antimalarial phytocompounds by structure based drug discovery" at 'National Symposium on Bioinformatics: Challenges in the Post-Genomic era' organised by University of Jammu, at Jammu, India (2nd Feb. 2012).

4. Poster presentation: "Polymorphism analysis of Lactate Dehydrogenase gene from *Plasmodium falciparum* and *Plasmodium vivax*" at global exchange lecture course on 'Molecular and evolutionary genetics of Malaria' organized by European Molecular Biology Organization(EMBO), at New Delhi, India (21st Nov – 4th Dec, 2010)

5. Poster presentation: "Cloning and Expression of *P. falciparum* specific L-lactate dehydrogenase gene" at IX international symposium on vectors and vector borne disease. (2008)

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Ethnopharmacology guided screening of traditional Indian herbs for selective inhibition of *Plasmodium* specific lactate dehydrogenase

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ABSTRACT

Ethnopharmacological relevance: Medicinal plants traditionally used to treat malaria can provide quality leads towards identifying novel anti-malarial drugs. Here we combined this approach with target based drug discovery and explored *Plasmodium* specific lactate dehydrogenase (LDH) inhibitory activity of 8 Indian plants which are ethnically used to treat malaria.

Methods: LDH from Indian *Plasmodium falciparum* and *Plasmodium vivax* strains, were cloned and expressed in *Escherichia coli*, followed by purification of recombinant enzymes (rPfLDH and rPvLDH respectively). Extracts of 8 plants in different organic and aqueous solvents, were screened for their inhibitory activity on rPfLDH, rPvLDH and mammalian LDHs. *Phyllanthus amarus* aqueous extract was further tested for *in vitro* parasiticidal activity.

Results: Aqueous extract of *Phyllanthus amarus* Schum. and Thonn. and chloroform extract of *Murraya koenigii* (L.) Spreng. exhibited profound and exclusive inhibitory effect on *Plasmodium falciparum* LDH (IC_{50}=11.2 μg/ml ± 0.4) and *Plasmodium vivax* LDH (IC_{50}=6.0 μg/ml ± 0.6) respectively. Moreover, *Phyllanthus amarus* aqueous extract also demonstrated antiplasmodial activity *in vitro*, on Chloroquine sensitive and resistant strains of *Plasmodium falciparum* (IC_{50}=7.1 μg/ml ± 0.5 and 6.9 μg/ml ± 0.7 respectively).

Conclusion: Target specific screening of traditional herbs used in malaria treatment has proffered *Phyllanthus amarus* and *Murraya koenigii* extracts as hits which can optimistically provide novel antimalarial drugs.

1. Introduction

Malaria has remained one of the most vicious infectious diseases with estimated 216 million cases worldwide (World Malaria Report, 2011). *Plasmodium* species, especially *Plasmodium falciparum* have acquired resistance to most of the present antimalarial drugs, thereby hindered the control and eradication of malaria (Wongsrichanalai et al., 2002). Recent reports indicating the emergence and spread of resistance to artemisinin which is a major drug for first line therapy of *falciparum* malaria, has triggered an alarm over the situation (Phyo et al., 2012). Therefore, one of the major points of the agenda, to contain a scourge of this disease, is a persistent search for novel drugs with more efficient targets (Hall and Fauci, 2009).

Development in the genomic research of major human malaria parasites has opened new prospects of reverse pharmacology based drug discovery wherein available databases and bioinformatics tools can be applied to determine parasite enzymes and receptors as potential drug targets (Guantai and Chibale, 2011). *Plasmodium* species specific L-lactate dehydrogenase (pLDH), the last enzyme of glycolytic pathway is one amongst such targets. It is expressed in the endoerythrocytic stage of a parasite and plays an indispensable role of providing NAD^+ to continue glycolysis (Royer et al., 1986). It has significantly different structural and kinetic properties compared to human LDH isoforms (Sessions et al., 1997) and several selective inhibitors of pLDH have demonstrated anti-malarial activity *in vitro* and *in vivo* (Choi et al., 2007; Vivas et al., 2005). Gene ontology studies have also espoused pLDH as a potential target in exterminating malaria (Wiwanitkit, 2007). A quantitative assay to screen pLDH inhibitors was designed by Xu et al. (2007) for developing novel antimalarial drugs targeting pLDH.

Medicinal plants, traditionally used to treat malaria, possess a repertoire of prospective antimalarial drugs and have been exploited globally since antiquity, some excellent examples being quinines and artemisinin (Klayman, 1985). Pharmacological analysis of such traditional plants can lead to novel, affordable and accessible antimalarial drugs (Karunamoorthi and Tsehaye, 2012). Several studies have screened these herbs for parasiticidal activity (Andrade-Neto et al., 2003; Chenniappan and Kadarkarai, 2010; Muthaura et al., 2011; Ruiz et al., 2011; Tona et al., 1999). Moreover Kantamreddi and Wright

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(in press) have recently confirmed efficacy of this approach over random screening of plants in the process of drug discovery. In the present study we have carried out ethnopharmacology guided screening in conjunction with target based drug discovery approach, wherein we have selected 8 herbs that are traditionally used to treat symptomatic malaria in India and screened for specific inhibition of pLDH. To this end, we obtained *Plasmodium* species specific pLDH by cloning recombinant enzyme from *Plasmodium falciparum* (rPfLDH) and *Plasmodium vivax* (rPvLDH) of Indian origin, in *Escherichia coli*.

2. Materials and methods

2.1. Cloning and sequencing of PfLDH and PvLDH from Indian strains

Blood samples used in the study were obtained from National Institute of Malaria Research, New Delhi. Samples were collected after getting necessary approvals and written consents of informed patients.The patients from Odisha and Karnataka states of India were confirmed for *Plasmodium falciparum* and *Plasmodium vivax* malaria respectively, by microscopy. Genomic DNA was extracted from both the samples using QIAamp mini DNA kit (Qiagen, Germany) as per the manufacturer's protocol. Species specific primers CCATGGCAC-CAAAAGCAAAAATC (PfF) and ATCGTCCTCGAGAGCTAATGCCTTCATTC (PfR) were designed based on *Plasmodium falciparum* 3D7 *ldh* sequence (GenBank ID: XM_001349953) to amplify 951 bp *Pf*LDH open reading frame (ORF). Likewise primers CCATGGCTATGACGCC-GAAACCCAAAATTG (PvF) and CTCGAGAATGAGCGCCTTCATCC (PvR) were designed based on *Plasmodium vivax* Sal1 *ldh* sequence (GenBank ID: XM_001615570.1) to amplify 951 bp PvLDH ORF. *Nco*I and *Xho*I restriction sites were incorporated in forward and reverse primers respectively (restriction sites are underlined in primer sequence). The PCR mixture (50 μl) contained 50 pmol of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, app.100 ng of genomic DNA and 2.5 U of *Pfu* polymerase. PCR program used was as follows: initial denaturation of 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 45 s at 54 °C for PfLDH and 58 °C for PvLDH, 2 min at 72 °C and a 10 min final extension at 72 °C. Amplified PCR products were cloned in *Escherichia coli* DH5α using CloneJET PCR cloning Kit (Fermentas Inc., USA). Clones harboring pLETLDH constructs were confirmed by PCR using PfF PfR and PvF PvR primer pairs. Putative clones were further confirmed by sequencing using Single Pass Analysis sequencing services (Bangalore Genei, India) using pJET1.2 sequencing primers.

2.2. Protein expression purification and characterization

PfLDH and PvLDH genes were subcloned in the expression vector pET 28a(+) (Merck Inc., India) using *Nco*I and *Xho*I restriction sites to get expression constructs pETPFL and pETPVL respectively. These constructs were transformed into *Escherichia coli* BL21 (DE3) lysogen. Transformant clones were induced by 1 mM IPTG and incubated at 20 °C for 12 h with agitation for protein expression. Cells were harvested by centrifuging at 6000 × *g* at 4 °C for 10 min and lysed by French press at 1000 psi. Cell free lysates were tested for protein expression on 12% SDS-PAGE gels and by immunochromatography based species specific *Plasmodium* LDH detection kit 'Vector screen' (IND Diagnostics Inc., Canada). LDH activity in the lyaste was determined as described by Bergmeyer and Bernt (1981). The assay mix contained 3 ml of 5 mM pyruvate in 50 mM phosphate buffer (pH 7.5) and 50 μl of 11.3 mM β-NADH. As an enzyme source, appropriately diluted cell free lysates (30 μl) were added in the assay mix, such that enzyme activity was maintained < 250 U/L. $O.D._{340\ nm}$ was measured for 4 min at 1 min interval and change in absorbance (ΔA)/min was calculated. Enzyme units were determined by formula 'Enzyme activity = 5064 × mean ΔA/min' and 1 unit was defined as 1 μmol of NADH oxidized to NAD per minute. Protein concentration was determined by Bradford method (1976). Histidine tagged recombinant enzymes were then purified using Ni-NTA (nitrilo triacetic acid) resin based kit (Invitrogen, USA) by following the manufacturer's protocol. Purification efficiency for both the enzymes was determined by enzyme assay and by silver staining of SDS-PAGE gels. Molecular weight estimation and densitometry analysis of protein bands was carried out using AlphaEase FC software version 6.0 (Alpha Innotech Corp., USA).

2.3. Medicinal plants

Herbs used in the treatment of recurrent fever or symptomatic malaria in the traditional medicinal practices of India were selected for this study (Table 1). Plant materials were collected from Vadodara district; Gujarat, India and were identified by Dr. Vinay Raole, Associate Professor at Department of Botany, Faculty of Science, M. S. University of Baroda and their voucher specimens were deposited at Maharaja Sayajirao University of Baroda Herbarium (Biodiversity Collection Index Code: BARO).

2.4. Preparation of plant extracts

Plant materials were desiccated by lyophilization and were stored in airtight containers at −20 °C until used for extraction. Petroleum ether, chloroform and ethanol extracts were prepared with 0.5 g dried biomass of each plant in 200 ml of respective solvent. Extraction was carried out in the Soxhlet apparatus for 12 h at 50 °C for petroleum ether, 55 °C for chloroform and 70 °C for ethanol. Aqueous extracts were prepared by heating 0.5 g of biomass in 50 ml of water at 80 °C for 1 h followed by filtration through Whattman number 3 filter paper. Using rotary vacuum evaporator, extracts were evaporated completely to form dry flakes or powder. These were analyzed gravimetrically and re-dissolved in suitable solvents (petroleum ether and chloroform extracts were re-dissolved in isopropanol, ethanol

Table 1
Plants used in the study.

Plants (botanical name; local name)	Herbarium code	Plant part	References [a]	% Yield (w/w) [b]			
				PE	C	E	A
Eclipta alba (L.) Hassk.; Mahabhringaraj	PKSI 01	Aerial parts	Bapna et al. (2007)	4.2	6.1	6.8	9.7
Azadirachta indica A. Juss.; Neem	PKSI 05	Leaves	Udeinya et al. (2008)	2.4	5.8	6.2	8.5
Andrographis Paniculata (Burm. *F.*) Wall. ex Nees; Kirayat	PKSI 10	Aerial parts	Mishra et al. (2009)	4.3	5.5	6.1	8.1
Murraya koenigii (L.) Spreng.; Currypatta	PKSI 14	Leaves	Patel et al. (2009)	3.1	6.5	6.7	8.6
Calotropis procera (Aiton) Dryand. ex W. T. Aiton; Akra	PKSI 18	Latex	Sharma and Sharma (1999)	7.6	14.5	16.1	18.3
Caesalpinia crista L.; Latakaranj	PKSI 21	Seed kernels	Linn et al. (2005)	9.3	12.3	14.2	20.6
Ocimum sanctum L.; Tulsi	PKSI 25	Leaves	Bagavan et al. (2011)	3.8	7.4	8.3	9.8
Phyllanthus amarus Schum. and Thonn.; Bhui Amla	PKSI 30	Aerial parts	Devi et al. (2001)	4.6	6.9	7.6	10.2

[a] Reference quoting antiplasmodial or antipyretic activity of respective medicinal plants.
[b] % yield obtained in PE, petroleum extracts; C, chloroform extracts; E, ethanol extracts; A, aqueous extracts.

extracts in ethanol and aqueous extracts in water) to attain 1% w/v solutions. Organic extracts were stored at ambient temperature while aqueous extracts were stored at 4 °C.

2.5. Screening of herbal extracts for pLDH inhibitory activity

Recombinant pLDH and bovine LDH were diluted to 18 kU/l concentration in phosphate buffered saline (pH 7.4) containing 1 mg/ml BSA and 1 mM PMSF and from this stock, 30 µl enzyme was used in the assay described in Section 2.2. Effect of plant extracts, on rPfLDH and rPvLDH, was determined by examining variation in the enzyme activity by adding 15 µl extract (to get final concentration 50 µg/ml) in the assay mix. The solvents, used for redissolving plant extracts (Section 2.4), were tested as controls. One way ANOVA with the Holm–Sidak method was performed to determine the extract with significant effect, compared to control. Extracts reducing pLDH activity to $\leq$50% were further tested for their specificity by examining their effect on bovine heart and muscle LDH (Sigma Aldrich, India). Effect of the extracts on parasite and mammalian enzymes were compared by calculating Pearson's correlation coefficient *r*.

The extracts reducing either or both rPfLDH and rPvLDH activity to $\leq$50% were tested for their 50% inhibitory concentration (IC_{50}). Gossypol (Sigma Aldrich, India) was also tested as positive control along with the extracts. IC_{50} values were determined by nonlinear regression analysis as described by Xu et al. (2007). Aqueous extract of *Phyllanthus amarus* was tested for *in vitro* anti-malarial activity at National Institute of Malaria Research, New Delhi, using a method described by Bagavan et al. (2011). All the enzyme and *Plasmodium falciparum* culture inhibition assays were performed in triplicates and statistical calculations were carried out using SigmaStat version 3.5 software (Systat Software Inc., USA).

2.6. Nucleotide sequence accession numbers

The sequences determined in this study are submitted in Genetic sequence database at the National Center for Biotechnology Information (NCBI).

PfLDH sequence GenBank ID: JN547218

PvLDH sequence GenBank ID: JN547225

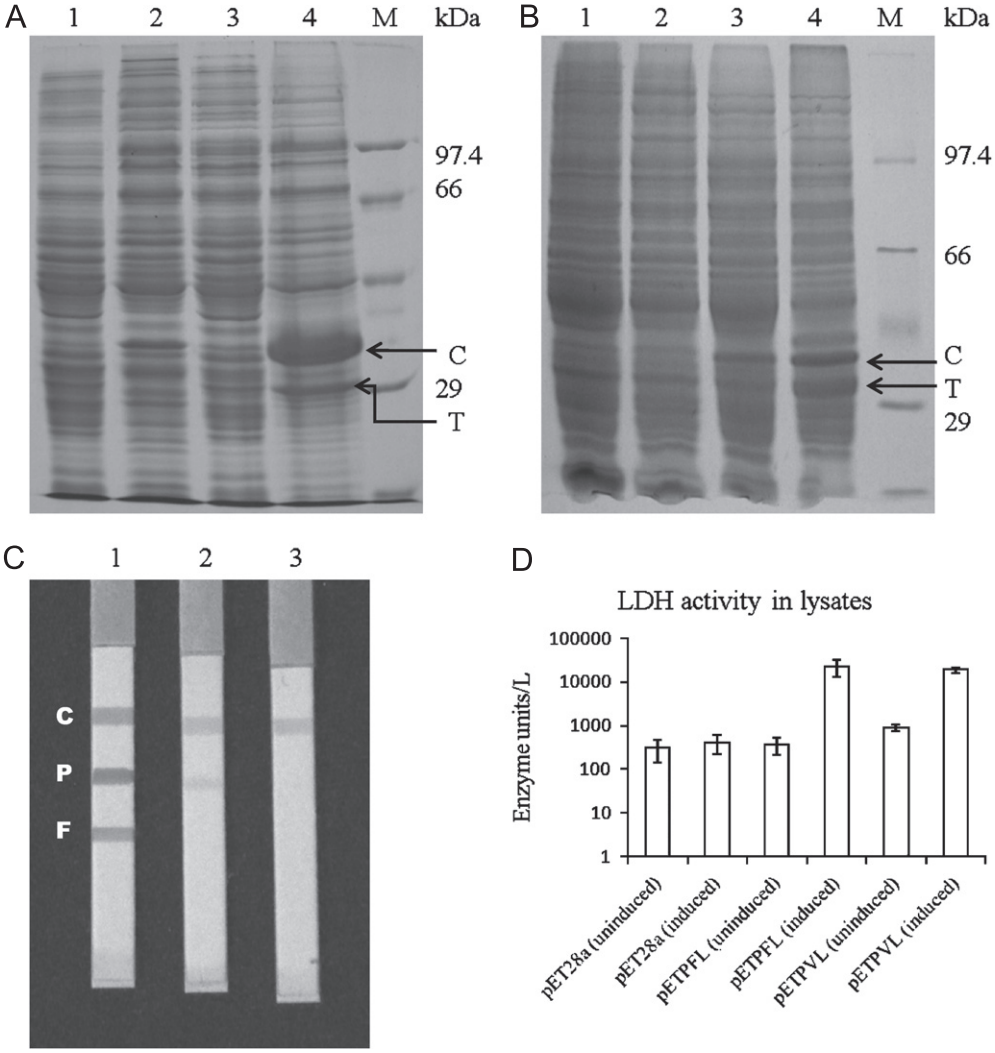

Fig. 1. Expression analysis of rPfLDH and rPvLDH in *Escherichia coli*. A and B, SDS-PAGE analysis of *Escherichia coli* cell lysate, with pET28a (only vector) under IPTG uninduced (Lane 1 of A and B)and induced conditions (Lane 2 of A and B), with pETPFL and pETPVL under uninduced conditions (Lane 3 of A and B respectively) and with pETPFL and pETPVL under induced conditions (Lane 4 of A and B respectively). Arrows indicate band positions of complete (C) and truncated (T) proteins, expressed in lysate. Protein bands in the gel were visualized by Coommassie Blue staining. Molecular weights of marker bands (M) are shown on the right hand side of the gel. C, Immumochromatography based dip stick assay of induced *Escherichia coli* lysate with pETPFL construct (Stick 1), pETPVL construct (Stick 2) and pET28a (Stick 3). Band development at *falciparum* (F) and pan specific (P) position indicate presence of rPfLDH, at only pan specific (P) position indicate presence of rPvLDH. Assay control bands (C) are developed at top of the stick. D, LDH activity of *Escherichia coli* cell lysate with specified constructs and conditions. Enzyme unit is 1 µmol of $NADH^+$ utilized per minute.

Table 2
Effect of plant extracts on rPfLDH and rPvLDH.

Plants	rPfLDH activity [mean U/L[a] ± SD] (Activity, % of solvent control)				rPvLDH activity [mean U/L[a] ± SD] (Activity, % of solvent control)			
	Petroleum ether extract	Chloroform extract	Ethanol extract	Aqueous extract	Petroleum ether extract	Chloroform extract	Ethanol extract	Aqueous extract
Solvent control	134.4 ± 2.6 (100.0)	134.4 ± 4.4 (100.0)	149.1 ± 7.3 (100.0)	152.4 ± 3.3 (100.0)	133.3 ± 10.2 (100.0)	133.3 ± 10.2 (100.0)	143.0 ± 6.0 (100.0)	149.9 ± 4.3 (100.0)
Eclipta alba	115.4 ± 2.6 (85.9)	87.5 ± 7.4[b] (65.1)	29.8 ± 1.1[b] (20.0)	116.2 ± 5.0[b] (76.3)	145.8 ± 1.9 (≥ 100)	85.0 ± 18.5[b] (63.8)	134.6 ± 0.8 (94.1)	130.4 ± 3.9 (87.1)
Azadirachta indica	119.7 ± 2.2 (89.1)	92.0 ± 8.3[b] (68.4)	118.4 ± 1.9[b] (79.4)	161.0 ± 9.6 (≥ 100)	149.8 ± 0.6 (≥ 100)	153.7 ± 4.5 (≥ 100)	139.5 ± 2.8 (97.6)	148.7 ± 3.3 (99.3)
Andrographis paniculata	124.8 ± 4.7 (92.9)	85.8 ± 6.0[b] (63.9)	62.3 ± 2.5[b] (41.8)	149.3 ± 8.8 (98.0)	132.3 ± 6.4 (99.3)	60.2 ± 29.4[b] (45.2)	134.1 ± 2.8 (93.8)	144.9 ± 2.0 (96.8)
Murraya koenigii	119.7 ± 1.0 (89.1)	41.5 ± 3.6[b] (30.8)	146.4 ± 1.1 (98.2)	150.3 ± 11.3 (98.7)	121.5 ± 2.3 (91.1)	5.2 ± 10.0[b] (3.9)	131.0 ± 3.5[b] (91.6)	137.9 ± 12.7 (92.0)
Calotropis procera	62.6 ± 27.2[b] (46.6)	135.9 ± 3.5 (≥ 100)	150.3 ± 0.8 (≥ 100)	134.0 ± 8.9 (88.0)	66.8 ± 8.8[b] (50.1)	108.5 ± 13.7 (81.4)	162.8 ± 2.4[b] (≥ 100)	148.7 ± 1.3 (99.2)
Caesalpinia crista	N/D[c]	114.3 ± 2.3[b] (85.0)	150.0 ± 0.7 (≥ 100)	158.9 ± 4.1 (≥ 100)	N/D[c]	168.3 ± 3.2 (≥ 100)	149.0 ± 1.5 (≥ 100)	164.0 ± 1.1 (≥ 100)
Ocimum sanctum	129.9 ± 3.1 (96.6)	114.4 ± 12.2[b] (85.1)	119.4 ± 0.6[b] (80.1)	103.8 ± 10.4[b] (68.1)	143.4 ± 3.9 (≥ 100)	139.7 ± 7.6 (≥ 100)	137.3 ± 7.3 (96.0)	146.6 ± 3.0 (97.9)
Phyllanthus amarus	123.7 ± 0.5 (92.0)	138.8 ± 1.7 (≥ 100)	13.6 ± 3.5[b] (9.1)	3.1 ± 5.4[b] (2.1)	128.0 ± 2.7 (96.0)	141.9 ± 5.2 (≥ 100)	135.2 ± 3.9 (94.5)	45.9 ± 19.4[b] (30.6)

[a] Enzyme unit, $U = 1$ μmol of $NADH^+$ utilized per minute.
[b] Significant variation compared to control using one way ANOVA by the Holm Sidak method ($P \leq 0.001$).
[c] Not determined.

3. Results

3.1. Cloning of PfLDH and PvLDH from Indian Plasmodium samples

Genomic DNA used in the study, to carry out PCR amplification of PfLDH and PvLDH contained both human and *Plasmodium* DNA as it was extracted from malaria patient's blood. However use of *Plasmodium* species specific primers and optimization of PCR conditions yielded intense amplification of 951 bp DNA fragment (the expected size of PfLDH and PvLDH genes) in both the samples (data not shown). Cloning of these amplicons in *Escherichia coli* DH5α using pJET vector had yielded putative clones which were confirmed by PCR and sequencing. BLASTn analysis of cloned sequences had confirmed their identity as PfLDH, with 100% sequence similarity to *Plasmodium falciparum* 3D7 LDH (GenBank ID: XM_001349953.1) and PvLDH, with T233C substitution compared to *Plasmodium vivax* SAL1 LDH sequence (GenBank ID: XM_001615570.1). This variation was not reported in available PvLDH sequences, moreover it was found to be nonsynonymous with V77A amino acid substitution in the protein sequence.

3.2. Protein expression, purification and characterization

Proper orientation of the pLDH genes was maintained in subcloning by *Nco*I and *Xho*I restriction digestion, such that they match with the expression ORF of pET28a vector. This strategy has effectively used strong translation signals of vector ribosome binding site and yielded efficient expression of rPfLDH and rPvLDH in *Escherichia coli* BL21 (DE3) in response to IPTG induction, as illustrated by the appearance of intense new bands in SDS-PAGE gels (Fig. 1A and B). The higher molecular weight bands were close to 35 kDa while sizes of the lower molecular weight bands were close to 30 kDa. Appearance of 2 bands indicate co-expression of truncated protein with complete rPfLDH and rPvLDH in *Escherichia coli* and was attributed to the presence of internal Shine Dalgarno sequence in the ORF as reported by Turgut-Balik et al. (2001). Band intensity was higher in the induced lysate of pETPFL clone compared to pETPVL suggesting variation in the expression levels of rPfLDH and rPvLDH. In immunochromatography based dip stick test, development of PfLDH specific and pan specific (Human *Plasmodium* species LDH) bands in the induced lysate of pETPFL clone had confirmed the presence of rPfLDH whereas development of pan specific band in the induced lysate of pETPVL clone had confirmed the presence of pan specific LDH, here particularly rPvLDH (Fig. 1C). A substantial increase in the LDH activities was observed in induced lyastes of pETPFL and pETPVL clones (22312 U/l and 18939 U/l respectively) as compared to uninduced clones (374 U/l and 897 U/l respectively) (Fig. 1D) indicating presence of functional rPfLDH and rPvLDH. *Plasmodium* LDH specific assay was not used in the study and hence enzyme activity was also observed in uninduced clones and vector controls, attributed to the *Escherichia coli* LDH (Fig. 1D).

Single step protein purification carried out by metal chelation chromatography yielded ≈ 10 fold increase in the specific activity of rPfLDH and rPvLDH (266 U/mg of protein and 236.6 U/mg of protein respectively) compared to crude lysate (25.1 U/mg of protein and 24.4 U/mg of protein respectively). Silver stained SDS-PAGE gel analysis confirmed ≥ 90% purity of both recombinant proteins. Their molecular weights (rPfLDH 34.5 kDa and rPvLDH 35 kDa) were close to calculated molecular weights using amino acid sequence (rPfLDH 34.9 kDa and rPvLDH 35.1 kDa).

3.3. Inhibition studies of rPfLDH and rPvLDH

Extracts of 8 plants in 4 solvents were prepared with varying yield of biomass (Table 1) and were tested at 50 μg/ml concentration for pLDH inhibitory activity. Petroleum ether extract of

Table 3
Effect of plant extracts on bovine heart and bovine muscle LDH.

Plant extracts	Enzyme activity [mean U/L[a] ± SD] (Activity, % of solvent control)	
	Bovine heart LDH	Bovine muscle LDH
Solvent control	149.6 ± 2.6 (100)	130.4 ± 1.8 (100)
Petroleum ether extract of *Calotropis procera*	150.8 ± 0.1 (≥ 100)	130.2 ± 2.2 (100)
Chloroform extract of *Murraya koenigii*	143.9 ± 2.1 (96.2)	125.2 ± 2.7 (96)
Chloroform extract of *Andrographis paniculata*	148.5 ± 1.3 (99.3)	127.2 ± 1.5 (97.6)
Solvent control	216.2 ± 3.1 (100)	201.6 ± 3.2 (100)
Ethanol extract of *Andrographis paniculata*	227 ± 2.9[b] (≥ 100)	200.2 ± 0.3 (99.3)
Ethanol extract of *Eclipta alba*	227.8 ± 3.8[b] (≥ 100)	211.7 ± 2.9[b] (≥ 100)
Ethanol extract of *Phyllanthus amarus*	227.5 ± 4.8[b] (≥ 100)	200.6 ± 2.5 (98.4)
Gossypol[c]	218 ± 0.5 (≥ 100)	198.3 ± 2.5 (98.4)
Solvent control	225.8 ± 4.8 (100)	210.7 ± 12.2 (100)
Aqueous extract of *Phyllanthus amarus*	216.4 ± 1.3 (95.8)	203.7 ± 4.7 (96.7)

[a] Enzyme unit, $U=1$ μmol of $NADH^+$ utilized per minute.
[b] Significant variation compared to control using one way ANOVA by Holm Sidak method ($P \leq 0.001$).
[c] Gossypol (positive control) is strong and selective inhibitor of parasite LDH.

Table 4
Inhibitory concentrations (IC_{50}) of plant extracts on rPfLDH and rPvLDH.

Plant extracts	IC50 [mean μg/ml ± SD]	
	rPfLDH	rPvLDH
Petroleum ether extract of *Calotropis procera*	42.8 ± 5.7	48.4 ± 1.9
Chloroform extract of *Murraya koenigii*	21.3 ± 1.9	6.0 ± 0.6
Chloroform extract of *Andrographis paniculata*	N/D[b]	39.3 ± 0.8
Ethanol extract of *Andrographis paniculata*	45.7 ± 1.2	N/D[b]
Ethanol extract of *Eclipta alba*	35.0 ± 0.4	N/D[b]
Ethanol extract of *Phyllanthus amarus*	14.6 ± 2.5	N/D[b]
Aqueous extract of *Phyllanthus amarus*	11.2 ± 0.4	34.9 ± 4.7
Gossypol[a]	2.6 ± 0.8 (5.1 μM)	10.4 ± 0.2 (20.1 μM)

[a] Gossypol (positive control) is strong and selective inhibitor of parasite LDH.
[b] Not determined.

Caesalpinia crista was not tested as it was precipitated in aqueous reaction system. Ethanol extract of *Eclipta alba*, *Andrographis paniculata* and *Phyllanthus amarus* reduced rPfLDH activity to $<50\%$, similarly chloroform extracts of *Andrographis paniculata* reduced rPvLDH activity to $<50\%$ whereas petroleum ether extract of *Calotropis procera*, chloroform extracts of *Murraya koenigii* and aqueous extracts of *Phyllanthus amarus* reduced activity of both pLDH to $\leq 50\%$. Effects of these 7 extracts were statistically significant at 0.001 levels (Table 2). Though few other extracts also changed pLDH activity to statistically significant level, activity were not reduced to $\leq 50\%$ suggesting their IC_{50} values would be more than 50 μg/ml and hence were not considered in further studies. The extracts, effectively inhibiting parasite LDH, were tested at same concentration on Bovine heart and muscle LDH. Both the enzymes retained $>95\%$ activity in presence of these extracts, compared to control (Table 3). Correlation was not observed between the effect of 7 plant extracts on the rPfLDH and rPvLDH activity (Pearson's coefficient, $r=0.2$, $P=0.6$), in contrast the effect of extracts on bovine heart and muscle LDH activity were strongly correlated ($r=0.8$, $P \leq 0.05$). The IC_{50} of all selected 7 extracts were <50 μg/ml (Table 4) and *Phyllanthus amarus* aqueous extract exerted strongest effect on rPfLDH ($IC_{50}=11.2$ μg/ml ± 0.4) and *Murraya koenigii* chloroform extract had strongest effect on rPvLDH ($IC_{50}=6.0$ μg/ml ± 0.6). Gossypol had effectively inhibited both rPfLDH and rPvLDH (Table 4) but was ineffective on mammalian LDH (Table 3). *In vitro* test of *Phyllanthus amarus* aqueous extract confirmed its parasiticidal activity on *Plasmodium falciparum* NE (chloroquine sensitive) and MRC-2 (chloroquine resistant) strains ($IC_{50}=7.1$ μg/ml ± 0.5 and 6.9 μg/ml ± 0.7 respectively).

4. Discussion

Overproduction of therapeutic target protein plays an important role by providing abundant enzyme for drug screening and in this view, cloning of LDH from major Indian malaria species *Plasmodium falciparum* and *Plasmodium vivax* was the first step in our search for pLDH inhibitors. rPfLDH was cloned using blood sample from Odisha state that has highest share of total malaria cases in India (25%) with high proportion of *Plasmodium falciparum* malaria cases. rPvLDH was cloned using blood sample from Karnataka state that has substantial share of total malaria cases in India (7%) but with more proportion of *vivax* malaria cases (Kumar et al., 2007).

In codified (Eg. *Ayurveda*, *Siddha*, *etc.*) and noncodified (Verbal folk remedies) traditional medicinal systems of India, plants are often used to treat recurrent fever or symptomatically diagnosed malaria (Unnikrishnan et al., 2004). We have tested 8 such plants, which are well documented for their antiplasmodial or antipyretic activity (For references see Table 1) and are readily available at Ayruvedic shops in India. Four solvents of different polarity were

used to extract maximum compounds from 8 plants and hence all 32 extracts were expected to contain different concoction of compounds on the basis of their polarity. Seven of these extracts had significantly reduced pLDH activity without having any inhibitory effect on bovine heart and bovine muscle LDH (Section 3.3). These results, being analogous to the effect of gossypol, have validated the specificity of extracts towards *Plasmodium* LDH and this can be accredited to structural variations between *Plasmodium* and mammalian LDH (Dunn et al., 1996). These extract had discrete effects on PfLDH and PvLDH (Section 3.3) suggesting that compounds present in these extracts bind to both enzyme with varying affinities and this can be justified by the conformational differences, mainly at the cofactor binding site, in structures of PfLDH and PvLDH (Brown et al., 2004). As utility of X-ray crystallography is rising in the discovery of new antimalarial drugs (Mehlin, 2005); this technique can be used to decipher actual molecular interactions of pLDH with these inhibitors and can be used further for structure based drug development.

Amongst tested extracts, *Phyllanthus amarus* aqueous extract and *Murraya koenigii* chloroform extract had inhibitory effects close to gossypol, which is known selective inhibitor of pLDH (Sessions et al., 1997; Section 3.3). Rationally, effective compound/s (responsible for pLDH inhibition) are merely a fraction of the total composition of the crude extract. Hence, if crude extract can inhibit the enzyme comparable to the pure inhibitor (gossypol), its component compound/s responsible for enzyme inhibition, in pure form; will have more effective inhibitory concentrations (IC50).

Aqueous extracts of *Phyllanthus amarus* had remarkable parasiticidal activity on chloroquine sensitive and resistant strains of *Plasmodium falciparum*. These results are analogous to reported PfLDH inhibitors which in turn kill parasite (Choi et al., 2007; Royer et al., 1986; Vivas et al., 2005). Interestingly *Phyllanthus amarus* extract had stronger parasiticidal activity than pfLDH inhibitory activity in terms of IC50 values (Section 3.3). This is possibly caused by presence of compounds with different mode of action on parasite, along with PfLDH inhibition, which synergistically act and kill parasites more efficiently. *Phyllanthus amarus* is used in several ethnomedical systems to treat malaria and other diseases (Bagalkotkar et al., 2006; Patel et al., 2011). Antimalarial activity of *Phyllanthus amarus* extract was also observed *in vivo* in mice model wherein parasitemia was reduced by 73% on 200 mg/kg of oral dose without any toxicological effects (Tona et al., 2001). The parasiticidal activity of the *Phyllanthus amarus* aqueous extract was comparable to the effect of individual compounds purified from *Phyllanthus amarus* (IC_{50} values ranging from 1.4 to 32) as reported by Subeki et al. (2005). Our results, being in conjunction with these reports, strongly indicate presence of potential antimalarial drug/s in *Phyllanthus amarus* with PfLDH inhibition as mode of action. Further detailed investigation can lead to inclusion of the effective drug in our present inadequate arsenal of antimalarial compounds.

Murraya koenigii chloroform extract had inhibitory effect on rPvLDH, more effective than gossypol (Section 3.3). These results have suggested presence of extremely efficient and selective rPvLDH inhibitor in the extract. Though *in vitro* activity of *Murraya koenigii* extract on *Plasmodium vivax* culture was not determined, owing to the importance of pLDH for survival of the parasite (Vivas et al., 2005), strong rPvLDH inhibition can presumably culminate in parasiticidal activity. *Murraya koenigii* holds medicinal properties such as antipyretic (Patel et al., 2009), antimicrobial (Rahman and Gray, 2005) and nephroprotective activity (Yankuzo et al., 2011). Besides it is a common ingredient of Southeast Asian and Indian cuisine, hence is safe for human applications.

5. Conclusion

Phyllanthus amarus and *Murraya koenigii* extracts were found to have selective and significant inhibitory effect on *Plasmodium falciparum* and *Plasmodium vivax* LDH respectively. Using combined approach of ethnopharmacology and reverse pharmacology, these extracts were obtained as hits by screening of as low as 8 plants. Further identification and analysis of effective molecules from these extracts may lead to new therapeutic molecule with known mode of action. In the light of urgent need for novel drugs against malaria and other drug resistant pathogens, this kind of approach can effectively translate rich ethnic medicinal repertoire throughout the globe into effective drugs. Nevertheless this study is more economical over high throughput screening and excludes need of post drug development studies on determination of target and mode of action. Thus in the context of tropical infectious disease like malaria, where limitation of funds is a major concern, this study introduce an efficient and economical approach for drug discovery.

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